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Identification of the genes expression profile associated with the *ex vivo* resistance to etoposide in childhood acute leukemias*

Identyfikacja profilu ekspresji genów związanego z opornością *ex vivo* na etopozyd w dziecięcych ostrych białaczkach

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Drug resistance and the gene expression profiles might discriminate the therapy outcome, and indicate the subgroup of patients with poor prognosis. In this study we analyzed the gene expression profile in correlation with the profile of *ex vivo* resistance to etoposide in children with acute leukemias.

Methods: The *ex vivo* drug resistance profile was determined by the MTT cytotoxicity assay performed on leukemic blasts of 56 patients. Gene expression profiles were obtained from the results of hybridization of cRNA to Human Genome U133A 2.0 ologonucleotide arrays. The following analyses were performed: correlation analysis, hierarchical clustering, the assignment of location and function. Verification of data for four selected genes (*MNDA*, *GH1*, *NUDT21*, *RHOG*) was performed by quantitative real time polymerase chain reaction in the studied population and in an independent group of 54 leukemic patients.

Results: Using the permutation Spearman correlation test, a set of 233 probes/209 genes was selected. The global test confirmed the significance of the correlation of gene expression profile and resistance to etoposide ($p<0.001$). The *NUDT21* (nudix, nucleoside diphosphate linked moiety X-type, motif 21) gene showed the strongest correlation with resistance to etoposide (FDR<0.0001%).

Conclusions: Profiling of transcriptome may help in assessing the sensitivity to drugs used in chemotherapy. Resistance to etoposide is possibly associated with a change of expression of a large number of biologically important genes that influence several cellular mechanisms.

Key words: **acute lymphoblastic leukemia (ALL) • acute myeloblastic leukemia (AML) • drug resistance • etoposide • VP16 • microarrays • gene expression profile • children**

Streszczenie

Wstęp: Oporność na terapię oraz związany z nią profil ekspresji genów dyskryminują wyniki leczenia, a także wskazują podgrupy pacjentów z niekorzystnym rokowaniem. Celem badań była analiza

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profilu ekspresji genów w korelacji z profilem chemiooporności *ex vivo* na etopozyd u dzieci z ostrymi białaczkami.

Materiał/Metody: Profil oporności *ex vivo* oznaczono w teście cytotoksyczności MTT na komórkach białaczkowych pobranych od 56 pacjentów. Profil ekspresji genów opracowano na podstawie wyników hybrydyzacji cRNA do macierzy oligonukleotydowych HGU133A 2.0 Chip. Do zidentyfikowanego zestawu sond przeprowadzono analizę korelacji, grupowanie hierarchiczne, przypisanie lokalizacji i funkcji. Weryfikację danych o poziomie ekspresji dla 4 wybranych genów (*MNDA*, *GH1*, *NUDT21*, *RHOG*) przeprowadzono techniką QPCR w badanej oraz niezależnej grupie 54 pacjentów.

Wyniki: Za pomocą permutacyjnego testu korelacji Spearmana wyselekcjonowano zestaw 233 sond/209 genów. Ekspozycja blastów białaczkowych na etopozyd inicjuje złożoną odpowiedź komórkową, będącą odzwierciedleniem globalnych zmian ekspresji genów. Globalny test istotności różnic potwierdził (p<0,001) związek profilu ekspresji genów z opornością na etopozyd. Gen *NUDT21* (Nudix, nucleoside diphosphate linked moiety X-type, motif 21) wykazywał najsilniejszą korelację z opornością na etopozyd (FDR<0,0001%).

Podsumowanie: Profilowanie ekspresji genów może pomóc w ocenie wrażliwości na leki stosowane w chemioterapii dużymi dawkami. Oporność na etopozyd prawdopodobnie jest związana ze znaczną liczbą biologicznie ważnych genów i jest konsekwencją współistnienia w komórce nowotworowej różnych mechanizmów.

Słowa kluczowe: **ostra białaczka limfoblastyczna • ostra białaczka mieloblastyczna • lekooporność • etopozyd • VP-16 • mikromacierze • profil ekspresji genów • dzieci**

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Background

The most important obstacle in cancer chemotherapy is the development of resistance. Except for the overexpression of ATP-binding cassette transporters and the selection of mutated cells capable of avoiding pro-apoptotic signals, the knowledge of the resistance mechanisms to various cytostatics is still unsatisfactory. Research on the scale of the whole genome/transcriptome is being used more frequently to identify genes important for the cellular sensitivity to drugs [4,16,17,27,33,40]. This approach offers the possibility of creating a better characterization of the multigene nature as well as understanding the pathways involved in the cellular response to chemotherapeutic agents [20].

Etoposide (VePesid – VP16) is an alkaloid – a semisynthetic analogue of podophyllotoxin. As a cytostatic it is used in chemotherapy of malignant tumors such as Ewing's sarcoma, glioblastoma, lung cancer, testicular cancer, lymphoma, non-lymphocytic leukemia, osteosarcoma and erythema multiforme [5,20,25,31,39,45]. It is most commonly used in therapy combined with other drugs. Its application also includes a fixing treatment before transplantation of bone marrow or blood stem cells.

The cytostatic effect is a consequence of cell cycle arrest after a disruption of single- and double-stranded DNA [7]. It belongs to the phase-specific drugs, operating mainly in the interphase (later in the S and G2 phase as well). The primary target of the medicine is an enzyme that repairs damaged DNA – topoisomerase II. Topoisomerases regulate DNA replication and transcription, and participate in chromosome segregation, cell cycle progression and RNA metabolism [32]. VP16 inhibits DNA synthesis after the creation of complexes of enzyme and Deoxyribonucleic acid [1,32]. Formation of such complexes induces the formation of breaks in double-stranded DNA and simultaneously prevents bound topoisomerase II from repairing them [22,23]. Accumulation of DNA breaks prevents cell entry into the mitosis phase of the cell cycle, thus leading to programmed cell death [8,10]. Etoposide-induced DNA damage leads to activation of the p53-dependent pathway, which in turn is associated with changes in stimulation of tens of genes such as Waf-1/p21, PCNA, GPX and S100A2 [36,44].

For the purpose of the study an assumption was made that the *ex vivo* drug susceptibility profile combined with the gene expression profile may provide new insights into the choice of drugs used in high-dose therapy prior to transplantation of hematopoietic stem cells.

Patients and Methods

In vitro sensitivity test. Bone marrow samples were collected from 56 pediatric patients (43 *de novo* ALL, 8 relapse

Table 1. Characteristics of patient group analyzed with microarray and QPCR technique

	AL	Microarray group				Validation group			
		Total	Sensitive	Intermediate	Resistant	Total	Sensitive	Intermediate	Resistant
Patients		56	16	20	20	54	4	28	22
	ALL	43	14	16	13	44	4	26	14
	rALL	8	2	3	3	7	0	2	5
	AML	5	1	1	3	3	0	2	1
Age (median)		8 yrs	9 yrs	9 yrs	5 yrs	7.5 yrs	6 yrs	7 yrs	8 yrs
	ALL	7 yrs	9 yrs	8 yrs	5 yrs	7 yrs	6 yrs	5 yrs	8 yrs
	rALL	11.5 yrs	12 yrs	14 yrs	9 yrs	9 yrs	–	14 yrs	6 yrs
	AML	11 yrs	11 yrs	18 yrs	8 yrs	14 yrs	–	16.5 yrs	5 month
Sex		29F: 27M	8F: 8M	12F: 8M	9F: 11M	22F: 29M	2F: 2M	9F: 18M	12F: 11M
	ALL	22F: 21M	7F: 7M	11F: 5M	4F: 9M	19F: 25M	2F: 2M	9F: 14M	8F: 9M
	rALL	2F: 6M	1F: 1M	0F: 3M	1F: 2M	3F: 4M	–	0F: 2M	3F: 2M
	AML	5F: 0M	1F: 0M	1F: 0M	3F: 0M	1F: 2M	–	0F: 2M	1F: 0M

AL – leukemia type; ALL – acute lymphoblastic leukemia; AML – acute myeloblastic leukemia; rALL – ALL relapse; F – female; M – male.

ALL, 5 AML *de novo*) and analyzed (Table 1). Patients with relapsed ALL and AML were entered into the study in order to increase the size of the group resistant to etoposide. At the same time, differences in expression between different subtypes of leukemia were not investigated (due to the very large disparities in the number of patients with ALL, relapsed ALL and AML). The profile of *ex vivo* resistance and sensitivity to etoposide (Bristol-Myers Squibb) was determined in a 4-daily cytotoxicity assay (MTT). The essence of the test is to reduce the yellow soluble bromide 3 [4.5-dimethyl-2-yl]-2.5-2,5-diphenyl tetrazolium to blue insoluble formazan. This reaction is characteristic for living cells. As a measure of resistance to etoposide the LC50 parameter was adopted (lethal concentration of the drug, in which 50% of the cells underwent apoptosis). Concentration used in the test was 0.048–50 mg/ml. Cells were classified as sensitive if the LC50 value was ≤0.04 mg/ml, or as resistant when the LC50 was ≥1.7 mg/ml.

Preparation of RNA. Samples (6–10×10^6 isolated blasts) were homogenized in RLT (Qiagen) buffer. Cell lysates were stored at –80°C. Total RNA was extracted using Trizol (Invitrogen) and then digested with DNase I (Fermentas). RNA integrity assessment was based on the RIN coefficient obtained after a capillary electrophoresis was performed (Agilent 2100 Bioanalyzer).

Microarray analysis. The procedure for nucleic acid preparation was carried out in accordance with the recommendations of the manufacturer (Affymetrix), and based on sets of reagents for hybridization reactions appropriate for the selected type of microarray. The baseline in each test was 5 µg of total RNA as a template. Fragmented and labeled cRNA was subjected to hybridization for Human Genome U133A 2.0 microarrays HG-U133A 2.0 Chip (Affymetrix).

Quantitative real time polymerase chain reaction (QRT-PCR). In order to confirm the data derived from microarray technique, for 4 selected genes (*MNDA*, *GH1*, *NUDT21*, *RHOG*) a verification by real time PCR was conducted. The synthesis of cDNA library and quantitative PCRs reactions were performed according to the manufacturer's reagents recommendation (Roche Diagnostics). Reactions were carried out in a Mastercycler ep realplex thermocycler (Eppendorf). To analyze the relative expression levels the REST© (QIAGEN) program was used. The expression of selected genes was evaluated in a study group and in an independent group of 54 patients.

Statistical analysis. Arrays were grouped according to the MTT assay resistance to etoposide profile. For each gene an expression median was defined. The median was calculated in 4 analyzed groups (sensitive, moderately sensitive, moderately resistant, resistant). As a measure of changes in the level of gene expression, the median ratio between resistant and sensitive patients was adopted. Comparative analysis was performed on the basis of the algorithms implemented in the MAS 5.0 program (Affymetrix). For each gene, the correlation coefficient (Spearman test), the significance of the difference (Wilcoxon test) and the significance adjusted for multiple comparisons (FDR, the Benjamin and Hochberg test) were calculated.

Results

Selection of relevant genes. Genes were ranked by fold change in expression level as up-regulation or down-regulation. Using the permuted Spearman correlation test, 233 probe sets significantly correlated with sensitivity to etoposide ($p<0.001$) were selected. In the global test of significance of differences, the probability of obtaining such a number of genes is low ($R=0.008$), which confirms the significance of the observed difference.

For 150 sets of probes/134 genes an increase in expression was observed ($p<0.001$, FDR<0.09, correlation coefficient from 0.418 to 0.625). The biggest changes in the level of expression were observed for the following genes: RAB32 (member of RAS oncogene family), HPGDS (hematopoietic prostaglandin D synthase), XCL1 (chemokine (C motif) ligand 1), CSF3R (colony stimulating factor 3 receptor (granulocyte)), PTGS2 (prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)) and CSTA (cystatin A (stefin A)). For 83 sets of probes/75 genes the expression silencing was observed ($p<0.001$, FDR <0.09, correlation coefficient from –0.418 to

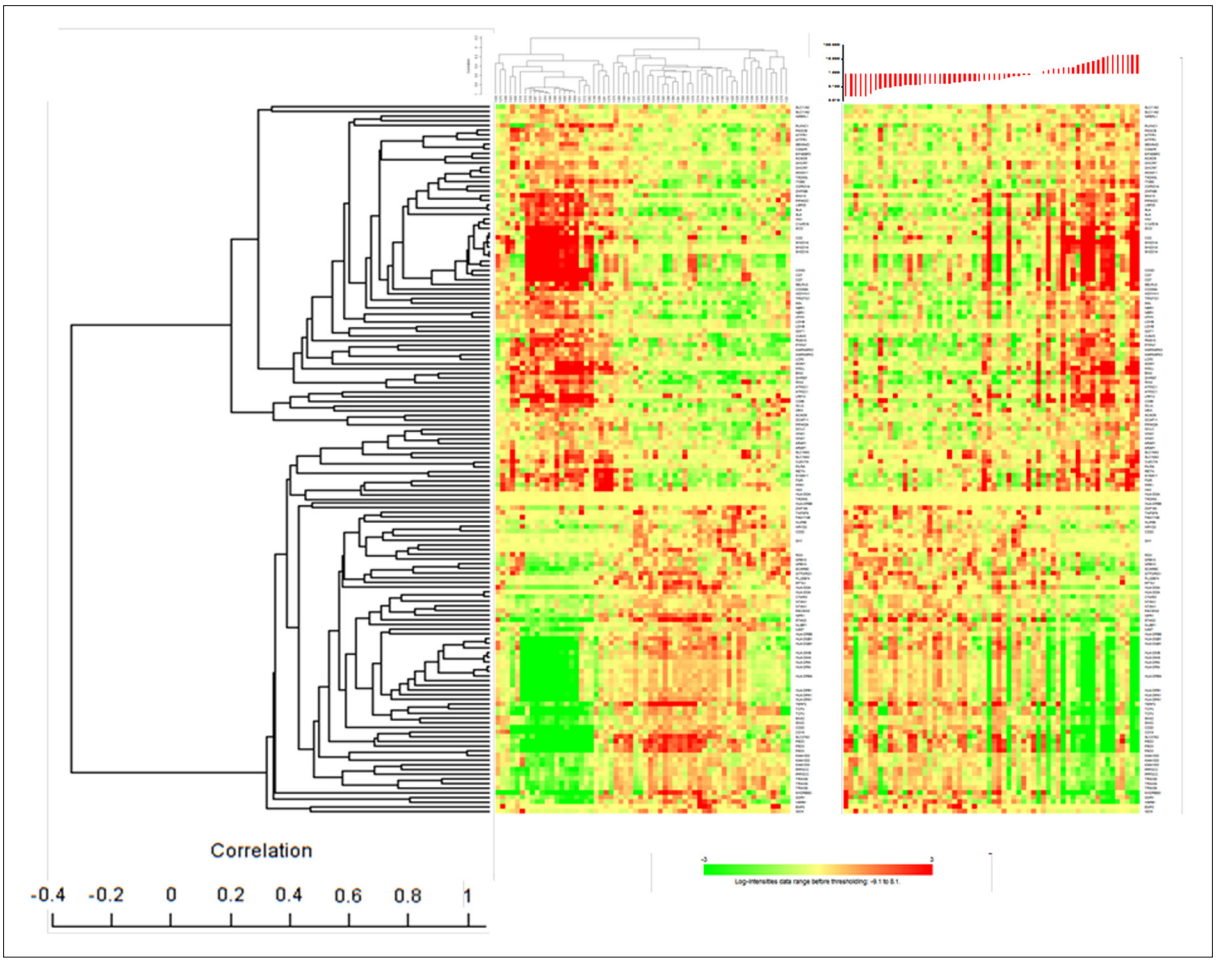

Figure 1. Supervised hierarchical clustering discriminating etoposide resistant and sensitive ALL patients (genes grouped in rows, patients in the columns). Left side – shows the grouping according to the similarity of the expression, right side – contains a grouping of patients by increasing the LC50 values

–0.611). The largest decrease in expression level was observed for: SMARCA4 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4), CELSR2 (cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)), TCF3 (transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)), QRSL1 (glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1), VASH2 (vasohibin 2), TPD52 (tumor protein D52), QRSL1 (glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1) and PLXNB1 (plexin B1).

Hierarchical clustering. On the resulting set of discriminatory genes a cluster analysis was performed (Figure 1). The set of tested probe was used for hierarchical clustering of both genes and patients. The results of this analysis, illustrated in the form of a dendrogram, are shown in Figure 1. On the basis of this analysis it was concluded that a specific set of genes shows a significant expression dependence on the value of chemoresistance of the samples.

Ontological analysis of molecular functions. Detailed analysis of each functional class within a designated set of genes revealed that the genes most strongly represented in the profile were those associated with nucleic acids (16.8%), receptor genes (13.2%) and genes participating in signal transduction (5.3%).

Diverse expression of genes was also observed for enzymes performing functions associated with the metabolism of proteins such as kinases, hydrolases and proteases. A decrease in expression levels was observed for genes of RNA binding proteins and for genes involved in post-transcriptional mRNA splicing such as *NUDT21* (nudix (nucleoside diphosphate linked moiety X)-type motif 21), FUSIP1 (FUS interacting protein (serine/arginine-rich) 1), SFRS3 (splicing factor, arginine/serine-rich 3). Stronger expression silencing (by approximately 40%) was observed in the case of 4 helicases: BRIP1 (BRCA1 interacting protein C-terminal helicase 1), DHX9, 34 (DEAH (Asp-Glu-Ala-His) box polypeptide 9, 34) and SMARCA4 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4). Topoisomerase II, a gene for the target protein for etoposide, also belongs to the analyzed functional group. For the TOP2B gene (topoisomerase (DNA) II beta 180 kDa) the analysis showed results indicating that the amount of transcripts in resistant cells is reduced by 10%. Changes in the transcription level show a strong correlation with the profile of resistance to VP16 (p=8.43 E-05, FDR=0.032).

Among the genes performing receptor functions, the biggest changes were observed for the following receptor genes: G protein-bound, cytokines and immunoglobulins.

Table 2. Statement of functional classes for which it has been observed 2-fold genes overrepresentation (molecular function)

GO ID	GO class	Observed	Expected	Observed/Expected
GO: 0030983	mismatched DNA binding	7	0.38	18.5
GO: 0003690	double-stranded DNA binding	8	1.27	6.31
GO: 0051082	unfolded protein binding	6	1.72	3.49
GO: 0019900	kinase binding	7	2.18	3.22
GO: 0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	6	2.10	2.86
GO: 0019901	protein kinase binding	5	1.76	2.84
GO: 0030414	peptidase inhibitor activity	8	2.95	2.71
GO: 0004866	endopeptidase inhibitor activity	5	1.99	2.52
GO: 0008022	protein C-terminus binding	5	2.00	2.49

GO – gene ontology.

The diverse expression within the class of genes performing regulatory functions focused mainly on genes of G proteins and their modulators. Overexpression was observed for small GTPases such as RAP2B (RAP2B, member of RAS oncogene family), RAB31 (RAB31, member of RAS oncogene family), *RHOG* (ras homolog gene family, member G (rho G)), RAB27A (RAB27A, member of RAS oncogene family), RAB32 (RAB32, member of RAS oncogene family) and their regulators: RGS10 (regulator of G-protein signaling 10), RIN3 (Ras and Rab interactor 3), HP (haptoglobin), REPS2 (RALBP1 associated Eps domain containing 2), ARAP1 (centaurin, delta 2). RAB32 and RIN3 belong to a group of genes for which the highest fold change in expression level ($p<0.001$, FDR<0.06) was obtained, which suggests a strong relationship with the profile of resistance to VP16.

The analysis of molecular functions in the resulting list of genes was narrowed down so as to select only classes where at least a double overrepresentation of genes – in relation to the number of expected genes based on the frequency of a given group of genes on the used oligonucleotide microarray – occurred. The results indicate that the most significant changes correlating with resistance to VP16 apply to genes performing functions associated with DNA metabolism and protein modifications (Table 2).

Analysis of biological processes. Classification of selected genes according to their participation in known biological processes showed that the largest group consisted of genes associated with signal transduction (23.7%), metabolism and protein modifications (20%), nucleic acids metabolism (17.9%), and immune processes (18.4%).

Among signal transducers, over a dozen genes were identified, including genes involved in cell communication, signal transduction from surface receptors and intracellular cascades. Overexpression was observed mainly for 9 genes involved in cell adhesion, 8 genes involved in signal transduction of chemokines and cytokines, 7 genes associated with the path of signals involving G proteins and 5 genes of the JAK-STAT cascade.

Diverse changes in expression level were observed for genes involved in regulating mRNA transcription, which accounted for the largest percentage of nucleic acid metabolizing genes. DNA metabolism genes including regulators of replication and damaged helix repair enzymes' genes, were numerously represented in the profile. For repair genes such as CSNK1E (casein kinase 1, epsilon), BRIP1 (BRCA1 interacting protein C-terminal helicase 1), ATR (ataxia telangiectasia and Rad3 related), and MSH3, 6 (mutS homologue 3, 6 (*E. coli*)), a decline in the level of expression was observed.

Analysis of cellular pathways. The strongest association and the largest number of genes were obtained for a trail associated with inflammatory processes, which are associated with chemokines and cytokines. The following genes belonging to this pathway were identified: ITGAM (integrin, alpha M (complement component receptor 3, alpha, also known as CD11b (P170), macrophage antigen alpha polypeptide)), STAT3 (signal transducer and activator of transcription 3 (acute-phase response factor)), IL8RB (interleukin 8 receptor, beta), FPR1 (formyl peptide receptor 1), FGR (Gardner-Rasheed feline sarcoma viral (v-FGR) oncogene homolog), ALOX5AP (arachidonate 5-lipoxygenase-activating protein), CISH (cytokine inducible SH2-containing protein), STAT4 (signal transducer and activator of transcription 4), ITGB2 (integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1, macrophage antigen 1 (mac-1) beta subunit)), IKBKB (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta).

Five overexpressed genes belonging to the integrin pathway were also identified, including the ITGB2 gene (integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1, macrophage antigen 1 (mac-1) beta subunit)). The increased activity of beta 2 integrin is associated with the reduction of drug-induced DNA damage reduction of caused by etoposide activity. Expression of this gene showed a strong correlation with the profile of resistance at high significance ($p=8.07$ E-05, FDR=0.032, $R=0.492$), which confirms the significance of the observed change.

Discussion

Studies in cell lines indicate that the causes of cellular insensitivity to etoposide may be overexpression of MDR-1 and anti-apoptotic BCL-2 genes, as well as topoisomerase IIα expression silencing [28]. It was shown that in cells resistant to etoposide an increase in drug efflux from cells and avoiding the apoptosis associated with BCL occurs. In cells insensitive to VP16, chromosomal instability and polyploidy induction were observed [9,28,29]. Another reason may be the changes in topoisomerase activity associated with changes in the phosphorylation of the enzyme (in a catalytic site) [3,9,10,12,26,34]. Changes in the cellular localization of the enzyme can also affect cellular resistance. Phosphorylation of the end of C terminus of the nuclear form of topoisomerase IIα plays a role in protein translocation into the cytoplasm [4]. The decline of the nuclear form affects the reduction of DNA damage under the influence of etoposide [1,30]. Cells with a resistant phenotype are also characterized by a decrease in intracellular calcium ion concentration [3]. Another cause of inhibition of the therapeutic effect of etoposide is the reduction of after drug-induced DNA damage, associated with β1 integrin pathway cell adhesion. Resistance associated with this pathway may be a consequence of the reduction in the amount of DNA strand breaks and increased cellular tolerance for these types of damage [15]. One of the mechanisms of cell response to VP16 is hypoxia and the associated variable expression of tens of genes, allowing the inhibition of p53-dependent apoptosis [37]. Changes in the level of expression of oncogenes from the RAS and SRC family have an indirect impact on the sensitivity of cells to VP16 [24,38].

In order to identify genetic mechanisms leading to the emergence of resistance in etoposide-induced leukemic blasts, expression arrays were used, which allowed us to define the changes in the level of gene expression. The study shows that the most significant changes of expression, correlating with resistance to VP16, apply to genes performing functions associated with DNA metabolism and protein modifications. Within the resistance profile a significant percentage consists of genes involved in the regulation of mRNA transcription and DNA metabolism genes, including those controlling replication as well as those belonging to the double helix damage repair enzymes. Reduction of gene expression was observed for RNA-binding proteins, helicases, and genes participating in post-transcriptional mRNA splicing. In the group of nucleic acid metabolizing genes we also observed changes in gene expression levels for the target protein for etoposide – topoisomerase II. A strong correlation with the profile of resistance was shown by a decrease in TOP2B gene (topoisomerase (DNA) II beta 180 kDa) expression. The consequence of lowering the level of topoisomerase II in cells is the reduction of the amount of so-called fissile complexes (covalent connections between enzyme and double-stranded DNA), which become susceptible to stabilization by etoposide. A cell with a smaller pool of target molecules for cytostatic is less susceptible to its effects.

Silencing of expression was also observed for a group of genes involved in DNA strand repair, such as CSNK1E, BRIP1, ATR, MSH3 and MSH6. Cysteine kinase 1 is one of the enzymes that affect the activity of topoisomerase II. It is also responsible for the phosphorylation of the enzyme in a catalytic spot in a Ser1106 position [3,9,10]. This post-translational modification is required for full activation of topoisomerase. Reduction of expression of CSNK1E causes a decrease in the amount of active enzyme and indirectly reduces the efficiency of VP16. Ten genes involved in protein phosphorylation and proteolysis were identified within the resistance profile, which suggests a significant effect of such modification of proteins in leukemic cells' defense mechanism against the cytostatic effect of etoposide.

Changes of expression in regulatory genes and their protein products from the BCL-2/BAX family are believed to be the cause of apoptosis abnormalities in cancer cells. Within the resistance profile to etoposide an increased expression of BCL2A1 (BCL2-related protein A1, p=1.70 E-06, FDR=0.00541, R=0.583) was observed. Overexpression of BCL-2 is related to resistance to many chemotherapeutic agents acting through the induction of programmed death of cancer cells. Silencing of BCL-2 significantly enhances the sensitivity of breast cancer cells to etoposide and doxorubicin [35]. Research by Hong et al. [18] showed a significant increase in expression of BCL-2 in leukemic line HL-60 cells with a multi-drug resistant phenotype. Bednarek et al. [2] confirmed that the combination of etoposide with the inhibitor gene (oligonucleotide anti-BCL-2) significantly increases the therapeutic effect by reducing cell proliferation.

Several genes from the signal transduction pathway connected with integrins were identified within our analyzed profile. Increased activity of integrins is associated with the reduction in after drug-induced DNA strand damage induced by etoposide [19]. The role of overexpression of genes for integrins in resistance to cytostatics has been previously demonstrated in studies on liver tumor by Zhang et al. [46]. It has also been proven that intracellular signaling pathways, associated with integrins, modulate Fas T lymphocyte-dependent apoptosis [11]. Overexpression of genes of this group in resistant leukemic blasts shows that they may play an important role in inhibition of the programmed death pathway.

Microarray technology (expression, cDNA, miRNA) is increasingly being used in studies aimed at identification of new markers of resistance of tumor cells to etoposide [5,13,14,21,25,43,45]. Several genes from our profile in common with the analysis of resistance to etoposide in cell lines described by Györffy et al. [14] were identified. In compared profiles, large groups of genes were genes of kinases and endopeptidases, and genes related to the metabolism of nucleic acids. Maximum compliance, both in terms of the number of selected genes and their functions, were found for the analysis conducted by Györffy et al. [14], although those studies were carried out on cell lines.

Recent studies of the past few years, performed using microarray technology, have led to the identification of new candidate genes responsible for cell resistance to etoposide. Re-expression of transcription factor TWIST2 resulted in increased sensitivity of lymphoblasts to this chemotherapeutic agent [41]. De Tayrac et al. [5] suggested PCDH9 and STARD13 as candidate tumor suppressor

genes, participating in resistance to etoposide, probably through changes in ceramide signaling to the RhoA pathway. Lawson et al. [25] indicated 2 genes as key genes for resistance to etoposide in small cell lung cancer: the DNA repair enzyme DNA polymerase β (Pol β) and the neuroendocrine transcription factor NKX2.2. Inhibition of Pol β potentiates the cytotoxicity of etoposide, by promotion of DNA double strand breaks. Resistance to etoposide in SCLC cell lines was related to higher levels of NKX2.2. Wong et al. [44] reported that changes in expression of the TOP2B gene are associated with histological grading, microvascular invasion, early age of onset of the malignancy response to etoposide and survival in hepatocellular carcinoma.

Despite the presence of the used HG-U133A 2.0 probe arrays for known genes linked to resistance (e.g., 93 probes for the ABC family of transporters, including MDR1 and MRP1), significant changes in their expression correlating with resistance to etoposide have not been found. Intensive studies of various mechanisms of resistance to therapy (including known multidrug resistance genes) prove their limited relevance, especially in the establishment of *de novo* drug resistance [6,42]. These studies stand in opposition to results presented by Iijima et al. [5], Walters et al. [42] and Wong et al. [44], which indicate a strong association of changes in expression level of ABC family genes with the lack of sensitivity of tumor cells to etoposide.

Studies on the scale of the whole genome or transcriptome are a relatively objective approach to identifying genetic determinants of cell responses to drugs and their resistance to them. The identification of genes contributing to resistance to etoposide and determining the role of those genes could help in characterizing patient responsiveness and overcoming resistance in children with leukemia.

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