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QUANTITATIVE REAL TIME PCR ANALYSIS OF APOPTOSIS-RELATED GENE EXPRESSION IN LEUKEMIAS IN UKRAINIAN PATIENTS

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***Background*: The complete medical consequences of the long-term exposure of population to ionizing radiation in post-Chernobyl period are still a controversial issue. The molecular biological analysis of malignant diseases of hematopoietic and lymphoid tissues in contaminated territories requires the precise diagnosis based on criteria of novel classifications. *Aim*: To analyze the relative gene expression of six apoptosis-related genes in different types of tumors of hematopoietic and lymphoid tissues in patients living in areas of Ukraine contaminated with radionuclides in post-Chernobyl period. *Material and methods*: The samples of the peripheral blood and bone marrow of 189 Ukrainian leukemia patients and 16 patients with reactive lymphocytosis were analyzed morphologically and immunocytochemically for precise delineation of the main forms and cytological variants of hematological malignancies according to new WHO classification. Expression of six apoptosis-related genes was analyzed in the individual samples of 9 different groups of malignant diseases of hematopoietic and lymphoid tissues and one group of patients with reactive lymphocytosis by quantitative RT-PCR. Expression of genes was assessed relative to that in control group of healthy donors. *Results*: Up-regulation of six analyzed apoptosis-related genes is observed in all groups of leukemia. In most groups of leukemia being analyzed, *BCL-2* up-regulation level is superior to that of *BAX*. Prominent *MYC* up-regulation is observed in B-lymphoblastic leukemia/lymphoma, non-Hodgkin's lymphoma, and T-lymphoblastic leukemia/lymphoma groups. In myelodysplastic/myeloproliferative neoplasms, the striking up-regulation of *Fas-1* and *P38MAPK* is evident. Practically all the groups of leukemia are characterized by stable high ratios of *P53* up-regulation. *Conclusion*: In Ukrainian patients, up-regulation of six analyzed apoptosis-related genes is observed practically in all types of malignant diseases of hematopoietic and lymphoid tissues under study. Microarray-based analysis of these samples would be of great importance in terms of elucidating genomic interactions in leukemias and their possible association with ionizing radiation.**
***Key Words*: leukemia, ionizing radiation, Chernobyl accident, apoptosis, gene expression.**

Chernobyl nuclear accident on April 26, 1986, led to a massive release of radionuclides into the environment. Although the vast areas of Europe were affected by Chernobyl-related ionizing radiation, the most contaminated territories are located in Ukraine, Republic of Belarus, and Russian Federation. In Ukraine, in particular, 2293 towns and villages in 74 administrative units of 12 administrative regions are considered as contaminated with radionuclides. As of the beginning of 2011, more than 2.15 million people inhabit the contaminated territories being subjected for a long time to the continuous exposure to low doses of ionizing radiation IR.

Up to present, the data on the possible medical consequences of irradiation due to Chernobyl accident are inconsistent. Nevertheless, IR is an ever-present hazard to humans primarily due to its mutagenic, carcinogenic, and cell killing ability [1]. Chromosome aberrations and gene mutations induced by IR are conventionally attributed to DNA being irreversibly changed immediately after exposure, either during the processing and enzymatic repair of the damage or during DNA replication. As malignant transformation is generally regarded as being initiated by a gene mutation or a chromosomal aberration, the initiating lesion for malignant transformation has been similarly attributed to DNA damage in a directly irradiated target cell [2]. IR is implicated in the development of some forms of acute and chronic leukemia (except for chronic lymphocytic leukemia — CLL, that until recently has not been thought to be associated with radiation exposure), multiple myeloma, primary myelofibrosis, erythremia, non-Hodgkin's lymphoma, myelodysplastic syndromes (MDS) [3] with the largest risk for acute myeloid leukemia (AML) [4].

Apoptosis plays a key role in the control of rapidly renewing tissues, such as the hematopoietic system. Leukemic cells invariably have abnormalities in one or more apoptotic pathways providing a survival advantage of these cells and the development of drug resistance. The susceptibility of normal and cancer cells to induction of apoptosis depends on the balance between proapoptotic and antiapoptotic genes. The analysis of mutations and gene expression of apoptosis-related proteins such as Bcl-2, Bax, Myc, Fas-1, p38 MAPK and p53 may be of importance in predicting the patient response to chemo- or radiotherapy as well as patient's survival. The different expression levels and post-translational modifications of these proteins may determine cell sensitivity to apoptosis.

The aim of the study was to analyze the relative gene expression of six apoptosis-related genes in different types of tumors of hematopoietic and lymphoid tissues in 189 patients living in areas of Ukraine contaminated with radionuclides in post-Chernobyl period. These results may be important to search for possible markers specific of the leukemogenic effects of IR.

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Abbreviations used: ALL – acute lymphoblastic leukemia; AML – acute myeloid leukemia; CLL – chronic lymphocytic leukemia; IR – ionizing radiation; MDS – myelodysplastic syndromes.

MATERIALS AND METHODS

The samples of the peripheral blood of 189 leukemia patients and 16 patients with reactive lymphocytosis upon fractionation in Ficoll-Verografin were obtained from R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine. All the patients were referred to the Reference Laboratory of Immunocytochemistry and Oncohematology Department of the Institute for verifying the diagnosis. Control group comprised the peripheral blood samples of 30 healthy donors from Kyiv city. The design of the study was approved by the ethic committees of both collaborating research institutions.

Bone marrow and peripheral blood smears stained by May-Grunwald-Giemsa were studied morphologically. Immunocytochemical techniques (APAAP, LSAB-AP) and a broad panel of monoclonal antibodies (MoAbs) against lineage specific, differentiation and activation antigens of leukocytes were employed for immunophenotyping pathological cells in blood and bone marrow. The main forms and cytological variants of hematological malignancies were diagnosed according to new WHO classification [5].

Total RNA was isolated from mononuclear cells of each patient using QIAamp RNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) and treated with DNase I according to the manufacturer's instructions. cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas Inc., Maryland, USA). Quantitative RT-PCR was performed at the Medical Genetics Department, Medicine Faculty of Kocaeli University, Turkey as we described previously [6, 7]. Standard curves were obtained using serial dilutions of the beta-globulin gene (DNA Control Kit, Roche). Gene-specific primers (Table 1) were obtained from Integrated DNA Technologies (Iowa, USA). Obtained gene expression values were normalized using a housekeeping gene of beta2 microglobulin. Gene expression ratios were compared in patient and control groups using REST (Relative Expression Software Tool).

Table 1. Primer sequences of the studied genes

Genes	Primer sequences
Beta2 microglobulin	(F) 5' TGA CTT TGT CAC AGC CCA AGA TA 3'
	(R) 5' AAT CCA AAT GCG GCA TCT TC 3'
BCL-2	(F) 5' AGG AAG TGA ACA TTT CGG TGA C 3'
	(R) 5' GCT CAG TTC CAG GAC CAG GC 3'
BAX	(F) 5' TGC TTC AGG GTT TCA TCC AG 3'
	(R) 5' GGC GGC AAT CAT CCT CTG 3'
MYC	(F) 5' GGC AAA AGG TCA GAG TCT GG 3'
	(R) 5' GTG CAT TTT CGG TTG TTG C 3'
FAS-1	(F) 5' CAA GGG ATT GGA ATT GAG CA 3'
	(R) 5' GAC AAA GCC ACC CCA AGT TA 3'
P38MAPK	(F) 5' GCC CAA GCC CTT GCA CAT 3'
	(R) 5' TGG TGG CAC AAA GCT GAT GAC 3'
P53	(F) 5' CTG GCC CCT GTC ATC TTC TG 3'
	(R) 5' CCG TCA TGT GCT GTG ACT GC 3'

RESULTS AND DISCUSSION

Expression of six genes (*BCL-2, BAX, MYC, FAS-1, P38MAPK, P53*) was studied in samples of 189 leukemia patients. These samples were divided into 9 different groups of malignant diseases of hematopoietic and lymphoid tissues. One further group comprises 16 patients with reactive lymphocytosis. Table 2 summarizes the specific gene expression levels of these groups.

In general, up-regulation of six analyzed genes is observed in all groups of leukemia. Low levels of down-regulation of *FAS-1* in B-cell prolymphocytic leukemia and *MYC* in T-cell leukemia from large granular lymphocytes are observed on small number of patients. Stable and high ratios of up-regulation of *P53* in all groups are conspicuous.

The high ratio of Bcl-2 to Bax proteins has been shown to confer an unfavorable prognosis with decreased rates of complete remission and overall survival. *BCL-2* down-regulation might lower the apoptotic threshold of leukemic cells and, through this mechanism, favors response to chemotherapy [8]. According to the previous studies, correlation between apoptosis and up-regulation of *BAX* gene was observed in pediatric acute lymphoblastic leukemia (ALL) samples [9]. In our study, *BCL-2* up-regulation level is superior to that of *BAX* for all groups being analyzed.

In our findings, *MYC* gene up-regulation is observed, in particular, in B-lymphoblastic leukemia/lymphoma, non-Hodgkin's lymphoma, and lymphocytosis groups. The highest values of *MYC* up-regulation are observable in T-cell lymphoblastic leukemia-lymphoma. Many forms of leukemia are associated with specific genetic events, often resulting in *MYC* proto-oncogene activation. *Myc* is very often found to be up-regulated in many types of cancers. The microarray study demonstrated up-regulation of c-*MYC* gene in B-CLL samples [10]. All these results are consistent with our findings and confirm that *MYC* gene has an important role in regulation of cell cycle.

In our study, a tendency to rising *FAS* expression in B-cell leukemia, lymphoma, and in the patients with reactive lymphocytosis is compatible with our expectations. A high rise of *FAS* expression in MDS group is remarkable. Cells from several hematological tumors are known to express Fas on their surface. Usually, in B-CLL Fas expression is not very high [11]

Table 2. Gene expression levels (relative to those in healthy controls) in cells of different types of tumors of hematopoietic and lymphoid tissues: U – up-regulation, D – down-regulation

	Number of pts	*BCL-2*	*BAX*	*MYC*	*FAS-1*	*P38MAPK*	*P53*
B-CLL/B-cell lymphoma from small lymphocytes	127	16.696 U	5.0265 U	4.15 U	4.536 U	3.263 U	20.3415 U
B-cell prolymphocytic leukemia	4	9.221 U	1.717 U	9.931 U	1.550 D	1.436 D	2567.236 U
B-cell non-Hodgkin's lymphoma	26	22.722 U	10.056 U	18.870 U	248.310 U	82.025 U	264.111 U
B-lymphoblastic leukemia/lymphoma	7	16.819 U	14.113 U	18.804 U	161.233 U	5.333 U	29.000 U
T-cell leukemia from large granular lymphocytes	4	1.936 U	1.221 U	1.324 D	1.871 U	1.166 U	3.401 U
T-lymphoblastic leukemia/lymphoma	6	227.229 U	45.443 U	210.256 U	15.043 U	2898.318 U	30828.990 U
Myelodysplastic / Myeloproliferative neoplasms	5	77.172 U	11.104 U	24.369 U	5326.603 U	1257.201 U	30.400 U
Chronic myelogenous leukemia	6	17.692 U	14.470 U	12.185 U	8.610 U	8.225 U	23.736 U
Hairy cell leukemia	4	29.243 U	12.711 U	10.883 U	9.113 U	12.623 U	69.358 U
Patients with reactive lymphocytosis	16	29.182 U	8.622 U	19.794 U	374.806 U	9.918 U	172.088 U

that coincides with our findings of relatively small up-regulation. It is of interest to note that even the low doses of radiation were sufficient to modulate Fas expression [12]. Although the relationship between Fas/FasL system and apoptosis were shown in peripheral T cells, FasL expression is practically absent in T-cell lymphoblastic lymphomas [13]. In our study the moderate level of up-regulation in T-lymphoblastic leukemia/lymphoma group was shown. In another study, in AML samples in Chernobyl clean-up workers, Fas receptor was expressed more rarely than in AML that was not associated with radiation exposure [14]. An increased Fas and FasL levels depending on the effect of irradiation was identified in B-CLL samples [15].

Up-regulation of *P53* is observed as a stable and high marker in almost all studied groups. In another study, an increase of radiation-induced P53 level was shown in several lymphoblastic cell lines [16]. Characteristic *P53* damage caused by IR was described in Chernobyl patient with Sezary syndrome [17]. *P53* gene defects stand out especially in B-CLL in multi-gene screening [18]. According to our study, all leukemia types exhibited a regular increase in expression of *P53* gene, an important protector of cells. These increased levels of gene expression should be examined in terms of diagnostic and prognostic values.

Furthermore, in our study a dramatic increase of *P38Mapk* levels is observed in B and T-cell lymphoma groups. Analysis of this gene pathway as a target area of follicular lymphoma treatment in a microarray study has been considered [19].

To summarize, up-regulation of six analyzed genes has been observed in all types of malignant diseases of hematopoietic and lymphoid tissues under study. The stable and high ratios of *P53* up-regulation in all groups are noticeable. Microarray-based analysis of these samples would be of great importance in terms of IR and genomic interactions in leukemias.

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