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IMMUNOLOGICAL MARKERS OF ANTI-TUMOR DENDRITIC CELLS VACCINE EFFICIENCY IN PATIENTS WITH NON-SMALL CELL LUNG CANCER

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***Aim*: To investigate the quantitative and functional status of peripheral blood lymphocytes in patients with non-small cell lung cancer during DC-vaccine therapy and identify the most informative immunological parameters which are associated with clinical outcome. *Materials and Methods*: The study was conducted within the framework of randomized phase III clinical trial of DC-vaccine efficacy in patients with non-small cell lung cancer. Quantitative composition of peripheral blood lymphocytes was determined by flow cytometry. Cytokines mRNA expression level was estimated using real-time RT-PCR. *Results*: In our study the most pronounced changes in the immune system have been defined after fourth DC-vaccine injection. Immunologic features such as reduction the MIP-1α mRNA expression level, increasing the RANTES mRNA expression level and NK-cells count, retention CD4/CD8 ratio at physiological level were associated with favorable clinical outcome after DC-immunotherapy. *Conclusions*: Immunological markers established in our investigation can be used for estimation of DC-immunotherapy efficiency. The results of our research are very promising, but these data should be confirmed in further studies with a large cohort of patients.**
***Key Words*: lung cancer, DC-vaccine, immunological monitoring, effector cells, chemokines.**

Lung carcinoma is the leading cause of cancer mortality worldwide. Although 5-year survival rate has tripled from 5% to 15% over the last 25 years, the estimated number of deaths still exceeds 1.3 million annually. The overall 5-year survival of lung cancer is only 10% in Europe and 15% in the United States [1]. In Ukraine lung cancer remains the prevalent nosological form of cancer with an estimated 16 413 new cases and 13 305 deaths in 2011. Non-small cell lung cancer (NSCLC) accounts 80–85% of all lung cancer cases [2].

Patients with early-stage NSCLC have relatively high long-term survival rates after surgical resection, but a substantial majority of patients (~75%) are diagnosed with advanced or metastatic stages (stage III–IV) [3]. Such patients are treated with a variety of therapies including surgery, radiation, and chemotherapy. Unfortunately, survival with even stage III disease is dismal by any measure and has been remarkably resistant to improvement despite more intensive standard chemotherapy, surgery, and/or radiation therapy. The poor prognosis in NSCLC is due to its high aggressiveness, early metastasis and high recurrence rate [4, 5].

New approaches in immunotherapy offer hope of prolonged survival in selected patients with advanced NSCLC. The great interest in this regard is the use of anti-tumor vaccines, in particular, dendritic-cell-based auto vaccines. Antigen presenting dendritic cells (DC) are used as powerful natural adjuvant to enhance the immune response to tumor antigens. This method aims to activate a specific anti-tumor immunity in order to prevent recurrence and metastasis after primary treatment of cancer [6].

The rationale behind DC-based immunotherapy is that injected DC induces a tumor-specific immune response resulting in tumor shrinkage/clearance. So, ideally we should be able to identify patients that respond to therapy by analyzing the anti-tumor immune response generated by the DC vaccine. However, to date, limited studies have shown a correlation between immune and clinical responders [7].

In contrast to chemotherapeutic agents, anti-tumor vaccines and other immunotherapies that target the patient's immune system need first to reprogram the pattern of interactions between the immune system and the tumor. Immunotherapy induces novel patterns of the antitumor response not captured by World Health Organization criteria or Response Evaluation Criteria in Solid Tumors (RECIST) which are commonly used for chemotherapy assessment. Thus, is a needed new evaluation criteria for immunotherapy efficiency assessment.

In late 2009 and 2010 the Society for Immunotherapy of Cancer (SITC), convened an "Immunotherapy Summit" with representatives from immunotherapy organizations of Europe, Japan, China and North America to discuss collaborations to improve development of cancer immunotherapy. The SITC Taskforce on Immunotherapy Biomarkers addressed the lack of validated biomarkers for monitoring the of immune response development of a following therapy and identified challenges critical for the translation of cancer immunotherapies. Eight of the nine challenges identified by this Taskforce were related to immunological monitoring considerations [8].

During last five years several immune response assays (ELISPOT, HLA-peptide tetramers, intracellular cytokine staining and CFSE assay) have been developed and become commonly used. However, results

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Abbreviations used: APC – antigen-presenting cells; DC – dendritic cell; NK – natural killer cells; NR-group – non relapsed patient; NSCLC – non-small cell lung cancer; PBMCs – peripheral blood mononuclear cells; R-group – relapsed patient.

from T-cell immune response assays are highly variable and often not reproducible. Therefore, the investigation of the cellular immune response as a biomarker and testing it for clinical surrogacy remains relevant problem of immune therapy in cancer. Moreover for estimation of immunological efficiency of anti-tumor immunotherapy, it is necessary to determine the quantity of lymphocyte subpopulation, as well as their functions, along with the cytokine and chemokine gene expression profiles [9].

The aim of our study was to investigate the quantitative and functional status of main lymphocyte subsets in peripheral blood of NSCLC patients at courses of DC vaccine administration in order to determine the most informative immunological criteria of vaccine efficiency. The study was conducted within the framework of randomized phase III clinical trial of DC-vaccine efficacy in NSCLC patients.

MATERIALS AND METHODS

Patient characteristics and treatment scheme. 120 patients with IIB–IIIA stage of NSCLC (median age: 61 years, range: 43–82; males: 55, females: 5) treated in the Department of Thoracic Oncology, National Cancer Institute of Ukraine from 2005 to 2012 were enrolled into the study. Patients were randomly divided into 2 groups: 1 — patients received standard treatment (only surgery); 2 — surgery and DC-vaccine. The DC-vaccine treatment was given 7–14 days after surgery. DCs in amount $4,62 \pm 0,37 \times 10^6$ per injection were administered intravenously in course consisted of 4 injections with one-month interval. The scheme of DC-vaccine immunotherapy is presented on Fig. 1.

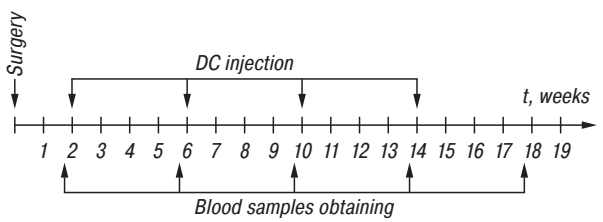

Fig. 1. Scheme of DC-vaccine immunotherapy

Adverse effects were evaluated according to the NCI Common Toxicity Criteria. Clinical and immunological monitoring of DC-vaccine immunotherapy was performed at the beginning of the treatment and before each vaccination. An informed consents were obtained from patients according to the the Ethical Commission requirements of the National Cancer Institute of Ukraine. According to the clinical outcome, patients who received DC — immunotherapy were divided into 2 groups: a group of patients who are alive without recurrence within 2 years period, and patients who developed recurrence during this observation period. Follow-up period was 6 years.

DC –vaccine preparation. Peripheral blood mononuclear cells (PBMCs) were isolated from 50–100 ml of heparinized whole blood samples by Ficoll ($\rho = 1,077$ g/см^3) density gradient centrifugation ("Bio-Clot", Germany). Autologous DCs were grown from PBMCs by *in vitro* cultivation in RPMI-1640 ("Sigma", USA) supplemented with 1% autologous plasma and cytokines GM-CSF, IL-4 and IFN-α at 37 °C in a humidified 5% CO_2 atmosphere. Mechanically activated microparticles of lyophilized autologous tumor cells were added on the 6-th day of DCs cultivation. Vaccine criteria include a negative bacterial contamination and a fully mature DC phenotype ($CD86^+$, $CD83^+$ and $HLA\text{-}DR^+$) that was confirmed by flow cytometry analysis. Autologous DCs with expression of surface markers CD86 and HLA-DR at least 70%, CD83 — 50% were used for treatment.

IMMUNOLOGICAL MONITORING

Lymphocyte phenotype analysis by flow cytometry. Peripheral venous blood (2–3 ml) was drawn into heparinized tubes. The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD3-PC5, anti-CD4-FITC, anti-CD8-PE, anti-CD16-FITC ("Beckman Coulter", USA). After immunofluorescent staining, the cells were fixed with 1% paraformaldehyde and then analyzed by a FACSCalibur flow cytometer using CellQuest-PRO software ("Becton Dickinson", USA). The acquisition and analysis gates were restricted to the lymphocyte gate as determined by their characteristics forward (FSC) and side-scatter (SSC) properties. FSC and SSC were set in a linear scale. For analysis 1×10^4 lymphocytes were acquired. Cell expressing CD markers were acquired and analyzed in the FL1 or FL2 logarithmic scale using the set gates.

Gene expression analysis by quantitative real-time RT-PCR. Freshly isolated PBMCs were washed three times with PBS, soaked overnight in RNAlater solution ("Ambion", USA) and kept frozen until use. Total RNA was extracted from 10^6 cells using NucleoSpin® RNA II ("Macherey Nagel", Germany) and treated with DNase ("Ambion", USA). Concentration of purified RNA was determined by measuring the absorbance at 260 nm using Nanodrop 1000 ("Thermo Scientific Inc", USA). cDNA was synthesized from 200 ng total RNA in a 20 µl reaction mixture using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor ("Applied Biosystems", USA). Quantitative Real-time PCR was performed in a total volume of 25 µl containing 5 µl of cDNA, 12,5 µl 2X Maxima SYBR Green qPCR Master Mix ("Thermo Scientific Inc", USA), 0,25 µM of each specific sense and anti-sense primers on ABI Prism 7500 Sequence Detection System ("Applied Biosystems", USA) using Software Version 2.0.1. The PCR cycling conditions included an initial denaturation at 50 °C (2 min) and 95 °C (10 min), followed by 40 cycles at 95°C (15 s) and 60 °C (1 min). Melt-curve analysis was performed immediately after the amplification protocol to determine if nonspecific products were amplified. The mRNA level was normalized by housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers were designed using Primer Express Software v2.0 and synthesized by Assay-by-Design service ("Applied Biosystems", USA): *IFN*- γ sense primer: CCA-ACG-CAA-

AGC-AAT-ACA-TGA, *IFN*-γ anti-sense primer: TTT-TCG-CTT-CCC-TGT-TTT-AGC-T; CCL5 sense primer: ACC-ACA-CCC-TGC-TGC-TTT-G; CCL5 anti-sense primer: GCG-GTT-CTT-TCG-GGT-GAC-A; MIP-1α sense primer: CAT-CAT-TTG-CTG-CTG-ACA-CG, MIP-1α antisense primer: TGT-GGA-ATC-TGC-CGG-GAG; *GAPDH* sense primer: TGC-ACC-ACC-AAC-TGC-TTA-GC, *GAPDH* anti-sense primer: CAC-GAT-ACC-AAA-GTT-GTC-ATG-GA. Quantitative Real-time PCR was performed in triplicate and repeated in at least three separate experiments using the previous conditions.

Statistical analysis. Statistical analyses were performed by STATISTICA 6.0. To determine data statistical significance for normal distribution we used Student's *t*-test, the values $p < 0.05$ were considered as significant. Mann — Whitney test was used for comparison of two independent groups and Wilcoxon test — for comparison of two dependent groups for nonparametric data.

RESULTS AND DISCUSSION

DC-immunotherapy was well tolerated without significant toxicity. Only 10% of patients demonstrated short time temperature increase (about 38 °C). It has been found that distant recurrences had occurred for 25% (15/60) of patients received only surgery (disease-free period — 9.55 ± 1.37 months) and only for 10% (6/60) of patients treated with DC-vaccine (disease-free period — 24.92 ± 2.39 months).

In further researches, patients treated with DC-vaccine were divided into 2 groups: non relapsed (NR) and relapsed (R) patients.

The analysis of quantitative status of lymphocyte subsets revealed that the total number of T and B cells for R-group didn't differ significantly from NR-group. Statistically significant differences between NR- and R-groups among subsets of $CD4^+$ T-lymphocytes and $CD16^+$-natural killer cells (NK) were observed at different stage of DC-vaccine therapy. It should be mentioned that the differences were determined in the absolute values of these cells but not in their percentages. The results are presented on Fig. 2.

Thus, for NR-group patients absolute counts of $CD4^+$-cells were $(0{,}96 \pm 0{,}08)\times10^9$/L before DC-vaccine therapy and $(1.27 \pm 0.35)\times10^9$/L after IV injection of DC-vaccine, whereas for R-group — $(0.61 \pm 0.02)\times10^9$/L against $(0.49 \pm 0.07)\times10^9$/L, respectively, $p < 0.05$. The number of $CD4^+$-cells prior to immunotherapy was significantly lower in R-group compared to NR-group patients, $p < 0.05$ (Fig. 2 *b*).

We have found that the number of main immune effectors of immune surveillance, NK-cells, was significantly higher for R-group compared to NR-group patients before the vaccine therapy. Multidirectional changes of lymphocytes subsets quantities during the courses of the DC vaccine therapy were observed. Thus the NR-group $CD8^+$-cells absolute counts slightly increased during the DC-vaccine therapy, while NK-cells increased significantly from $(0.28 \pm 0.04)\times10^9$/L to $(0.65 \pm 0.18)\times10^9$/L, $p < 0.02$. Meanwhile, for R-group patients an increase of $CD8^+$- and abrupt increase NK-cells counts after I injection of DC-vaccine was noticed and followed by a decrease after II injection. The amount of $CD8^+$-cells before vaccination was $(0.94 \pm 0.33)\times10^9$/L against $(0.62 \pm 0.10)\times10^9$/L after IV injection, and the amount of NK-cells — $(0.60 \pm 0.02)\times10^9$/L against $(0.53 \pm 0.06)\times10^9$/L, respectively.

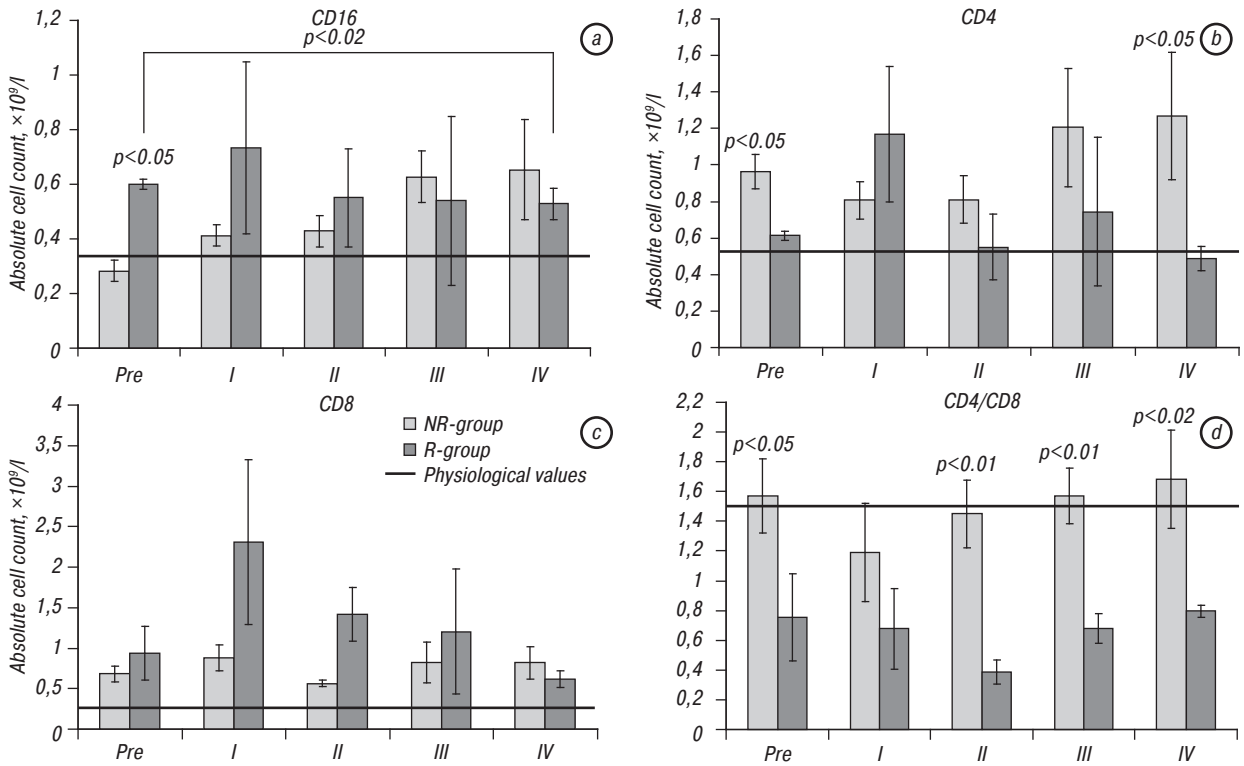

Fig. 2. Quantitative values of lymphocyte subsets in peripheral blood of NSCLC patients. $CD16^+$ (*a*), $CD4^+$ (*b*) $CD8^+$ (*c*) absolute cell counts and $CD4^+/CD8^+$ ratio (*d*) before vaccine therapy (Pre) and after DC-vaccine injections (I, II, III and IV respectively)

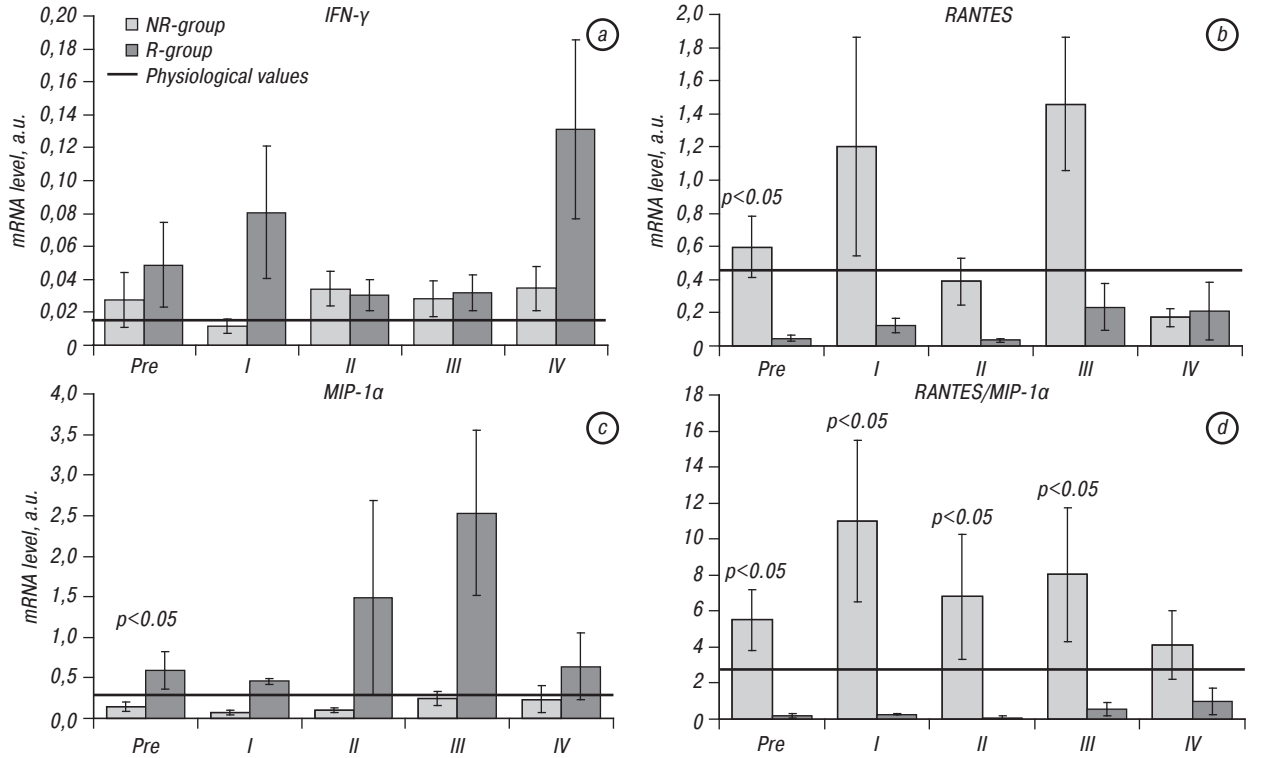

Fig. 3. Cytokines mRNA expression in PBMCs of NSCLC patients. IFN-γ (*a*), RANTES (*b*) MIP-1α (*c*) mRNA levels and RANTES/MIP-1α ratio (*d*) before vaccine therapy (Pre) and after DC-vaccine injections (I, II, III and IV respectively); a.u. — arbitrary units

Patients with disease progression (R-group) during the treatment had initially low $CD4^+/CD8^+$ ratio, which didn't increase during the treatment. Whereas for the NR-group patients $CD4^+/CD8^+$ rate increased after the first course of DC-vaccine administration.

For the anti-tumor immune response monitoring the analysis of lymphocyte counts is not sufficient as it doesn't reveal their functional activity. Estimation of the cytokine production levels of PBMCs as a functional state of the cells is essential. Hence one of the tasks of our research was to examine the functional status of PBMCs for the patients during DC-vaccine therapy.

It is known that IFN-γ provides a connection between lymphocytes and macrophages and stimulates of antigen-presenting cells (APC) functioning, increases secretion of IL-2 and IL-12, adjusts the activity of cellular and humoral links of immune system, specifically enhances cellular and inhibits humoral immune response. In addition, IFN-γ is a key factor for differentiation of $CD4^+$-lymphocytes to Th1-cells. According to several authors, the elevated serum level of IFN-γ during vaccine therapy may consider as predictive factor for recurrence risk assessment in patients with colorectal cancer, prostate cancer.

We have observed that mRNA expression level of IFN-γ for R-group patients was significantly higher at almost all vaccine therapy stages in comparison with NR-group. The IFN-γ mRNA expression level is shown on Fig. 3 *a*.

Immune responses against cancer rely upon leukocyte trafficking patterns that are coordinated by chemokines, small cytokines with selective chemoattractant properties that coordinate tissue homeostasis and inflammation. The C-C motif chemokines, such as CCR5 ligands — RANTES, MIP-1α and MIP-1β (CCL5, CCL3 and CCL4) play a crucial role in chemoattraction and activation of immune cells. CCR5 and its ligands regulate communication between $CD4^+$, APC and $CD8^+$ cells in the draining lymph nodes, drive tumor infiltration by T and NK-cells, enhance the effector cells activity which leads to the elimination of tumor cells [10].

CCR5 ligands exert major regulatory effects on $CD4^+$- and $CD8^+$ T cell-mediated immunity. CCR5 ligation boosts T-cell tumor response by modulating helper-dependent $CD8^+$ T-cell activation with guiding these cells to productive $CD4^+$/APC complexes [11].

Despite both RANTES and MIP-1α are potent proinflammatory chemokines and share common receptors, their effects on the immune cells have some peculiarities. RANTES is considered as a T cell-specific chemokine, it acts as a potent chemoattractant for many cell types such as monocytes, NK-cells, memory T cells and DCs. RANTES is also known to regulate T cell differentiation toward Th1 subtype and required for normal T cell functions as well as for recruiting $CD8^+$ and $CD4^+$ T cells to inflammation sites [11, 12]. Although MIP-1α (as well as RANTES) is a CCR5 ligand involved in T cell traffic, it is a potent chemoattractant for immature (but not mature) DCs and macrophages. Moreover, MIP-1α governs B-cells chemotaxis and modulates CD40, CD80, and CD86 expression by $B220^+$ cells, promoting humoral response [13, 14]. In this manner, it can be assumed that prevalence of RANTES against MIP-1α leads to selective enhancement of Th-1 cells, cytotoxic T lymphocytes, and NK-cell-mediated type 1 immunity versus humoral type 2 immunity.

We have shown a statistically significant difference in the expression levels of RANTES and MIP-1α for both studied groups prior to the immunotherapy. Thus, RANTES mRNA expression level for NR-group patients was 16-fold higher than for R-group ($p < 0.05$), whereas MIP-1α mRNA expression level was 3 times lower, respectively ($p < 0.01$). It should be mentioned an unidirectional change of chemokine levels for both groups after DC-vaccine administration. For NR-group of patients a highest RANTES expression was observed after III injection of DC-vaccine administration, comparable dynamics were found for R-group.

The level of MIP-1α expression for NR-group remained at physiological values, whereas for R-group patients it increased after each subsequent administration of DC-vaccine, and reached maximum values after III injection of DC, $p < 0.05$. The analysis results of chemokines RANTES and MIP-1α mRNA expression levels are shown on Fig. 3 *b, c*.

Analyzing RANTES and MIP-1α mRNA expression levels we have found that RANTES/MIP-1α ratio was 14–39 times higher for NR-group patients compared to R-group after I-III DC vaccine administrations, $p < 0.05$ (Fig. 3 *d*).

Thus, changes in the immune system induced by the DC-vaccine which are associated with favorable clinical outcome consist in reduction the MIP-1α mRNA expression level, increasing the RANTES mRNA expression level and NK-cells count, retention CD4/CD8 ratio at physiological level during DC-vaccine immunotherapy. Immunological markers established in our investigation can be used for estimation of DC-immunotherapy efficiency. The results of our research are very promising, but these data should be confirmed in further studies with a large cohort of patients.

In conclusion, the most pronounced changes in the immune system have been defined after fourth DC-vaccine injection. In our study no association between IFN-γ mRNA expression level and clinical outcome for NSCLC patients who received DC-vaccine have been found. The number of NK-cells, CD4/CD8 ratio, MIP-1α and RANTES mRNA expression levels are the most important immunological markers which are associated with favorable clinical outcome after DC-vaccine therapy for NSCLC patients. RANTES/ MIP-1α mRNA expression level ratio at initial stage of DC-vaccine therapy could be considered as the most substantial marker for prognosis of its effectiveness for NSCLC patients.

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