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Nrf2 Expression in CML and AML Patients' Peripheral Blood Mononuclear Cells Treated by Vitamin D, Carnosic Acid and Curcumin

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The following study demonstrated the influence of 1,25D/CA and 1,25D/CUR combinations on Nrf2 expression in primary leukemic cells derived from newly diagnosed CML and AML patients of hematological department of Karaganda Regional Clinical Hospital (Karaganda, Kazakhstan). Methods. The research was conducted by using quantitative real-time PCR. Mononuclear cells were isolated by centrifugation in a Ficoll-PaqueTMPREMIUM density gradient. Cell cultures were incubated in RPMI 1640 medium with test agents. RNA extracted from mononuclear cells was used for cDNA molecules synthesis by reverse transcription. Quantitative real-time PCR was carried out in a DT-322 PCR machine. The relative expression level was quantified by using the 2-ΔΔCt method. The statistical analysis was carried out by GraphPadPrism 6.0 program. Results. It was found 1.25D, CUR, CA alone and their combinations do not lead to an increase in the level of expression of the Nrf2 gene in peripheral blood mononuclear cells of AML and CML patients. The expression of NQO1 gene did not change much in the all cell groups of CML patients, but is pronounced in cells of AML patients grown in a medium supplemented with combinations of 1.25D/CUR, 1.25D/CA and CUR, CA alone. 1.25D/CUR and 1.25D/CA combinations do not enhance the expression level of the Nrf2 gene, but increase the amount of its protein, which in turn augments the expression level of the NQO1 gene.

Keywords: Vitamin D (1.25D), curcumin, carnosic acid, Nrf2, NQO1, leukemia patients.

Leukemia is the most common malignant disease. In Kazakhstan 6741 new cases of leukemia were registered for the period from 2003-2012 (Igissinov et al. 2013). The main methods of leukemia treatment are bone marrow transplantation, radiotherapy and chemotherapy. Each of these methods has a number of limitations, including immunity suppression, radiation poisoning and development of secondary tumors which is much more resistant to all kinds of treatment. In the present day many laboratories search innovative approach of leukemia treatment. One of these ways is the differentiation therapy. It is based on the induction of leukemic blasts to mature beyond the differentiation block and, thus, to restore the normal cellular phenotype. Vitamin D, converted in the body to its hormonal form 1α,25-dihydroxyvitamin D_3 (1,25D), is a potent differentiation agent which can induce maturation and/or apoptosis in different types of cancer cells (Gocek & Studzinski 2009). Although many laboratories have used 1,25D to differentiate various subtypes of AML cells in culture, the required concentrations would be lethal *in vivo*, principally due to hypercalcemia (Brown & Slatopolsky 2008). One approach to

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overcoming this problem to combine relatively low doses of 1,25D with the administration of another agent that augments the differentiation-inducing action of 1,25D but that does not enhance the levels of circulating calcium. Danilenko M. and others have previously shown that plant-derived polyphenolic antioxidants, such as carnosic acid (CA) isolated from the rosemary plant, curcumin (CUR) from turmeric and silibinin from milk thistle, markedly enhance the differentiation effects of low nanomolar concentrations of 1,25D in AML cell lines, both human (HL60) (Sokoloski, Shyam & Sartorelli 1997; Liu et al. 1997; Danilenko, Wang & Studzinski 2001; Kang et al. 2001; Danilenko et al. 2001) and murine (Sharabani et al. 2006; Shabtay et al. 2008) as well as in leukemic blasts obtained from AML patients (Zhang et al. 2010). They have shown for the first time that the transcription factor Nrf2 largely mediates the potentiating effect of plant polyphenols on 1,25D-induced differentiation in human AML cells. The enhancement of the differentiation caused by CA in U937 cells correlates with its ability to activate the Nrf2/ARE transcription system and to increase the redundant pathway for the synthesis of cellular glutathione in these cells. Activated Nrf2 binds to ARE sequences located in the promoters of many cytoprotective and antioxidant genes, the induction of which leads to increased detoxification and antioxidant potential. One of these genes is the NAD (P) quinone oxidoreductase 1 (NQO1) gene (Danilenko et al. 2001).

The aim of the research is to study the influence of 1,25D and CA, CUR alone and in the combination on the Nrf2 expression in CML and AML patients' peripheral blood mononuclear cells.

MATERIALS AND METHODS

The material of the study was 8 newly diagnosed patients peripheral venous blood from hematological department of Karaganda Regional Clinical Hospital (Karaganda, Kazakhstan) suffering from CML and AML. The diagnosis was made on the basis of blood and bone marrow examination.Written informed consent was obtained from all patients participated in this study in compliance with the guidelines of the Helsinki Committee's approved protocol.

Mononuclear cells were isolated by centrifugation in a Ficoll-Paque^{TM}PREMIUM density gradient (GE HealthcareBio-SciencesAB, Uppsala, Sweden). Cell cultures were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum treated with test agents. Cells were seeded at 1-8 $\times$ 10^6 cells/mL in plates and incubated for 120 hours with CA (10 mM), 1,25D (2.5 μM) and 1,25D (100 μM), CUR (5 mM) and combinations 1,25D (2.5 μM) with CA and CUR. The cells grown in medium supplemented with 0.1% ethanol were used as a control.

Total RNA was extracted from mononuclear cells by using a Ribo-zol-A reagents kit (AmpliSens, Moscow, Russia). The RNA concentration and purity were measured spectrophotometrically on a NanoVueplus instrument (BiochromLTD, Cambridge, England). cDNA molecules were synthesized from RNA by reverse transcription using REVERTA L reagents kit (AmpliSens, Moscow, Russia). Quantitative real-time PCR was carried out in a DT-322 PCR machine (DNA technology, Moscow, Russia). The primers for the reaction were synthesized by Sigma-Aldrich (Rehovot, Israel). The cDNA samples (5 μl) were diluted 25-fold, mixed with the specific primers (0.2mM) and then added to reaction mixture. Standard cycling conditions for this instrument were 3 min initial enzyme activation at 94°C then 42 cycles as follows: 15 sec at 94°C, 30 sec at the annealing temperature (62°C). The results were normalized by GusB mRNA content. The relative expression level was quantified by using the $2^{-}\Delta\Delta$ Ct method.

All measurements were performed in triplicate. The statistical analysis was carried out by GraphPadPrism 6.0 program (Graph-PadSoftware, San Diego, USA).

RESULTS AND DISCUSSION

The study was conducted from September 2016 to March 2017. Half of the patients were diagnosed with CML (4 people), which is 50%. All these patients showed expression of the chimeric *bcr-abl* gene, which is allowed doctors to diagnose. Also in all CML patients were observed chronic stage of the disease and leukocytosis. Four patients were diagnosed with AML (50%) in the bone marrow of which more than 45% of blast cells were detected. One of the patients with AML had

a subtype M0 with co-expression of CD7. The sex ratio was as follows: 6 females and 2 males. The mean age was 61.1 ± 10.9 years.

Nrf2 gene expression

In order to study the expression of the Nrf2 gene in leukemia cells of CML patients a

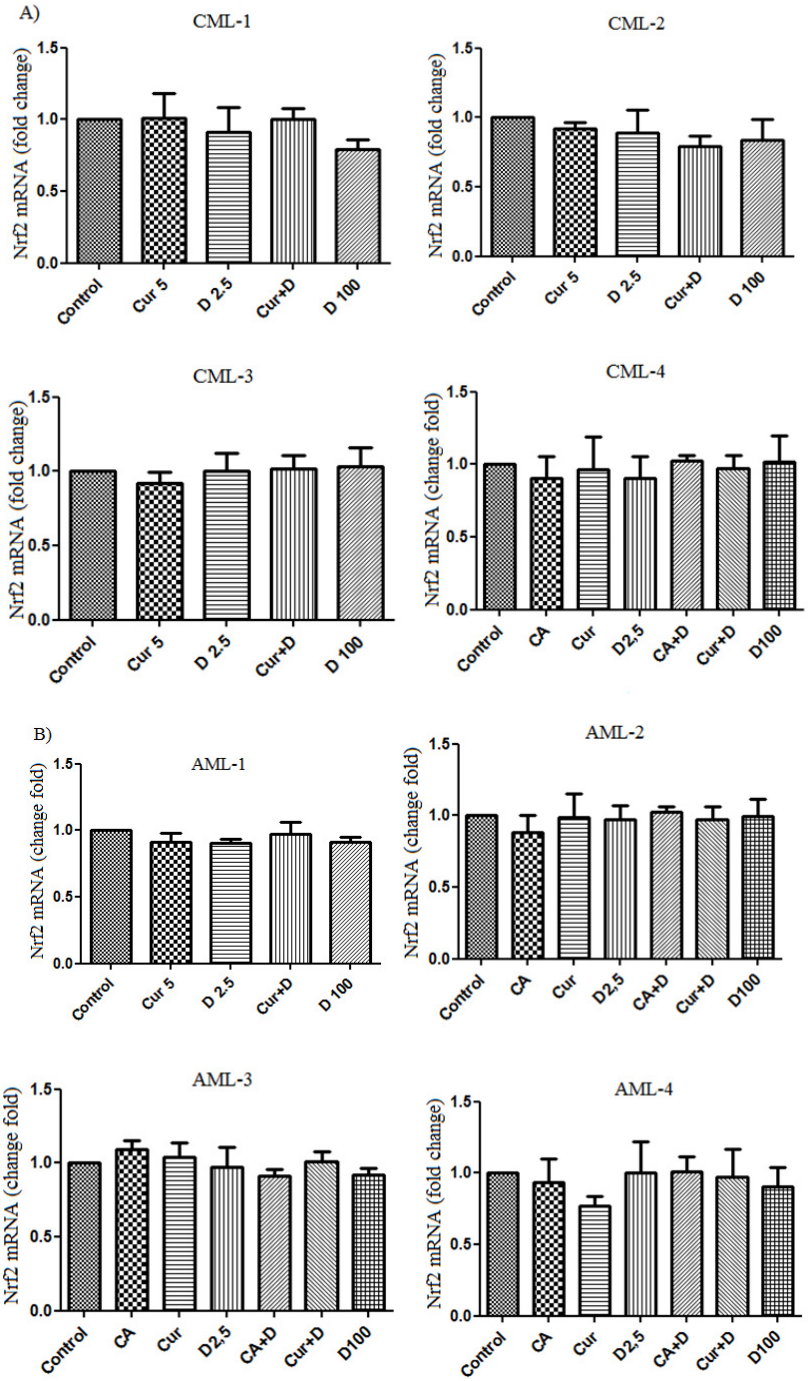

Fig. 1. Nrf2 mRNA expression level in CML (A) and AML (B) patients leukemic cells treated with CA (10 µÀM), 1,25D (2.5 nM) and 1,25D (100 nM), CUR (5 µÀM) and combinations of 1,25D (2.5 nM) with CA and CUR. Data are the means ± SD

quantitative polymerase chain reaction in real time was conducted with cDNA obtained from cells treated with test agents. The expression level the studied gene in the control group of cells was taken as 1. Expression in the remaining groups was calculated in comparison to the control. Nrf2 gene expression in all studied cell groups of all CML patients treated with 1.25D (2.5 nM), 1.25D

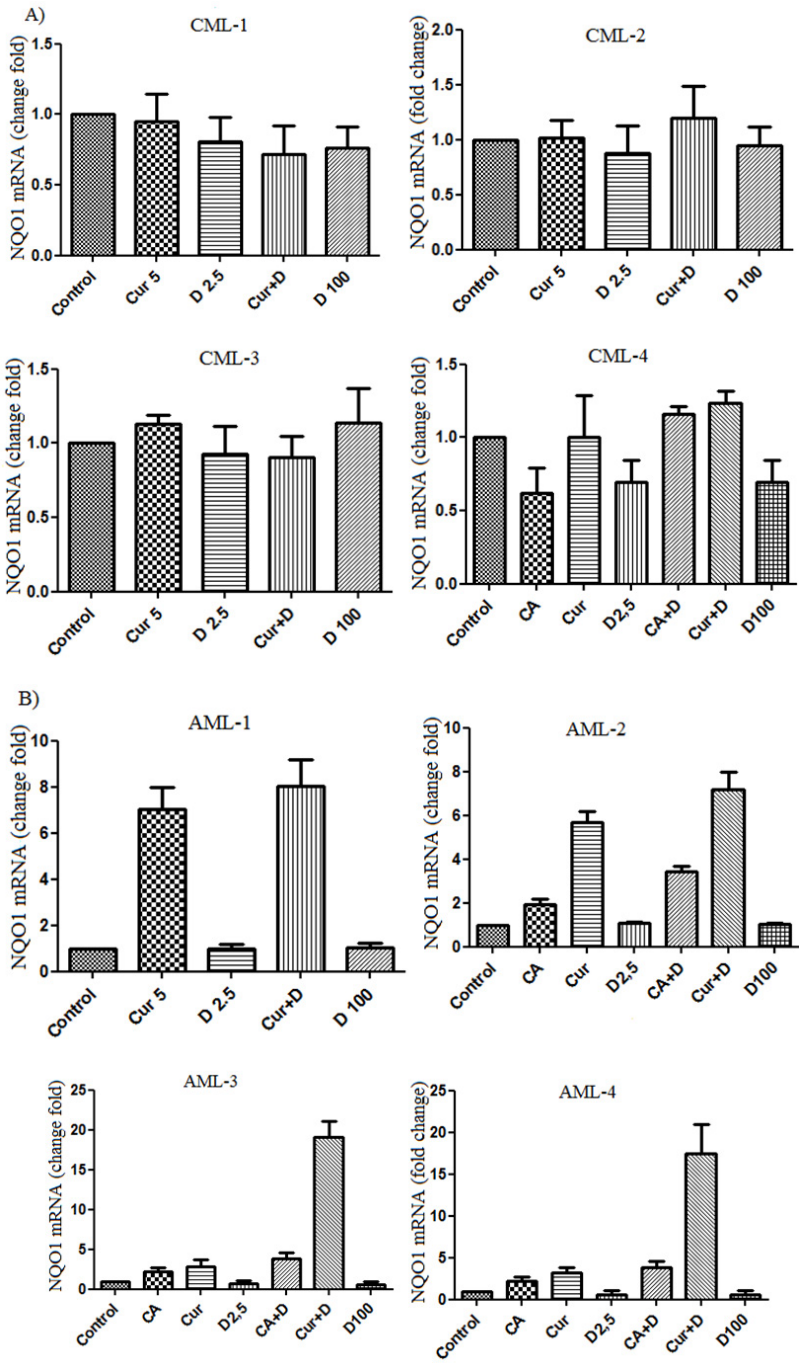

Fig. 2. NQO1 mRNA expression level in CML (A) and AML (B) patients leukemic cells treated with CA (10 µÀM), 1,25D (2.5 nM) and 1,25D (100 nM), CUR (5 µÀM) and combinations of 1,25D (2.5 nM) with CA and CUR. Data are the means ± SD

(100 nM), CUR, CA, and their combinations was approximately similar or lower than control group (Figure 1A).

Further experiments were performed with treated cells of AML patients (Figure 1B). Analysis of the data obtained from the study shows that the groups of cells treated with 1.25D (2.5 nM), 1.25D (100 nM), CUR, CA, as well as and their combinations did not respond to treatment with an increase in the level of expression of the gene being studied. The values of the expression level obtained in all groups were the same or lower in comparison with the control group. All of the above suggests that 1.25D (100 nM), CUR, CA alone and their combinations do not lead to an increase in the level of expression of the Nrf2 gene in peripheral blood mononuclear cells of AML and CML patients.

According to research of M.Danilenko, treatment of U937 cells with 1.25D does not affect the level of the Nrf2 protein, however, treatment with a combination of 1.25D and CA increased it (Huang, Nguyen & Pickett 2000; Martin et al. 2014; Xu et al. 2006). In our case, treatment with combinations of vitamin D with plant polyphenols did not show a change in the expression level of the Nrf2 gene. Our results suggest that regulation the Nrf2 gene expression occurs at the post-translational level. To confirm this hypothesis, it is necessary to study the expression level of one of the Nrf2 effectors genes. We selected the NAD(P) H gene of quinone oxidoreductase 1 (NQO1), as the induction of NQO1 gene expression occurs through the Nrf2-Keap1/ARE pathway.

NQO1 gene expression

In order to determine the NQO1 gene expression level qPCR in Real Time was performed with the same cDNA samples. The NQO1 gene expression level in CML patients' cells is shown in Figure 2 A. The analysis of the figure suggests that the expression of the gene under study did not change much in the all cell groups. Subsequent experiments were performed with groups of cells obtained from AML patients.

The results are given in Figure 2B show that the NQO1 gene expression is most pronounced in cells grown in a medium supplemented with combinations of 1.25D/CUR and 1.25D/CA. However, the cells of these patients also reacted to a slight degree to the treatment with CUR and CA. Expression in groups of cells treated with 1.25D (2.5 nM), 1.25D (100 nM) did not change in comparison with the control group.

CONCLUSION

Thus, the obtained results make it possible to conclude that the peripheral blood mononuclear cells of AML patients were more sensitive to treatment with 1.25D/CUR and 1.25D/CA and to a lesser extent and not in all patients - with CUR and CA. This study allows suggest that 1.25 D in combinations with CUR or CA do not affect the expression level of the Nrf2 gene, but affects the amount of its protein, which in turn affects the expression level of the NQO1 gene. However, the effect of the above agents on the expression of the NQO1 gene was detected only in the cells of patients with AML. The data require further studies involving more patients to obtain statistically reliable results.

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