

Increased temperature-related adeno-associated virus vectors transduction of ovarian cancer cells - essential signatures of AAV receptor and heat shock proteins

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Abstract. Recombinant adeno-associated viruses (rAAVs) are becoming more commonly used in clinical trials involving gene therapy. Additionally AAV-based drugs have already been registered. Gene therapy aims to increase transduction efficiency, increase *in vivo* selectivity and reduce side effects. One approach to achieve this is the use of physical factors, such as temperature or more specifically, hyperthermia, which is already utilized in oncology. The aim of the present study was to investigate the effect of hyperthermic conditions (40°C and 43°C) on the rAAV transduction efficiency of ovarian cancer cells (Caov-3 and NIH:OVCAR-3) and non-cancerous cells (AAV-293). The present study was designed to identify functional associations between the level of gene transfer and the expression of representative genes for rAAV transmission (AAVR (AAV receptor), heparan sulfate proteoglycan (HSPG) 1 and HSPG2) and heat shock proteins (HSPs). The expressions of selected genes were measured via reverse transcription-quantitative PCR and cell adhesion/invasion chamber tests were also performed. The results revealed that ovarian cancer cell lines were more efficiently transduced with rAAV vectors at an elevated temperature. Additionally, the expression patterns of AAVER, HSPG1 and HSPG2 genes were different between the tested lines. The expression of certain receptors in ascites-derived NIH:OVCAR-3 ovarian cancer cells was higher compared with tumor-derived Caov-3 cells at 37, 40 and 43°C, which indicates a higher transduction efficiency in the formerly mentioned cells. Ascites-derived

ovarian cancer cells were characterized by high expressions of HSP40, HSP90 and HSP70 families. Lower levels of HSP expression were demonstrated in less-effectively transduced Caov-3 cells. Furthermore, expressions of the examined genes changed with increasing temperature. The results indicated that temperature-dependent transduction is associated with the expression of the rAAV receptor and HSP genes. The results of the current study may aid the design of effective protocols for ovarian cancer gene therapy.

Introduction

Adeno-associated viruses (AAV) are small (a diameter of approximately 25 nm), non-encapsulated, icosahedral viruses. They are classified as members of the *Parvoviridae* family, the *Dependovirus* genus. AAV can replicate only in the presence of helper viruses, such as adenovirus, herpes simplex virus and human papilloma virus (1). The contribution of genotoxic factors in the activation of AAV replication is also indicated (2). The AAV genome is single-stranded DNA (approximately 4.7 kb) consisting mainly of two reading frames: the rep and cap genes, and ITR flanking sequences. The expression cassette in recombinant vectors, containing the promoter and the transgene, is cloned in place of deleted rep and cap genes, between 145 nucleotides ITR sequences (1). Due to the non-pathogenic nature and occurrence of serotypes with defined organ tropism, recombinant AAV vectors are increasingly used in gene therapy trials (3). There already are registered AAV-based drugs (3). The safety of rAAV vectors as well as their presence in medicine result in a number of studies revealing critical points in the pathway of gene transfer and intracellular events involving rAAV (1,4). The role of the miRNA signature (5) and the expression of AAV membrane receptors (6-8) in the AAV cellular transmission are in the course of documentation. Discovering cellular mechanisms of rAAV transduction helps to understand the AAV biology and makes it possible to design new vectors-synthetic AAV mosaic serotypes (9), vectors with dsDNA (10), AAV chemo-conjugates (11). There is research indicating the possibilities of optimizing the efficiency of rAAV transduction by various

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physicochemical treatments. The increase of rAAV transduction efficiency is observed as a result of hyperthermia (12). Also, the use of proteasome inhibitors and the degradation of proteins associated with endoplasmic reticulum induce the same effect (13,14). The possibility of physicochemical manipulation of transduction efficiency significantly increases the use of bio-safe AAV vectors in gene therapy.

The normal temperature of the human body, around 37°C, is a condition for maintaining homeostasis and is necessary for the course of physiological processes. Thermoregulation is crucial in the context of maintaining the continuity of human life, and it is based on many well-defined and complex physiological processes controlled by the function of the thermoregulation center, the vasomotor system and the skin. Incorrect temperature fluctuations, which go beyond the range of the menstrual cycle or the aging of the body, cause changes in the functioning of relevant cellular biomolecules, including protein denaturation and irreversible DNA damage. Exceeding the thermoregulatory thresholds results in disturbances in the essential function of cardiovascular, nervous and respiratory systems (15-18). Temperature modulation is also the basis for the development of new biomaterials and therapeutic technologies (19). The synthesis of thermally sensitive hydrogels allows the design of controlled drug release systems in response to an external stimulus, such as temperature (20). Controlled temperature increase is also the basis of oncological hyperthermia (21). Local or whole-body hyperthermia is considered a complementary therapy in oncology, in combination with chemotherapy or radiotherapy (21,22).

Cell profiling in terms of their temperature sensitivity, e.g., by evaluating expression of heat shock proteins (HSP) or transient receptor potential (TRP) channels, is important for the design of new temperature-regulating drugs and medical technologies, also in the field of gene therapy (23,24). The temperature response of cells is closely related to the heat shock protein family gene expression pattern. HSPs are classified based on molecular weight and function (e.g., HSP27, HSP70, HSP90). HSP biosynthesis is precisely regulated by the activity of transcription factors (HSF) that recognize heat shock elements in the promoter regions of genes. HSPs are involved in the folding, maturation, functioning and degradation of many crucial proteins (being chaperones they interact with many proteins). Therefore, they perform regulatory functions in the fundamental biological processes such as proliferation, apoptosis, drug resistance, stress response (25-27). HSP signatures in cells are different, there are significant differences in the constitutive expression of HSP genes in tumor cells, which are associated with the pathomechanisms of cancer development (28,29). In ovarian cancers, the important role of HSP27, HSP70 and HSP90 is stressed (29). It is documented that HSP expression changes under stress conditions, and individual HSPs can be expressed at different levels. The role of HSP90 in AAV transduction to cells is emphasized, underlining functional relationships between FKBP52 and HSP90 (12,27,30). The phosphorylated form of the FKBP52 chaperone protein has been shown to interact with the D-sequence of the ITRs in the AAV genome. It inhibits the synthesis of the second viral DNA strand and thereby leads to inefficient expression of the transgene from the rAAV vector. The heat shock leads to the dephosphorylation of

FKBP52, stabilization of the FKBP52-HSP90 complex, which results in the increase of rAAV transduction efficiency (12,30). It seems that the transduction efficiency of rAAV depends on the cells HSP signatures. The level of selected HSPs in the cells promotes an increase the transduction efficiency with the use of AAV vectors. It is worth considering that the determination of the HSP signature in tumors may be helpful in planning efficient gene therapy trials.

At present, gene therapy of ovarian cancers is based primarily on the use of suppressor gene (e.g., WWOX) strategies, suicide therapy (e.g., HVS-TK), anti-angiogenic strategy (e.g., VEGF) and a strategy directed towards multi-drug resistance genes (e.g., MDR1) or based on oncolytic virotherapy (31). Despite progress in the field of ovarian gene therapy, there are too few clinical trials on oncology patients and there is still a lack of efficient and safe gene transfer systems. The aim of this study was to investigate the effect of hyperthermia conditions (40 and 43°C) on the rAAV transduction efficiency of ovarian cancers with various clinical origins. Moreover, the purpose was to evaluate the expression of representative genes involved in rAAV transmission with special emphasis on heat shock protein genes. Notably, depending on the origin of ovarian cancer cells, the increase of rAAV transduction efficiency at elevated temperature was subsequently the cause of the search for functional linkages between the high sensitivity of ascites-derived ovarian cancer to rAAV, and for the expression of representative genes for rAAV transmission and HSP.

Materials and methods

Cell lines. The ovarian cancer cell lines with a different clinical origin (32,33): Caov-3 (ATCC[®] HTB-75), NIH:OVCA3 (ATCC[®] HTB-161, American Type Culture Collection) were cultured in the Dulbecco's Modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and RPMI Medium-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS and 0.6 mg insulin (Insulin solution from bovine pancreas; Sigma-Aldrich; Merck KGaA)/500 ml medium, respectively. Cell line AAV-293 (Agilent Technologies) derived from human embryonic kidney cells was cultured in DMEM supplemented with 10% FBS. Human fibroblasts CCD-18Co (ATCC[®] CRL-1459) were cultured in Minimum Essential Media (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. The media were also supplemented with 1% Antibiotic-Antimycotic (Gibco; Thermo Fisher Scientific, Inc.), and the cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. Mycoplasma test was performed in our laboratory, and its shown no contamination. The analyzed cells were verified in the International Cell Line Authentication Committee and ExPASy Cellosaurus databases (34,35), in order to exclude their contamination with other cell lines or their incorrect identification.

rAAV transduction at hyperthermia conditions. Cells were seeded at the density 1x10⁵ for Caov-3, AAV-293 and 1.5x10⁵ for NIH:OVCA3 per a 6 cm diameter dish in the appropriate medium and incubated for 24 h in a normal culture condition. Just before transduction, cell culture media were

replaced with adequate ones (AAV-293, Caov-3-DMEM; NIH:OVCAR-3-RPMI-1640 with insulin) heated up to 37, 40 or 43°C with FBS reduced to 2%. To transduce the ovarian cancer cells, the recombinant AAV vector rAAV/DJ-cmv-eGFP (cat. no: 7101; Vector Biolabs) was added to the new, heated media with the multiplicity of infection (MOI) of 4×10^4 genome copies or 0 genome copies (non-transduction control group). The vector rAAV/DJ-cmv-GFP expresses eGFP gene under a control of cmv promoter. To test the promoters' transcriptional activity, AAV-293 cells were transfected with rAAV/DJ-CAG-GFP (cat. no: 7078; Vector Biolabs) at the same conditions and MOI. The vector rAAV/DJ-CAG-GFP expresses eGFP gene under a control of CAG promoter. The cells were incubated for 3 h in hyperthermia conditions (40 or 43°C) in a humidified 5% CO₂ atmosphere. Then the cells were moved to 37°C. After 48 and 96 h of transduction, the cell culture media were replaced with fresh 2% FBS media. Finally, the cells were harvested on the 6th day after transduction.

Non-transduction cells were examined to test HSP (Caov-3 and NIH:OVCAR-3) and AAV receptor (CCD-18Co, AAV-293, Caov-3 and NIH:OVCAR-3) expression. They were harvested after 24 h from exposure to hyperthermia conditions.

Transduction efficiency measurement. The Countess II FL Automated Cell Counter (Invitrogen; Thermo Fisher Scientific, Inc.) with EVOS Light Cube-GFP (470/22 nm Excitation; 510/42 nm Emission) was used to determine the percentages of Green Florescent Protein-positive (GFP+) cells. The images of GFP-expressing cells were obtained using an inverted fluorescence microscope (Olympus IX53; Olympus) with the pE-300^{white} (CoolLED) illumination system. Pictures of non-transduced (in BF) and transduced (in FITC) cells were taken at x10 magnification. All measurements were performed on the 6th day after transduction, before the cells harvesting for DNA isolation to analyze the transduction efficiency by qPCR.

Quantitative (q)PCR for AAV genome copy number determination. Total DNA was isolated using the High Pure Viral Nucleic Acid kit (Roche Life Science) from AAV-293, Caov-3 and NIH:OVCAR-3 cell lines. The amount of DNA was quantified by 260/280/230 nm absorbance measurements using spectrophotometer Quawell Q5000 UV-Vis (Quawell). TaqMan assays for rAAV genome copy number were developed using probe and primers for the ITR region. The fluorescent probe (5'-CACTCCCTCTCTGCGCGCTCG-3') featured 6-FAM and TAMRA. The forward primer was 5'-GGA ACCCCTAGTGATGGAGTT-3' and the reverse primer was 5'-CGGCCTCAGTGAGCGA-3' (36). Total volume of qPCR reactions was 10 μ l, contained 50 ng DNA and ran under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 60 sec. qPCR was performed in StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Absolute quantification analysis with the StepOne Software v2.3 (Thermo Fisher Scientific, Inc.) was performed to determine transcripts numbers. Data were quantified using the standard curve (37, 38). Briefly, standard curves were generated using serial dilutions of plasmid pAAV-hrGFP (Agilent Technologies) used to

calculate the number of genome copies in the tested samples. The rAAV genome copy number was normalized as viral genome copy number per 50 μ g of total genomic DNA.

Reverse transcription qPCR. To test gene expression, total RNA was isolated (39) using the TRI Reagent (Sigma-Aldrich; Merck KGaA). To examine the miRNA profile, RNA was isolated with the used of the TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). DNA-free™ DNA Removal Kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to remove the contaminating DNA from RNA samples. Single-stranded cDNA was synthesized with the High Capacity RNA-to-cDNA kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). All primers listed below are available on Thermo Fisher Scientific website as appropriate TaqMan Assays (Thermo Fisher Scientific Website; <https://www.thermofisher.com/pl/en/home.html>).

The expression of genes-encoding receptors for AAV was examined with the use of the following TaqMan Assays (assay ID; Thermo Fisher Scientific, Inc.): AAVR (Hs00967343_m1), HSPG1 (Hs01081432_m1), HSPG2 (Hs01078536_m1), and ACTB (Hs01060665_g1) as endogenous control. The expressions were measured after 24 h from exposure at 40 and 43°C for 3 h. The ovarian cancer cells, AAV-293 and CCD-18Co were used. CCD-18Co was selected as a reference sample in $\Delta\Delta C_q$ method (37).

The expression of miRNA responsible for silencing AAVR, HSPG1 and HSPG2 was examined on the designed TaqMan Custom MiRNA Array Card (TaqMan Low-Density Array-TLDA card; Thermo Fisher Scientific, Inc.) for Caov-3, NIH:OVCAR-3 and AAV-293 cell lines maintained in normal culture condition. The following human miRNA were analyzed (assay ID, Thermo Fisher Scientific): miR-15b-5p (000390), miR-133a-3p (002246), miR-195-5p (000494), miR-23a-3p (000399), miR-23b-3p (000400), miR-27a-3p (000408), miR-30a-5p (000417), miR-34a-5p (000426), miR-34c-5p (000428), miR-101-3p (002253), and U6 snRNA (001973). Functional links between the designated miRNAs and the AAVR, HSPG1 and HSPG2 genes were searched in miRNA databases (40, 41).

The expression of HSP was examined on TaqMan Array 96-Well FAST Plate Human Heat Shock Proteins (cat. no. 4418733; Applied Biosystems; Thermo Fisher Scientific, Inc.) in ovarian cancer cells 24 h after exposure to hyperthermia condition. Results for cells maintained at 37°C were selected as reference samples and used for the comparison of constitutive levels of HSP in ovarian cancer cell lines.

The expression of genes associated with the molecular mechanisms of cancer was examined on TaqMan Array 96-Well FAST Plate Human Molecular Mechanisms of Cancer (MMoC; cat. no. 4418806; Applied Biosystems; Thermo Fisher Scientific, Inc.) for Caov-3 and NIH:OVCAR-3 maintained in normal culture condition.

In all gene expression tests total volumes of qPCR reactions were 10 μ l, contained 100 ng cDNA and ran under the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The FAM was used as fluorophore and NFQ-MGB as a quencher (Thermo Fisher Scientific, Inc.). Transcript levels of target genes were normalized to the level of housekeeping genes encoding: β -actin (ACTB) for AAV

receptors, U6 snRNA for miRNA, β -glucuronidase (GUSB) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) for HSP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for MMoC. All reactions were performed according to manufacturer instructions in the StepOnePlus™ Real-Time PCR System. Relative gene expression levels were calculated using the $\Delta\Delta C_q$ method (37) by Expression Suite Software v1.1 (Thermo Fisher Scientific, Inc.). The Morpheus matrix visualization and analysis software was used to present results of miRNA and MMoC expression as heat maps (in the form of $2^{-\Delta C_q}$) (Morpheus-versatile matrix visualization and analysis software; <https://software.broadinstitute.org/morpheus>).

Protein CA 125 measure. To measure the level of CA 125 marker, Caov-3, NIH:OVCAR-3 and AAV-293 cells were harvested, washed with PBS and lysed in lysis RIPA buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS and protein inhibitor cocktail (cat. no. P8340; Sigma-Aldrich; Merck KGaA)] for 30 min on ice. The probes were centrifuged (20 min, 4°C, 12,500 rpm), and the supernatants were collected for further tests. CA 125 was measured in protein samples using solid phase, two-site sequential chemiluminescent assays (CLIA) with paramagnetic microparticle solid phase that was fully processed on an automated randomaccess immunoassay analyzer LIAISON XL (DiaSorin, Saluggia, Italy). The intra- and interassay CV for CA 125 ranged from 1.4-2.2 and 4.6-5.8%, respectively.

Cell adhesion. The adhesion potency of the tested cells was determined using laminin coated plates (cat. no. 354404; 6-well plates; BD BioCoat™ Laminin). The experiment was performed for transduced and non-transduced cells incubated at a different temperature (37, 40, 43°C). The examined cell lines on the 6th day after transduction were trypsinized (Trypsin-EDTA (0.25%), phenol red; Gibco; Thermo Fisher Scientific, Inc.), counted (in an automatic cell counter) and seeded on laminin plates. From a 6 cm diameter dish, the cells were transferred to 1 well in laminin coated plate. A control was performed, i.e., cells were seeded on 6-well plates without laminin (Nunc; Thermo Fisher Scientific, Inc.). After 4 h of incubation under standard culture conditions, the attached cells were trypsinized and the number of viable cells was counted. Subsequently, the percentage of adhered cells was estimated due to laminin presence.

Invasion chamber assay. To verify how hyperthermia and transduction modify cell invasion, invasion chamber assay was performed using a 24-well cell culture insert 8 μ m pore (cat. no. 354480; Corning Matrigel Invasion Chamber; Corning). The examined cell lines on the 6th day after transduction were trypsinized, cell suspensions in 2% FBS media at a concentration of 1×10^5 /ml for AAV-293, Caov-3 and 1.5×10^5 /ml for NIH: OVCAR-3 were prepared. Cell suspensions in volume 0.5 ml were added per well into the upper chambers, the lower chambers were filled with 0.5 ml of complete media. Cells which migrated to the lower chamber were counted after 24 and 48 h. The cells from the upper membrane were aspirated by gentle pipetting. The GFP+ cells from the bottom side of the Matrigel chamber were visualized with the fluorescence microscope (with FITC) after 24 h.

After 48 h of incubation, cells which migrated to the bottom of the Matrigel surface were fixed with methanol and stained with 0.5% crystal violet.

Statistical analysis. The transduction efficiency, receptor expression (AAVR, HSPG1, HSPG2), cell adhesion and cell invasion results expressed as mean and standard deviation. HSP results were performed as mean and standard error. A one-way analysis of variance (ANOVA, $\alpha=0.05$) with a post-hoc Bonferroni ($\alpha=0.05$) test using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) was used to calculate the statistical significance of differences between mean values of compared samples. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

rAAV transduction of ovarian cancer cells at hyperthermia conditions. The effect of elevated temperature on rAAV transduction was investigated using ovarian carcinoma cell lines of different clinical origin and pathomechanism of carcinogenesis. The NIH:OVCAR-3 cell line was originally derived from the malignant ascites of a patient with progressive adenocarcinoma of the ovary. NIH:OVCAR-3 cells are resistant to cytostatics at concentrations used in clinics and they are sensitive to hormonal therapy (32). Caov-3 cells are derived from the ovarian solid tumor (33). The cells' functional variability was visualized through the analysis of the expression of Molecular Mechanisms of Cancer (MMoC) genes that are essential for carcinogenesis. The MMoC gene expression pattern of functionally differentiating ovarian cancer lines is shown in Table I and Fig. 1. Ascites-derived NIH:OVCAR-3 cells were characterized by a higher expression of almost all genes involved in proliferation, cell cycle, cell differentiation, metastasis, invasion, angiogenesis, apoptosis and signal transduction processes, than the solid-derived Caov-3 cells (Table I). Additionally, the estimated ovarian cancer marker CA 125 confirmed the origin of ovarian cancer cells, as well provided information about the protein expression in the studied cells. CA 125 is a tumor marker, which is mainly used in the diagnosis of ovarian cancer and monitoring treatment. As shown in Table II, the level of CA 125 for NIH:OVCAR-3 and Caov-3 was $>68,590.0$ and $2,220.0$ U/ml, respectively. In the case of control line AAV-293, marker was determined on the level <0.3 U/ml. The obtained results showed that ascites-derived NIH:OVCAR-3 cells were characterized by increase expression of this cancer marker, which may indicate a higher invasiveness and progress in carcinogenesis of this line, compared to Caov-3 derived from a solid tumor.

Transductions were carried out under conditions of 37, 40 and 43°C, defined as hyperthermia conditions (21). To determine the transcriptional activity of the CAG and cmv promoters, the cells were transduced with the rAAV/DJ mosaic vector with the GFP reporter gene under the control of the hybrid CAG promoter (AAV-293) or the conventional cmv promoter (Caov-3 and NIH:OVCAR-3). The transduction efficiency was determined by direct counts of GFP+ cells and measuring genome copies of rAAV in transduced cells with qPCR. As Fig. 2A shows, as the temperature

Table I. Expression of representative genes involved in cancer.

NIH:OVCAR-3				
Fold change ($2^{-\Delta\Delta Cq}$)	Proliferation, cell cycle, cell differentiation	Metastasis, invasion, angiogenesis	Apoptosis	Signal transduction
0.00		CDC42, ITGB3, COL1A1	BCL2	KIT
<0.50	TGFB1 CCND3, CDKN1A			FDZ1
0.51-1.00	EGFR JUN, CCND1, CDK2	FYN, ITGB1, SPP1, VEGFA	BID	FAS, IGF1R, MAP2K1, PIK3CA, SHC1
1.01-1.50	ELK1, FGF2 MYC		BAX, FADD	AKT1, MAPK1, NFKB2, NRAS, RAC1
1.51-2.00	FOS BCAR1, PTEN			RAF1, MAPK8
2.01-2.50		PTK2B		MAP3K5, MAPK14, MAPK3, NFKB1, RELA, SMAD4, SOS1
2.51-3.00	MDM2	CRK, RHOA	BCL2L1, CASP8, GSK3B	ERBB2, GRB2, TGFBR2
3.01-4.00	TCF3 CDH1, E2F1	CTNNB1, ITGAV		APC, BRAF, TGFBR1
4.01-5.00	DVL1 CDKN1B	FN1	BCL2L11	HRAS, NFKBIA, PTK2
5.00-10.00	ABL1, SRC CDK4, CDKN2A	ITGA2B	CYCS	
10.01-15.00	CCNE1			AKT2, PIK3R1
15.01-50.00	CDKN2B			KRAS
50.01-300.00	TP53			LEF1
>300.01	RB1			IGF1

The molecular status of two ovarian cancer cell lines of different origin are presented. The expression of genes involved in cell proliferation, the cell cycle, cell differentiation, metastasis, invasion, angiogenesis, apoptosis and signal transduction were presented as $2^{-\Delta\Delta Cq}$ values, with Caov-3 as a reference.

elevates, the transduction efficiency increases. Furthermore, there were differences in the transduction efficiency between the lines. The highest efficiency of transduction was demonstrated for NIH:OVCAR-3 cells. At 37°C, approximately 23% of cells were GFP+. In comparison only 2% of Caov-3 cells, and 6% of AAV-293 cells under the same conditions were transduced. The temperature effect was visible for all lines. As the temperature raised, the percentage of GFP+ cells increased significantly. Despite the lower level of transduction efficiency, Caov-3 cells responded to temperature stimulation in both 40 and 43°C. In 40°C, a 50% increase in GFP+ cells was observed, and in 43°C, GFP+ cells showed an increase of 200% compared to 37°C. The percentage of fluorescent NIH:OVCAR-3 cells was significantly increased in 40°C (about 65% compared to normal temperature; Fig. 2A).

The transduction efficiency was also determined by the qPCR method (Fig. 2B). The percentage of GFP+ cells corresponds to the number of rAAV genome copies (gc) in all transduced cells. As shown in Fig. 2B, at 40°C a higher level of gc was demonstrated than at 37°C, which reflects the increase in transduction efficiency due to response to the rise in temperature. Interestingly, at 43°C an increase in the number of GFP+ cells was observed, with the gc number in all lines being lower than at 40°C and even 37°C (Fig. 2B).

AAV receptor expression. The expression of crucial genes of the extracellular rAAV transmission was analyzed in the study. The AAVR, HSPG1 and HSPG2 genes were selected for evaluation. The qPCR method was determined by normalizing the results to the level of ACTB gene expression, the human fibroblasts (CCD-18Co cells) were used as reference. As shown in Fig. 3, the expression of the examined genes changes with increasing temperature. Additionally, the expression pattern for AAVR, HSPG1 and HSPG2 genes was different in the lines tested. In NIH:OVCAR-3 cells, AAV receptor signature was revealed as a high expression of AAVR and HSPG1, and as a low expression of HSPG2. On the other hand in Caov-3 cells, the signature of selected genes occurred as a comparable expression level of all three genes tested. However, the expression of AAVR dominated in AAV-293 cells. Expression of receptors in NIH:OVCAR-3 ovarian cancer cells was higher than in Caov-3 cells at 37, 40, and 43°C, which corresponds with the higher transduction efficiency in NIH:OVCAR-3 (Fig. 2). Caov-3 cells, despite lower expression of AAV receptors, respond better to the increase of temperature. The expression of AAVR, HSPG1 and HSPG2 at 40°C has increased by 29, 51 and 82% respectively, compared to 37°C. In the case of AAVR and HSPG1 after 43°C, the expression normalized to the level observed in 37°C, but for HSPG2 the expression remained at a level

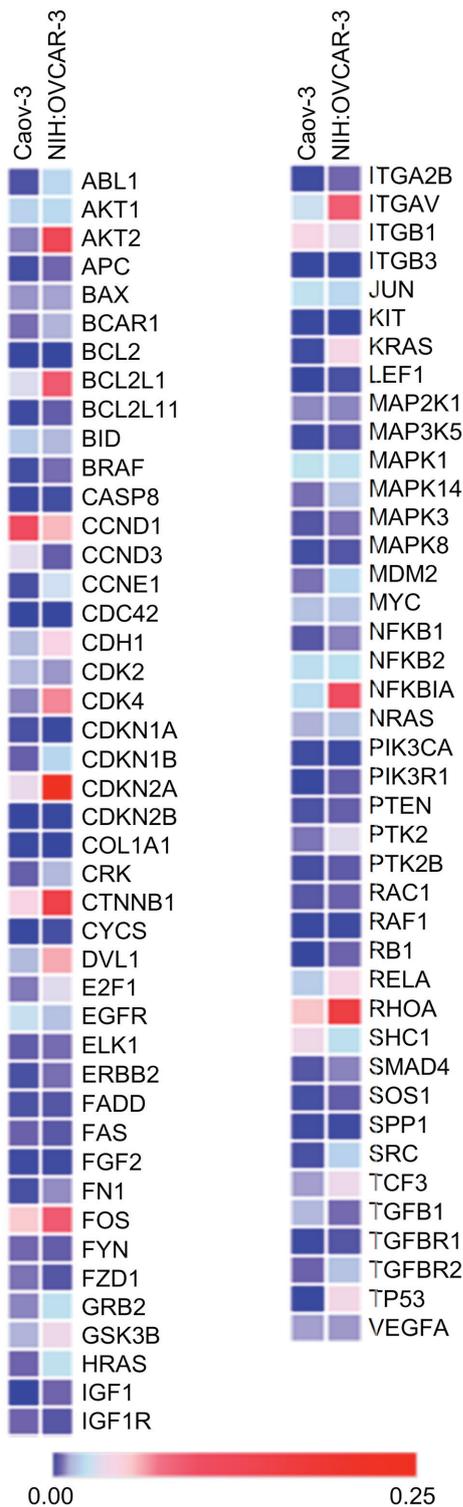


Figure 1. Heat map of the representative gene expression ($2^{-\Delta Cq}$) revealed in two ovarian cancer cell lines of different origin.

similar to 40°C. For NIH:OVCAR-3 cells, there was an increase in AAVR expression in 43°C of about 5% and HSPG2 of about 33%, vs. 37°C. The most proportional increase in the expression of AAVR and HSPG1 to the temperature elevation was observed for AAV-293 cells. They responded with the increase of AAVR and HSPG1 by approx. 6, 7% at 40°C, and 37 and 92% at 43°C, respectively. Interestingly, despite an increase in the number of GFP+ cells (Fig. 2A), a lower

Table II. CA 125 protein levels in AAV-293, Caov-3 and NIH:OVCAR-3 cell lines.

Cell lines	CA 125 (U/ml)
AAV-293	<0.3
Caov-3	2,220.0
NIH:OVCAR-3	>68,590.0

level of receptor expression as well as the rAAV copy number was observed in cells exposed to 43°C (Fig. 2B).

miRNA expression panel. During the search for mechanisms to increase the efficiency of rAAV transduction in cells exposed to elevated temperature, particular attention was paid to the expression of selected miRNAs. MiRNAs involved in the silencing processes of rAAV transmission were analyzed. Fig. 4B shows the mean expression of silencing miRNAs in the cells tested at 37°C and indicates that the level of miRNA corresponds to the rAAV transduction efficiency. The mean values were calculated due to underline the miRNA expression differences between studied cell lines. The lowest level of miRNAs responsible for silencing AAV transmission genes was observed for NIH:OVCAR-3. This ovarian cancer cell line was transfected at the highest level. The level of silencing miRNAs was about 35% higher in Caov-3 cells which transduced almost 12 times less efficiently than NIH:OVCAR-3. In the case of AAV-293 cells there was no functional coherence between the miRNA level and the transduction efficiency. The level of miRNA in AAV-293 cells was about 60% lower than in NIH:OVCAR-3, although the AAV-293 transduction efficiency (Fig. 2A) was lower than NIH:OVCAR-3.

HSP expression. The result of HSPs expression in NIH:OVCAR-3 and Caov-3 ovarian cancer cells are shown in Figs. 5 and 6. Fig. 5 shows the constitutive level of HSP expression in lines at 37°C, expressed in $2^{-\Delta Cq}$ form, while Fig. 6 shows changes in HSP level ($2^{-\Delta \Delta Cq}$, fold range) in cells exposed to elevated temperature (40 and 43°C). As shown in Fig. 5, in NIH:OVCAR-3 22 out of 42 HSP genes tested had higher expression than in Caov-3 cells. NIH:OVCAR-3 cells compared to Caov-3 are characterized by higher expression of the HSP40 family (DNAJA2, DNAJB13, DNAJB2, DNAJB4, DNAJC7), HSP90 family (HSP90AA1, HSP90B1), and HSP70 family (HSPA14, HSPA1A, HSPA1L, HSPA4, HSPA4L, HSPA6). NIH:OVCAR-3 cells also had a higher expression of HSP than Caov-3: AHSA1, HSPB8, HSPB9, HSPBAP1, HSPD1, HSPE1, STUB1, and TRAP1. Based on the highest expression HSP (3-10x higher than in Caov-3), the form of AHSA1+, HSP90AA1+, HSPA1A+, HSPD1+, STUB1+, and TRAP1+ can be proposed as constitutive signature for NIH:OVCAR-3 cells. On the other hand, the characteristic HSP signature for Caov-3 (3-10x higher HSP levels than in NIH:OVCAR-3) is DNAJB1+, HSP90AB1+, HSPA5+, HSPA8+, and HSPB1+. The obtained results (Fig. 5) may explain the differences between the NIH:OVCAR-3 and Caov-3 cell lines in the transduction efficiency (Fig. 2). Results presented in Fig. 6 show that the selected hyperthermia conditions caused the increase of

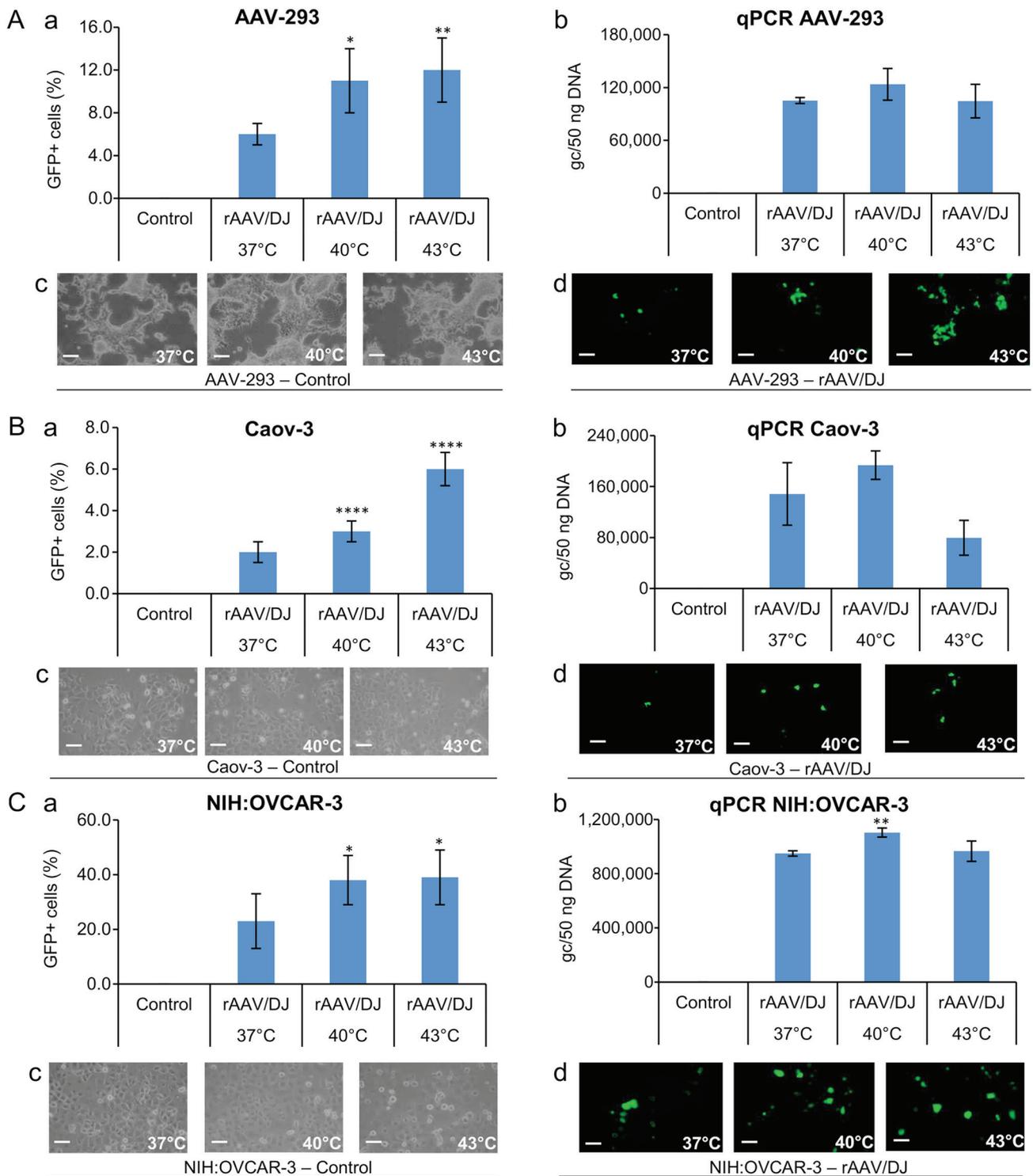


Figure 2. Transduction efficiency of AAV-293, Caov-3 and NIH:OVCAR-3 cells. (A) AAV-293, (B) Caov-3 and (C) NIH:OVCAR-3 cells were transduced with the rAAV/DJ vector under normal (37°C) and hyperthermic conditions (40 and 43°C) for 3 h. The green fluorescent protein positive cells were analyzed using (a) the Countess II FL Automated Cell Counter, (b) qPCR and visualized with an inverted fluorescence microscope under (c) bright field and (d) FITC (magnification, x10; scale bar, 100 μm). Results are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ****P<0.0001 vs. 37°C. rAAV/DJ, recombinant adeno-associated viral hybrid serotype DJ; qPCR, quantitative PCR.

selected HSP expression. NIH:OVCAR-3 cells demonstrated an increase expression at 40 and 43°C for AHSA1, DNAJA3, DNAJB2, DNAJC3, HSP90AA1, HSPA2, HSPA5, HSPA6, and HSPB1 (Fig. 6). A particularly high (>10x) increase in expression for DNAJB13, HSPA6, and HSPB1 was observed. Caov-3 cells were much less responsive to hyperthermia

through increased HSP expression (Fig. 6). Of the 42 HSP genes examined, only 5 genes showed increased expression at elevated temperature (DNAJB2, HSPA1L, HSPB1, HSPB6, HSPBAP1). In conclusion, the various ovarian cancer cells responded significantly differently to both the constitutive HSP expression level at 37°C and after the exposure cells to

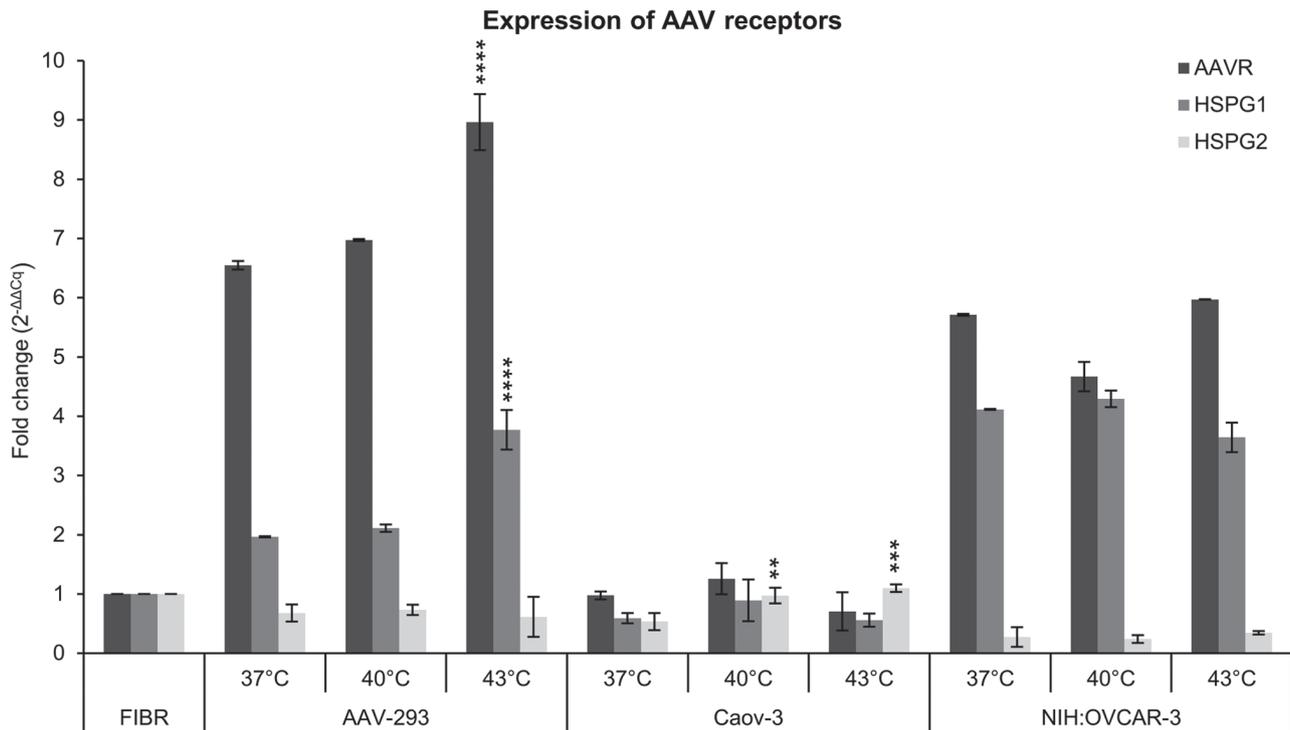


Figure 3. AAVR, HSPG1 and HSPG2 expression in AAV-293, Caov-3 and NIH:OVCAR-3 cells. The influence of heat shock on the expression of genes in ovarian cancer cells and normal cells was determined via reverse transcription-quantitative PCR. The CCD-18Co fibroblast cell line was used as reference to calculate fold change ($2^{-\Delta\Delta Cq}$). Data are presented as the mean \pm standard deviation. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ vs. 37°C. AAVR, adeno-associated virus receptor; HSPG, heparin sulfate proteoglycan.

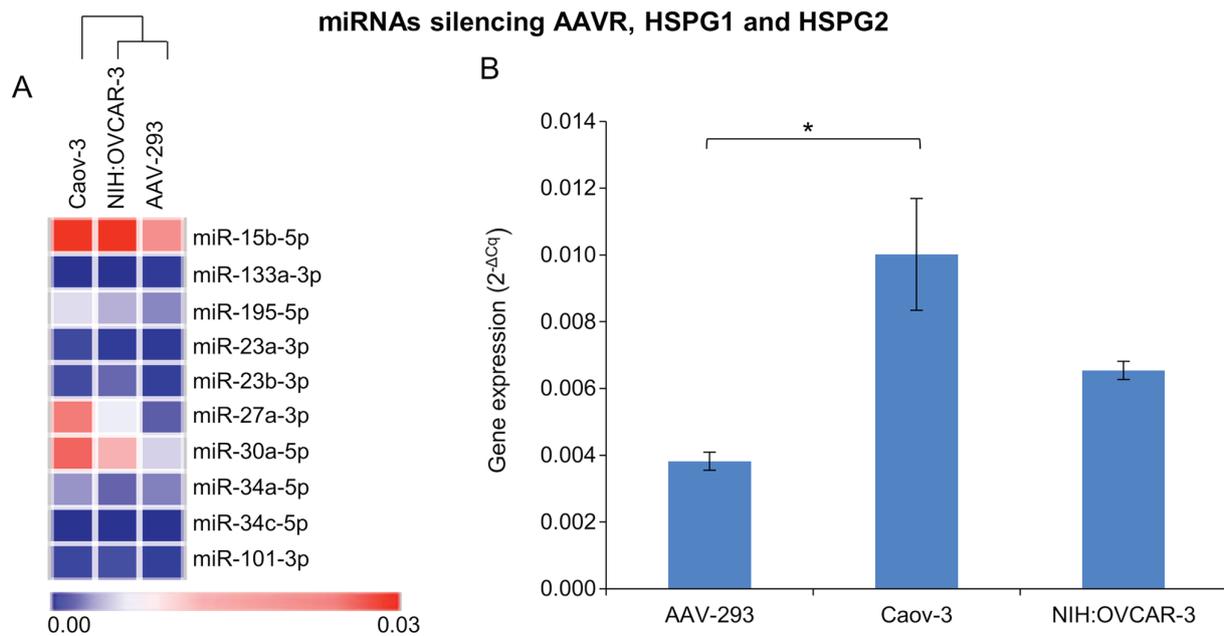


Figure 4. AAV-293, Caov-3 and NIH:OVCAR-3 cell miRNA analysis at normal temperatures. The selected miRNA responsible for silencing the cell transmission genes of rAAV (AAVR, HSPG1 and HSPG2) were assessed via reverse transcription-quantitative PCR. (A) Heat map represents the $2^{-\Delta Cq}$ data obtained from the studied miRNA. The distance clustering of the examined cells was elucidated. (B) Mean value of all analyzed miRNAs ($2^{-\Delta Cq}$) characteristic of AAV-293, Caov-3 and NIH:OVCAR-3 cell lines. Mean values were calculated to better visualize the miRNA expression differences between studied cell lines. Data are presented as the mean \pm standard deviation. * $P < 0.05$ as indicated. miRNA, microRNA; rAAV, recombinant adeno-associated viruses; AAVR, adeno-associated virus receptor; HSPG, heparin sulfate proteoglycan;

40 and 43°C. Temperature-dependent changes in HSP expression appear to be helpful to understand the rAAV transduction efficiency increase in cells exposed to elevated temperature.

Cell adhesion. To investigate the influence of rAAV transduction and hyperthermia on the biology of the studied cells, adhesion assays were performed. The cells were

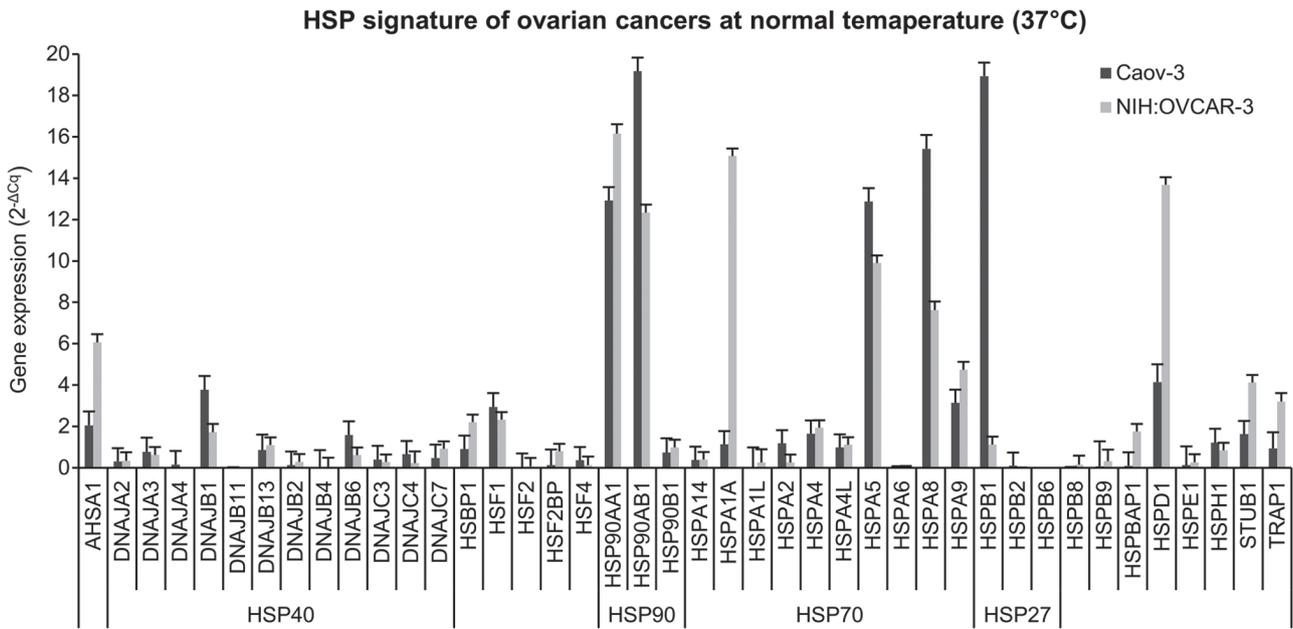


Figure 5. HSP expression profiles of ovarian cancer in Caov-3 and NIH:OVCAR-3 cell lines at 37°C. Relevant differences between constitutive expressions of HSP (2^{-ΔCq}) are presented. Data are presented as the mean ± SE. HSP, heat shock protein.

plated on dishes covered with laminin which is described as crucial protein for extracellular matrix maintenance. Generally, as shown in Fig. 7, the greater number of cells attached to the control plates (without laminin) was noted. The results demonstrated that hyperthermia induced the decrease of the number of adhered cells. In the case of AAV-293 line, in plate without extracellular matrix protein were small differences in the percentage of viable, adherent cells for each temperature. Whereas, for the ovarian cancer cell lines a decrease in attached cells exposed to hyperthermia was observed for both panels, with and without laminin. Additionally, the NIH:OVCAR-3 cell line showed the lowest adhesion potency after incubation at 43°C. The control AAV-293 cells were characterized by a greater ability to adhere compared to ovarian cancer cells. In all the tested cell lines no effect of rAAV/DJ transduction for adhesion was observed.

Invasion chamber assay. To determine how transduction and hyperthermia can change the invasiveness of tested cells, Matrigel chamber assays were performed. The invasive cells were counted, and crystal violet stained. Results presented on Fig. 8 showed that hyperthermia increased the invasiveness of all tested cells in both groups (transductants and non-transductants) in temperature-dependent manner. The highest cell numbers in the lower chambers were observed at 43°C. Moreover, the NIH:OVCAR-3 was indicated as the most invasive cell line. The non-cancerous AAV-293 line revealed the lowest number of cells which pass through the chamber membrane. Number of the cells migrated to the lower chamber through the Matrigel, collected after 24 and 48 h were similar, which may indicate significant changes in cellular physiology that occurred after incubation at hyperthermia. The rAAV/DJ transduction did not affect the invasive potential of the examined cells.

Discussion

As we read in one of the last issues of the prestigious journal *Science* (42), gene therapy, thanks to translational research, is strengthening its position in clinical trials. The authors of the review, leaders in the field of gene therapy, conclude that clinical trials, which started in the 1990 with varying degrees of success, enabled that gene therapy to now play a significant role in medicine thanks to advanced basic and translational research. The authors of the *Science* article emphasize the main, extremely crucial solutions in the field of DNA vectorology, which form the basis for the development of gene therapy. Moreover, researchers suggest further advances in gene therapy will be determined by three approaches. These are strategies based on rAAV vectorology, lenivirus vectors and gene editing technologies (42). In each of these strategies, intensive research is conducted to increase the efficiency of transduction and *in vivo* selectivity, and to reduce side effects. Research aimed at optimizing the dose of used viral vectors is important. Having the tools to reduce the doses of the vectors used, while maintaining high efficiency of gene transfer, will lead to increase in the biosafety of viral vectorology and consequently facilitate the introduction of viral vectors to patients. The use of physicochemical solutions in gene transfer methods is gaining attention. One of the approaches is the use of physical factors, such as temperature, pH, light, sounds for precise activation/deactivation of gene expression and to improve the efficiency of transduction (19).

Recently observed interest in using temperature for gene therapy purposes is partly due to the development of innovative, oncological patient treatment methods based on temperature modulation. The hyperthermia described in the literature assumes local or comprehensive (whole body) heating of tissues to produce a therapeutic effect. Hyperthermia is primarily considered a therapeutic strategy

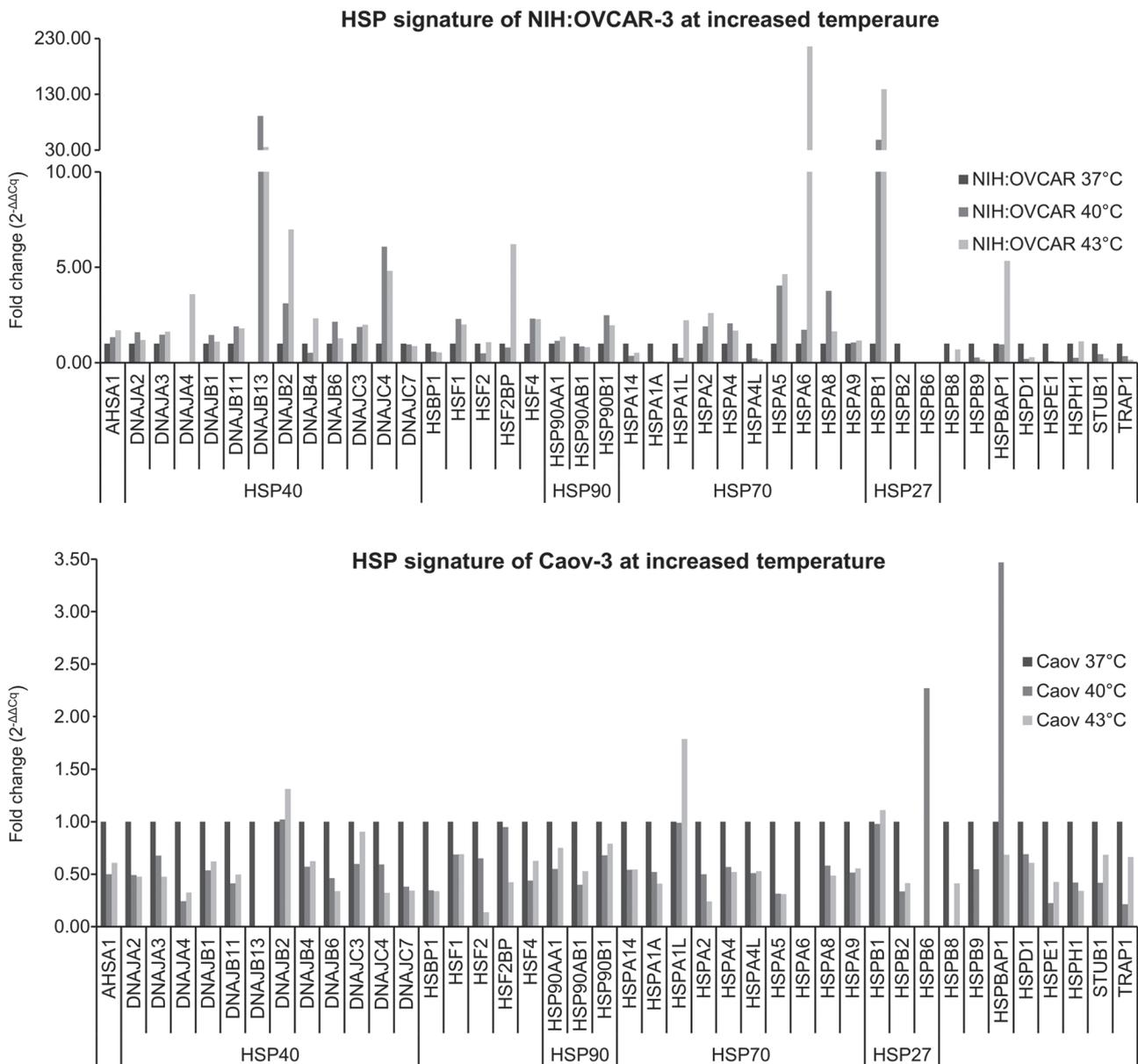


Figure 6. Temperature-associated HSP expression profiles of ovarian cancer cell lines (Caov-3 and NIH:OVCAR-3). Cells were exposed to hyperthermic conditions (40 and 43°C). Under heat shock conditions, differences were revealed between the sensitivity of ovarian cancer cells to increased temperature. Expression values ($2^{-\Delta\Delta Cq}$) indicate the specific HSP signature for ovarian cancers of different origin. Samples at 37°C were not exposed to hyperthermic conditions and were used as reference to calculate fold change ($2^{-\Delta\Delta Cq}$). HSP, heat shock protein.

complementing or supporting classical protocols of oncological treatment (21). In addition to the direct cytotoxic effect on cancer cells, the influence of hyperthermia to vascular permeability and drug penetration is highlighted (19,21). In the field of oncological hyperthermia, an interesting solution is Hyperthermic Intraperitoneal Chemotherapy-HIPEC. The importance of HIPEC in the treatment of intraperitoneal dissemination of tumors originating from the gastrointestinal tract and the female genital organs, including ovarian cancer, is emphasized (22,43,44). Individual selected cytostatics (e.g., mitomycin C, doxorubicin, cisplatin) administered to oncological patients by perfusion under elevated temperature (hyperthermia conditions; e.g., 0.5-1.5 h, 41-43°C) (43) may significantly increase the patients' lifetime (22,44). The use of hyperthermia in clinics may be associated with some

complications. Generally, healthy tissues are not destroyed during hyperthermia. The differences into tissue characteristics implicate to the changes between heat distribution in patient's body. This may result in burns, discomfort or pain. Complications associated with the method of achieving high temperature of tissue, in the case of perfusion techniques may appear tissue swelling, blood clots, bleeding, and other damage in the perfusion area, however, most of these side effects are temporary. Whole body hyperthermia may cause more serious side effects, such as cardiological and vascular disorders, but these are rare events. However, in comparison with the results achieved by the combination of chemotherapy and hyperthermia (significantly increase the patients' lifetime), it is worth using this method of treatment in oncology (22,43-46). Increasing the temperature is usually accompanied by a change

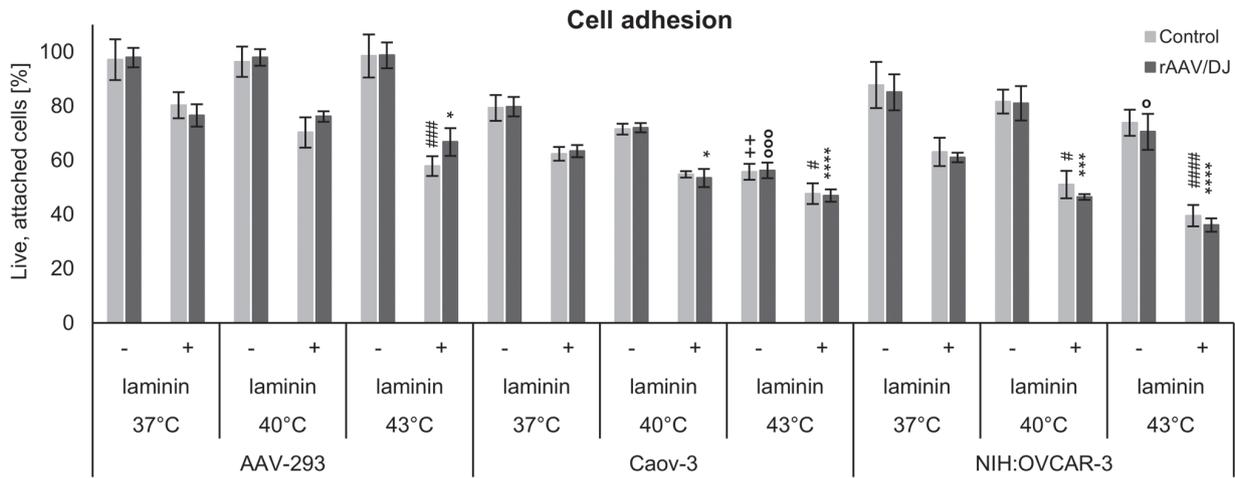


Figure 7. Adhesion of ovarian cancer cell lines (Caov-3 and NIH:OVCAR-3) and AAV-293 under hyperthermic conditions. Plates were coated with laminin. Statistically significant differences were observed between cells transduced at 37°C and hyperthermia conditions and similarly between control cells incubated at 37°C and cells exposed to higher temperatures. Data are presented as the mean ± standard deviation. °P<0.05 vs. 37°C rAAV/DJ NIH:OVCAR-3; °°P<0.001 vs. 37°C rAAV/DJ Caov-3; *P<0.05 vs. 37°C rAAV/DJ AAV-293 or Caov-3; **P<0.001 vs. 37°C rAAV/DJ NIH:OVCAR-3; ***P<0.0001 vs. 37°C rAAV/DJ Caov-3 or NIH:OVCAR-3; **P<0.01 vs. 37°C control Caov-3; *P<0.05 vs. 37°C control Caov-3 or NIH:OVCAR-3; ###P<0.001 vs. 37°C control AAV-293; ####P<0.0001 vs. 37°C control NIH:OVCAR-3.

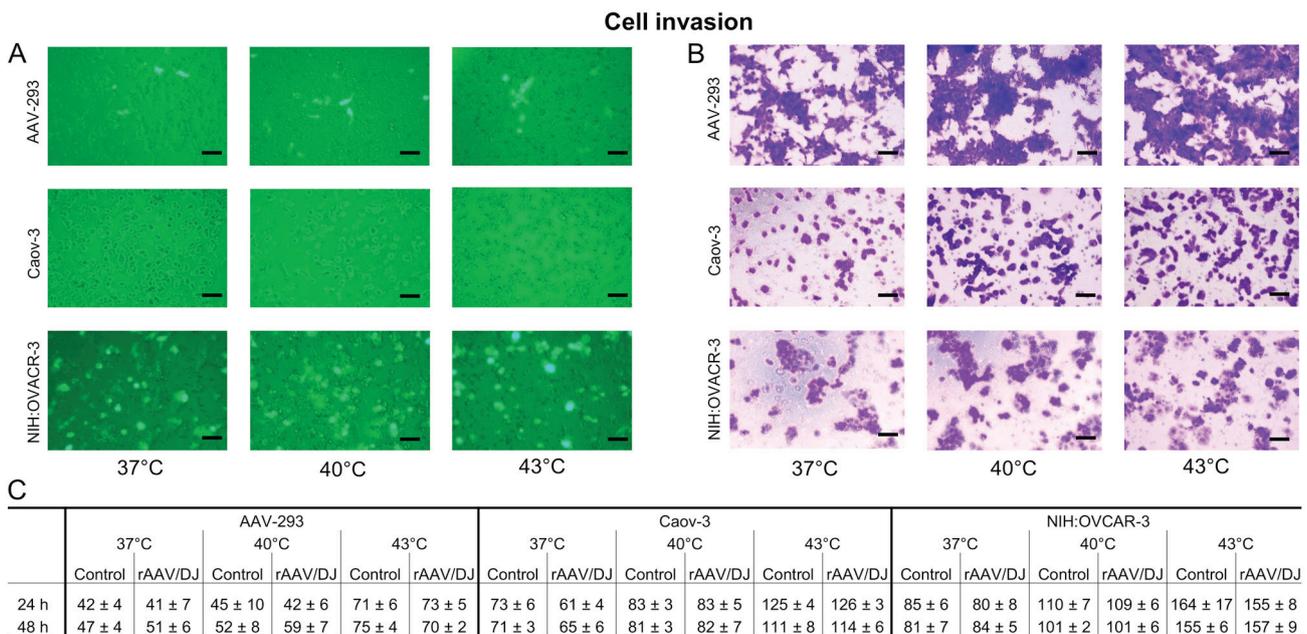


Figure 8. Caov-3, NIH:OVCAR-3 and AAV-293 cell invasion assays under hyperthermic conditions. (A) transduced green fluorescent protein + cells observed on the lower Matrigel chamber after 24 h of incubation (FITC). (B) Fixed and stained (0.5% crystal violet) cells on the lower Matrigel chamber after 48 h of incubation (magnification, x10; scale bars, 100 μm). (C) Number of migrating cells. Data are presented as the mean ± standard deviation. rAAV/DJ, recombinant adeno-associated viral hybrid serotype DJ.

in the pattern of HSP gene expression. Grimmig *et al* (26) emphasize the functional meaning of the increase in HSP expression in the course of chemotherapy in hyperthermia. Modern treatment of ovarian cancer is also based on the participation of gene therapy (31).

The study attempts to determine the effect of temperature on the transduction efficiency the ovarian cancer cells of various clinical origin by substantial gene therapy vectors-rAAV. Research done in the present study is a continuation of the work carried out by our team aimed at introducing genes into cancer cells (47,48). Some researchers

are practicing physicians and are directly involved in treating patients through hyperthermia. As shown in Fig. 2A, we reveal that increasing temperature from 37 to 40°C or even to 43°C leads to the increase of the ovarian cancer cell transduction efficiency by the rAAV/DJ vectors. In order to check the transcriptional activity of promoters, the rAAV/DJ mosaic vector with the CAG hybrid promoter for AAV-293 cells was used in the work, while the conventional cmv promoter was used for ovarian cancer lines. In the case of AAV-293 (Fig. 2), 11% GFP+ cells correspond to approx. 123000 gc, while extrapolating the transduction results for the Caov-3, 11% of

GFP+ cells would correspond to as much as 600000 gc. The obtained results indicate higher transcriptional activity of CAG promoters, compared to cmv, which is consistent with results in other studies (49,50). Although the used rAAV mosaic vector is considered to have a wide range of tropism (51), in the present study, as shown in Fig. 2A, substantial differences in the transduction of tested cell lines by rAAV/DJ are evident. For ascites-derived ovarian cells, the transduction efficiency in 37°C was at 23%, and for solid-derived cancer cells-Caov-3 at only 2%. In the context of gene therapy reasons, the differences between cell transduction efficiency may have clinical importance and define the necessary selectivity of drug delivery due to development of the personalized treatment. In fact, the transduction differs depending on the cell types. In the present study, an universal vector (rAAV/DJ) was used, which is generally characterized by good transduction efficiency, regardless of the cells' origin. It is worth noting that in the gene therapy protocols the different vectors are used. The examples of efficient vectors for cells poorly transduced by rAAV are known (e.g., lentiviral vectors, adenoviral vectors). Our research is innovative, and we decided to choose one vector, which will transduce different cell lines. Differences between rAAV/DJ transduction efficiency are not obstacle for the develop further gene therapy and hyperthermia-based treatment, because gene therapy engineering propose various types of effective vectors.

The use of elevated temperatures significantly increased the efficiency of rAAV transduction in both lines. For NIH:OVCAR-3, a 65% increase in transduction was observed at 40°C and 70% at 43°C, while for Caov-3 cells, a 50 and a 200% increase, respectively. The number of copies of the vector in the transductants corresponded to the number of GFP+ cells, but only at the temperatures of 37 and 40°C. Unexpectedly, the Caov-3 cells exposed to 43°C were characterized with high number of GFP+ cells and lower level of rAAV genome copies. This may suggest that hyperthermia (at higher temperatures, like 43°C) activates the intracellular mechanism of vector degradation in Caov-3. On the other hand, the other lines appear to be more resistant to potential temperature-stimulated degradation.

The stimulatory effect of the elevated temperature on the performance of rAAV transduction was also demonstrated in the work of other authors. In the publication of Zhong *et al* (12) showed that 4 h exposure of Hela cells to 42,5°C leads to about 6x increase in rAAV transduction efficiency. In this study, 3 h stimulation was used at 40 and 43°C. The viability cells in response to heat shock was determined at 95-98% (results not shown in the paper). The works of Zhong *et al* (12) and Zhao *et al* (52) it was revealed that the increase in rAAV transduction efficiency at a higher temperature is associated with the expression of selected HSP genes and the existence of functional connections between HSP and FKBP52 protein. The authors of the works showed that phosphorylated FKBP52 interacts with D-sequences in inverted terminal repeats of adeno-associated virus 2 genome, which in turn leads to inhibition of second strand synthesis of AAV DNA and further to inefficient expression of reporter transgenes, i.e. a decrease in transduction efficiency. In this study, attention was paid to the expression of a comprehensive panel of HSP (HSP expression plate) genes including 42 genes predominantly from the HSP40,

HSP60, and HSP90 families. The constitutive HSP levels in cells cultured under standard conditions (37°C) as well as in cells exposed to 40 and 43°C for 3 h were examined. The study allowed us to define HSP expression signature cells transduced with different levels of efficiency. Differences in constitutive and temperature-stimulated HSP expression between the cell lines were demonstrated in Figs. 5 and 6. It can be postulated that HSP specific signatures may promote rAAV transduction. Efficiently transducing NIH:OVCAR-3 cells are characterized by highly expressed HSP40 (DNAJA2, DNAJB13, DNAJB2, DNAJB4, DNAJC7), HSP90 (HSP90AA1, HSP90B1), and HSP70 (HSPA14, HSPA1A, HSPA1L, HSPA4, HSPA4L, HSPA6). Lower levels of HSP expression, mainly DNAJB1+, HSP90AB1+, HSPA5+, HSPA8+, and HSPB1+, were observed in less-effectively transduced Caov-3 cells.

In 2016, a study published in *Nature* was the key to determine the concept of the cellular transmission of rAAV (6). Pillay *et al* (6) pointed to the significant role of AAV transmembrane protein (KIAA0319L), defined as AAVR, in the transmission of rAAV. Subsequent team work expanded the knowledge of AAV transmission with the participation of AAVR (7,8). This study also analyzed the expression of crucial genes of the rAAV transmission to cells. The AAVR, HSPG1 and HSPG2 genes were selected for evaluation. Differences were found in the expression pattern between the cell lines and the effect of elevated temperature on the level of expression (Fig. 3). The obtained results correspond with the literature data indicating that there are specific patterns of rAAV transmission for various cells, which are sometimes even, as recently reported by Dudek *et al* (7), which are independent of the expression of AAVR. The expression of the AAV transmission protein genes was also associated with the miRNA pattern in transduced cells. Based on the information available in miRNA databases and using the proposed algorithms, functional links were found between the designated miRNAs and the AAVR, HSPG1 and HSPG2 genes (40,41). Based on preliminary studies, it was established that a high rAAV transduction efficiency and high levels of AAVR, HSPG1 and HSPG2 expression (Fig. 3), occur together with a lower level of miRNAs (Fig. 4) that silence the expression of AAV receptors and genes. On the other hand, in the cells with a high level of miRNA silencing (Caov-3 cells, Fig. 4), lower transduction efficiency and a lower 3-5x expression level of the tested receptors was observed. Moreover, considering our other studies on miRNA profiling in tumors, it seems that the miRNA signatures can be a valuable complement to the information panel, which should be analyzed before attempting gene therapy. In studies that are the subject of another work (based on TLDA cards that allow the evaluation of the expression of more than 700 miRNA) we have demonstrated that, for example, in breast cancer cells there is a specific miRNA signature that is helpful in choosing a vector for gene therapy trials. The clinical benefits of miRNA profiling can also be found in the work of other authors (53,54).

To better understand the changes occurring in the studied cells transduced at hyperthermia, experiments for adhesion and invasion were performed. As shown in the Fig. 7, laminin reduces cell adhesion. The results demonstrated that hyperthermia induced the decrease of the number of adhered

cells. Luchetti *et al* (55) showed that in cells of neuroblastoma exposed to hyperthermia, a floating population of cells increased, which had down-regulation expression of CD11a integrin molecule. Perhaps the analogous mechanism occurred for ovarian cancer and AAV-293 cells. Probably, the incubation at the increased temperature can cause change of integrins expression in cells, which in turn promoted their detachment. Furthermore, hyperthermia increased the invasiveness of all tested cells (Fig. 8), which may suggest the relation to HSPs essential for cell adhesion and invasion. For example, HSP27 is involved in the regulation of the cytoskeleton, which may affect the migration of tumor cells (56). Voll *et al* (57) showed that HSP27 increased in prostate cancer cell motility and invasion. Another instance can be over-expression of HSP70 enhances tumor growth, cancer cell migration and metastasis. It is postulated that the expression of HSP correlates with poor prognosis of treatment (58). In our study the expression of HSP27 in cells exposed to hyperthermia was increased in NIH:OVCAR-3, interestingly this line after incubation at 43°C was also characterized by the smallest percentage of cells adhered to laminin. Furthermore, NIH:OVCAR-3 showed the greatest invasion potency and had increased expression of HSP70, both constitutively and after exposed to hyperthermia. On the other hand, in study Xie *et al* (59) and Jin *et al* (60) indicated that hyperthermia could inhibit cancer cells invasion *in vitro*. However, the methodology details in the mentioned publications were different. It is worth underlining that the time of measurement can be an important factor, it seems that the ability to invasion/migration of cells measured directly after the exposure of cells to the high temperatures is decreased. But after a longer interval of time from hyperthermia (as documented in our study) cell migration and invasion may achieve higher results than these from original state. The time required to expression of HSP seems to be a crucial factor in cancer invasiveness.

The study showed that at elevated temperature ovarian cancer cells were more efficiently transduced with rAAV. It was also indicated that temperature-dependent transduction is in relation to the expression of the rAAV receptor genes and HSP genes. Moreover, hyperthermia modified the invasiveness of the studied cells. The obtained information can be helpful in the design and implementation of effective protocols for ovarian cancer gene therapy. Ovarian cancer is currently the primary cause of death among women suffering from gynecological cancer. The classic treatment protocols are not very effective. Among patients, there is a low 5-year survival rate and a high percentage of chemo-resistant cancer recurrence (31,61). The need to develop new therapeutic solutions is extremely pressing. Pre-clinical studies and weak effects of clinical trials of ovarian cancer force the search for new solutions in the field of gene transfer methods (31). In the context of the results obtained in this study, it seems that the hyperthermia strategy present in oncology clinics, as a method supporting oncological treatment, may also be helpful in increasing the effectiveness of gene therapy of ovarian cancer based on rAAV vectors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AB and MM conceived and designed the experiments of the present study. AB, MD, MO, ŽS and OC performed the experiments. AB, JJ and MM analyzed the data. AB and MM wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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