VILNIUS UNIVERSITY

Miglė Janeliūnienė

THE SIGNIFICANCE OF ABERRANT PHENOTYPES AND MULTIDRUG RESISTANCE FOR EVALUATION OF THE DYNAMICS OF MINIMAL RESIDUAL DISEASE IN ACUTE LEUKEMIA PATIENTS

Summary of the Doctoral Dissertation
Biomedical sciences, medicine (06 B)

Vilnius, 2012

Dissertation has been prepared in the Department of Physiology, Biochemistry and Laboratory Medicine of Vilnius University Faculty of Medicine during the period of 2004 -2011.

Scientific supervisor:

Zita Aušrelė Kučinskienė, M.D., Ph.D., Professor habil. (Vilnius University, biomedical sciences, medicine – 06B).

Scientific consultant:

Laimonas Griškevičius, M.D., Ph.D., Professor (Vilnius University, biomedical sciences, medicine – 06B).

Dissertation to be defended at the Medical Research Council of Vilnius University Faculty of Medicine:

Chairman:

Prof. Janina Didžiapetrienė (Vilnius University, Biomedical sciences, Medicine – 06 B)

Members:

Assoc. Prof. Aušrinė Barakauskienė (Vilnius University, Biomedical sciences, Medicine – 06 B)
Dr. Astra Vitkauskienė (Lithuanian University of Health Sciences, Biomedical sciences, Medicine – 06 B)
Dr. Eiva Bernotienė (State Research Institute, Innovative Medicine Center, Biomedical sciences, Medicine – 06 B)
Dr. Radvilė Malickaitė (Vilnius University, Biomedical sciences, Medicine – 06 B)

Opponents:

Prof. Dainius Characiejus (Vilnius University, Biomedical sciences, Medicine – 06B)
Dr. Audronė Eidukaitė (State Research Institute, Innovative Medicine Center, Biomedical sciences, Medicine – 06 B)

The official defense of the dissertation will be held at the open session of the Medical Research Council on February 29, 2012 at 14:00 in the Red Conference Hall of Vilnius University Hospital Santariškių Klinikos. Address: Santariškių str. 2, LT-08661, Vilnius, Lithuania.

A summary of dissertation was distributed on January 29, 2012.
The dissertation in full text is available in the library of Vilnius University. Address: Universiteto str. 3, LT-01122, Vilnius, Lithuania.

VILNIAUS UNIVERSITETAS

Miglė Janeliūnienė

SERGANČIŲJŲ ŪMINE LEUKEMIJA ABERANTINIŲ FENOTIPŲ IR DAUGINIO ATSPARUMO VAISTAMS NUSTATYMAS IR SVARBA VERTINANT MINIMALIOS LIEKAMOSIOS LIGOS DINAMIKĄ

Disertacijos santrauka
Biomedicinos mokslai, medicina (06 B)

Vilnius, 2012

Disertacija rengta 2004 – 2011 metais Vilniaus universiteto Medicinos fakulteto Fiziologijos, biochemijos ir laboratorinės medicinos katedroje.

Mokslinis vadovas :

Prof. habil. dr. Zita Aušrelė Kučinskienė (Vilniaus universitetas, biomedicinos mokslai, medicina – 06B).

Mokslinis konsultantas:

Prof. dr. Laimonas Griškevičius (Vilniaus universitetas, biomedicinos mokslai, medicina – 06B).

Disertacija bus ginama Vilniaus universiteto Medicinos mokslo krypties taryboje:

Pirmininkas:

Prof. dr. Janina Didžiapetrienė (Vilniaus universitetas, biomedicinos mokslai, medicina – 06 B)

Nariai:

Doc. dr. Aušrinė Barakauskienė (Vilniaus universitetas, biomedicinos mokslai, medicina – 06 B)

Dr. Astra Vitkauskienė (Lietuvos sveikatos mokslų universitetas, biomedicinos mokslai, medicina – 06B)

Dr. Eiva Bernotienė (Valstybinis mokslinių tyrimų institutas Inovatyvios medicinos centras, biomedicinos mokslai, medicina – 06 B)

Dr. Radvilė Malickaitė (Vilniaus universitetas, biomedicinos mokslai, medicina – 06 B)

Oponentai:

Prof. dr. Dainius Characiejus (Vilniaus universitetas, biomedicinos mokslai, medicina – 06 B)

Dr. Audronė Eidukaitė (Valstybinis mokslinių tyrimų institutas Inovatyvios medicinos centras, biomedicinos mokslai, medicina – 06 B)

Disertacija bus ginama viešame Medicinos mokslo krypties tarybos posėdyje 2012 m. vasario 29 d. 14:00 val. Vilniaus universiteto ligoninės Santariškių klinikų Raudonojoje auditorijoje. Adresas: Santariškių 2, LT-08661, Vilnius, Lietuva.

Disertacijos santrauka išsiuntinėta 2012 m. sausio 29 dieną.
Disertaciją galima peržiūrėti Vilniaus universiteto bibliotekoje. Adresas: Universiteto 3, LT-01122, Vilnius, Lietuva.

CONTENT

ABBREVATIONS………………………………………………………………… 6
1. INTRODUCTION……………………………………………………………… 7
1.1. Study problem………………………………………………………… 7
1.2 Objective of study …………………………………………………… 9
1.3. Tasks of study ………………………………………………………… 9
1.4. Statements to defend…………………………………………………… 9
1.5. Novelty and importance of study……………………………………… 10
2. MATERIALS AND METHODS………………………………………….. 11
2.1. Study design………… ……………………………………………... 11
2.2. Investigative population……………………………………………... 12
2.3. Methodology of flow cytometric assays…………………………….. 12
2.3.1. Assessment of aberrant leukemia associated phenotypes………… 12
2.3.2 Investigation of minimal residual disease ………………………… 14
2.3.3 Assessment of multidrug resistance ……………………………… 15
2.4. Statistical methods……..……………………………………………….. 16
3. RESULTS…………………………………………………………………….. 17
3.1. Incidence of leukemia associated phenotypes …………………….…..... 17
3.2. Optimization of minimal residual disease detection ……………........... 19
3.2.1. Selection of markers for minimal residual disease panels……….. 20
3.2.2. Selection of sample preparation technique and the dilution experiment…………………………………………………….. 20
3.2.3. Data analysis system development……………………………… 21
3.2.4. Analysis of healthy bone marrow………………………………… 22
3.2.5. Stability of leukemia associated phenotypes…………………….. 24
3.3. Influence of leukemia associated phenotypes to the dynamics of minimal residual disease……………………………………………………… 25
3.4. Optimization of multidrug resistance detection………………………… 29
3.4.1. Examination of normal bone marrow…………………………... 29
3.4.2. Correlation between structural and functional MDR parameters…. 31
3.4.3. Multidrug resistance parameters in different leukemia subtypes…. 32
3.5 Influence of aberrant leukemia associated phenotypes to the level of multidrug resistance…………………………………………………… 33
3.6. Association of MDR parameters with a level of minimal residual disease 33
4. CONCLUSIONS……………………………………………………………… 34
5. PRACTICAL RECOMMENDATIONS……………………………………… 35
LIST OF PUBLICATIONS ON THE TOPIC OF THE DISSERTATION……... 36
ABOUT THE AUTHOR……………………………………………………… 37
SUMMARY IN LITHUANIAN………………………………………………… 38
REFERENCES……………………………………………………………….. 44

ABBREVIATIONS

AL – acute leukemia
ALL – acute lymphoblastic leukemia
AML – acute myeloid leukemia
AML/MD - acute myeloid leukemia with minimal differentiation
AML/M – acute myeloid leukemia with maturation
AMMoL – acute myelomonocytic leukemia
AMoL – acute monocytic leukemia
AML/MDS – acute myeloid leukemia with myelodyspastic features
AML/NOS – acute myeloid leukemia, not otherwise classified
AML/WM - acute myeloid leukemia without maturation
APC – allophycocyanin
B-ALL – acute B-lymphoblastic leukemia
CD – cluster of differentiation
CNS – central nervous system
DNR – deoxyribonucleic acid
FAB – French American British system for classification of acute leukemia
FITC – fluoresceine isothiocyanate
HLA – human leukocyte antigens
LAP – leukemia associated aberrant phenotype
MDR – multidrug resistance
MDS – myelodyspastic syndrome
MLL – mixed lineage leukemia
MPO – myeloperoxydase
MRD – minimal residual disease
MRP1 – multidrug resistance protein 1
NK cells – natural killer cells
PBS – phosphate buffered saline
PCR – polymerase chain reaction
PE - phycoerythrin
PerCP – peridinine chlorophyll protein
p-gp – permeability glycoprotein
RNR – ribonucleic acid
T-ALL – acute T-lymphoblastic leukemia
TdT – terminal deoxynucleotidyl transferase
WHO – World Health Organisation

1. Introduction

1.1. Study problem

Acute leukemia is a neoplasm of a hematopoietic system, characterized by uncontrolled proliferation of immature blood cells (blasts) in bone marrow and blood. It is a heterogeneous group of disorders with a rapid progression of the disease and clinical signs attributed to a suppression normal hematopoiesis. Acute leukemia is divided into two main categories - acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) [1, 2].

According to the National Cancer Institute (US), the incidence of acute myelogenous leukemia, which is the most common form of acute leukemia in the adult population, in 2004 - 2008 was 3.5 per 100 000 inhabitants. Mortality from this disease was 2.8 per 100 000. The overall five-year survival in 2001 - 2007 was 22.6%. Incidence of acute lymphoblastic leukemia, the most prevalent malignant disease of children, in 2004 - 2008 was 1.7 per 100 000 and mortality rate was 0.5 per 100 000 inhabitants. A five-year survival for ALL in 2001 - 2007 was 64.4%. In children, this rate is even higher and usually is above 85% [3, 4].

Modern treatment of acute leukemia is based on a combination of chemotherapeutic agents with different chemical structure and action mechanisms. For the induction of ALL treatment vincristine, asparaginase and corticosteroids are used. Once the clinical and morphological remission is achieved consolidation therapy which includes medications that do not show cross-resistance to drugs used for induction (cytarabine, methotrexate, anthracyclines, alkylating agents, epipodophylotoxins) is given for several months. Later less intensive maintenance therapy with 6-mercaptopurine and methotrexate is applied for up to 36 months. For the induction therapy of AML cytarabine, a pyrimidine analog and one of the anthracyclines are given. After achieving the remission, treatment with high-dose cytarabine is given. Depending on risk factors for some ALL and most AML patients allogeneic hematopoietic stem cell transplantation is considered [5-7].

However, intensive treatment of acute leukemias has many side effects. Early side effects arise from the rapid disintegration of tumor cells and the destruction of cells of skin, gastrointestinal epithelium, pancreas, bone marrow and other tissues. Late side effects include infertility, liver and lung damage, osteoporosis or even secondary tumors. So because of numerous side effects the most intensive treatment is not the option suitable for all patients. With a wide consolidation treatment options, patients are divided into risk groups: low-risk patients are given a less intensive and high risk patients are given a more intensive treatment or even allogeneic hematopoietic stem cell transplantation [5-8].

Over the past decade, a completely individualized treatment based on biological properties of each patient's tumor cells became available. Medications, directly reducing multiple drug resistance, for example, Valspodar (PSC 833) can be included in the treatment regimens. Since the end of the eighties immunotherapy has been used. Chemotherapy drugs are conjugated with antibodies against the target antigens on cancer cell membrane, for example, Rituximab (anti-CD20) or gemtuzumab (anti-CD33). Cancer vaccines made from an individual patient's cancer cells may be used to enhance the immune response. Treatment that acts on a “target” principle is focused on the

processes that affect cancer cell growth, division, spread and natural death. For example, Imatinib, which blocks tumor cell growth signals, acts against the BCR-ABL hybrid protein and is widely used to treat chronic myelogenous leukemia [9, 10].

To chose an optimal for each patient treatment method objective and reliable indicators that allow to assign a particular patient to a certain risk group are needed. White blood count was a first laboratory parameter used to stratify patients to risk groups. With a development of laboratory equipment more sophisticated techniques were used to predict course of leukemia. Certain immunophenotypic features detected by flow cytometry were proven to have a predictive value. Also genetic abnormalities were shown as strong indicators of the aggressiveness of the disease. What's more, studies of gene expression profile and proteomics are started to use for identification of new biomarkers and potential therapeutic targets [11-13].

Taking into account the sensitivity and the applicability for clinical samples two principle methods, multiparameter flow cytometry and polymerase chain reaction are used both to predict the course of acute leukemia and to evaluate actual reduction of its mass. The advantage of flow cytometry is the speed of test (results are obtained within a few hours), a moderate cost, sufficient sensitivity (detection of one cell with a specific phenotype among 10,000 or even 100,000 others), the ability to analyze several parameters of each cell, the possibility to investigate cell viability. With the development of flow cytometric technologies, the number of cell parameters that could be simultaneously analyzed on a single cell increases. On the one hand, multiparameter flow cytometry allows to determine even very small quantities of cells, which may be called "rare events" (minimal residual disease, circulating plasma cells, lymphoma cells or even cells of solid tumors). On the other hand, the discovery of new cell subpopulations (leukemic subpopulations, leukemic stem cells) became possible [14].

The role of flow cytometry in diagnostics of acute leukemia is not any more questionable. Flow cytometric features of blasts are used as diagnostic criteria in WHO classification of acute leukemia. The value of flow cytometry in prognostics of this disease is still under investigation. One of most widely studied flow cytometric assays used to predict a relapse of acute leukemia patients is minimal residual disease detection. Minimal residual disease is a submicroscopic level of leukemic blasts that can be found in bone marrow of a patient in remission. It is proven that a level of minimal residual disease and speed of its reduction can help to predict relapses and to stratify patients to different risk groups [15]. Yet the methodology of the assay is not unified: different laboratories use different sample preparation techniques, different antibody combinations and data analysis strategies. One of tasks of this study was to create a standardized, evidence based and cost effective method of flow cytometric minimal residual disease estimation.

Detection of minimal residual disease is based on identification of leukemia associated phenotypes. These are phenotypes found on leukemic cells, but not on normal hematopoietic precursors. Leukemia associated phenotypes also can be used as predictors of the course of leukemia even at the time of diagnosis. Some of them are associated with certain genetic abnormalities (e.g., CD19+CD56+ AML is associated with t(8;21), others can be independent prognostic factors (e.g. CD7+ in AML). Until now only single phenotypic abnormalities of leukemic cells and their predictive value have been investigated [16, 17]. In this study we analyzed all main leukemia associated phenotypes of B-ALL, T-ALL and AML.

Further more, flow cytometry can help to look inside mechanisms of persistence of leukemic cells. Multidrug resistance is the main reason of treatment failure. The principle mechanism of multidrug resistance is the extrusion of chemotherapeutic agents from the malignant cell through the ATP dependent channels. In case of acute leukemia two main MDR related membrane "pumps" (p-gp and MRP1) are shown to have a predictive value. The external epitopes of these proteins can be detected by flow cytometry using fluorochrome labeled monoclonal antibodies. Also the activity of MDR proteins can be evaluated, directly measuring the accumulation or extrusion of fluorescent dyes with similar structure to chemotherapeutic agents [18].

In this work we united all three flow cytometric prognostic elements: we evaluated the impact of leukemia associated phenotypes to the development of multidrug resistance and to the level of minimal residual disease during treatment.

1.2. Objective of the study

The objective of this study was to identify aberrant leukemia associated phenotypes and multidrug resistance in acute leukemia patients and to evaluate the influence of these parameters to the dynamics of minimal residual disease.

1.3. Tasks of the study

1) To identify the prevalence of aberrant leukemia associated phenotypes in patients with acute leukemia.
2) To optimize the methodology of a flow cytometric assay for the detection of minimal residual disease.
3) To evaluate the influence of aberrant leukemia associated phenotypes to the number of blasts at early stages of treatment (the dynamics of minimal residual disease).
4) To optimize the assessment of multidrug resistance by flow cytometry.
5) To evaluate the influence of aberrant leukemia associated phenotypes to the level of multidrug resistance.
6) To evaluate the correlation of multiple drug resistance and the number of blasts in early stages of treatment.

1.4. Statements to defend

1) The phenotype of acute leukemias is not identical to the phenotype of normal hematopoietic precursors of the same cell line and maturational stage, in most cases aberrant leukemia associated phenotypes are found.
2) Some aberrant phenotypes are associated with the presence of multidrug resistance and through this pathway lead to the higher levels of minimal residual disease.

1.5. Novelty and importance of the study

The prevalence of the leukemia-associated phenotypes in Lithuanian population of patients with acute leukemia was examined and these data were compared with the prevalence of leukemia associated phenotypes described in literature. This information is relevant assessing the applicability of flow cytometry in investigation of minimal residual disease which is one of the most important factors predicting the course of acute leukemia.

We examined how certain phenotypic characteristics of blasts are associated with the actual leukemia reduction process, which can be estimated evaluating the dynamics of minimal residual disease. So far, only the predictive value of single leukemia associated phenotypes has been studied and it was estimated by survival rates of patients, which can be influenced by other factors (patient's general condition, functioning of his organ systems).

An original standardized minimal residual disease assay, the principle steps of which are the selection of multicolor antibody combinations based on the analysis of leukemia associated phenotypes, lysed whole blood sample preparation technique and a unified system of the analysis was developed. The bone marrow of healthy subjects was investigated and the phenotypes of normal hematopoietic precursors were described as well, as the incidence of leukemia associated phenotypes was estimated. Analysis of healthy bone marrow in order to detect the pathways of normal hematopoietic maturation is now very intensively carried out worldwide. Our data complement a rapidly growing base of information on phenotypes of normal hematopoietic cells in different maturity stages.

Multidrug resistance of leukemic cells is the major reason for their persistence and a subsequent relapse of the disease. Its estimation by laboratory methods is now also gaining importance. The expression of different proteins responsible for drug resistance, their function and a genetic basis is studied. Some researchers have tried to link multiple drug resistance with a level of leukemia's maturation, eg, CD34+ phenotype or leukemic stem cell detection. However, we were the first to link all major leukemia associated phenotypes with the multidrug resistance and the response to treatment. Were systematically analyzed how leukemia associated phenotypes are associated with the detection of multidrug resistance *in vitro*, and how these features reveal themselves *in vivo,* how effectively the number of blasts decreases during the treatment.

Once the correlation of leukemia associated phenotypes and the dynamics of minimal residual disease is demonstrated, useful prognostic information at the diagnosis moment without significant additional costs could be provided. Multidrug resistance testing could help to explain an accurate blast survival mechanism, to predict and to modify it. New medicines directly lowering multidrug resistance appear in the market. Multidrug resistance assays could help to identify groups of patients who would benefit from them.

In the perspective of globalization and an increasing mobility of patients the standardization of novel and sophisticated flow cytometric techniques is important in order to provide objective, independent from the investigator assessment of the results and their comparability between different institutions.

2. Materials and methods

2.1. Study design

This is a prospective analytical study. We tried to verify the hypothesis that aberrant leukemia associated phenotypes showing "distancing" of leukemic cells from healthy hematopoietic progenitor cells have an impact on the course of the disease: some of them might be related to multidrug resistances, and through this mechanism affect the dynamics of minimal residual disease.

All of the three variables (leukemia associated phenotypes, multidrug resistance and minimal residual disease) were examined by the flow cytometric method.

At the moment of diagnostics of acute leukemia 18 B-ALL, 18 T-ALL and 25 AML leukemia associated phenotypes were assessed. According to the presence or absence of each leukemia associated phenotype patients were divided into two groups. For every group the average values of multidrug resistance parameters (p-gp, MRP1 and calcein fluorescence) were calculated. Also for every group an average number of blasts at different treatment stages was estimated (at the diagnosis moment, during the induction of chemotherapy, after the induction, during the consolidation, after the consolidation, and in 4th, 5th and 6th month of treatment). Average values of multidrug resistance parameters and number of blasts at different stages of treatment were compared by statistical methods.

Tests were carried out form patients' and healthy individuals' bone marrow samples, anticoagulated with K_3EDTA. All investigations were carried out at the Laboratory of Hematology and General Cytology of Vilnius University Hospital Santariškių Klinikos.

Assays were performed according the following scheme:

1) At the moment of diagnosis of acute leukemia:
   a. Blast immunophenotype studies:
      - The establishment of phenotypic diagnosis of leukemia,
      - Assessment of aberrant leukemia associated phenotypes.
   b. Multiple drug resistance testing:
      - Analysis of p-gp and MRP1 expression,
      - Calcein test for the evaluation of MDR function.
2) Minimal residual disease studies:
   a. For patients with acute lymphoblastic leukemia:
      - During the induction of intensive therapy (11-15 days),
      - After the induction (29-35 days),
      - During the consolidation of therapy (36-76 days),
      - After the consolidation (77-90 days),
      - 4th, 5th and 6th months of treatment
   b. For patients with acute myelogenous leukemia: after the first induction and the 1st, 2nd, 3rd, 4th, 5th, 6th month of treatment according to the clinical situation.

2.2. Investigative population

114 patients with newly diagnosed acute leukemia, during the period of 2005 – 2009 admitted to the Center of hematology, oncology and transfusion of Vilnius University Hospital Santariškių Klinikos and the Center of oncohematology of Vilnius University Children Hospital were enrolled in the study. 35 patients had B-ALL (28 adults, mean age 33.6 y.o., 7 children, mean age 3.4 y.o.), 29 patients had T-ALL (14 adults, mean age 28. y.o., 15 children, mean age 8.2 y.o.) and 50 patients had AML (all adults, mean age 44.6 y.o.). Only patients who were eligible for an intensive chemotherapy with a purpose to cure the disease and those whose minimal residual disease was planned to monitor were included. If for some reason (the patient's death, treatment failure or lack of phenotypic aberrations) minimal residual disease was not possible to estimate, the patient was excluded from the study. The control group was formed from 12 healthy bone marrow donors (mean age 34,4 y.o.). The study received the agreement from Lithuanian Bioethics Committee.

2.3. Methodology of flow cytometric assays

2.3.1. Assessment of aberrant leukemia associated phenotypes

Leukemia associated phenotypes were evaluated along with the definition of phenotypic diagnosis. Samples were prepared by lysed whole blood technique. To stain cell surface antigens 100 µl of anticoagulated bone marrow were incubated with 20 µl of pretitrated (1:4) antibodies for 20 minutes in the dark at room temperature. Then 100 µl of lysing medium (*BD FACS Lysing solution*) were added and samples were incubated for 15 minutes in order to lyse red cells. Afterwards cells were washed in 3 ml of a wash buffer (*BD CellWash*) at 350 g for 5 minutes. If needed, 100 µl of permeabilization medium *(BD FACS Permeabilizing solution 2)* and 20 µl of pretitrated (1:10) antibodies for intracellular or intranuclear staining were added and samples were incubated for 20 minutes followed by another wash in 3ml of a the same wash buffer at 350 g for 5 minutes. After the aspiration of a supernatant cells were resuspended in 500 µl of prediluted (1X) paraphormaldehyde based *CellFix (BD)* solution. Samples were analyzed within 24 hours with *FACS Canto* flow cytometers and *FACS Diva* software. Marker panel applied at the time of diagnosis is presented in table 1.

Table 1. Marker panel for the diagnostics of acute leukemia.

Markers	CD classification
Early and non specific markers	CD34, CD38, CD45, CD99, CD117, HLA-DR, TdT, CD10, CD56
Myeloid markers	CD13, CD14, CD15, CD33, CD11c, CD64, CD66c, MPO
T-lymphoid markers	CD1a, CD2, CD3, CD4, CD5, CD7, CD8
B-lymphoid markers	CD19, CD20, CD22, CD79a, IgM
Megakaryocytic markers	CD41a, CD61
Erythroid markers	CD71, CD235a

Leukemia associated phenotype was considered as existing in a blast population when it was found in more than 50% of cells. Evaluating aberrancies in the intensity of marker expression, the fluorescence of isotypic control was considered as a negative threshold and the fluorescence of normal cells of the same origin as blasts was considered as a positive threshold. The list of aberrant phenotypes is presented in table 2.

Table 2. List of evaluated leukemia associated aberrant phenotypes.

	Asynchronous antigen expression	**Antigen hypo-expression**	**Antigen hyper-expression**	**Cross lineage antigen expression**	**Ectopic antigen expression**
B-ALL	CD10++TdT+ CD10- TdT- CD10- CD34+ CD10++CD20++ CD22++CD34+ CD45-TdT- CD45-CD34- CD10-CD20-CD34+	CD10 CD22 CD38 CD45 TdT	CD10 CD34	CD13 CD33 CD15	-
T-ALL	-	CD2 sCD3 cCD3 CD5 CD7 CD45	CD7	CD13 CD33 CD56 CD117	TdT CD34 CD99 CD10 CD1a CD4+CD8+ CD4-CD8-
AML	CD34+HLA-DR- CD117+CD33+HLA-DR- CD117+CD34-CD15- CD34+CD11c+ CD34+CD15+ CD34+CD14+ CD34+CD56+ CD34+CD64+ CD117+CD11c+ CD117+CD64+ CD117+CD56+ CD45 bl CD4+ CD13+CD33+ CD56+	CD13 CD33 CD38 HLA-DR	CD33 CD34	CD2 CD5 CD7 CD19 CD22 TdT	-

2.3.2. Investigation of minimal residual disease

The original standardized minimal residual disease assay was developed. Samples were prepared by lysed whole blood technique. Original four color (for ALL patients) and six color (for AML patients) marker panels were applied (tables 3, 4, 5).

Table 3. Marker panel for B-ALL minimal residual disease detection.

FITC	PE	PerCP-Cy5.5	APC
TdT	CD10	CD34	CD19
CD38	CD10	CD20	CD19
TdT	CD38	CD34	CD19
TdT	CD22	CD34	CD19
TdT	CD19	CD34	CD45

Table 4. Marker panel for T-ALL minimal residual disease detection.

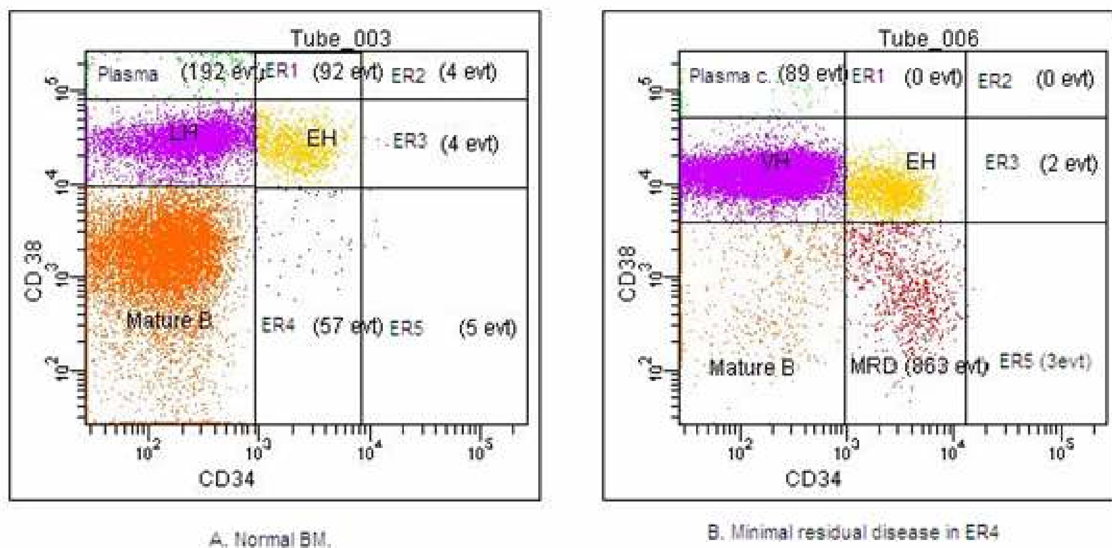
FITC	PE	PerCP-Cy5.5	APC
TdT	CD7	citoplasmic CD3	CD19
CD99	CD7	citoplasmic CD3	CD2

Table 5. Marker panel for AML minimal residual disease detection.

FITC	PE	PerCP-Cy5,5	APC	PE-Cy7	APC-H7
CD33	CD117	CD34	CD11c	CD56	CD45
CD33	CD117	CD34	CD13	CD56	CD45
CD15	CD117	CD34	CD33		CD45
CD64	CD117	CD34	CD33	CD38	CD45
HLA-DR	CD117	CD34	CD33	CD4	CD45
CD7	CD5	CD34	CD33	CD2	CD45
TdT	CD22	CD34	CD33	CD19	CD45

Acquiring the sample up to 1 million cells were collected or acquisition was stopped when sample ran out (at least 100,000 events needed for an informative resut). For ALL a “live – gate” was set on cells expressing CD19 or cytoplasmic CD3. Analysis was performed using the original standard "empty region" strategy. At population consisting of ten cells found in one or more empty regions and in whole or in part consistent with the diagnostic phenotype was referred as minimal residual disease (fig.1). The result was calculated dividing the number of cells evaluated as leukemic to the total number of collected bone marrow cells and expressed as a percentage.

Fig. 1. Minimal residual disease detection analysis: “empty region” method (B-ALL).



A. Normal BM.

B. Minimal residual disease in ER4

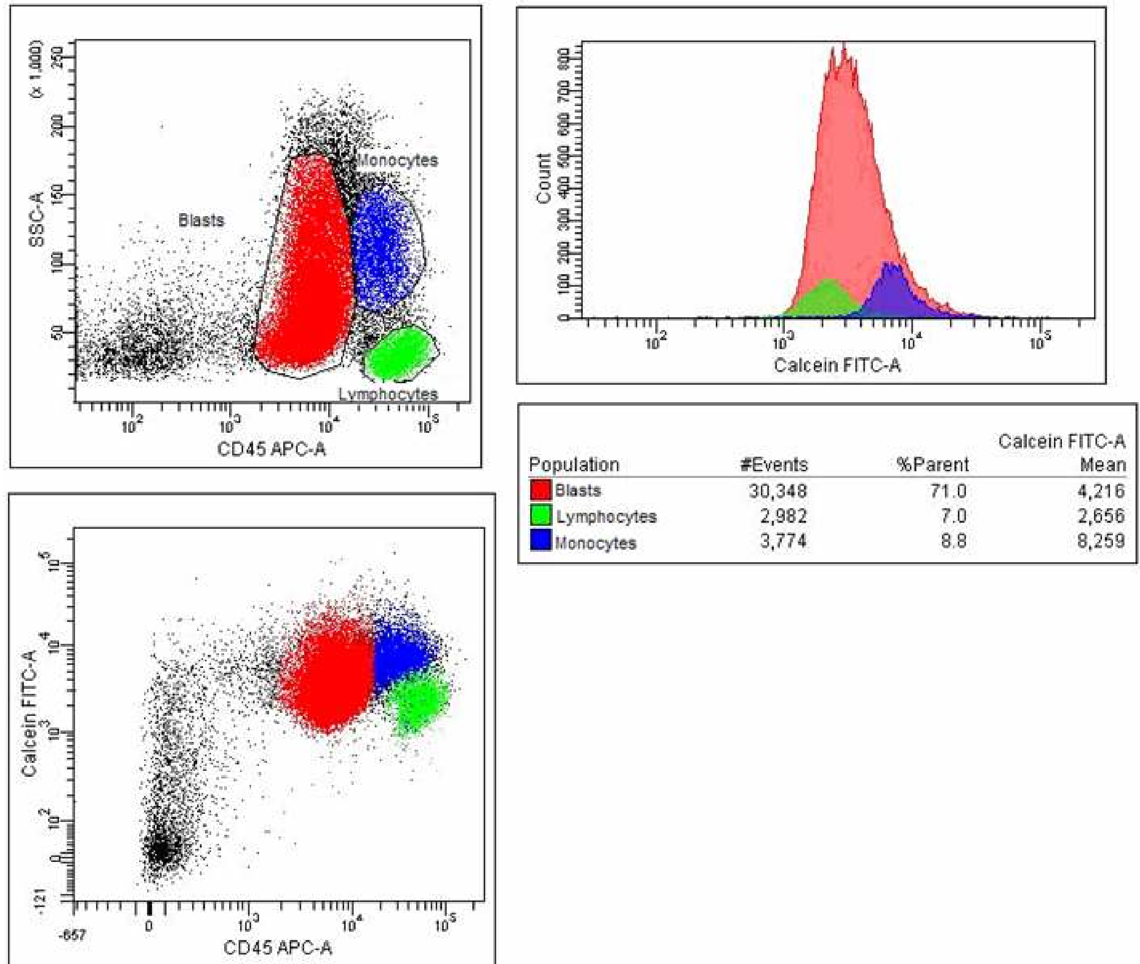
The expression of every antigen was divided into three levels: negative/dim, positive, bright. 9 regions were formed, some of them were filled with normal cells, other were referred as “empty regions” (ER). In picture B minimal residual disease is found in region ER4.

2.3.3. Assessment of multidrug resistance

For multidrug resistance assays samples were prepared using mononuclear density separation technique. Bone marrow sample was diluted 2:1 with *PBS* buffer. 4 ml of *Lymphoprep* (*Nycomed*) medium was placed in 15 ml conical tubes and 6 ml of diluted bone marrow was overlaid on the top. Tubes were centrifuged for 30 minutes at 400 g in room temperature. Mononuclear layer was aspirated and washed twice in *BD CellWash* for 10 minutes at 300 g. Cell count was measured with a hematological analyzer and a cell suspension containing approximately 10 000 cells per µl prepared.

100 ml of prepared cell suspension was put into three test tubes. 20 µl of 1:10 pretitrated APC labeled monoclonal antibody against human CD45 (BD, USA) were added to each tube and samples were incubated for 20 minutes at room temperature. Then 20 µl of 1:10 pretitrated calcein AM solution were put and cells incubated for exactly 10 minutes at 37^0C temperature. Afterwards the reaction of calcein accumulation was terminated by rapid centrifugation (1 min 2000 g, 15 sec acceleration and deceleration). The supernatant was discarded and the cells were resuspended in 0.5 ml reaction buffer with propidium iodide. The samples were tested immediately after preparation. 50 000 events were collected from each tube. Mean calcein fluorescence intensity was determined in a viable (negative for propidium iodide) blast cell population and average value of all three tubes was calculated (fig.2).

Fig.2. Detection of calcein fluorescence in blast population.



P-gp and MRP1 expression was investigated by direct immunofluorescence staining, the same way, as markers for leukemia diagnostics. PE labeled antibodies against P-gp (clone 17F9, Pharmingen, USA) and FITC labeled antibodies against MRP1 (clone QCRL-3, Pharmingen, USA) were used. Mean fluorescence intensities of both markers were determined.

2.4. Statistical methods

Statistical analysis was carried out on SPSS for Windows v.12.0 program. The influence of aberrant leukemia associated phenotypes to the level of multidrug resistance and to the size of minimal residual disease was studied using Mann-Whitney-Wilcoxon test. Correlation between multidrug-resistance parameters and the size of minimal residual disease as well as between different indices of multidrug resistance was estimated using Spearman rank correlation coefficient. The results of the dilution experiment when two sample groups were prepared by different sample preparation techniques was evaluated applying paired (dependent samples) t-test. Multidrug resistance parameters of acute leukemia patients and healthy individuals were compared by independent sample t-test. ANOVA test was used to examine whether there was a

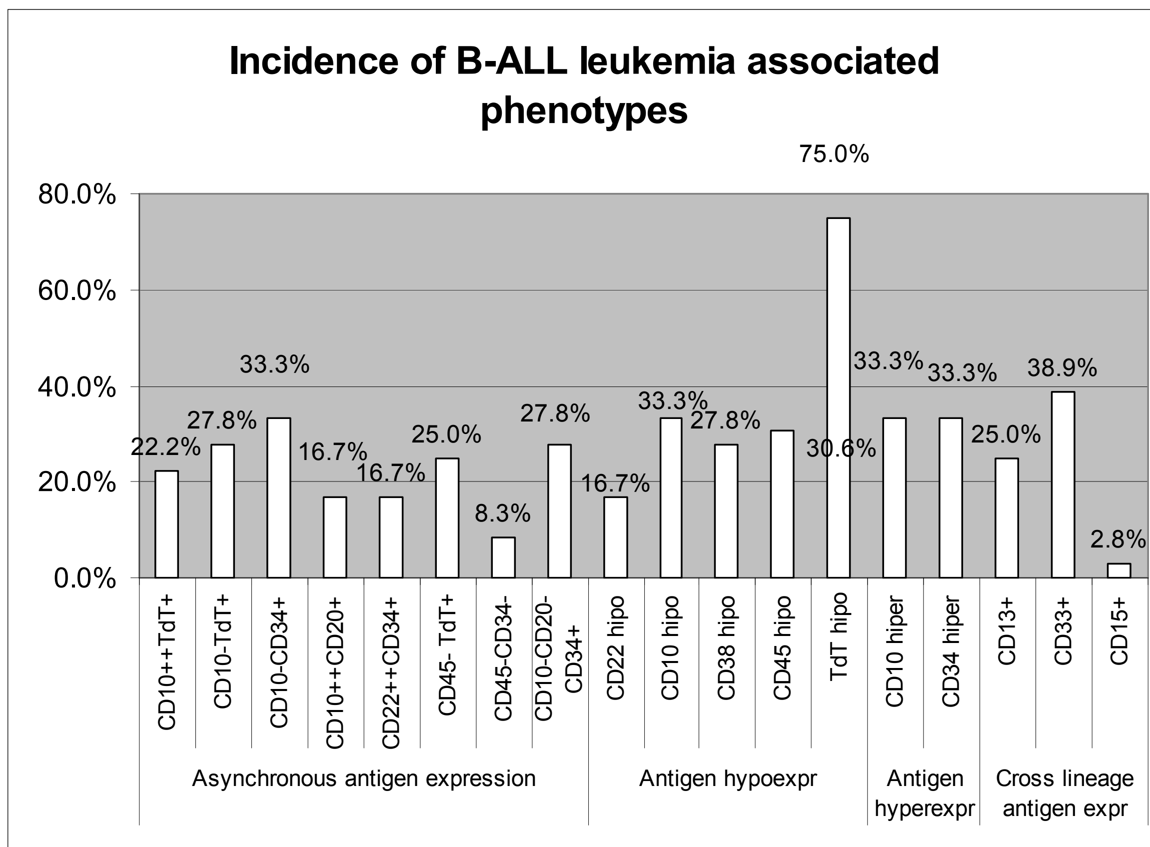
statistically significant difference in drug resistance between patients with different acute leukemia types.

3. Results

3.1. Incidence of leukemia associated phenotypes

At least one phenotypic aberration was found in all studied patients with B-ALL (in average 5.1, from 1 to 10). The most common group of leukemia associated phenotypes was antigen hypoexpression. In 75% of cases TdT hypoexpression was found. A significant number of patients had a decreased expression of CD10, CD38 and CD45 (33.3%, 27.8%, 31%). Coexpression of myeloid marker CD33 was detected in 38.9% of cases. Other frequent findings were asynchronous phenotype CD10-CD34+, hyperexpression of CD34 and CD10 (33.3%). All leukemia associated phenotypes are presented in the graph (Figure 3).

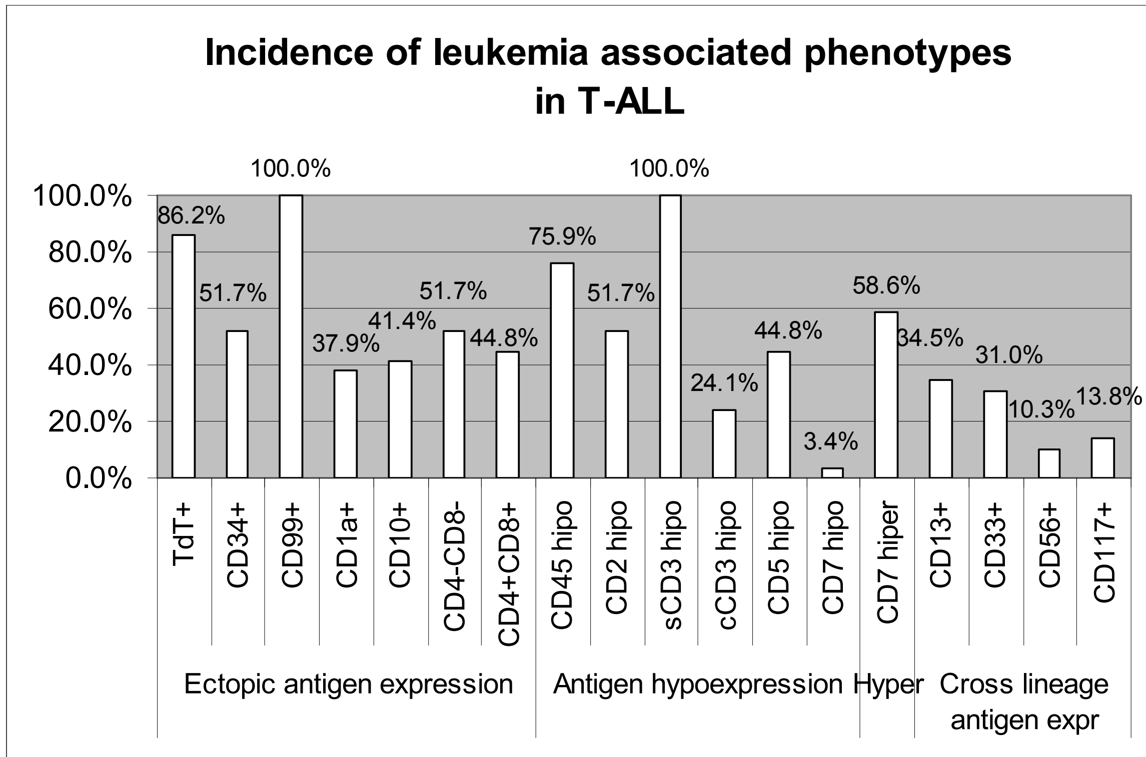
Fig 3. Incidence of B-ALL leukemia associated phenotypes.



In T-ALL number of phenotypic aberrations was even higher: on average 8.6 aberrations were found per patient (from 5 to 12). The most common phenotypic aberrations belonged to the group of ectopic marker expression: early differentiation marker TdT was found in T-lymphoid cell population in 86.2% and CD99 was detected in 100% of cases. Another marker of early differentiation CD34 expression was detected

in slightly more than half of patients (51.7%). Another common aberration group was antigen hypoexpression, for example, CD45 and CD3 markers were hypoexpressed in 75.9% and 100% of cases respectively. All detected T-ALL leukemia associated phenotypes are presented the graph (Figure 4).

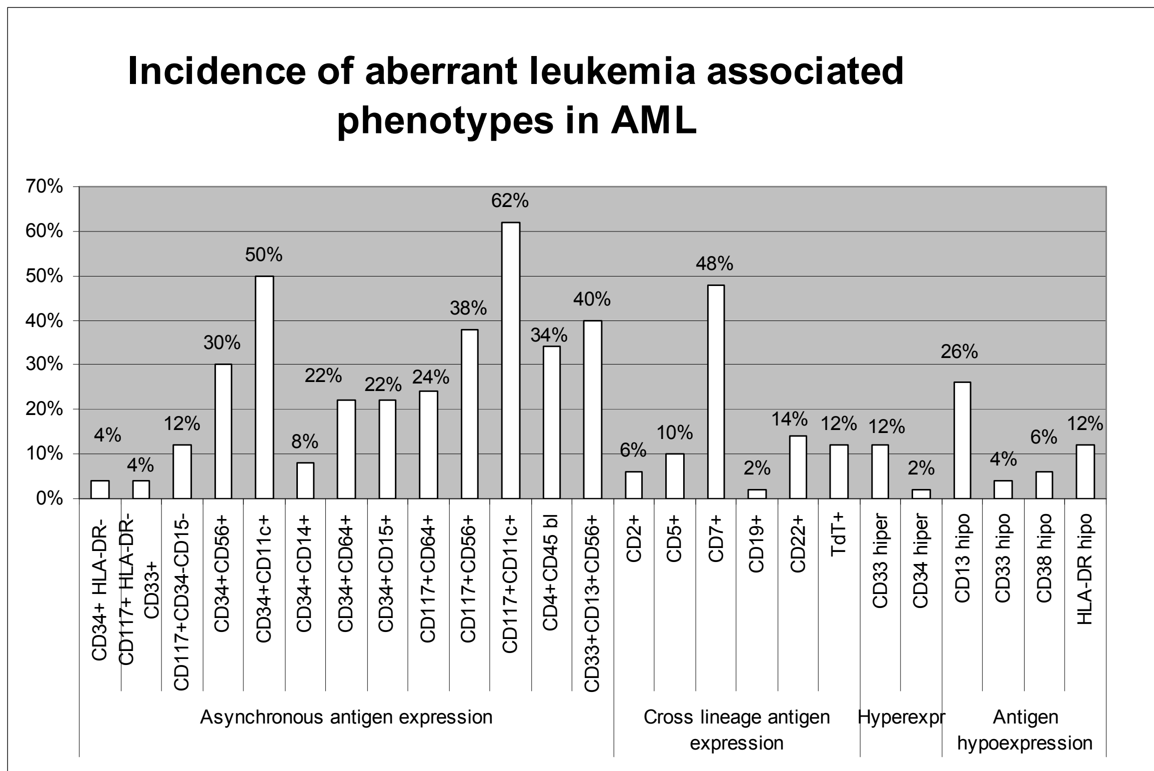
Fig. 4. Incidence of leukemia associated phenotypes in T-ALL.



At least one of the phenotypic aberration was found in 71.4% of AML patients (averagely 5.0, from 1 to 11). Patients with acute myelogenous leukemia are characterized by a high diversity of phenotypic aberrations. In contrast to patients with acute lymphoblastic leukemias, the predominant group of phenotypes in AML patients was asynchronous expression of markers. Expression of marker CD11c, which appears during late stages of myeloid maturation, with early markers CD117 and CD34 was found in about 62% and 50% of AML cases. Marker CD56 is not typical of normal myelopoiesis, but it is often found in granulocyte or monocyte population in non leukemic states, including those associated with chemotherapy. In addition, although associated with NK cells, this marker is not specific to them and belongs to the group of adhesion molecules. Analyzing leukemia associated phenotypes we ascribed expression of this marker to asynchronous expression. CD56 expression with CD34 marker was found in 30%, with CD117 in 38%, and in combination with myeloid markers CD13 and CD33 in 40% of AML cases. With monocytic differentiation associated marker CD4 in blast population was found in 34% of cases. The most frequent expression of other lineage markers was the expression of T-lymphoid marker CD7 expression (48%). Hyperexpression the markers was quite rare, only the myeloid marker CD13 was

hypoexpressed in 26% of cases. All aberrant phenotypes of patients with AML is presented in the graph (Fig. 5).

Fig 5. Incidence of leukemia associated phenotypes in AML



3.2. Optimization of minimal residual disease detection

The size and dynamics of minimal residual disease was considered as an indicator of the course of acute leukemia and was used to determine the prognostic value of other flow cytometric parameters. Before the beginning of this work minimal residual disease was not tested yet, so the test had to be developed and standardized. The standardization of minimal residual disease was carried out in several stages:

- Based on the analysis of the incidence of aberrant phenotypes four color B-ALL and T-ALL and six color AML panels were developed.
- The dilution experiment of leukemia samples was carried out in order to detect the sensitivity of the test and to select the optimal sample preparation technique (mononuclear isolation by density gradient and lysed whole blood the sample preparation techniques were compared).
- A standardized data analysis system was developed.
- Healthy individuals' bone marrow was examined to evaluate the specificity of leukemia associated phenotypes.
- Stability of the leukemia associated phenotypes was assessed.

3.2.1. Selection of markers of minimal residual disease detection

For B-ALL patients a specific B lymphoid marker CD19 was included into all combinations of markers. For the detection of blast population an early marker CD34 was also necessary. It was not used only in a combination CD38/CD10/CD20/CD19 because its purpose was also to evaluate the regularity of differentiation of B-lymphoid progenitor cells which can be characterized by a bright and homogeneous CD38 marker expression. Since TdT hypoexpression was the most common aberration in B-ALL, this marker was included to all remaining combinations. To avoid false positive or false-negative results, blasts for the definition of minimal residual disease more than one phenotypic aberration was always assessed. Additional frequently aberrant markers were added: CD10 (hyper and hypoexpressed in 66% of B-ALL cases), CD45 (hypoexpressed in 31% of cases), CD38 (hypoexpressed in 28% of cases) and CD22 (asynchronous expression CD22++CD34+ in 17%of cases and hypoexpression in 17% of cases).

Since blasts of the majority of patients with T-ALL expressed CD99 and TdT and no T-cell precursors were found in the bone marrow of healthy individuals two four-color combinations of markers were developed: TdT/CD7/cCD3/CD19 and CD99/CD7/cCD3/CD2. In all T-ALL cases the surface expression of specific T cell marker CD3 was reduced, so this marker has been studied in the cytoplasm. CD7 marker was included because it was hyperexpressed in as many as 59% of patients. B-lymphoid marker CD19 was included to exclude TdT positive B lymphoid progenitor cells, due to technical or biological reasons entering into cCD3+ region and mimicking T lymphoblasts. CD2 was included because it was hypoexpressed in a significant number of T-ALL cases (52%).

Acute myelogenous leukemia is characterized by a high phenotypic diversity. We created 7 combinations of markers to choose according to the initial blast phenotype of each patient. Blast population was identified by the expression of markers CD45, CD33, CD34 and CD117. At least three of these markers were included in all combinations. The remaining two fluorescence channels were used to investigate two additional aberrations.

3.2.2. Selection of sample preparation technique and the dilution experiment

To maximize the sensitivity of minimal residual disease detection as many cells as possible shoud be collected. Mononuclear isolation by density gradient sample preparation method allows to concentrate cells, however, it has several drawbacks: the red blood cells may remain in the sample, efficiency of mononuclear separation depends on the proportion of anticoagulant and storage conditions. Applying lysed whole blood technique, smaller, but still sufficient number of cells cal be collected, and minimal residual disease can be expressed as the ratio to all nucleated bone marrow cells, as it is done at the diagnosis.

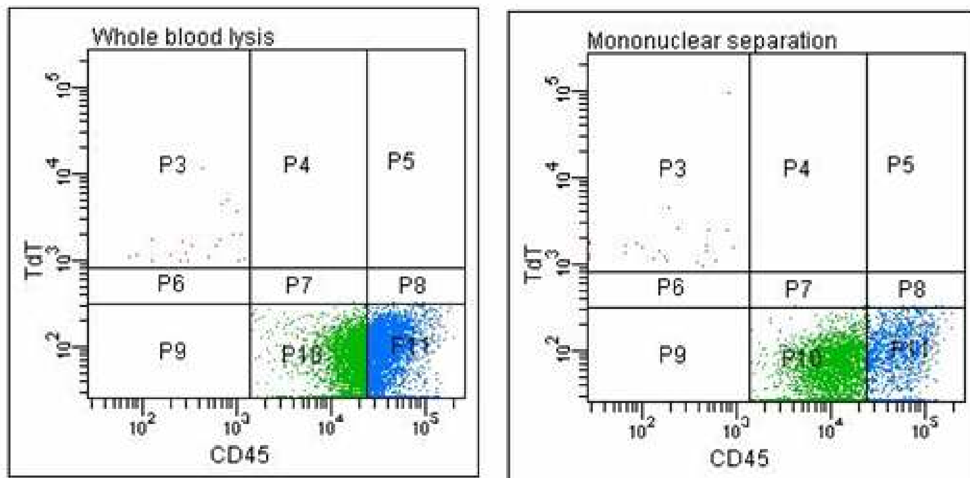
In this study, lysed whole blood and mononuclear separation by density gradient sample preparation techniques were compared analyzing samples of acute leukemia dilution experiment.

For a dilution experiment, random samples of B-ALL, T-ALL and AML were chosen. Samples were diluted with venous blood of a healthy donor 100, 1,000, 10,000 and 100,000 fold. Samples were prepared by lysed whole blood and mononuclear isolation techniques, using originally developed B-ALL, T-ALL and AML marker

panels. Data collection and analysis was performed as described in "Materials and Methods".

In case of B-ALL and T-ALL leukemic cells were detected in all diluted samples. In the highest dilution (100,000x) B-ALL leukemic cells comprised 0.007% and 0.008% of all cells in samples, prepared by lysed whole blood and mononuclear separation techniques respectively (Fig. 6).

Fig 6. Comparison of lysed whole blood and mononuclear separation sample preparation techniques (B-ALL sample diluted 100,000x). Leukemic blasts still visible in region M3.



In 100,000-fold diluted T-ALL samples leukemic cells comprised 0.009% of all cells by lysed whole blood and 0.008% by mononuclear separation technique. In 100,000-fold diluted AML sample, leukemic cell were not detected. In 10,000-fold diluted sample leukemic cells comprised 0.07% of all cells by lysed whole blood and 0.05% by mononuclear separation technique.

The results show that in case of acute lymphoblastic leukemia even higher than indicated in the literature sensitivity of 0.01% was reached. In case of acute myelogenous leukemia such sensitivity has not been achieved, but as the limit value for the detection of minimal residual disease in AML is 0.1%, our sensitivity (0.07% and 0.05%) was sufficient.

Since sensitivity of lysed whole blood technique and mononuclear separation did not differ, the former was chosen for subsequent assays as more consistently defining blast number.

3.2.3 Data analysis system development

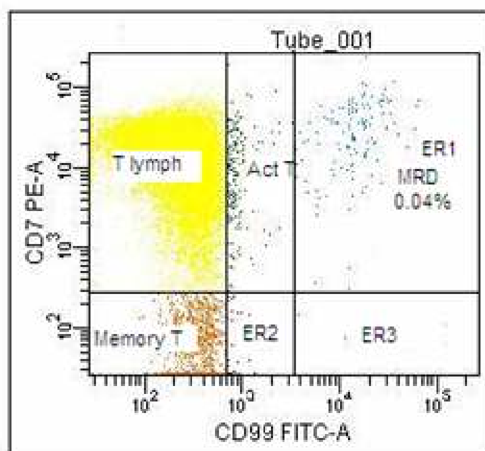
Data analysis system was developed regarding most common phenotypic aberrations of blasts and the examination of normal bone marrow.

For B-ALL minimal residual disease detection “3x3 empty region" analysis system was created. In a healthy person's bone marrow normal precursors of B-lymphoid cells are found. After examining the antigen expression pattern of healthy individuals, “empty regions” were defined. Each dot plot axis according to the expression level of the marker was divided into three levels: negative/dim, positive, bright. Nine rectangular

regions were formed. Some of them were filled with regenerating or mature B-cells, others were called "empty regions" and used for minimal residual disease detection.

For T- ALL CD7/TdT and CD7/CD99 dot plots were divided into 6 regions according to the antigen expression level: CD7 positive and CD7 negative, TdT and CD99 negative, dim and bright. Some of the blocks were filled with populations of normal T-cells, while the rest were ascribed to "empty regions" ("2x3 empty region" analysis strategy) (Fig.7).

Fig.7. "2x3 empty region" analysis in case of T-ALL: regions of mature T lymphocytes, memory (CD7-) T lymphocytes and activated (CD99dim) T lymphocytes are seen and minimal residual disease is found in empty region 1.



For AML minimal disease analysis "2x3 empty region" analysis was also applied. Expression of aberrant markers was assessed only as positive and negative, and early and myeloid markers were evaluated as negative, positive or bright. Six rectangular regions were formed, some of them are filled with normal myeloblast population, while others were consistent with the "empty region" description.

3.2.4. Analysis of healthy bone marrow

12 healthy individuals (bone marrow donors) were tested using a standardized methodology of minimal residual disease.

Following B-lymphoid cell subpopulations were detected in bone marrow of healthy subjects:

1) Early B lymphoid progenitor cells, representing 0.08 ± 0.04% of bone marrow cells. Their phenotype is: TDT+, CD10+, CD19+, CD22+dim, CD34+, CD38+, CD45+dim, CD20-.
2) Late B lymphoid progenitor cells, representing 0.60 ± 0.03% of bone marrow cells. Their phenotype is: CD10+, CD19+, CD20+dim, CD22+dim, CD38+, CD45+dim, TDT-, CD34-.
3) Mature B-lymphocytes, constituting 1.57 ± 0.53% of bone marrow cells. Their phenotype is: CD19+, CD20+, CD22+, CD45+, TDT-, CD10-, CD34-, CD38-/+.

Number of cells with aberrant leukemia associated phenotypes in healthy bone marrow representing specificity of these pphenotypes is presented in a table 6.

Table 6. Number of cells with B-ALL associated phenotypes in bone marrow of healthy subjects.

	Leukemia associated phenotypes																
	Asynchronous antigen expression								**Antigen hypoexpression**					**Antigen hyper-expression**		**Cross lineage antigen expression**	
	CD10r++TdT+	CD10-TdT+	CD10- CD34+	CD10++CD20++	CD22++CD34+	CD45- Td+	CD45-CD34-	CD10-CD20- CD34+	CD22 hypo	CD10 hypo	CD38 hypo	CD45 hypo	TdT hypo	CD10 hyper	CD34 hyper	CD13+	CD33+
Number of cells	0,001%	0,001%	0,003%	0,001%	0,012%	0,000%	0,004%	0,000%	0,010%	0,015%	0,006%	0,002%	0,003%	0,000%	0,000%	0,021%	0,019%

Most of aberrations were found in quantities less than 0.01%, except CD22++ CD34+ (0.012%), CD10 hypoexpression (0.015%), CD33+ (0.021%), CD13+ (0.019%).

In BM of healthy controls we identified 3 populations of normal T-cells:

1) CytoCD3+CD7+TdT-CD99- T lymphocytes (3.81% ± 2.67% of all BM cells)
2) CytoCD3+CD7+TdT-CD99+ T lymphocytes (0.18% ± 0.09% of all BM cells)
3) CytoCD3+CD7-TdT-CD99- T lymphocytes (0.17% ±0.16% of all BM cells)

The amount of cells in TdT positive (0.009% ± 0.004%) and bright (0.000%) and CD99 bright (0.004± 0.004) regions was lower than 0.01% thus making these regions applicable for minimal residual disease detection.

In healthy individuals' bone marrow myeloblast population constituted 0.421% ± 0.193% of all nucleated cells and had this phenotype: CD45+ dim, CD34+, CD117+, CD38+, HLA-DR+, CD13+, CD33+. The number of cells with characteristic for AML phenotypic aberrations was determined (Table 7).

Table 7. The quantity of cells with AML associated phenotypes characteristic for in bone marrow on healthy individuals.

	Leukemia associated phenotypes																								
	Asynchronous antigen expression													**Cross lineage expression**						**Antigen hyperex pression**		**Antigen hypo-expression**			
	CD34+HLA-DR-	CD11+HLA-DR-	CD117+CD34-CD15-	CD34+CD56+	CD34+CD11c+	CD34+CD14+	CD34+CD15+	CD34+CD64+	CD117+CD64+	CD117+CD56+	CD117+CD11c+	CD4+CD45+dim	CD33+CD13+CD56+	CD2+	CD5+	CD7+	CD19+	CD22+	TdT+	CD33 hyper	CD34 hyper	CD13 hypo	CD33 hypo	CD38 hypo	HLA-DR hypo
Number of cells	0,053%	0,089%	0,133%	0,055%	0,163%	0,002%	0,106%	0,031%	0,132%	0,040%	0,092%	0,070%	0,039%	0,059%	0,044%	0,048%	0,028%	0,035%	0,028%	0,078%	0,017%	0,144%	0,169%	0,068%	0,075%

The results show that the majority of AML aberrations are less specific than the aberrations of lymphoblastic leukemia. However, the standard detection limit of minimal residual disease in AML is also higher (0.1%). More cells than this value we found only with asynchronous marker expression CD117+CD34-CD15- (0.133%, possibly because of admixture of mast cells), CD34+CD11c+ (0.163%), CD117+CD64+ (0.132%, probably promyelocytes), CD13 and CD33 hypoexpression (0.144% and 0.169% respectively). These leukemia associated phenotypes should not be used for minimal residual disease detection alone, but can be used in combination with other more specific aberrations.

3.2.5 Stability of leukemia associated phenotypes

Changes of blast phenotype during treatment is one of the most important factors that could influence the accuracy of minimal residual disease detection. Although the patients were monitored for a short time, we tried to evaluate the stability of leukemia associated phenotypes.

Minimal residual disease was detected for 28 out of 36 (77.8%) patients with B-ALL. Most stable with leukemia associated phenotypes were asynchronous antigen expression CD10-CD20-CD34+ and CD38 marker hypoexpression (did not change for any patients). Relatively stable were phenotypes CD10++CD20++, hyperexpression of CD10 and coexpression of CD34, CD13 and CD15 (changed only 3.57% of cases). Quite unstable appeared to be CD22 ++CD34+, hypoexpression of CD45 and CD34 (changed in 14.29% of patients). TdT hypoexpression which was the most common B-ALL aberration was modulated in a relatively small number of subjects (7.14%). Conversely,

in 32.15% of all B-ALL cases TdT expression, compared to the diagnosis, decreased even more.

In case of T-ALL and AML, expression of aberrant markers fluctuated during treatment, but remained higher than expression on normal cells of the same lineage.

3.3. Influence of leukemia associated phenotypes to the dynamics of minimal residual disease

The impact of leukemia associated phenotypes to the size of minimal residual disease was estimated separating patients into two groups according each aberrant phenotype (phenotype present or absent). Statistical differences among these groups were investigated applying Mann-Whitney-Wilcoxon test.

Differencies in the number of blasts between groups of aberrant B-ALL phenotypes are presented in tables 8, 9.

Table 8. Mann-Whitney-Wilcoxon test results showing differencies in blast number in groups of aberrant B-ALL phenotypes.

Period	**CD10++ TdT +**	**CD10- TdT+l**	**CD10- CD34+**	**CD10++ CD20++**	**CD45- TdT+**	**CD45- CD34-**	**CD22 hypo**	**CD10 hypo**	**CD45 hypo**	**CD34 hyper**
Diagn.	0,923	0,701	0,578	1,000	0,151	0,498	**0,030**	0,578	0,110	0,531
11-15d.	**0,049**	0,689	0,316	0,171	0,334	0,168	0,412	0,316	*0,078*	**0,049**
29-35d.	0,229	0,315	*0,065*	**0,039**	*0,142*	*0,063*	0,376	*0,065*	0,*095*	0,195
36-76d.	0,145	0,296	0,203	0,114	*0,367*	*0,184*	0,504	0,203	0,331	0,745
77-90d.	*0,064*	0,532	0,400	0,403	*0,123*	*0,193*	0,310	0,400	0,126	0,820
4 mo	0,873	0,671	0,893	0,525	-	*0,525*	-	0,893	0,631	0,671
5 mo	0,572	*0,094*	0,180	0,576	*0,576*	*0,576*	-	0,180	0,287	1,000
6 mo	*0,058*	0,946	0,870	0,540	*0,058*	*0,219*	*0,072*	0,870	0,671	0,951

Table 9. Comparison of blast number in different groups of aberrant B-ALL phenotypes during treatment (average number of blasts).

Period	CD10++TdT+		CD10- TdT+		CD10-CD34+		CD10++CD20++		CD45- TdT +	
	0	1	0	1	0	1	0	1	0	1
Diagn.	66.93%	63.38%	65.40%	67.90%	65.30%	67.67%	65.97%	66.83%	62.23%	77.33%
11-15d.	**7.51%**	**0.32%**	5.33%	6.98%	2.69%	12.34%	7.00%	0.27%	7.24%	1.85%
29-35d.	2.21%	0.19%	1.53%	2.14%	*0.66%*	*4.26%*	**2.00%**	**0.00%**	1.77%	1.35%
36-76d.	1.94%	0.01%	1.43%	1.61%	1.27%	1.80%	1.76%	0.00%	1.32%	2.06%
77-90d.	*1.45%*	*0.00%*	1.23%	0.67%	1.25%	0.73%	1.25%	0.24%	0.67%	2.16%
4 mo	0.48%	0.04%	0.45%	0.25%	0.31%	0.47%	0.43%	0.00%	0.38%	-
5 mo	0.16%	0.00%	*0.00%*	*0.32%*	0.00%	0.25%	0.16%	0.00%	0.16%	0.00%
6 mo	*12.75%*	*0.00%*	13.03%	1.07%	13.75%	0.93%	11.19%	4.00%	*12.75%*	*0.00%*
Period	**CD45- CD34-**		**CD22 hypo**		**CD10 hypo**		**CD45 hypo**		**CD34 hyper**	
	0	**1**	**0**	**1**	**0**	**1**	**0**	**1**	**0**	**1**
Diagn.	66.66%	60.33%	**62.69%**	**82.67%**	65.30%	67.67%	60.92%	77.45%	68.43%	61.67%
11-15d.	6.35%	0.30%	5.58%	6.91%	2.69%	12.34%	*7.60%*	*2.08%*	**2.55%**	**12.64%**
29-35d.	*1.84%*	*0.00%*	1.79%	0.79%	*0.66%*	*4.26%*	*2.22%*	*0.15%*	1.17%	2.72%
36-76d.	1.69%	0.00%	1.77%	0.33%	1.27%	1.80%	1.64%	1.00%	1.82%	1.04%
77-90d.	1.20%	0.00%	1.16%	0.73%	1.25%	0.73%	1.54%	0.23%	1.45%	0.40%
4 mo	0.43%	0.00%	0.38%	-	0.31%	0.47%	0.28%	0.75%	0.25%	0.45%
5 mo	0.16%	0.00%	0.13%	-	0.00%	0.25%	0.21%	0.00%	0.21%	0.01%
6 mo	11.09%	0.00%	*7.15%*	*24.43%*	13.75%	0.93%	8.91%	11.81%	10.70%	8.59%

Several phenotypes that have an influence on the number of blasts during the therapy can be distinguished.

Leukemia associated phenotypes, leading to a smaller number of blasts usually involve changes in CD10 and CD45 expression:

1) CD10 hyperexpression:

A. Patients whose blasts display a phenotype CD10++TdT+ on day 11-15 have a lower number of blasts than patients without this phenotype.

B. CD10++CD20++ phenotype is associated with a reduced number of blasts on day 29-35.

2) CD45 hypoexpression: the influence of phenotype CD45-TdT+ to a smaller number of blasts on 6th month, the influence of phenotype CD45-CD34- to a smaller number of blasts on day 29-35 and the influence of isolated CD45 hypoexpression to a smaller number of blasts on day 11-15 and 29-35 is close to statistical significance.

Leukemic phenotypes associated with larger number of blasts include changes in CD10, CD22 and CD34 marker expression:

1) CD10 hypoexpression: influence of phenotypes CD10-TdT+, CD10-34 + and a single CD10 hypoexpression is almost statistically significantly associated with an increased number of blasts on 5th month and day 29-35 respectively.

2) Influence of a single CD22 hypoexpression to an increased number of blasts is statistically significant at the time of diagnosis and almost statistically significant on 6th month of treatment.

3) Single hyperexpression of CD34 identifies patients who have a higher number of blasts on day 11-15.

Differencies in the number of blasts between groups of aberrant T-ALL phenotypes are presented in tables 10, 11.

Table 10. Mann-Whitney-Wilcoxon test results showing differencies in the number of blasts in groups of aberrant T-ALL phenotypes during treatment.

Period	CD34+	CD1a+	CD2 hypo	CD3 hypo	CD5 hypo	CD7 hyper	CD4- CD8-	CD4+ CD8+	CD13+	CD33+
Diagn.	0,710	0,216	0,136	0,345	0,658	0,658	0,471	0,469	0,148	*0,085*
11-15d.	**0,031**	**0,005**	**0,006**	0,724	0,575	0,286	**0,025**	*0,058*	**0,040**	*0,087*
29-35d.	*0,080*	**0,005**	**<0,001**	0,382	0,191	0,673	**0,006**	**0,006**	**0,050**	**0,034**
36-76d.	0,408	0,157	**0,042**	**0,037**	**0,017**	0,487	*0,061*	*0,061*	0,333	0,465
77-90d.	0,306	**0,026**	**0,002**	0,305	*0,086*	0,690	**0,026**	**0,026**	0,174	0,163
4 mo	0,775	0,513	0,663	0,186	*0,087*	1,000	0,775	0,513	0,513	0,513
5 mo	0,513	0,513	0,513	0,383	**0,016**	*0,079*	0,513	0,513	0,663	0,775
6 mo	0,427	**0,024**	**0,001**	0,151	0,128	0,933	*0,054*	*0,093*	**0,017**	0,160

Table 11. Comparison of blast number in different groups of aberrant T-ALL phenotypes (average number of blasts).

Period	CD34+		CD1a+		CD2 hypo		Cyto CD3 hypo		CD5 hypo	
	0	1	0	1	0	1	0	1	0	1
Diagn.	73.93%	72.2%	68.94 %	79.73 %	77.33 %	61.75 %	76.15 %	66.11%	71.60%	82.00%
11-15d.	**0.45%**	**8.97%**	**7.74%**	**0.02%**	**0.53%**	**17.23%**	3.46%	7.82%	5.27%	1.33%
29-35d.	*0.21%*	*0.54%*	**0.59%**	**0.00%**	**0.01%**	**1.40%**	0.12%	0.89%	0.39%	0.47%
36-76d.	0.71%	0.01%	0.53%	0.00%	**0.00%**	**1.92%**	**0.00%**	**1.15%**	**0.00%**	**1.44%**
77-90d.	2.43%	0.16%	**1.46%**	**0.00%**	**0.02%**	**3.13%**	0.05%	2.05%	*0.11%*	*4.32%*
4 mo	0.00%	0.02%	0.02%	0.00%	0.02%	0.02%	0.00%	0.03%	*0.00%*	*0.10%*
5 mo	0.00%	0.03%	0.03%	0.00%	0.03%	0.00%	0.01%	0.05%	**0.00%**	**0.09%**
6 mo	2.24%	1.99%	3.58%	0.00%	**0.00%**	**6.65%**	0.11%	6.43%	1.19%	8.01%

Period	CD7 hyper		CD4-CD8-		CD4+ CD8+		CD13+		CD33+	
	0	1	0	1	0	1	0	1	0	1
Diagn.	75.90%	66.67%	75.14%	71.07%	70.94%	75.62%	76.05%	67.30%	*78.10%*	*61.78%*
11-15d.	**0.32%**	**13.98%**	**0.44%**	**8.98%**	*8.38%*	*0.47%*	**1.16%**	**6.39%**	*3.60%*	*7.48%*
29-35d.	0.49%	0.23%	**0.06%**	**0.66%**	**0.66%**	**0.06%**	**0.09%**	**0.86%**	**0.13%**	**0.97%**
36-76d.	0.48%	0.01%	*0.00%*	*0.65%*	*0.65%*	*0.00%*	0.01%	1.15%	0.42%	0.00%
77-90d.	1.33%	0.07%	**0.00%**	**1.46%**	**1.46%**	**0.00%**	0.05%	2.31%	1.33%	0.25%
4 mo	0.03%	0.01%	0.00%	0.02%	0.02%	0.00%	0.02%	0.00%	0.02%	0.00%
5 mo	0.04%	0.00%	0.00%	0.03%	0.03%	0.00%	0.02%	0.04%	0.02%	0.00%
6 mo	2.82%	0.26%	*0.06%*	*4.18%*	*3.83%*	*0.07%*	**0.07%**	**5.08%**	1.85%	2.51%

T-ALL leukemic phenotypes leading to a larger number of blasts are associated with the earlier stage of differentiation, hypoexpression of specific T-lymphoid markers and coexpression of myeloid markers:

1) Expression of CD34 is related to a higher number of blasts in the early phase of treatment (11-15 day and possibly 29-35 day).

2) Phenotype of early differentiation CD4-CD8- is related to an increased number of blasts in almost all stages of treatment.

3) Hypoexpression of T lymphoid marker CD2 leads to a larger number of blasts in almost all stages of treatment and hypoexpression of another T lymphoid marker CD5 leads to a larger number of blasts from day 36. Hypoexpression of cytoplasmic CD3 is associated with a higher residual disease on day 36-76.

4) In contrast to the findings in B-ALL, expression of myeloid markers CD13 and CD33 was related to an increased number of blasts on certain stages of treatment.

And on the contrary, phenotypes associated with the later differentiation resulted in a lower number of blasts during the treatment: patients who had an expression of CD1a and phenotype CD4 +CD8+ also had a lower number of blasts during treatment.

Differencies in number of blasts between groups of aberrant AML phenotypes are presented in tables 12, 13.

Table 12. Mann-Whitney-Wilcoxon test results showing differencies in number of blasts in groups of aberrant AML phenotypes.

Period	**CD34+CD15+**	**CD117+ CD64+**	**CD117+ CD11c+**	**CD13+CD33+ CD56+**	**CD5+**	**CD7+**	**CD22+**	**CD33 hypo**	**HLA-DR hypo**
Diagn.	0,406	0,214	0,734	0,902	0,131	*0,075*	*0,096*	*0,088*	0,462
Up to 1 mo	0,339	*0,073*	0,749	0,478	0,675	-	0,160	0,330	0,160
1 mo	*0,061*	0,181	0,749	0,227	0,583	0,132	-	0,595	*0,077*
2 mo	0,403	0,342	**0,043**	0,659	0,655	0,484	0,440	0,545	1,000
3 mo	0,172	1,000	0,621	0,352	**0,042**	0,295	-	0,534	-
4 mo	0,293	0,854	0,594	0,424	*0,096*	**0,030**	-	1,000	0,515
5 mo	0,176	0,570	1,000	1,000	0,322	-	-	0,803	-
6 mo	0,199	0,358	0,744	**0,005**	0,193	1,000	-	0,893	-

Table 13. Comparison of blast number in different groups of aberrant AML phenotypes (average blast number)

Period	CD34+ CD15+		CD117+ CD64+		CD117+ CD11c+		CD13+CD33+ CD56+	
	0	1	0	1	0	1	0	1
Diagn.	40.18%	46.82%	39.44%	49.45%	41.19%	42.37%	42.21%	40.53%
Up to 1 mo	12.40%	1.38%	*13.15%*	*0.93%*	11.53%	9.88%	14.23%	4.01%
1 mo	*5.34%*	*1.36%*	5.34%	1.37%	5.76%	1.76%	5.33%	2.46%
2 mo	3.19%	15.52%	7.24%	0.13%	**7.40%**	**2.48%**	6.41%	4.19%
3 mo	0.93%	2.03%	1.04%	1.52%	1.33%	1.02%	0.97%	1.47%
4 mo	0.96%	0.00%	1.09%	0.03%	1.19%	0.02%	1.08%	0.06%
5 mo	0.00%	3.22%	2.11%	0.10%	3.17%	0.05%	2.71%	0.06%
6 mo	2.89%	4.79%	3.96%	1.98%	2.32%	4.62%	**1.00%**	**7.80%**
Period	**CD5+**		**CD7+**		**CD33 hypo**		**HLA-DR hypo**	
	0	1	0	1	0	1	0	1
Diagn.	40.21%	64.00%	*43.71%*	*23.00%*	*37.03%*	*54.77%*	40.68%	56.67%
Up to 1 mo	11.30%	6.26%	10.82%	-	11.54%	9.02%	8.86%	50.00%
1 mo	4.42%	2.76%	3.93%	13.00%	3.91%	5.55%	*2.65%*	*49.00%*
2 mo	5.38%	9.90%	5.63%	6.64%	4.20%	10.43%	6.15%	1.50%
3 mo	**1.17%**	**1.36%**	0.68%	7.25%	1.80%	0.01%	1.18%	-
4 mo	*0.79%*	*0.17%*	**0.02%**	**14.00%**	1.02%	0.04%	0.85%	0.00%
5 mo	1.90%	0.15%	1.61%	-	0.04%	3.80%	1.61%	-
6 mo	3.11%	10.00%	3.27%	3.99%	2.74%	4.68%	3.35%	-

In case of acute myelogenous leukemia only several leukemia associated phenotypes influenced the number of blasts during the treatment. Asynchronous expression CD117+CD11c+ was associated with a reduced number of blasts on the 2nd month of treatment. Phenotype CD33+CD13+CD56+ leaded to a higher number of blasts on the 6th month. Coexpression of T-lymphoid marker CD5 was associated with a higher minimal residual disease on 3rd month, and coexpression of CD7 marker was related to the size of the minimal residual disease on 4th month of treatment.

3.4. Optimization of multidrug resistance detection

After several experiments defining the level of the expression of MDR related proteins we abandoned the standard 20% threshold for a positive result, because it was reached only in single cases. Instead, mean fluorescence intensity (MFI) was assessed. Higher MRP1 and P-gp MFI value than in normal mononuclear cells and lower calcein MFI value was considered "positive" and these blasts were evaluated as having a structural or functional drug resistance.

3.4.1. Examination of normal bone marrow

Multidrug resistance related parameters of acute leukemia patients were compared to the parameters of healthy individuals. Results are presented in Table 14.

Table 14. Comparison of calcein, MRP1 and P-gp MFI values between AL patients and healthy individuals.

	Group	Average MFI	SD	p
Blast calcein	AL	5242.533	2044.126	0.080
	Control	6856.143	2685.027	
Blast MRP1	AL	121.500	47.042	0.919
	Control	123.429	34.058	
Blast p-gp	AL	170.167	80.514	0.158
	Control	125.286	25.766	
Lymphocyte calcein	AL	4400.800	1589.829	0.311
	Control	4826.842	1087.813	
Lymphocyte MRP1	AL	76.800	10.580	0.260
	Control	71.789	44.120	
Lymphocyte p-gp	AL	131.267	44.120	0.002
	Contrl	96.316	16,941	
Monocyte calcein	AL	9719.464	4513.511	0.013
	Control	12806.579	3147.798	
Monocyte MRP1	AL	253.400	123.731	0.004
	Control	164.842	24.548	
Monocyte p-gp	AL	282.967	142.416	0.079
	Control	222.526	42.891	

We found the following statistically significant differences in acute leukemia patients and healthy individuals :

In the group of patients lymphocyte p-gp was higher (p = 0.002);

In the group of patients monocyte calcein was higher (p = 0.013),

In the group of patients monocyte MRP1 was higher (p = 0.04).

As values of calcein, MRP1 and p-gp in blast population of patients did not differ significantly from the control group, we compared blasts with other cell populations (lymphocytes and monocytes) (Tables 15, 16). Almost all parameters of blasts were significantly different from parameters of other mononuclear cells.

Table 15. Comparison of MRP1, P-gp and MRP1 expression values between blast and lymphocyte populations.

Parameter	**Blasts**		**Lymphocytes**		p
	MFI	**SD**	**MFI**	**SD**	
Calcein	5242.533	2044.126	4400.800	1589.829	0.075
MRP1	121.500	47.042	76.800	10.580	0.0008
p-gp	170.167	80.514	131.267	44.120	0.024

Table 16. Comparison of MRP1, P-gp and MRP1 expression values between blast and monocyte populations.

Parameter	Blasts		Monocytes		p
	MFI	SD	MFI	SD	
Calcein	5242.533	2044.126	9719.464	4513.511	0.0008
MRP1	121.500	47.042	253.400	123.731	0.0002
p-gp	170.167	80.514	282.967	142.416	0.04

3.4.2. Correlation between structural and functional MDR parameters

Results of correlation between structural and functional MDR characteristics are presented in Table 17.

Table 17. Relationship between multidrug resistance parameters (B-ALL).

		Calcein	MRP1	p-gp
Calcein	Spearman correlation coefficient	1	0,452	0,567
	p	<0,0001	0,222	0,112
MRP1	Spearman correlation coefficient	0,452	1	**0,929**
	p	0,222	<0,0001	**0,000**
p-gp	Spearman correlation coefficient	0,567	**0,929**	1
	p	0,112	**0,000**	<0,0001

Only strong statistically significant association between structural elements of drug resistance was demonstrated.

In T-ALL group no statistically significant relationships between MDR parameters have been identified.

Relations between MDR parameters in AML group is presented in Table 18.

Table 18. Relationship between multidrug resistance parameters (AML).

		Calcein	MRP1	p-gp
Calcein	Spearman correlation coefficient	1	-0,103	0,105
	p	<0,0001	0,694	0,687
MRP1	Spearman correlation coefficient	-0,103	1	**0,767**
	p	0,694	<0,0001	**0,000**
p-gp	Spearman correlation coefficient	0,105	**0,767**	1
	p	0,687	**0,000**	<0,0001

Once again, only association between parameters of structural resistance has been proven.

3.4.3. Multidrug resistance parameters in different leukemia subtypes

Table 19 shows differences in MDR parameters of patients with different leukemia subtypes.

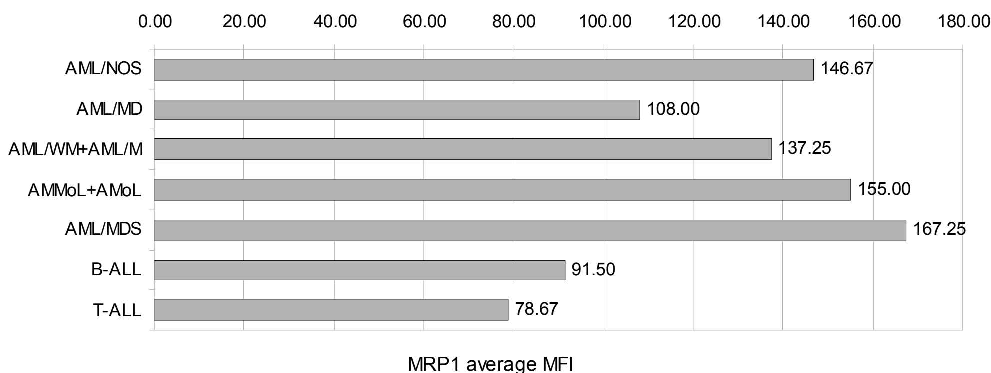
Table 19. MDR parameters in different leukemia subgroups.

	Diagnosis	MFI	SD
Calcein	AML/NOS	4630,333	1809,0579
	AML/MD	3560,500	1344,210
	AML/WM+AML/M	4508,000	774,330
	AMMoL+AMoL	7570,500	1921,591
	AML/MDS	6288,000	3071,996
	B-ALL	4638,100	1633,819
	T-ALL	5472,333	2098,601
MRP1	AML/NOS	146,667	47,816
	AML/MD	108,000	1,414
	AML/WM+AML/M	137,250	52,766
	AMMoL+AMoL	155,000	30,078
	AML/MDS	167,250	29,182
	B-ALL	91,500	40,045
	T-ALL	78,667	21,502
p-gp	AML/NOS	228,333	100,401
	AML/MD	147,000	1,414
	AML/WM+AML/M	183,500	107,090
	AMMoL+AMoL	231,000	12,490
	AML/MDS	218,000	98,031
	B-ALL	120,200	65,518
	T-ALL	131,333	7,506

ANOVA test results showed that only values of MRP1 differed significantly between subtypes of acute leukemia ($p = 0.013$). In cases of p-gp and calcein p values were 0.100 and 0.142 respectively.

As seen, patients with acute lymphoblastic have a lower MRP1 expression (MFI 91.50 in B-ALL and 78.67 in T-ALL), while patients with acute myelogenous leukemia, particularly with acute myelogenous leukemia with myeloidysplasia have much higher MRP1 expression (167.25) (Fig. 8).

Fig. 8. MRP1 expression in acute leukemia subtypes.



3.5 Influence of aberrant leukemia associated phenotypes to the level of multidrug resistance

Leukemia associated phenotypes showed influence on multidrug resistance parameters only in AML group.

Asynchronous expression CD13+CD33+CD56+ was associated with lower mean fluorescence intensity of calcein (Mann-Whitney-Wilcoxon test value 0.036, average calcein fluorescence intensity 5382 in phenotype positive group and 6128 in phenotype negative group). Reduced intracellular accumulation of calcein shows functional activity of MDR transporters.

Coexpression of T lymphoid marker CD5 on myeloid blast population was also associated with lower calcein mean fluorescence intensity (Mann-Whitney-Wilcoxon test value 0.020, average fluorescence intensity in CD5 positive group 4217, in CD5 negative group 5845). Coexpression of another T lymphoid marker CD7 had influence on higher p-gp value (Mann-Whitney-Wilcoxon test value was 0.049, average fluorescence intensity in CD7 positive groupe was 3991, in CD7 negative group 6211).

3.6. Association of MDR parameters with a level of minimal residual disease.

In acute B lymphoblastic leukemia group several statistically significant relations between MDR parameters and the number of blasts were found:

- Very strong correlation between calcein MFI and the number of blasts on day 35-76 (Spearman correlation coefficient 1.000, $p < 0.0001$),

- A strong correlation between MRP1 expression and the number of blasts on day 29-35 and 77-90 (Spearman correlation coefficient respectively 0.712, $p = 0.048$ and 0.706, $p = 0.034$),

-A strong correlation between p-gp expression and the number of blasts on day 77-90 (Spearman correlation coefficient = 0.703, $p = 0.035$).

Patients with acute T-lymphoblastic leukemia had strong link between MRP1

expression and the number of blasts at the diagnosis of acute leukemia, and between p-gp expression and the number of blasts on day 77-90 (Spearman correlation coefficient 1.000, $p < 0.0001$). However, the number of subjects was not sufficient for reliable interpretation.

Investigation of patients with AML revealed a strong correlation between p-gp expression and the number of blasts on the 4th month of treatment (Spearman correlation coefficient 1.000, $p < 0.0001$).

4. Conclusions

1) In all cases of acute lymphoblastic leukemia and in most cases of acute myelogenous leukemia aberrant leukemia associated phenotypes are found.

2) Minimal residual disease detection based on four color ALL marker panels and six color AML marker panels, lysed whole blood sample preparation technique and a standardized analysis strategy can be applied to virtually all patients and provides a sufficient sensitivity and specificity.

3) Aberrant B-ALL phenotypes CD10++TdT+ and CD10++CD20++ are related to a lower minimal residual disease and CD34 hyperexpression is related to a higher minimal residual disease. Aberrant T-ALL phenotypes consistent with later maturation (CD1a+, CD4+CD8+) are associated with a lower minimal residual disease and phenotypes consistent with earlier maturation (CD34+, CD4-CD8-), hypoexpression of CD2 and CD5 and coexpression of myeloid markers are associated with a higher minimal residual disease. AML phenotype CD117+CD11c+ is related to a lower minimal residual disease while phenotypes CD13+CD33+CD56+, CD5+ and CD7+ are related to a higher minimal residual disease.

4) Multidrug resistance can be studied determining expression of P-gp and MRP1 (structural resistance) and calcein accumulation (functional resistance) and comparing their fluorescence intensity with the corresponding parameters of healthy cells. Structural multidrug resistance is not necessarily associated with functional multidrug resistance.

5) Aberrant phenotypes of acute myeloid leukemia CD13+CD33+CD56+, CD5+ and CD7+ are associated with a higher level of multidrug resistance.

6) P-gp expression influences the size of minimal residual disease in B-ALL and AML, while MRP1 expression and calcein accumulation has this impact only in B-ALL.

5. Practical recommendations

1) Diagnosing acute leukemia it is important to evaluate leukemia associated phenotypes as having a prognostic significance:
   - In B-ALL: CD10++TdT+, CD10++CD20++, CD34 hyperexpression,
   - In T-ALL: CD34+, CD4-CD8-, hypoexpression of CD2 and CD5, coexpression of CD13 and CD33, expression of CD1a, CD4+CD8+,
   - In AML: CD117+CD11c+, CD13+CD33+CD56+, CD5+, CD7+.

2) For the detection of minimal residual disease in ALL 4-color marker combinations can be used (for a higher sensitivity 6-color or 8-color combinations are desirable). For AML minimal residual disease detection at least 6-color marker panel should be applied. Introducing minimal residual disease assays it is essential to investigate normal bone marrow in order to define specificity of aberrant phenotypes. Analysis of minimal residual disease should be standardized and based on investigation of healthy bone marrow. It is important to keep in mind the possibility of false negative results when phenotypic shifts occur. To avoid them more than one phenotypic aberration shoud be monitored.

3) Evaluation of the expression of multidrug resistance related proteins and studies of their function may be included in the diagnostic algorithm of acute leukemias, or at least performed when aberrant phenotypes related to multidrug resistance are detected. With the emergence of drugs that inhibit multidrug resistance, the value of laboratory tests of MDR inhibition should be studied.

4) Further studies of the influence of aberrant phenotypes and features of multidrug-resistance to the distant results of treatment are needed. It is also important to explore other mechanisms how aberrant phenotypes influence the level of minimal residual disease (e.g., links to genetic anomalities and other prognostic factors).

.

List of publications on the topic of dissertation

1. Publications in ISI Master list journals

Janeliūnienė M, Matuzevičienė R, Griškevičius L, Kučinskienė Z.A. Monitoring of T-cell acute lymphoblastic leukemia by flow cytometry. Central European Journal of Medicine 2010; 5(6): 651-658.

2. Publications in Lithuanian peer-reviewed periodicals

Janeliūnienė M, Matuzevičienė R, Griškevičius L, Kučinskienė Z. Optimizing detection of minimal residual disease in B-precursor acute lymphoblastic leukemia by multiparameter flow cytometry. Acta Medica Lituanica 2007; 4(14): 257-266.

Janeliūnienė M, Matuzevičienė R, Kučinskienė Z. Blastų dauginio atsparumo vaistams tyrimo tėkmės citometru reikšmė diagnozuojant ūminę leukemiją. Laboratorinė medicina 2011; 2(50): 65-75.

About the author

Miglė Janeliūnienė was born in on March 5, 1976 in Vilnius. During the period of 1983 – 1993 she studied at the Vilnius 31st secondary school, graduated from it with the gold medal. In 1993 she entered the Faculty of Medicine of Vilnius University. In 1999 graduated from it with honors. In 2000 Miglė Janeliūnienė finished the primary residency program, and during 2001 - 2003 she was enrolled in laboratory medicine specialty residency. After successful completion of it, she obtained a license of laboratory physician. During the period 2004 – 2011 Miglė Janeliūnienė studied in a doctoral program of Medicine at Vilnius University.

During 2001 - 2003 Miglė Janeliūnienė worked at the laboratory of Hematology and general cytology of Vilnius University Hospital Santariškių Klinikos as an assistant doctor. Since 2003 she works at the same laboratory as a laboratory physician. Main area of interest of Miglė Janeliūnienė is flow cytometry and its application in the field of hematology, particularly in diagnostics and prognostics of hematopoietic neoplasms. Miglė Janeliūnienė regularly participates in international events in the area of her specialty, prepares theses, and publishes articles. She has published 7 scientific publications in peer reviewed Lithuanian and international periodicals, three of them concern a topic of a dissertation, one publication is in the journal with the ISI citation index. In 2006 she was enrolled in a visiting fellowship program in St.Jude Children Research Hospital (Memphis, US).

Miglė Janeliūnienė is married; her husband Tomas is a political scientist, an associate professor of Vilnius University. She has two children.

Summary in Lithuanian

Įvadas

Taikant šiuolaikinį gydymą, daugumai sergančiųjų ūminėmis leukemijomis pasiekiama pilna klinikinė ir morfologinė remisija. Šis gydymas nėra vienodas visiems ligoniams: remiantis tam tikrais kriterijais, jie skirstomi į rizikos grupes ar net taikomas individualizuotas gydymas. Standartiniai, pacientų biologinėmis savybėmis (amžius, leukocitų skaičius) paremti ligonių skirstymo į rizikos grupes kriterijai ne visada yra patikimi: daliai ligonių skiriamas nepakankamai intensyvus gydymas ir liga atsinaujina, kita dalis gauna pernelyg intensyvų ir pernelyg toksišką gydymą, kurio pašaliniai reiškiniai gali lemti net ir letalią išeitį. Tėkmės citometrijos metodu galima įvertinti keletą rodiklių, galinčių turėti įtakos ligos eigai: su leukemija susijusius aberantinius fenotipus (tai blastų fenotipai, aptinkami tik leukeminiuose, bet ne sveikuose kraujodaros ląstelių pirmtakuose) ir dauginį atsparumą vaistams. Atsakas į taikomą gydymą taip pat gali būti vertinamas tėkmės citometrijos metodu, tiriant minimalią liekamąją ligą (submikroskopinį leukeminių blastų kiekį).

Darbo tikslas

Nustatyti sergančiųjų ūminėmis leukemijomis blastų su leukemija susijusius aberantinius fenotipus ir dauginį atsparumą vaistams bei įvertinti jų įtaką minimalios liekamosios ligos dinamikai.

Darbo uždaviniai

1) Nustatyti su leukemija susijusių aberantinių fenotipų paplitimą sergančiųjų ūminėmis leukemijomis populiacijoje.
2) Optimizuoti minimalios liekamosios ligos tyrimo tėkmės citometru metodologiją.
3) Nustatyti su leukemija susijusių aberantinių fenotipų įtaką minimalios liekamosios ligos dydžiui ankstyvajame leukemijų gydymo etape (periode iki 6 mėn.).
4) Optimizuoti dauginio atsparumo vaistams tyrimo tėkmės citometru metodologiją.
5) Nustatyti su leukemija susijusių aberantinių fenotipų įtaką dauginio atsparumo vaistams rodikliams.
6) Nustatyti dauginio atsparumo vaistams rodiklių koreliaciją su minimalia liekamąją liga ankstyvajame leukemijų gydymo etape (periode iki 6 mėn.).

Ginamieji teiginiai

1) Ūminių leukemijų fenotipas nevisiškai atitinka sveikų tos pačios kraujodaros ląstelių linijos pirmtakų fenotipą, daugeliu atvejų aptinkama su leukemija susijusių aberantinių fenotipų.
2) Kai kurie su leukemija susiję aberantiniai fenotipai turi ryšį su dauginiu atsparumu vaistams ir per šį mechanizmą daro įtaką minimalios liekamosios ligos dydžiui, todėl gali būti naudojami ūminių leukemijų prognozei vertinti.

Tyrimo naujumas ir aktualumas

Pirmą kartą ištirtas su leukemija susijusių aberantinių fenotipų paplitimas Lietuvos ligonių, sergančių ūminėmis leukemijomis populiacijoje ir palygintas su literatūroje pateikiamais jų paplitimo duomenimis.

Įdiegtas originalus standartizuotas minimalios liekamosios ligos tyrimo metodas: remiantis aberantinių fenotipų analize, parinkti žymenų deriniai ūminių B ir T limfoblastinių leukemijų ir ūminės mielogeninės leukemijos minimaliai liekamajai ligai tirti, parinktas optimaliausias mėginių ruošimo būdas, sukurta vieninga analizės sistema. Įdiegtas standartizuotas blastų struktūrinio ir funkcinio atsparumo vaistams tyrimas tėkmės citometru.

Mes pirmieji sistemiškai pabandėme susieti visų pagrindinių su leukemija susijusių fenotipų nustatymą, kuris rodytų leukeminių ląstelių fenotipinį „nutolimą“ nuo normalių kraujodaros pirmtakų, su dauginiu atsparumu vaistams bei su atsaku į taikomą gydymą – minimalios liekamosios ligos dinamika. Nuosekliai ištyrėme, ar su leukemija susiję aberantiniai fenotipai turi įtakos dauginio atsparumo vaistams rodikliams *in vitro*, ir kaip šios savybės pasireiškia *in vivo* – kaip efektyviai blastų skaičius kinta gydant. Studijų, apjungiančių visus tris prognostinius tėkmės citometru tiriamus ūminių leukemijų rodiklius, iki šiol neatlikta. Tirtas tik pavienių blastų fenotipo ypatybių (pvz., CD34 žymens ekspresijos) sąsajos su dauginio atsparumo vaistams rodikliais ar minimalia liekamąją liga. Mes pirmieji nustatėme, kad sergančiųjų ūmine mielogenine leukemija fenotipai CD33+CD13+CD56+ ir CD5 bei CD7 žymenų koekspresija turi įtakos dauginio atsparumo vaistams atsiradimui, kuris savo ruožtu lemia minimalios liekamosios ligos persistavimą.

Tyrimo medžiaga

Į tyrimą buvo įtraukta 114 pirmą kartą ūminėmis leukemijomis susirgusių ligonių, 2005 – 2009 m. gydytų VULSK Hematologijos, Onkologijos ir Transfuziologijos centre bei VU Vaikų ligoninės Onkohematologijos centre. Įtraukti tik tie ligoniai, kuriems buvo numatoma skirti intensyvią chemoterapiją ir tirti minimalią liekamąją ligą. 35 ligoniai sirgo ūmine B-limfoblastine leukemija, 29 – ūmine T limfoblastine leukemija, 50 – ūmine mielogenine leukemija. Kontrolinė grupė sudaryta iš 12 sveikų kaulų čiulpų donorų.

Tyrimo metodai

Su leukemija susiję aberantiniai fenotipai, dauginis atsparumas vaistams ir minimali liekamoji liga tirta tėkmės citometrijos metodu. Mėginių paruošimui taikyta lizuoto „viso" kraujo technika: 100 µl mėginio 20 min. inkubuojama su 20µl fluorochromais žymėtų monokloninių antikūnų prieš tiriamus žymenis. Mėginys dukart nuplaunamas, jį užpylus tam skirtu tirpalu ir centrifuguojant 5 min 300g režimu, o paskui fiksuojamas paraformaldehido turinčiu tirpalu. Mėginys tiriamas *FACS Canto (BD)* tėkmės citometru, analizuojama *FACS Diva* programa. Aberantiniams su leukemija susijusiems fenotipams nustatyti ir daugini atsparumą vaistams vertinti buvo rinkta po 50 000 ląstelių, minimalios liekamosios ligos tyrimams – 1 mln. ląstelių. Aberantiniai su leukemija susiję fenotipai vertinti kokybiškai (0/1 sistema), minimali liekamoji liga vertinta procentiškai, santykyje su visomis mėginio branduolėtomis ląstelėmis. Dauginis atsparumas vaistams buvo tiriamas dvejopai: nustatant nuo ATF priklausančių membraninių nešiklių p-gp ir MRP1 ekspresiją ląstelių paviršiuje ir tiriant švytinčio jų substrato - kalceino susikaupimą ląstelėse (abiem atvejais buvo vertinamas santykinis kiekybinis rodiklis - vidutinis fluorescencijos intensyvumas). Su leukemija susijusių aberantinių fenotipų įtaka minimalios liekamosios ligos dinamikai ir dauginio atsparumo vaistams rodikliams tirta taikant statistinį Mano-Vitnio-Vilkoksono kriterijų, dauginio atsparumo vaistams rodiklių koreliacija su blastų skaičiumi įvairiais gydymo periodais tirta taikant Spirmeno koreliacijos koeficientą.

Rezultatai

Visiems tirtiems sergantiems B-ŪLL ligoniams buvo nustatyta bent po vieną fenotipo aberaciją (vidutiniškai 5,1, nuo 1 iki 10). Labiausiai paplitusi su leukemija susijusių fenotipų grupė buvo žymenų hipoekpresija. Dažniausiai (75% atvejų) aptikome ląstelės branduolio žymens TdT hipoekspresiją. Žymenų CD10, CD38 ir CD45 ekspresijos sumažėjimas aptiktas atitinkamai 33,3%, 27,8%, 31% ligonių. T-ŪLL pacientams vidutiniškai rasta po 8,6 aberacijos (5-12). Dažniausios fenotipo aberacijos priklausė ektopinės žymenų ekspresijos grupei: ankstyvosios diferenciacijos žymens TdT ekspresija kaulų čiulpų T limfoidinių ląstelių populiacijoje aptikta 86,2%, o CD99 – net 100% atvejų. Kita dažna aberacijų grupė buvo žymenų hipoekspresija: CD45 ir CD3 žymenų hipoekspresija rasta 75,9% ir 100% atvejų. Bent viena fenotipo aberacija buvo nustatyta 71,4% sergančiųjų ŪML (vidutiniškai 5,0, nuo 1 iki 11). Vyraujanti šių fenotipų grupė buvo asinchroninė žymenų ekspresija. Žymens CD11c ekspresija kartu su ankstyvaisiais žymenimis CD117 ir CD34 rasta atitinkamai 62% ir 50% ŪML atvejų. CD56 ekspresija kartu su CD34 žymeniu aptikta 30%, su CD117 – 38%, o derinyje su mieloidiniais žymenimis CD13 ir CD33 - 40% ŪML atvejų. Kitų linijų žymenų ekspresijos grupėje dažniausiai nustatyta T limfoidinio žymens CD7 ekspresija (48%).

Buvo optimizuota ir įdiegta tėkmės citometrinio minimalios liktinės ligos tyrimo metodika:

1) Remiantis aberantiniais su leukemija susijusiais fenotipais sudaryti keturspalviai žymenų deriniai ūminių limfoblastinių leukemijų minimaliai liekamajai ligai tirti ir šešiaspalviai žymenų deriniai ūminės mielogeninės leukemijos minimaliai liekamajai ligai tirti.

2) Atlikus praskiedimo eksperimentą, nustatytas vienodas lizuoto „viso“ kraujo ir mononuklearų išskyrimo pagal tankio gradientą mėginių paruošimo technikų jautrumas (atitinkamai 0,007% ir 0,008% B-ŪLL, 0,009% ir 0,008% T-ŪLL ir 0,07% ir 0,05% ŪML atveju). Tolesniems tyrimams parinktas lizuoto „viso“ kraujo metodas.
3) Sukurta standartizuota analizės sistema, pagrįsta sveikų asmenų kaulų čiulpų tyrimu: B-ŪLL – „3x3” tuščių regionų, T-ŪLL ir ŪML – „2x3” tuščių regionų metodas.

Nustatyta šių su leukemija susijusių aberantinių fenotipų įtaka minimalios liekamosios ligos dydžiui:

1) B-ŪLL ligoniams, turintiems CD10++TdT+, CD10++CD20++ fenotipą, stebėjimo eigoje nustatytas mažesnis blastų skaičius, o tiems, kurių blastai hiperekspresuoja CD34, nustatytas didesnis blastų skaičius.
2) Sergantiesiems T-ŪLL, CD34+ ekspresija, fenotipas CD4-CD8-, CD2 ir CD5 hipoekspresija bei mieloidinių žymenų CD13 ir CD33 koekspresija turėjo įtakos didesniam blastų skaičiui, o CD1a ekspresija ir CD4+CD8+ fenotipas turėjo įtakos mažesniam blastų skaičiui.
3) ŪML atveju ligoniams, kuriems rastas CD117+CD11c+ fenotipas, nustatytas mažesnis blastų skaičius ankstyvuoju gydymo periodu, o fenotipas CD13+CD33+CD56+ ir CD5 bei CD7 žymenų koekspresija turėjo įtakos didesniam blastų skaičiui.

Optimizuotas dauginio atsparumo vaistams tyrimas. P-gp ir MRP1 ekspresija vertinama, nustatant vidutinį fluorescencijos intensyvumą ir palyginant jį su analogiško sveikų asmenų mononuklearų rodiklio reikšme. Nustatyti dauginio atsparumo vaistams rodiklių tarpusavio ryšiai: B-ŪLL ir ŪML atveju nustatytas stiprus ryšys tarp p-gp ir MRP1 ekspresijos (Spirmeno koreliacijos koeficientas 0,929 ir 0,767, $p<0,0001$). Ryšio tarp p-gp ir MRP1 ekspresijos (struktūrinio atspraumo) ir kalceino fluorescencijos (funkcinio atsparumo) neįrodyta.

Nustatyta, kad blastų struktūriniai ir funkciniai dauginio atsparumo vaistams rodikliai (p-gp ekspresija, MRP1 ekspresija ir kalceino fluorescencija) statistiškai patikimai skyrėsi nuo kitų mėginio ląstelių rodiklių ($p<0,05$). Skirtinga ūminės leukemijos forma sergantiems ligoniams patikimai skyrėsi tik MRP1 ekspresijos reikšmė: mažiausia ji buvo sergančiųjų T-ŪLL (78,67 sant.vnt.), didžiausia – sergančiųjų ŪML su mielodispaziniais požymiais (167,25 sant vnt.).

Nustatytas stiprus ryšys tarp dauginio atsparumo vaistams rodiklių ir minimalios liekamosios ligos. Sergantiesiems B-ŪLL kalceino fluorescencijos intensyvumas koreliavo su blastų skaičiumi 35-76 gydymo dieną (Spirmeno koreliacijos koeficientas 1,000, $p<0,0001$), MRP1 ekspresija – su blastų skaičiumi 29-35 d. ir 77-90 d. (koreliacijos koeficientas atitinkamai 0,712, $p<0,05$ ir 0,706, $p<0,05$), p-gp ekspresija – su blastų skaičiumi 77-90 d. (koreliacijos koeficientas 1,000, $p<0,0001$). Sergantiesiems ŪML nustatytas stiprus ryšys tarp p-gp ekspresijos ir blastų skaičiaus 4-ą gydymo mėnesį (koreliacijos koeficientas 1,000, $p<0,0001$).

Su leukemija susijusių aberantinių fenotipų įtaka dauginio atsparumo vaistams rodikliams statistiškai reikšminga buvo tik ŪML ligonių grupėje. CD13+CD33+CD56+ fenotipas turėjo įtakos mažesniam kalceino vidutiniam fluorescencijos intensyvumui. Ligoniams, kuriems nustatyta T limfoidinio žymens CD5 koekspresija, kalceino

fluorescencija taip pat buvo mažesnė. CD7 žymens koekspresija buvo susijusi su didesne p-gp ekspresija. Būtent šie fenotipai siejosi ir su didesne minimalia liekamąja liga.

Išvados

1) Absoliučiai daugumai sergančiųjų ūmine limfoblastine leukemija ir žymiai daliai sergančiųjų ūmine mielogenine leukemija randama su leukemija susijusių aberantinių fenotipų, kurie gali būti taikomi tiriant minimalią liekamąją ligą.

2) Tėkmės citometrinis tyrimo metodas, kuomet sergantiesiems ūmine limfoblastine leukemija taikomi keturspalviai žymenų deriniai, o sergantiesiems ūmine mielogenine leukemija - šešiaspalviai žymenų deriniai, lizuoto kraujo mėginių paruošimo technika ir standartizuota analizės sistema, leidžia pakankamu jautrumu ir specifiškumu tirti daugumos sergančiųjų minimalią liekamąja ligą.

3) Sergantiesiems ūmine B-limfoblastine leukemija asinchroninė žymenų ekspresija CD10++TdT+ ir CD10++CD20++ turi įtakos mažesniam, o CD34 hiperekspresija – didesniam blastų skaičiui gydant. Sergantiesiems ūmine T-limfoblastine leukemija su ankstyvąja diferenciacija susiję fenotipai CD34+ ir CD4-CD8-, taip pat žymenų CD2 ir CD5 hipoekspresija bei mieloidinių žymenų CD13 ir CD33 koekspresija turi įtakos didesniam blastų skaičiui, o vėlesnės diferenciacijos fenotipai CD1a+ ir CD4+CD8+ – mažesniam blastų skaičiui gydant. Ūminės mielogeninės leukemijos atveju asinchroninė žymenų ekspresija CD117+CD11c+ turi įtakos mažesniam blastų skaičiui, o fenotipas CD13+CD33+CD56+ ir CD5 bei CD7 žymenų koekspresija - didesniam blastų skaičiui gydant.

4) Blastų atsparumas vaistams gali būti tiriamas nustatant p-gp ir MRP1 ekspresiją (struktūrinis atsparumas) ir kalceino susikaupimą (funkcinis atsparumas) ir šių žymenų vidutinį fluorescencijos intensyvumą lyginant su atitinkamais sveikų ląstelių rodikliais. Struktūrinis atsparumas vaistams nebūtinai susijęs su funkciniu.

5) Sergančiųjų ūmine mielogenine leukemija fenotipas CD13+CD33+CD56+ ir T limfoidinių žymenų CD5 bei CD7 koekspresija susijusi su didesniu dauginiu atsparumu vaistams.

6) P-gp ekspresija patikimai susijusi su minimalia B-ŪLL ir ŪML liekamąja liga, o MRP1 ekspresija ir kalceino susikaupimas – tik su B-ŪLL minimalia liekamąja liga.

Apie autorę

Miglė Janeliūnienė gimė 1976 m. kovo 5 d. Vilniuje. Nuo 1983 m. mokėsi Vilniaus 31-ojoje (dabar Tuskulėnų) vidurinėje mokykloje, kurią 1993 m. baigė aukso medaliu. 1993 – 1999 m . studijavo Vilniaus Universiteto Medicinos fakultete, jį baigė su pagyrimu. 2000 m. šiame universitete baigė pirminę rezidentūrą, o 2001 – 2003 m. mokėsi laboratorinės medicinos specialybės rezidentūroje. Sėkmingai ją pabaigusi, įgijo laboratorinės medicinos gydytojos specialybę. 2004 – 2011 m. studijavo Vilniaus universiteto Medicinos krypties doktorantūroje.

2001 – 2003 m. Miglė Janeliūnienė dirbo Vilniaus Universiteto ligoninės Santariškių klinikų Hematologijos ir bendrosios citologijos laboratorijoje gydytojo asistente, o nuo 2003 m. iki šiol šioje laboratorijoje dirba laboratorinės medicinos gydytoja. Miglės Janeliūnienės pagrindinė interesų sritis – tėkmės citometriniai tyrimai ir jų taikymas hematologinių ligų diagnostikoje ir prognozuojant šių ligų eigą. Miglė Janeliūnienė nuolat dalyvauja tarptautiniuose savo specialybės srities renginiuose, rengia stendinius pranešimus, straipsnius. Ji yra išspausdinusi 7 publikacijas recenzuojamuose periodiniuose Lietuvos ir tarptautiniuose leidiniuose, 3 jų – disertacijos tema, vieną – ISI sąrašo žurnale. 2006 metais stažavosi St. Jude Vaikų ligoninėje (Memfis, JAV).

Miglė Janeliūnienė yra ištekėjusi, vyras Tomas – politologas, VU docentas. Turi du vaikus.

References

1. Greer JP, Foerster J, Rodgers GM, Paraskevas F, Glader B. Wintrobe's Clinical Hematology, 12th ed. Philadelphia: Lippincott Williams & Wilkins, 2008.
2. Longo D. Malignancies of Lymphoid Cells. In: Kasper DL, Braunwald E, Hauser S, Longo D, Jameson JL, Fauci AS. Harrison‘s Principles of Internal Medicine. 16th ed. New York: McGraw-Hill Professional; 2004.
3. Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA, Edwards BK (eds). SEER Cancer Statistics Review, 1975-2008, National Cancer Institute. http://seer.cancer.gov/csr/1975_2008 (2011 09 28).
4. Cortelazzo S, Ponzoni M, Ferreri AJ, Hoeltzer D. Lymphoblastic lymphoma. [Journal article]. Crit Rev Oncol Hematol 2011, Jan 25.
5. Pui CH, Boyett JM, Rivera GK, Hancock ML, Sandlund JT, Ribeiro RC, Rubnitz JE, Behm FG, Raimondi SC, Gajjar A, Razzouk B, Campana D, Kun LE, Relling MV, Evans WE. Long-term results of Total Therapy studies 11, 12 and 13A for childhood acute lymphoblastic leukemia at St Jude Children's Research Hospital. Leukemia 2000; 14(12): 2286-94.
6. Kamps WA, Veerman AJ, van Wering ER, van Weerden JF, Slater R, van der Does-van den Berg A. Long-term follow-up of Dutch Childhood Leukemia Study Group (DCLSG) protocols for children with acute lymphoblastic leukemia, 1984-1991. Leukemia 2000; 14(12):2240-6.
7. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, Dombret H, Fenaux P, Grimwade D, Larson RA, Lo-Coco F, Naoe T, Niederwieser D, Ossenkoppele GJ, Sanz MA, Sierra J, Tallman MS, Löwenberg B, Bloomfield CD, European Leukemia Net. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European Leukemia Net. Blood 2010; 115(3): 453-74.
8. Barrett AJ, Horowitz MM, Pollock BH, Zhang MJ, Bortin MM, Buchanan GR, Camitta BM, Ochs J, Graham-Pole J, Rowlings PA, et al. Bone marrow transplants from HLA-identical siblings as compared with chemotherapy for children with acute lymphoblastic leukemia in a second remission. N Engl J Med 1994; 331(19): 1253-8.
9. Kris MG, Benowitz SI, Adam S, Diller L, Ganz P, Kahlenberg MS,Le QT,Markman M, Masters GA, Newman L,. Obel JC, Seidman AD, Smith SM,Vogelzang N, Petrelli NJ. Clinical Cancer Advances 2010: Annual Report on Progress Against Cancer From the American Society of Clinical Oncology. J Clin Oncol 2010; 28(36): 5327-47.
10. Meyer L. Timeline: Milestones in Cancer Treatment. http://www.curetoday.com/index.cfm/fuseaction/article.show/id/2/article_id/631 (2011 09 28).
11. Mastrangelo R, Poplack D, Bleyer A, Riccardi R, Sather H, D'Angio G. Report and recommendations of the Rome workshop concerning poor-prognosis acute lymphoblastic leukemia in children: biologic bases for staging, stratification,and treatment. Med Pediatr Oncol 1986; 14(3): 191-4.

12. Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. Blood Rev 2004; 18(2): 115-36.
13. Peters JM, Ansari MQ. Multiparameter flow cytometry in the diagnosis and management of acute leukemia. Arch Pathol Lab Med 2011; 135(1): 44-54.
14. Shapiro HM. Flow cytometry: problems, parameters, probes and principles. In: Shapiro HM. Practical flow cytometry. 4th ed. New Jersey: Willey-Liss; 2003, p. 18 - 48.
15. Campana D, Pui CH. Detection of minimal residual disease in acute leukemia: methodological advances and clinical significance. Blood 1995; 85: 1416-34.
16. Saxena A, Sheridan DP, Card RT, McPeek AM, Mewdell CC, Skinnider LF. Biologic and clinical significance of CD7 expression in acute myeloid leukemia. American Journal of Hematology 1998; 58: 278–84.
17. Hurwitz CA, Raimondi SC, Head D, Krance R, Mirro J Jr, Kalwinsky DK, Ayers GD, Behm FG. Distinctive immunophenotypic features of t(8;21)(q22;q22) acute myeloblastic leukemia in children. Blood 1992; 80(12): 3182-8.
18. Hrycyna CA, Zhang S, Ramachandra M, Ni B, Pastan I, Gottesman MM. Functional and molecular characterization of the human multidrug transporter. In: Gupta S, Tsuruo T. Multidrug Resistance in Cancer Cells. Chichester, England: Wiley, 1996; p. 29.