

Research Article

Joint Action of the Nano-sized System of Small Non-coding RNAs with DDMC Vector and Recombinant IL-7 Reprograms A-549 Lung Adenocarcinoma Cells into CD4⁺ Cells

Oxana V. Klimenko*

SID ALEX GROUP, Ltd. Kyselova 1185/2, Prague, Czech Republic

*Corresponding author: Oxana V. Klimenko, SID ALEX GROUP, Ltd. Kyselova 1185/2, Prague, Czech Republic, E-mail: o_klimenko@hotmail.com

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Abstract

Small non-coding RNAs, as class of small regulatory molecules, control normal development and differentiation of cells. Micro-RNAs and piwi-interacting-RNAs are members of epigenetic regulators family. In previous studies researches investigated the role of different small non-coding RNAs in pathophysiology of cancer.

Objective: In this study A-549 lung adenocarcinoma cells were firstly epigenetically reprogrammed and transformed into CD4⁺ cells.

Method: I used new non-viral carrier the complex of DDMC vector with antago-miR-155 and piR-30074 for longlasting transfection of lung cancer cells.

Results: The transformation of A-549 lung cancer cells was proved by morphological and genetic changes (AltAnalyze Platform) in dynamics. I observed CD4⁺ lymphocytes phenotypic marker and OCT4 marker of pluripotent cells by immunofluorescence microscopy in transformed cells.

Keywords: Lung adenocarcinoma; Antago-miR-155; piR-30074; DDMC vector; Reprogramming

Introduction

Lung cancer, which is leading cause of cancer death, is increasing in incidence. The SEER Stat Fact Sheets data statistics for lung cancer provide the following numbers: 221,200 estimated new cases in 2015 (% of all new cancer cases 13.3%); 158,040 estimated deaths in 2015 (% of all cancer deaths 26.8%); and 17.4% of patients surviving 5 years (2005-2011) [1]. Non-small-cell lung cancers (NSCLC) account for 85%-90% of all lung cancers [2]. More than 55% of patients who are newly diagnosed with NSCLC have distant metastases. The current lung cancer therapy has severe side effects and is often ineffective against tumors as the result of late diagnostics and/or metastases in the lymphatic system and in other organs [3-6]. For these reasons, it is necessary to investigate new anti-lung-cancer drugs. Small non-coding regulatory RNAs may be a new effective tool for lung cancer gene therapy. Previously, I studied action of piR-30074 and two miRs on the cervical adenocarcinoma [7]. In this study, complex of DDMC vector with two sncRNAs as candidate for future complex anti-lung cancer therapy was proposed and investigated in in vitro experiments.

Material and Methods

Cells

A549 (ATCC^{\circ} CCL-185^m) are human lung non-small adenocarcinoma cell line, was proceeded in accordance with the ATCC protocol. Cells were thawed in accordance with the standard method.

Cell culture was routinely maintained in tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM) medium, with 10% Fetal Bovine Serum, 2 mM l-glutamine, 100 mg/ml penicillin, and 100 ME/ml streptomycin (GE Healthcare) and cultivation parameters at 37°C with 5% CO₂. After 2 days cells were prepared for further cultivation in in the growth concentration of cells 0.5 x 106/ml. After accumulation of cells number 1 x 10⁶/ml, cells were prepared for transfection with nanoparticles. Day before transfection the medium was changed and cells were left to incubation.

Carriers

The cationic graft-copolymer (DDMC Vector® (Non-viral Transfection reagent) from Ryujyu science co., Aichi, Japan was purchased by SID ALEX GROUP, Ltd. (Prague, Czech Republic). The procedure of cells treatment was: The wash solution (PBS) and the DDMC Vector were warmed to 37°C. Transfection solutions were prepared as (for 35 mm plate): in sterile tube 10 µg of RNA was diluted in 270 µl of PBS then 14 µl of the DDMC was added. The tube was vortexed. Culture medium was removed from the cells, and cells were washed twice with 2×2.0 ml per 35 mm plate with PBS. Then the RNA/ DDMC vector complex was added to cells, and incubated plates at 37°C for 30 minutes with rocking and gently added 3.0 ml of growth medium per 35 mm plate. Cells split ratio of saturated culture about 1:2 every 2-3 days and doubling time approximately 48-72 hours. The medium usually changed every 48-72 hours by aspirating off old media and replace it with new medium. Cells were incubated for 30 days and they were analyzed after 11 days, 21 days and 31 days. One part of cells was removed on the 11th day, 21st and 31st days, and was treated with lysis buffer of RNAeasy mini kit (Qiagen, USA) for further isolation of total RNA, reverse transcription reaction and specific cDNA transcript

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amplification. Another part of cells was stained using the Leishman-Romanowsky method. For the control were used cells without any treatment, and cells, which were treated with unloaded nanoparticles. All manipulations with cell cultures were repeated for three times.

In the preliminary studies were used bioinformatics tools to choose most reliable candidates from small non-coding RNAs families for lung adenocarcinoma treatments. SncRNA targets are predicted mainly by three computational algorithms: 1) Lewis BP, and co.; 2) Krek A, and co.; 3) Griffiths-Jones S, and co. [8-10].

Manipulations with cells: In the pilot experiments with A-549 cells separate sncRNAs were experimentally chosen. In these series of experiments two sncRNAs sequences were used for further investigation. Oligonucleotide sequences were 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' – AAAGCTTTAAGTGTGTGGCGTGCTTC – 3'.



Figure 1A: A549 cells viability (ANOVA) detected by MTT-test on 7 days after treatment with naked a-miR-155 10 μ l/ml (1), DDMC vector 5 mg/ml (2), DDMC vector 7 mg/ml (3), DDMC vector 10 mg/ml (4), and complexes of DDMC vector 5 mg/ml with a-miR-155 10 μ l/ml (5), complexes of DDMC vector 7 mg/ml with a-miR-155 10 μ l/ml (6) and complexes of DDMC vector 10 mg/ml with a-miR-155 10 μ l/ml (7) (p<0.05, \$ - increase of statistically significant interactions F>Fcrit).

Statistics

Data are presented as the mean \pm SEM (p<0.05). MTT assay data were analysed using two-way analysis of variance (ANOVA) based on the concentrations of DDMC vector and complex of DDMC vector and a-miR-155 (p<0.05). All gene expression data were normalized to internal control gene expression levels of beta-actin. For external control were used culture of cells without any treatment in the same moment of time as experimental cells. All samples were prepared in triplets. Experiments were repeated three times (N=9). For gene expression analysis, I used AltAnalyze software.

To obtain the control for reprogramming of lung adenocarcinoma cells into CD4+ cells recombinant IL-7 was added. It is well known that IL-7 is CD4⁺ differentiation-regulating factor. On the 31st day of incubation a third part of the cells were treated with recombinant IL-7 (AbDSerotec, UK, Kidlington) and incubated for 14 days with routine change of the medium. Another third of the cells were stained using

the Leishman-Romanowsky method. The final third were treated the Dynabeads[®] CD4 Positive Isolation Kit (Invitrogen, Life Technologies, USA) and photos were taken under a microscope. Some of the cells were stained using the Leishman-Romanowsky method and photographed under a microscope. The remaining cells were treated with DETACHaBEAD[™] DYNAL[™] Dynabeads[™] (Invitrogen, Life Technologies, USA). Separated cells were stained with a CD4⁺/FITC staining reagent (R&D, USA) and were observed and photographed under fluorescent microscopy (AxioVertA1, Zeiss, Germany).

MTT assay

x600).

The A549 (ATCC^{*} CCL-185^{*}) human lung adenocarcinoma cell line was used for the MTT test and toxicity was detected 7 days after treatments. In MTT experiments on toxicity investigation were used DDMC vector, and complex of DDMC vector with a-miR-155. This delivery system was DDMC vector, which was used in all experiments. The concentration of DDMC vector was 5, 7 and 10 mg/ml of medium. The concentration of naked RNA oligonucleotide was 10 μ l/ml of medium. The concentration of the DDMC vector complexes was 7 mg/ml of medium plus 10 μ l/ml of medium RNA oligonucleotide.



MTT method was made as described previously [11]. As crystal solvent, I used DMSO (dimethyl sulfoxide) for detection of cell density. The absorbance was read in a spectrophotometer (ELx808 – Biotek, Winooski, USA) at 570 nm, 30 min after the solubilization of the crystals.

Gene expression analysis: Total RNA was extracted from cell culture using the RNAeasy Mini kit (Qiagen, USA) according to the manufacturer's protocol. In these series of experiments standard twostep reverse transcriptase-PCR procedure was used. All reaction components were from Invitrogen Co. (USA). Amplification of Caspase8, mTOR, PIWI1L1, EBI3, ICOS1B, FLT3, GITR3A, HMOX1, BACH2, CKIT1B, NOTCH1, KIR2DL1, DICER1 and beta-actin cDNA (as an internal control) was performed with an automatic thermocycler (TProfessional, Biometra, Germany). The primers sequences 5'-3'were: 1) Caspase8 NG_029188.1 5'- TCCAGATTGACGACAAGTGC -3', 5'-CACTCAGGAAGACGCGTTAC -3'; 2) mTOR ENST00000376838 5'-CCACCTATCCCAAGACCTCA -3'. 5'-GTGATCCCCTCTGTGCATCT -3'; 3) PIWI1L1 ENST0000245255 5'-TGCTATTCACCGGCTTCCTT -3' 5'-TGCTCACTCCTGAAAGTACGT -3'; 4) EBI3 ENST00000221847 5'-AGTCGGTCATCTGAGGTTGC 5'--3', CAATGAGCCTGGGCAAGTAG -3': 5 ICOS1B OTTHUM00000256369 5'- GCTTTGAAGCATCTCCCTTG -3', 5'-ACGTGTGCTTTTACCCCAAG -3'; 6) FLT3 OTTHUM00000044319

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5'-5'-CTTCTGACTGGCCCTGAGTC -3', ACGTGTGCTTTTACCCCAAG -3'; 7) GITR3A ENST00000328596 5'-ACACAGCCTCCCGTCCTAA 5'--3'. 5'-GAAGTGGGTGCAGGAAGGT -3'; 8) HMOX1 ENST00000216117 ACATCTATGTGGCCCTGGAG 5'-5'--3', 5'-TGTTGGGGAAGGTGAAGAAG -3'; 9) BACH2 ENST00000343122 GCGAGGAGGAGAACTCACAG 5'--3'. 5'actin CCCAGTACATCTCAGCAGCA -3'; 10) CKIT1B ENST00000288135 5'-GACTCATGGGCTTGGGAATA 5'--3', ACTTCAGGGGGCACTTCATTG -3': 11)NOTCH1

ENST00000277541 5'- GCGTCCCAAGATGTTGATTT -3', 5'-CTGGCTCCCTCAGAGCATAG -3'; 12) KIR2DL1 ENST00000611849 CTGGAATCTGAAGGCGTGA -3', 5'-GGCAGGGGTCAAGTGAAAT -3'; 13) DICER1 ENST000000526495 -3', 5'-GCCCCGTTAATTATGCTTGA ACTCGCACAGAGGCATTTCT -3'. Sequences for inner control betagene (ENST00000331789) 5'primers were: 5'-TCCCTGGAGAAGAGCTACGA-3', AGCACTGTGTTGGCGTACAG-3'.



Figure 2: Microscopic photos of A-549 lung adenocarcinoma cells on 31 days control group (A) group after addition of DDMC vector with amiR-155 (B.), cells after treatment using DDMC vector with piR-30074 (C) A-549 cells after treatment using complex of DDMC vector with piR-30074 and a-miR-155 (D) Expression of Kate2 far-red fluorescent protein on the 31st day after transfection using complexes of DDMC vector with Kate2, piR-30074 and a-miR-155 in A-549 cells. Kate2 vector for fluorescent protein expression (λ =588 nm) was added in cell culture for control of transfection efficiency (E) (Magnification x600).



Figure 3: Heat maps of the 13 differentially expressed genes levels in two independent microarray studies in A-549 lung adenocarcinoma cells after treatment using complexes of DDMC vector with piR-30074 (A.), complexes of DDMC vector with a-miR-155 (B.), and complexes of DDMC vector and both a-miR-155 and piR-30074 (C.) on 11th, 21st and 31st days after transfection. The heat map was produced by hierarchical clustering of the probeset data. Probesets for genes are represented by rows with the gene dendrogram at left. Green color indicates decreased expression and red colour indicates increased expression of genes (AltAnalyze Platform).

The PCR protocol was made as described previously, all reagents were purchased from Invitrogen, Co. (USA) [12]. I added 6.25 μ M primer (sense and antisense) for reaction mix. Amplification proceeded according standard protocol. PCR products were loaded on 1.5% agarose gel and electrophoresed then stained with Ethidium Bromide, exposed to a gel doc system (Syngene) and quantified with Quantity One Software (Bio-Rad). For comparison inner control – expression of beta-actin gene, and external control for which were used A549 cells without any treatments. Staining of the A549 cells was made using the Leishman-Romanowsky method [13].

Results

In the following experiments the DDMC Vector• (Non-viral Transfection reagent) from Ryujyu science co. was used. The DDMC vector was toxic to cells at a concentration more than 10 µg/ml (Figures 1A and 1B). In my experiments, I used concentration of DDMC vector 7 mg/ml. The toxicity was decreased after treatment with a complex of the DDMC vector and oligonucleotide (F observed \leq F crit). The transfection efficiency of the DDMC vector was lower than that particles of poly-N-vinylpyrrolidone (PNVP), which were used in previous studies; however, the transfection maximum was reached 11-14 days faster than that of the PNVP complexes (21 days) (Figure

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2E) [14]. DDMC vector based particles in non-toxic concentrations had greater stability and increased cell transfection with low sncRNA and DDMC vector concentrations. The DDMC vector is stable after

sterilization and is easier to use. The addition of oligonucleotides decreased the toxic effect of the DDMC vector compared to the addition of pure DDMC vector in cells (Figure 1).



Figure 4: Photo of agarose gel with separate genes expression in A-549 cells on the 31st day after treatment using complexes of DDMC vector with a-miR-155, DDMC vector with piR-30074, and complexes of DDMC vector with both a-miR-155 and piR-30074 (A.): 1. beta-actin, 2. Caspase8, 3. mTOR, 4. SOX2, 5. VMAF, 6. PIWIL1, 7. SOX2, 8. EBI3, 9. ICOS1B, 10. FLT3, 11. GITR3A, 12. FOXP3, 13. GATA1, 14. HMOX1, 15. BACH2, 16. CKIT1B, 17. RUNX1, 18. KLF4, 19. NOTCH1, 20. KIR2DL1. B. Gene expression levels in transformed cells on the 14th day after addition of recombinant IL-7 in A-549 cells.



Figure 5: Microscopic photos of transformed A-549 lung adenocarcinoma cells on 31st day after addition of complexes of DDMC vector with both a-miR-155 and piR-30074 and 14 days after treatment with recombinant IL-7 in fluorescence (anti-Oct-4-FITC)(A.), stained with the Leishman-Romanowsky method (B.), in fluorescence (anti-CD4-FITC)(C.). (Magnification x600).

Three weeks after a complex of sncRNAs was added, A-549 cells were morphologically changed compared to non-treated control cells. In the microscopic photos was observed an increase in number of sharp large cells (approximately 50 μ m in diameter) with single nuclei, displaying different morphological forms. These cells had vesicles of varying sizes on the periphery of the cytoplasm with properties of suspension cells. Un-stained cells and cells full of DDMC crystals were microscopically detected. These cells were large (approximately 60 μ m in diameter) and sharp with polymorphic nuclei that took 1/3 – 2/3 of the cell volume (Figures 2A-2E). Giant dendritic-like cells with multiple pseudopodia approximately 90 μ m in diameter were also detected (Figure 2B, 2D). Gene expression in A-549 cells was changed after addition of DDMC vector with a-miR-155, DDMC vector with

piR-30074 or DDMC vector with a-miR-155 and piR-30074 compared to the control group (Figures 3A-3C and 4A-B). Differences were observed in separate genes' expression levels. The expression of some genes was increased on different days, whereas that of other genes was fully inhibited after the transfection procedure on other to compared to the control cells. In the control A-549 cells, the expression of PIWIL1L1, ICOS1B, GITR3A, cKIT1B and KIR2DL1 genes was not detected. However, the expression of these genes was obtained in transfected cells. It is known that these genes are expressed in T-cells only [15-19].

The expression levels of HMOX1 and EBI3 were increased more than two-fold compared controls. The BACH2 gene expression levels

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were found to be variable in the control cells. NOTCH1 gene expression was fully inhibited after transfection with the complex of amiR-155 and piR-30074. NOTCH1-KRAS is the main marker of a pathway involved in lung adenocarcinoma [20]. cKIT1B gene expression was detected only after transfection with a-miR-155 or piR-30074 on day 21. However, gene cKIT1B expression was not observed after transfection with the complex of both sncRNAs on days 11, 21 or 31. The expression of this gene is a marker of progression of disease and poor prognosis for patients with lung cancer [21]. On day 14 after the addition of recombinant IL-7 genes FLT3 and DICER1 increased in expression (Figure 4B). Other genes were also investigated, but did not exhibit a response.



Figure 6: The general scheme of transformation of A-549 lung adenocarcinoma cells into CD4+ cells after treatment using complexes of DDMC vector with small non-coding RNAs (all microscopic photos of displayed cells were made by author).

In normal bone marrow, gene FLT3 is selectively expressed in CD34⁺ hematopoietic stem cells and immature hematopoietic progenitors, but it is virtually absent from the erythroid progenitors, which suggests that it plays an important role in regulating early haematopoiesis. Gene FLT3 is expressed in all hematopoietic organs, such as the spleen, liver, thymus, lymph nodes and placenta [22].

A summary of the morphological and genetic changes was obtained in cells that were treated with a complex of a-miR-155 and piR-30074 and a further addition of recombinant IL-7 (Figure 6). Other cell groups such as those, treated with a-miR-155 and piR-30074 separately but with further treatment with recombinant IL-7 exhibited not as significant changes in morphology and genetics compared to the controls. In these experiments, three types of cells were visualized microscopically. The first type was characterized by sharp and small (7 μ m in diameter) cells with large (3/4 of the cell volume), sharp nuclei and light blue cytoplasm. The second type included sharp cells approximately 13 μ m in diameter with single, sharp nuclei (2/3 – ¾ of the cell volume) and light blue cytoplasm. The third type contained large, sharp cells that were 100-120 μ m in diameter and had nuclei 2/3 - ¾ the size of the cell volume and light blue cytoplasm.

The bright blue nuclear concentrates were also microscopically detected (Figures 5A-C). To identify cells, the immune-fluorescent detection method was used and photos were taken using a microscope. In these experiments CD4 was used as a phenotypic marker of lymphocytes, CD117 as a phenotypic marker of thymocytes and early lymphocyte progenitors, and OCT4 as a marker of pluripotency [23-26]. The experimental cells expressed CD4 and OCT4 markers;

however, the CD117 marker was not detected by fluorescent microscopy (Figures 5A and 5C).

Discussion

In these experiments full transformation of lung cancer cells was firstly accomplished by using a new complex of DDMC vector as carrier for two sncRNAs and recombinant IL-7. The CD4+ cells were detected and identified. The reprogrammed cells had morphological, genetic and protein marker changes compared to control lung cancer cells. A two-step process was used to fully reprogram cells. First, sncRNAs reprogrammed genetic program of lung cancer cells and transformed them into intermediate, ready-to-differentiate form. Second, after the primary genetic and morphological transformation, recombinant IL-7 was added as a factor for lymphocytes differentiation. After these steps, the cells was morphologically changed compared to the control cells. In a previous study, the transformation of acute myeloid leukemia M1 cells into platelet-like cells using complex of polymer carrier for a-miR-155 was observed both microscopically and in the gene expression analyses [12].

The majority of the primary genetic transformations occurred due to action of sncRNAs that modified the cellular genome and structure. The main hallmarks of cancer are: genetic and epigenetic modifications, changes in the cell cycle with inhibition of apoptosis, metastatic activity of cancer cells, unlimited proliferation and division, and regulation of the tumor microenvironment. In tumor cells, genes can be regulated by epigenetic modifications and underlying genomic mutations that due to reversible and irreversible changes of cellular genome, which can result in the activation of oncogenes and the inhibition of tumor-suppressor genes. Tumorigenesis is a complex process that is driven by active and passive mutations. The resultant tumors generally comprise heterogeneous tissues with tumor cell characteristics [27].

Genetic and epigenetic modifications are enabling characteristics that generates random mutations including chromosomal rearrangements. Rare genetic changes can occur that orchestrate hallmark capabilities of cancer growth and progression. Because cancer initiation and development are based on genetic information, the uncontrolled behavior of cancer cells is probably due to the combined deregulation of genetic and epigenetic programs. Artificial epigenetic reprogramming, or remodeling of the tumor cells and microenvironment, can modify the functional capability, tumor growth and clinical relapse. Given that the number of parallel signaling pathways supporting a given hallmark must be limited, it may become possible to target all of these supporting pathways therapeutically, thereby preventing the development of adaptive resistance [28-31]. The transformation of cancer cells is possible by the combination of genetic modifications and full reprogramming of the intracellular genome. Therefore, only epigenetic regulators could be effective drugs for tumor treatment. Small non-coding RNAs have been suggested as possible candidates for this role [32,33].

In my study, I used antago-miR-155. Previously miR-155 was identified as metastatic sncRNA in lung cancer. Increased levels of miR-155 correlated with increased cancer invasion and migration and, correlated with poor prognosis in patients with NSCLC. Moreover, it was shown that miR-155 promoted tumor formation in the lung when cells were injected directly in the bloodstream [34-36]. MiR-155 also inhibits apoptosis in lung cancer cells by inhibiting Apaf-1 expression [37]. Among the microRNAs that have been linked to cancer, it is the most commonly overexpressed miRNA in malignancies of the breast, lung, liver, and lymphatic system. It down-regulates BCL6, which modulates the STAT-dependent IL-4 responses of B-cells and increases their function. The reduction of BCL6 is due to the up-regulation of inhibitors of differentiation such as IL-6, cMYC, Cyclin D1, and Mip1a/Ccl3, all of which promote cell survival and proliferation. MiR-155 also upregulates the Mxd1/Mad1 transcription factors that mediate cellular proliferation, differentiation, and apoptosis through the regulation BCL6. Thus, miR-155 leads to the resistance of cell death and enables replicative immortality.

HDAC4 is also a target for BCL6. HDAC alters chromosome structure and affects the access of transcription factors to DNA. BCL6 acts with MEF2C and MEF2D. MiR-155 activates metastatic processes in breast cancer cells by activating Rho. It also represses SHIP, which is a negative regulator of myeloid cell proliferation and survival. MiR-155 suppresses BACH1 and SOCS-1 and induces G-2 arrest through the CD40 ligand (CD154) and further represses of BCL2. MiR 155 also targets casein kinase (CK1a) which enhances beta-catenin signaling and cyclin D1 expression, thereby promoting tumor growth [38-42]. All of these genetic and morphological changes are due to cell death or a cell's irreversible transformation. As the result, structural modification of cancer tissues occurs. Treatments using long-living DDMC vector complexes with molecular epigenetic regulators such as sncRNAs and theirs antagonists would be effective, un-complicated and cost-effective tool for complex anti-lung-cancer therapy.

Conflict of interest

The author declare no conflict of interest.

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