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The full recovery of mice (Mus Musculus C57BL/6 strain) from virus–induced sarcoma after treatment with a complex of DDMC delivery system and sncRNAs

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HIGHLIGHTS

- Complex of a-miR-155, piR-30074, and miR-125b with DDMC vector induced full recovery from virus-induced sarcoma in mice.
- Complex of DDMC/a-miR-155, piR-30074, and miR-125b transformed sarcoma cells into other types non-cancerous cells.
- Tumor regression was connected with transformation of cancer cells.
- Complex of the DDMC vector with a-miR-155, piR-30074, and miR-125b induced apoptosis in transformed cells.

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ABSTRACT

Background: Virus-induced cellular genetic modifications result in the development of many human cancers.
Methods: In our experiments, we used the RVP3 cell line, which produce primary mouse virus-induced sarcoma in 100% of cases. Inbreed 4-week-old female C57BL/6 mice were injected subcutaneously in the interscapular region with RVP3 cells. Three groups of mice were used. For treatment, one and/or two intravenous injections of a complex of small non-coding RNAs (sncRNAs) a-miR-155, piR-30074, and miR-125b with a 2-diethylaminoethyl-dextran methyl methacrylate copolymer (DDMC) delivery system were used. The first group consisted of untreated animals (control). The second group was treated with one injection of complex DDMC/sncRNAs (1st group). The third group was treated with two injections of complex DDMC/sncRNAs (2nd group). The tumors were removed aseptically, freed of necrotic material, and used with spleen and lungs for subsequent RT-PCR and immunofluorescence experiments, or stained with Leishman-Romanowski dye.
Results: As a result, the mice fully recovered from virus-induced sarcoma after two treatments with a complex including the DDMC vector and a-miR-155, piR-30074, and miR-125b. In vitro studies showed genetic and morphological transformations of murine cancer cells after the injections.
Conclusions: Treatment of virus-induced sarcoma of mice with a-miR-155, piR-30074, and miR-125b as active component of anti-cancer complex and DDMC vector as delivery system due to epigenetic-regulated transformation of cancer cells into cells with non-cancerous physiology and morphology and full recovery of disease.

1. Introduction

More than one hundred years ago, the viral origin of cancer was proposed by Francis Peyton Rous and lately finally proven by other researchers [1–4]. In 1966 year, Rous received the Nobel Prize in Physiology or Medicine for this finding. He demonstrated the experimental transmission of sarcomas using cell-free filtrates of tumor extracts in healthy chickens and investigated processes of tumor formation [5]. Lately, this retrovirus (Rous sarcoma virus – RSV) was isolated and were obtained RSV-induced sarcomas in ducks, hamsters and mice [6–9]. The tumor-inducing gene of RSV and RSV-induced tumors is the viral src oncogene (v-src) [10]. The v-src oncogene is a truncated and active form of the wild-type proto-oncogene c-src. The src gene codes a non-receptor protein tyrosine kinase that is a member of the Src family kinases (SFKs). SFKs are involved in processes of tumor progression, invasion, and metastasis. Src kinase activity and protein levels are elevated in several human cancers, including those of the colorectal, prostate, lung, breast and brain [11–15]. In this study, we investigated

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the action of small non-coding RNAs a-miR-155, piR-30074, and miR-125b in a murine model of RSV-induced cancer.

SncRNAs are short oligonucleotides with lengths shorter than 200 bps. SncRNAs are Major classes of sncRNAs are microRNAs, Piwi-interacting (piRNAs), small nuclear, nucleolar, cytoplasmic (sn-, sno-, scRNAs, respectively), transfer (tRNAs), and ribosomal RNAs (rRNAs) [16]. Recently, was proven role of sncRNAs in regulation of cellular metabolism, breath, life cycle and death. SncRNAs can promote processes tumorigenic transformation, metastatic activity, inhibition of apoptosis, and stimulation of proliferation [17–25]. SncRNAs may regulate expression of src gene in cells and promote tumorigenic effect in cells [26–32]. In previous in vitro experiments, treatment with different sncRNAs, combinations of sncRNAs, and combinations of sncRNAs with different regulatory factors (interleukins (ILs), colony-stimulating factors (CSFs), etc.) in complex with a polymer delivery system induced full recovery in different human and murine cancer cell lines, and/or induced the transformation of cancer cells in different types of normal cells. The most effective in our experiments was using of the complex of DDMC delivery system with a-miR-155, piR-30074, and miR-125b for treatment of colorectal, lung, cervix, and glioblastoma cancer cell lines [33–36]. We performed a series of in vivo experiments in a very rare mouse model of virus-induced human cancer with the highest transformation efficiency in mouse cells. [9,37]. We used the most effective in preliminary in vitro experiments sncRNAs a-miR-155, piR-30074, and miR-125b in complex with DDMC vector.

2. Methods

2.1. Cells

In our experiments, we used the RSV (Prague C strain)-induced (NCBI_TaxID: 11888) RVP3 cell line (RRID: CVCL_L978) to produce primary sarcoma in mice (NCIt: C21603). Schwartz et al., 1983 characterized the nucleotide sequence of RSV (Prague C strain) [38]. Cells were maintained before injection into mice in accordance with protocols in previous studies [39]. RVP3 epithelioid cells are grown as monolayer adhesive culture in DMEM with 10% fetal calf serum, penicillin-streptomycin solution (100 μg/ml) and 32 μg/ml gentamicin in 37 °C. Split ratio was 1:3–1:6. After 5–7 days of cultivation, growing cells were harvested for inoculation in mice [40]. These cells have the possibility to produce primary mouse sarcoma in 100% of cases, and the number of transformation-defective cells is minimal compared with that of other RSV cell line strains [41–44]. The Prague strain of RSV is competent for viral replication and transforms mammalian cells but does not produce virus [40]. The cell line was purchased from the Russian Academy of Sciences (RAS) (MWIIW, Moscow) by SID ALEX GROUP, Ltd. (Prague, Czech Republic).

2.2. Animals and passage of tumors

2.2.1. Preliminary experiments

Inbreed 4-week-old female C57BL/6 (n = 15) mice were used for RVP3 cell inoculation to investigate the tumor-inducing activity of the cells, select the optimal concentration of cells for inoculation and the cell concentration/tumor growth rate for further therapy, determine the lifespan of the mice after tumor inoculation, and measure the changes in tumor size. Animals were purchased from the Research Centre of Biomedical Technology of the State BioMedical Agency (RAS, Russia). Animals were injected subcutaneously in the interscapular region with an optimal concentration of 5×10^4 of cells in 1 ml of culture medium (DMEM, 10% FCS, and an antibiotic mixture of 100 μg/ml penicillin-streptomycin and 32 μg/ml gentamicin). The tumors were removed aseptically, freed of necrotic material, minced with scissors and frozen in culture medium with 20% DMSO [40]. Spleen, lungs, kidneys, heart, blood, liver, and enlarged lymph nodes were washed with sterile PBS, cut into small pieces and frozen in culture medium with 20% DMSO for subsequent RT-PCR and immunofluorescence experiments. Some samples were very finely minced in PBS for the staining of obtained cells with Leishman-Romanowski dye [45]. All mice were kept in a temperature-controlled environment with a 12-h light/dark cycle and free access to food and water. Animal care and experimental procedures were conducted in compliance with the World Medical Association statement on animal use in biomedical research (adopted by the 41st World Medical Assembly, Hong Kong, September 1989 and reaffirmed by the 203rd WMA Council Session, Buenos Aires, Argentina, April 2016) and were approved by an institutional animal care committee [https://www.wma.net/policies-post/wma-statement-on-animal-use-in-biomedical-research].

2.2.2. Design of in vivo experiments

For the main experiments, 3 groups of mice (n = 10 in each group) were used. All three groups were inoculated with RVP3 cells. The first group consisted of untreated animals (control). The second group was treated with one injection of complex 24 h after tumor inoculation (1st group). The third group was treated with one injection of complex 24 h after tumor inoculation and with the second injection one week after the first injection (2nd group). The complexes of sncRNAs with the DDMC polymer were injected intravenously in the tail vein of the mice.

2.2.3. SncRNAs

In the preliminary in vitro studies, bioinformatics tools were used to choose the most reliable candidates from sncRNAs families for the treatment of human colorectal cancer and RVP3 mouse cell lines. SncRNA targets are predicted mainly by three computational algorithms: 1) Lewis et al., 2003; 2) Krek et al., 2005; 3) Griffiths-Jones et al., 2006 [46–48]. In these studies, three sncRNAs were used, and the oligonucleotide sequences were as follows: for a-miR-155 (antisense for miR-155 (MIMAT0004658)): 5′- GAG GAU GUA UAA UCG UAA UUG U-3′, and for piR-30074 (DQ569962.1): 5’ – AAAGCTTTAAGTGTGTT GGCGTGCTTC – 3′ and for miR-125b (MIMAT0004592): 5’ – ACGGG UUAGGCUCUUGGGAGCU – 3’.

2.2.4. DDMC vector

The cationic graft-copolymer (DDMC Vector® (2-diethylaminoethyl-dextran methyl methacrylate copolymer, nonviral transfection reagent from Ryujyu Science Co., Aichi, Japan was purchased by SID ALEX GROUP, Ltd. [49].

Transfection solutions were prepared as follows. In a sterile tube, 40 μg of each sncRNA and 6 μl of pmKate2-N vector were diluted in 155 μl water for injection, and 15 μl of the cationic graft-copolymer was added. The solution of the DDMC vector in water was preliminarily sterilized at 120 °C for 15 min, after which oligonucleotides were added. The mixtures of sncRNAs and the DDMC vector were warmed to 37 °C before the treatment of mice [49].

2.3. Treatment of tumor cells from non-surviving animals from the 1st group and the control group

Transfection solutions for tumor cells were prepared per 1 mL of culture medium: 10 μg of each sncRNA, and 4 μl of the DDMC vector were mixed in 60 μl water for injection. The solution of the DDMC vector in water was preliminarily boiled at 120 °C for 15 min.

2.4. Immunofluorescence labeling of tumor cells from the control group and 1st group with pmKate2-Annexin4A

A portion of the tumor cells from the animals in the control group and the 1st group was washed with PBS 3 times and put in culture medium. Twenty-four hours later, 10 μl/ml of pmKate2-Annexin vector (mouse) and 3 μl/ml DDMC vector were added to the culture medium. Fluorescence was visualized every 72 h, and images were acquired using fluorescence microscopy for the quantification of labeled cells

(AxioVertA1, Zeiss, Germany). The percentage of cells expressing pmKate2-Annexin deep-red fluorescent protein was calculated as previously described (magnification 40× and 60×) [50]. The pmKate2-Annexin vector (mouse) was purchased from Evrogen (Russia) [51–53].

2.5. Immunofluorescence labeling of tumor cells from animals in the 1st group with CD4+, CD117 + and Oct4+

One portion of the tumor cells from the animals in the control group and the 1st group was washed with PBS 3 times and air-dried in the air. Then, immunofluorescence labeling was performed. Separated cells were stained with a CD4+/FITC staining reagent (R&D, USA), CD117 purified mouse anti-human antibodies (Caltag Laboratories by Invitrogen, USA), and Oct4+ (Santa Cruz Biotechnology, Inc., USA), and were observed and imaged with fluorescence microscopy (AxioVertA1, Zeiss, Germany).

2.6. Another portion of cells was stained using the Leishman-Romanowsky method

2.6.1. RT-PCR

One portion of the cells obtained from different organs after mouse dissections was treated with lysis buffer from the RNeasy mini kit (Qiagen, USA) for the further isolation of total RNA, reverse transcription reaction and specific cDNA product amplification. Tumor samples obtained from the animals in the control group and the 1st group, as well as spleens and lungs from all mice, were used for the RT-PCR analyses.

Gene expression analysis: In these series of experiments, a standard two-step reverse transcription-PCR standard procedure was used. All products were obtained from Invitrogen Co. The amplification of casp8, casp3, piwil1, v-src, oct4, c-myc, tgfbr1, lin28a, sca-1, dicer, cdh2, nanog, c-src, and actb cDNA (as an internal control) was performed with an automatic thermocycler (TProfessional, Biometra, Germany). The primer sequences from 5′-3'were as follows: 1) casp8 (ENSMUST00000027189.14) 5′- TCTGGAGACAGAGGCAGGA -3′, 5′-TTCACCCCATTCTGCTGAC -3'; 2) casp3 (ENSMUST00000211115.1) 5′-TTCCCAGAGGGCTGAGAGT -3′, 5′- TGACTCAGCACCCCCATAA -3'; 3) piwil1 (ENSMUSE00000536439) 5′- GCATCCACAGAGAGCCAAA-3′, 5′-GCCGCTACACACCAACAAC -3'; 4) v-src (ENSMUST00000029175.13) 5′- ATGGCCACAGGCTCTCTCT-3′, 5′- AAGCCTCCAACAAGGATGG -3'; 5) oct4 (ENSMUST00000025271.16) 5′- TCTGTTCCCGTCACTGCTC -3′, 5′- TGAGCTTCTTTCCCCATCC -3'; 6) c-myc (ENSMUST00000022971.7) 5′- GAGGGCCAAGTTGGACAGT-3′, 5′-GCTCGTCTGCTTGAATGGA -3'; 7) tgfbr1 (ENSMUST00000007757.14) 5′- GTGGTTGAGCCTGGGTTTT-3′, 5′- CCCAGCTGCTTCAGATCAA -3'; 8) lin28a (ENSMUST00000051674.2) 5′- TGGAGGGAAGGAAAGG ATG-3′, 5′- GAGTCCCTTCCCCAGAACA -3'; 9) sca-1 (ENSMUST00000187994.6) 5′- ATGACCTCCACCCTTGTCC -3'; 5′-TGA GCAGCAATCCACAACA -3'; 10) dicer (ENSMUSE00000395197) 5′-AAGGATCACGTGCTGTTGC-3′, 5′- ACAATCCCACCCCACCTTA -3'; 11) cdh-2 (ENSMUST00000025166.13) 5′- AGCTCCCTCAACTCCTCCA-3′, 5′- CAAACTGGTTCGCAGCCTA -3'; 12) nanog (ENSMUST00000012540.4) 5′- GAAAGCCATGCGCATTTTA-3′, 5′-GGTGGCTCACAACCATACG -3'; and 13) c-src (ENSMUST00000034863.7) 5′- TGGTAGGCCTCTTGCCTCT-3′, 5′- GCC CATTTGCTGGGTACTT -3'.

PCR products were loaded on a 2% agarose gel, electrophoresed, stained with ethidium bromide, exposed in a gel doc system (Syngene, India) and quantified using Quantity One software (Bio-Rad, Germany). For expression comparison, the beta-actin gene was used as the internal control. The primer sequences for the internal control beta-actin gene (ENSMUST00000100497.10) were: 5′- CTGTCCACCTTCCAGCAGA-3′, 5′- GCCTTCACCGTTCCAGTTT -3'.

2.6.2. Statistics

Kaplan-Meier plots were generated using InVivoStat software (ver. 3.7.0.0), a freely available statistical analysis system for the analysis of in vivo data (developed by Bate S. and Clark R.). The lifespan of the controls was compared with the lifespan of the mice from the 1st and the 2nd groups ($p < .05$).

The differences in the treatments performed in the 1st and 2nd groups and in tumor progression compared to the treatments and tumor progression in the control group were calculated as the means ± SEMs ($p < .05$).

The transfection efficiency data were analyzed using two-way analysis of variance (ANOVA) based on the cell means and dynamics of transfection of the DDMC vector ($p < .05$). For the control, untreated control group mice at the same time point as the experimental groups were used. In all groups, 10 animals were used.

All gene expression data were normalized to the expression of the beta-actin internal control. Cells from the control group were used as an external control. All samples were prepared in triplicate. Quantification of the gene expression intensity was performed using Quantity One software (Bio-Rad, Germany). AltAnalyze software, used for gene expression analysis (ver. 2.1.0), is an open-source, freely available application covered under the Apache open-source license (developed by the research group of Dr. N. Salomonis, Cincinnati Children Hospital Medical Center). The data are presented as the means ± SEMs (the observed differences between the study control and experimental animals were considered statistically significant for $P = .05$).

3. Results

3.1. Influence of the DDMC/a-miR-155, piR-30074, and miR-125b complex on the lifespan of mice with virus-induced sarcoma

In the series of experiments, we observed a two-fold increase in the lifespan of mice with Rous sarcoma after one injection of the complex of the polymer delivery system with a-miR-155, piR-30074, and miR-125b (the 1st group). After two intravenous injections of the DDMC vector/sncRNAs complex, the full recovery of mice inoculated with RVP3 virus-induced sarcoma cells was attained (the 2nd group). Animals from the 2nd group were killed with the decapitation [54]. The Kaplan-Meier plots indicated a significant difference between the lifespan of the controls and the mice from the 1st and 2nd groups. (Fig. 1).

A significant difference was found in the tumor growth in the animals from the 1st group compared with that in the control animals (Fig. 2). Tumor growth in animals from the 1st group was significantly decreased compared with that in animals from the control group (Figs. 2 and 3). We did not find visual or palpable signs of tumors in animals from the 2nd group. The animals looked physically healthy until they were euthanized.

Using the complex of DDMC with a-miR-155, piR-30074, and miR-125b for two intravenous treatments of mice with sarcoma due to full recovery of mice. One injection of complex resulted in significant increase of lifespan of mice with sarcoma.

3.2. In vitro experiments with cells from tumors of dead mice with sarcoma, treated with one injection of DDMC/a-miR-155, piR-30074, and miR-125b

It was interesting to observe the changes in the tumor cells from the mice with one injection of the DDMC/sncRNA complex. These cells were labeled with Annexin A4 for the detection of changes in the tumor cells.

In a series of studies, a portion of the tumor cells from non-surviving animals in the 1st group and the control group were labeled with pmKate2-Annexin A4. In tumor cells from animals in the 1st group, significant nuclear translocation of Annexin A4 was detected on the 24th day after the start of the in vitro experiments (Fig. 4). In the control group, Annexin A4 was diffusely distributed in the cells.

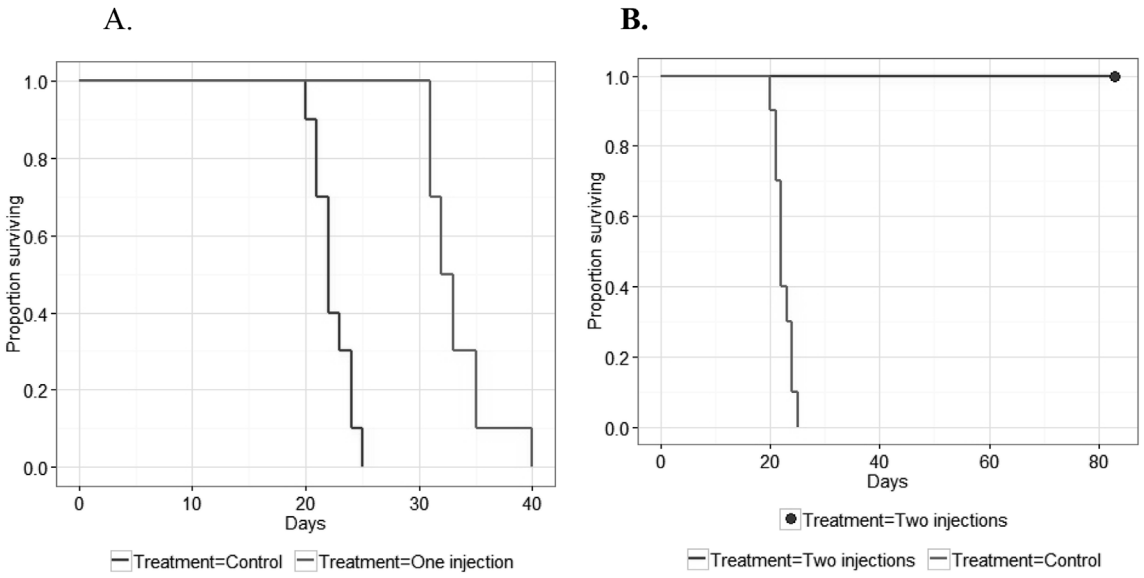

Fig. 1. Kaplan-Meier plots of the lifespan of animals from the 1st (n = 10) (A.) and 2nd (n = 10) (B.) groups compared to that of the controls (n = 10) ($p < .05$).

To investigate the morphologic and phenotypic changes in the tumor cells from non-surviving animals from the 1st group, the cells were stained with the Leishman-Romanowsky method. These cells were labeled with antibodies against $CD4^+$, CD117+, and Oct4. Cells with morphologies corresponding to tumor cells, tumor stem cells, and apoptotic tumor cells were obtained (Fig. 5). Immunofluorescence showed the expression of Oct4 and CD117+; however, $CD4^+$ was not expressed in tumor cells from animals in the 1st group (Fig. 5).

Next, we used scissors to mince tumors from the 1st group and put the tumor cells in culture medium. Then, we added the complex of a-miR-155, piR-30074 and miR-125b with the DDMC delivery system at the same concentration per milliliter of culture medium to the tumor cells. On the 11th day, we assessed morphological changes in these cells. We found ubiquitous small oval cells with round nuclei and dark, blue, small, round cells (Fig. 6). Immunofluorescence labeling showed the expression of $CD4^+$, CD117 + and Oct4 in the cells (Fig. 6).

In addition, the gene expression profiles of the treated and untreated mice showed differences. The expressions of v-src, c-src, lin-28, nanog, c-myc and stat3 were decreased in animals after treatment, and the expression of casp8 was increased (Fig. 7, Fig. 8).

Thus, in this series of in vitro experiments, we obtained morphologically tumor cells, tumor stem cells, and apoptotic tumor cells, nuclear translocation of Annexin A4, and expression of Oct4 and CD117 + in tumor cells of dead mice with one treatment with the complex of DDMC/a-miR-155, piR-30074, and miR-125b. Adding of the complex DDMC/a-miR-155, piR-30074, and miR-125b in cell culture of dead mice from the 1st group due to appearance of majority of morphologically same types of cells, which had had morphological characteristics of non-cancerous cells and expressed $CD4^+$ protein. In these cells was increased expression level of caspase 8 gene and decreased expression levels of c-src and v-src genes and genes, associated with Src tyrosine kinase pathways.

4. Discussion

In this series of experiments, a mouse model of retrovirus-induced cancer with high growth potential and metastatic activity was generated by the subcutaneous inoculation of RVP3 cells in the interscapular region of mice. The rare RVP3 cell line was obtained by the Czech scientist Dr. J. Svoboda, who, with his colleagues, obtained the strain of

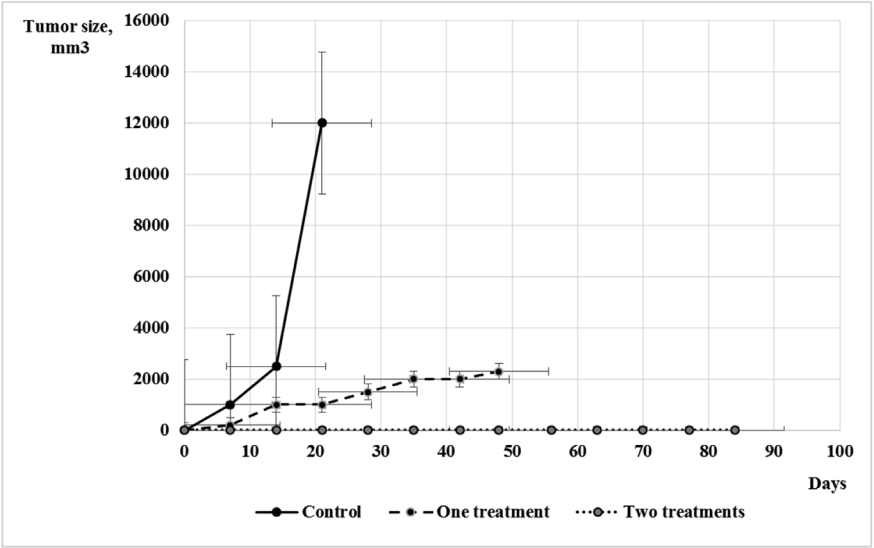

Fig. 2. The dynamics of tumor growth in mice from all three groups (means ± SEMs ($p < .05$)).

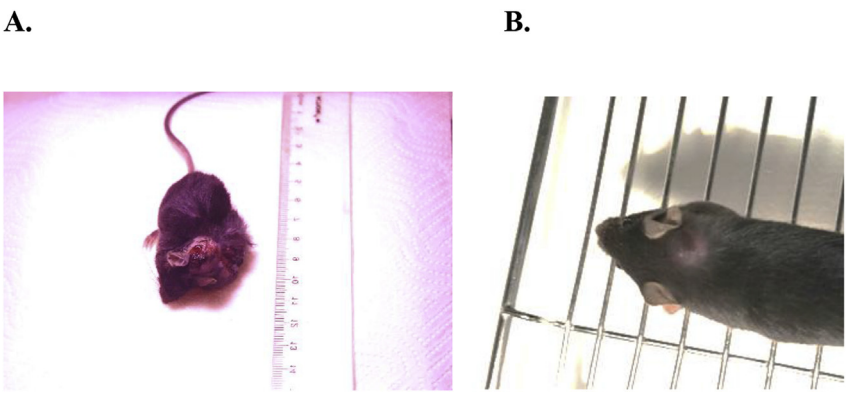

Fig. 3. Photographs of mice from the control (A.) and 1st (B.) groups on the 20th day after tumor inoculation.

RSV that triggers the transformation of normal cells into tumor cells in mammals. Because of these and other investigations, RSV-induced tumor formation has become a preeminent method in animal models of human virus-induced cancers [55–60].

The RVP3 cell line harbors tumor-specific transplantation antigen (TSTA) but lacks any capacity to yield a rescued virus that is non-productive, suggesting that these cells may contain only the viral transforming gene [39]. RVP3 causes the rejection of RSV-induced tumors, but the TSTA characteristic of RSV-induced tumors is present [61]. TSTA is encoded by the transforming v-src gene, and its formation triggers an 11 amino-acid insertion into v-src. The tumorigenic activity of retroviruses is connected to the expression of the v-src oncogene [62–65]. c-Src is the cellular homologue of the v-src viral oncogene.

c-Src gene activity is associated with processes of cellular proliferation, motility, differentiation, and survival, which are four main "whales" mediating cancer development. Recently, c-src activation was found in colon, liver, lung, breast, pancreas, and brain cancers [66]. Oncogenic activity of the src gene is connected with the activation of the Src protein kinase after interactions with other receptor tyrosine kinases: epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), colony-stimulating factor-1 receptor (CSF-1R), HER2/neu, HER1, hepatocyte growth factor receptor (c-Met), and vascular epithelial growth factor (VEGF). These relationships eventually result in cancer development, progression, and metastasis. Activated src interacts with ras/MAPK pathways, it activates stat1 and stat3, which promote cell transformation and tumor progression. Src is associated with the regulation of adhesion factors such as, α-, β-, and γ-catenins; cadherin; and plakoglobin (JUP). Src activates the anti-apoptotic factor Bcl-x_L by inducing Stat3 expression. Stat3 activation leads to the transcriptional regulation of cyclin D1 and c-Myc. The expression of these genes results in the stimulation of proliferative processes [40,67,68]. We observed significant down-regulation of src genes and src-dependent pathway genes (v-myc and stat3) after the treatment of animals with the complex of DDMC/a-miR-155, piR-30074 and miR-125b. Moreover, the expression of caspases was significantly increased in the cells from the animals from the 1st group.

Different classes of Src inhibitors have been synthesized. Of these, the most heavily clinically investigated are the dual inhibitors of Src and Bcr-Abl. In 2011, 100 years after Rous's discovery, one such inhibitor, dasatinib, gained U.S. Food and Drug Administration (FDA) approval for the complex therapy of patients with chronic myelogenous leukemia. Other inhibitors of SFKs are still under investigation. However, none of the investigated SFK inhibitors has shown appreciable activity in the monotherapy of patients with solid tumors [69,70].

In this study, we investigated a combination of a-miR-155, piR-30074 and miR-125b in complex with a DDMC polymer delivery system as a new class of anti-cancer treatment. We used one miRNA, one antago-miR and one piRNA. In our previous in vitro experiments, we obtained encouraging results [33–36]. In the current experiments, we used our complex as a treatment in a virus-induced mouse model of human cancer. In preliminary experiments, we established the toxic doses for the delivery system but not for the sncRNAs. All treatments were administered at the optimal doses of the complex. All animals were divided into three groups: the 1st group was the untreated control group, the 2nd group was composed of animals with 1 intravenous treatment, and the 3rd group was composed of animals with two intravenous treatments. A significant decrease in tumor progression was observed in animals after one injection; however, all animals from this group died. However, the animals with two injections had no visible tumors and were in good physiological condition. These animals were killed on the 83rd day after the start of the experiments. We could not find any similar experiments or results in the literature.

We would like to determine why tumors did not develop in mice receiving two injections of our complex. To detect changes in tumor cells, we took tumor tissue from freshly non-surviving animals from the group with one injection and continued our experiments in vitro. In this assay, we added our complex at the same concentration per milliliter of culture medium. We examined the cells every 72 h during culture. We observed apoptotic changes in one part of the tumor-like cells, aggregated and independent stem cells, and lymphocyte-like cells with oval shapes and round nuclei. Furthermore, these results were also obtained in our previous in vitro experiments. Phenotypic markers for $CD4^+$ were found in these cells.

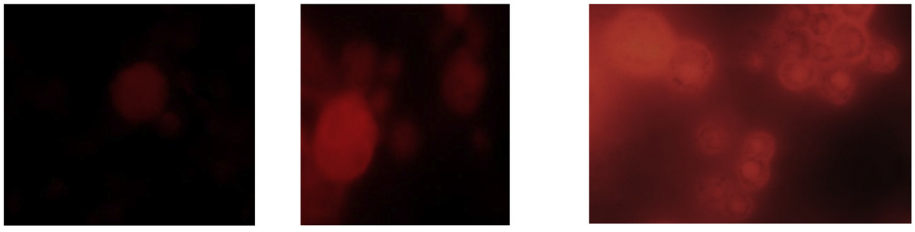

Fig. 4. Annexin A4 translocation to the nucleus in labeled tumor cells from animals in the 1st group. A. Tumor cells from the control group (24 days after beginning the experiment); B. tumor cells from mice in the 1st group (16 days after beginning the experiment); C. tumor cells from mice in the 1st group (24 days after beginning the experiment). Magnification 60x.

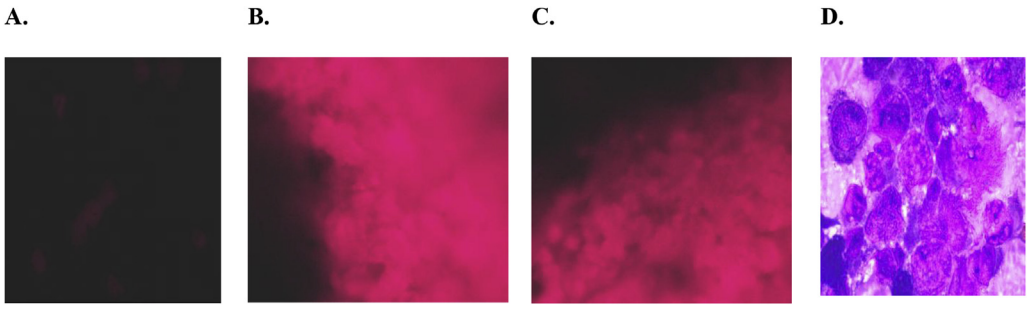

Fig. 5. Tumor cells from animals in the 1st group. Immunofluorescence labeling of cells with $CD4^+$ (A.), CD117+ (B.), and Oct4 (C.). Magnification 40x. Cells from tumors from animals in the 1st group were stained with the Leishman-Romanowsky method (D.). Magnification 60x.

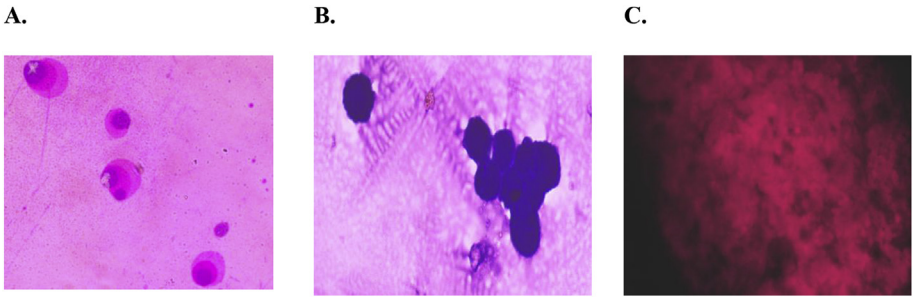

Fig. 6. Cells from animals in the 1st group were treated in vitro with the complex of sncRNAs with the DDMC delivery system 11 days after treatment. Cells were stained with the Leishman-Romanowsky method. Magnification 60x (A., B.). Cells labeled with anti-$CD4^+$ antibodies and analyzed by immunofluorescence. Magnification 40x (C.).

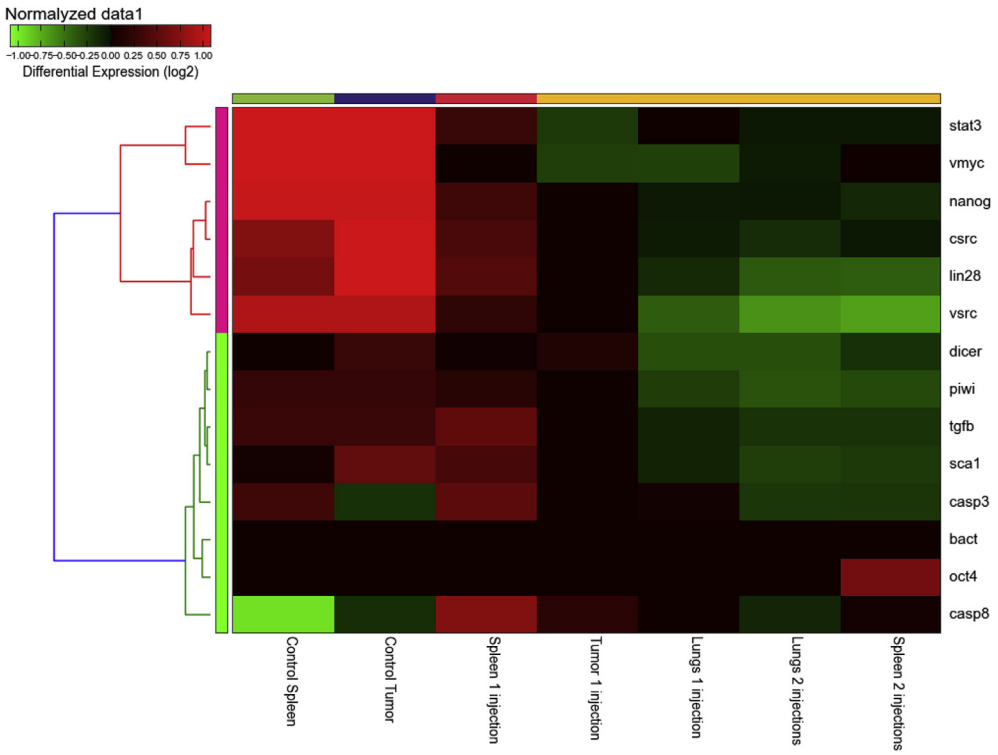

Fig. 7. Heat map of the hierarchical clustering of gene expression profiles in different cells from mice in all investigated groups.

In cells from animals with one injection, Annexin A4 was expressed; Annexin A4 was also diffusely expressed in tumor cells from control animals. Recently, Annexin A4 was observed to be significantly expressed in different epithelial cancers. In our experiments, we observed the translocation of Annexin A4 into the nucleus of cells from animals treated with one injection. Recently, an association between Annexin A4 expression and cancer progression, chemo- and radio-resistance and metastatic activity of tumor cells was investigated. The physiological activity of this molecule is related to cellular membrane permeability, exocytosis and ion channels regulation [71–74]. Annexin A4 also modulates the transcriptional activity of nuclear factor (NF)-kB in a Ca2+- dependent manner [75,76]. We observed the translocation of Annexin A4 from the cytosol to the nucleus of cells, a new finding in the study of the effects of miRs on cancer cells.

In the series of experiments, we used miR-125b, a-miR-155, and piR-30074. MiR-125b and miR-155 directly or not-directly regulate stat3/c-myc pathways. As known, miR-125b is the double-faced miRNA, which is down-regulate expression of oncogenes in solid

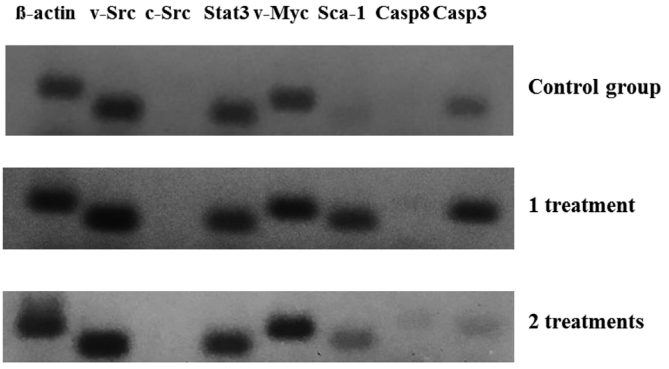

Fig. 8. Images of RT-PCR products obtained after electrophoresis on 2% agarose gels, stained with ethidium bromide.

tumors, and promote development of hematological malignancies. In solid tumors this mi-RNA regulates expression of genes, which promote cell proliferation, differentiation and apoptotic activity. Among these genes there are Bcl-2, c-jun, c-raf, p53, ERBB2/HER2, MUC1, PIK3CD [77–81]. MiR-125b regulates src/stat3/c-myc pathways. Recently were observed reciprocal negative relationships between c-Myc and miR-125b [82,83]. MiR-125b affect cofactors of STAT3 through gene-specific silencing of JunD [84]. MiR-125b decreases cell growth, migration and invasion of laryngeal squamous cell carcinoma, and suppresses the proliferation and migration of osteosarcoma cells by directly targeting STAT3 [85,86].

MiR-155 act as proto-oncogene involved in carcinogenesis, development, and invasion [87–91]. MiR-155 directly and not directly regulates c-Src/Stat3 pathways in hematological and non-hematological malignances. In breast cancer, oral squamous cell carcinoma, non-small cell lung cancer, prostate and pancreatic cancer miR-155 can target SOCS proteins, which modulate activity STAT3 [92–98]. In patients with colon cancers expression of miR-155 is up-regulated and promote cancer progression [99]. In our previous studies, we observed transformation of colorectal adenocarcinoma, Hela cells combined with right atrium cancer, and acute myeloid leukemia cells into other non-cancerous cells after treatment with a-miR-155 alone or in combination with factors of differentiation [33–36].

PiRNAs are direct regulators in cancer genetics. These sncRNAs interact with PIWI proteins, epigenetically block expression of transposable elements (TE) and regulate expression of genes in the nucleus [100]. TEs can induce genetic instability, which result in gene deregulation, chromosome rearrangement, and deleterious mutation caused to a number of cancers, such as leukemia, breast, ovarian, and colon cancers [101–106]. PiRNA/PIWI complex promotes repressive chromatin state at the transcriptional level due to enrichment of H3K9me3 mark and HP1, and reduction of RNAPII [107–111]. Increased activity of TEs is associated with inhibition of piRNAs expression, which can due to mutagenic transpositions and genomic instability result in carcinogenesis [112]. Aberrant expression of piRNAs is obtained in different cancer types, which possible indicate tissue-specific dependence of separate cluster piRNAs expression [113–119].

PiRNAs can regulate expression of different genes. For example, piRNA derived from Growth Arrest Specific 5, a tumor-suppressive long non-coding RNA, potently upregulates the transcription of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a pro-apoptotic protein, by inducing H3K4 methylation/H3K27 demethylation. The PIWIL1/4 proteins, which bind with this piRNA, directly interact with WDR5, resulting in a site-specific recruitment of the hCOMPASS-like complexes. These processes due to consequently inhibition of tumor growth [120]. PiR-L-163 (piRNA-like-163) binds to active site of p-ERM gene, which due to decrease of invasion capability and cell migration in non-small lung cancer cells. The ERM proteins (ezrin, radixin and moesin) are critical in connecting transmembrane proteins, such as EBP50 (ERM-binding phosphoprotein 50), and the cytoskeleton to play important role in regulating signal transduction pathways [121]. PIWIL2 facilitates the binding of NME2 to the promoter region of the MYC gene [122,123]. piR-54265/PIWIL2 complex interact with STAT3/phosphorylated-SRC and promotes proliferation, metastasis and chemoresistance of colorectal carcinoma cells. Treatment with piR-54265 inhibitor significantly suppressed the growth and metastasis of inoculated tumor in vivo [124]. Treatment with piR-8041 of mice with glioma xenograft mouse model significantly inhibited tumor growth. Functionally, piR-8041 inhibited expression of ERK1/2/

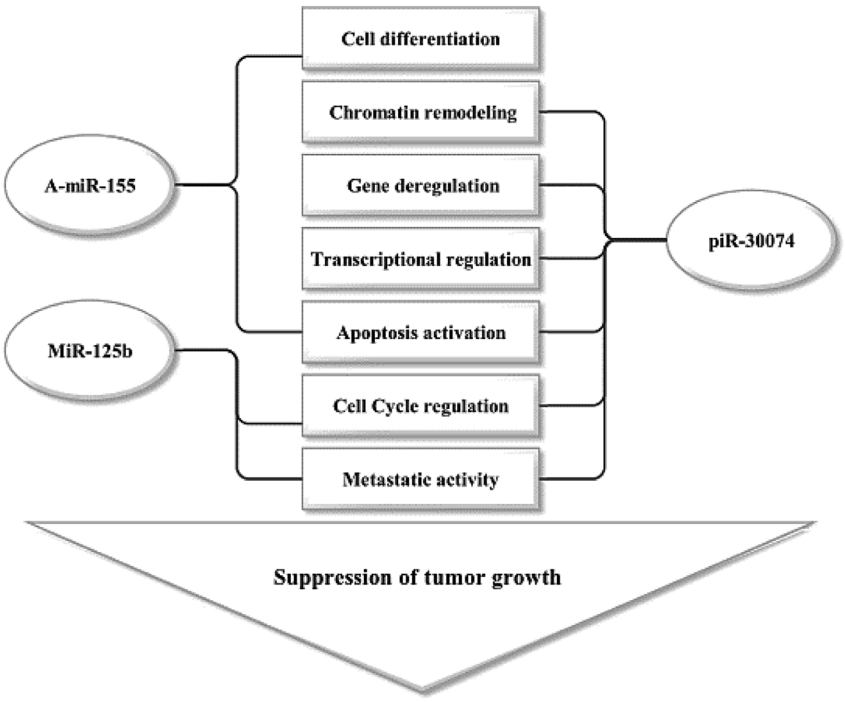

Fig. 9. Summary figure of influence of complex a-miR-155, miR-125b, and piR-30074 with the DDMC delivery system on the processes of tumor suppression.

MAPK signaling and reduced cell proliferation. This piRNA transcriptionally inhibited MAP3K7 and promoted apoptosis in U-87 glioblastoma cells [125].

Taken together, complex therapy with miRNAs, piRNAs, and antago-miRs with properly selected delivery system may induce intracellular and intranuclear changes of cancer cells due to activation of apoptosis, reduce of proliferation, stimulation of differentiation, decrease of invasive capability and metastatic activity. As the result, using of different schemes of separate sncRNAs may be powerful tool for complex and personalized therapy of cancers in the dependence of type of tumor, stage of disease, characteristics of tumorous cells (Fig. 9).

5. Conclusions

1. Two intravenous injections of the complex of a-miR-155, miR-125b, and piR-30074 with the DDMC delivery system induced a full recovery from virus-induced cancer in mice.
2. One intravenous injection of DDMC/a-miR-155, piR-30074 and miR-125b resulted in increase of lifespan of mice with sarcomas.
3. Treatment of animals with DDMC/a-miR-155, piR-30074 and miR-125b due to activation of apoptosis and the inhibition of Src-associated pathways.
4. Treatment of mice with the sncRNAs/DDMC vector complex transformed cancer cells into morphologically and genetically non-cancerous types of cells, which expressed $CD4^+$.

Additional information

Animal care and experimental procedures were conducted in compliance with the Declaration of Helsinki and were approved by an institutional animal care committee [53].

Conflict of interest statement

The authors declare no competing financial interests.

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Authors contributions: OVK planned, designed, performed, analyzed experiments, and wrote the manuscript. AS supported financially all experiments and helped writing the manuscript.

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