

Molecular Genetic Markers in Detection and Follow-up of Hematological Malignancies



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Biomedical Science



- Fascinating development and accumulation of knowledge in the field of molecular genetics made a great impact on medical practice. It has been shown that the majority of human diseases is determined by genetic factors.

Laboratory for Molecular Hematology...

and other diseases

Topics:

- **Monogenic Diseases**

Thalassemia syndromes

Phenylketonuria

- **Inflammatory Diseases**

Inflammatory Bowel Diseases

- **Cancer**

Hematological malignancies

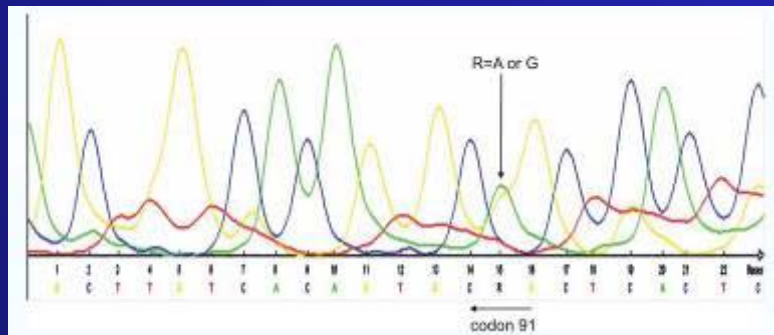
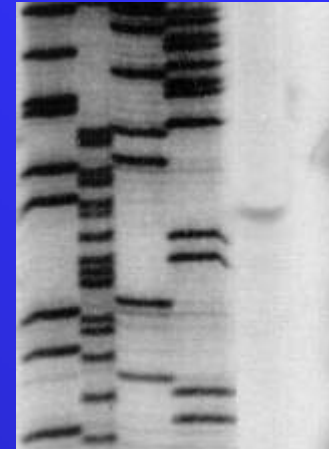
- **Pharmacogenetics**

One way...

From fundamental research to practical application in health care

Regulation of transcription of rat globin genes

Pavlovic S, Mitrovic T, Nikcevic G, Grujicic N, Lazic D, Glisin V, Popovic Z. The rat β miny-globin promoter: nuclear protein factors and erythroid-specific induction of transcription. Cellular and Molecular Life Sciences 56, 871-881. (1999)

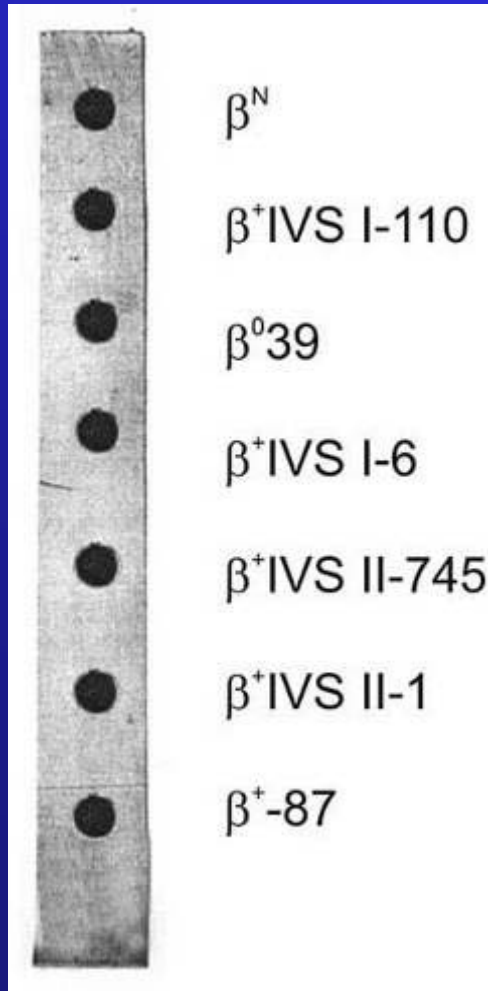


Thalassemia syndromes

Pavlovic S, Urosevic J, Poznanić J, Perišić Lj, Petručev B, Tošić N, Krivokapić-Dokmanović L, Janić D, Čvorkov-Dražić M, Bunjevački G. Molecular basis and origin of thalassemia syndromes in Serbia.

Acta Haematologica 2005; 113: 175-180.

Serbian diagnostic chip for β -thalassemia



From fundamental research to application

- Benefits:
- Improvement of health care sector
- Development, standardization and implementation of new approaches in medicine in the field of prevention, diagnosis, prognosis and therapy
- Self-funding science
- Clinical studies

...or another

From application in health care to basic
research and new knowledge

Benefits:

Biobanks

Interesting Cases

Medical phenomena explained at
molecular level

Cooperation between research and medical institutions- common project proposals in the field of Biomedicine

Our Projects funded by MSTD RS:

- 1. Genomic elements in phenotype modulation (Biology, with participation of medical scientists)**
- 2. Clonal transformation of hematopoietic stem cells (Medicine, with participation of biologists)**

Modern biomedical science requires engagement of various experts in basic research, laboratory practice and clinics. Therefore, mobilization of the critical human and material resources in Serbia is indispensable.

Integrative, multidisciplinary approach should be promoted.

Molecular Genetic Markers in Detection and Follow-up of Hematological Malignancies

- Nowadays, research in the field of molecular hematology is very intense, resulting in successful application of molecular genetic methods in diagnostics, follow-up and treatment of hematological diseases.

What are molecular genetic markers?

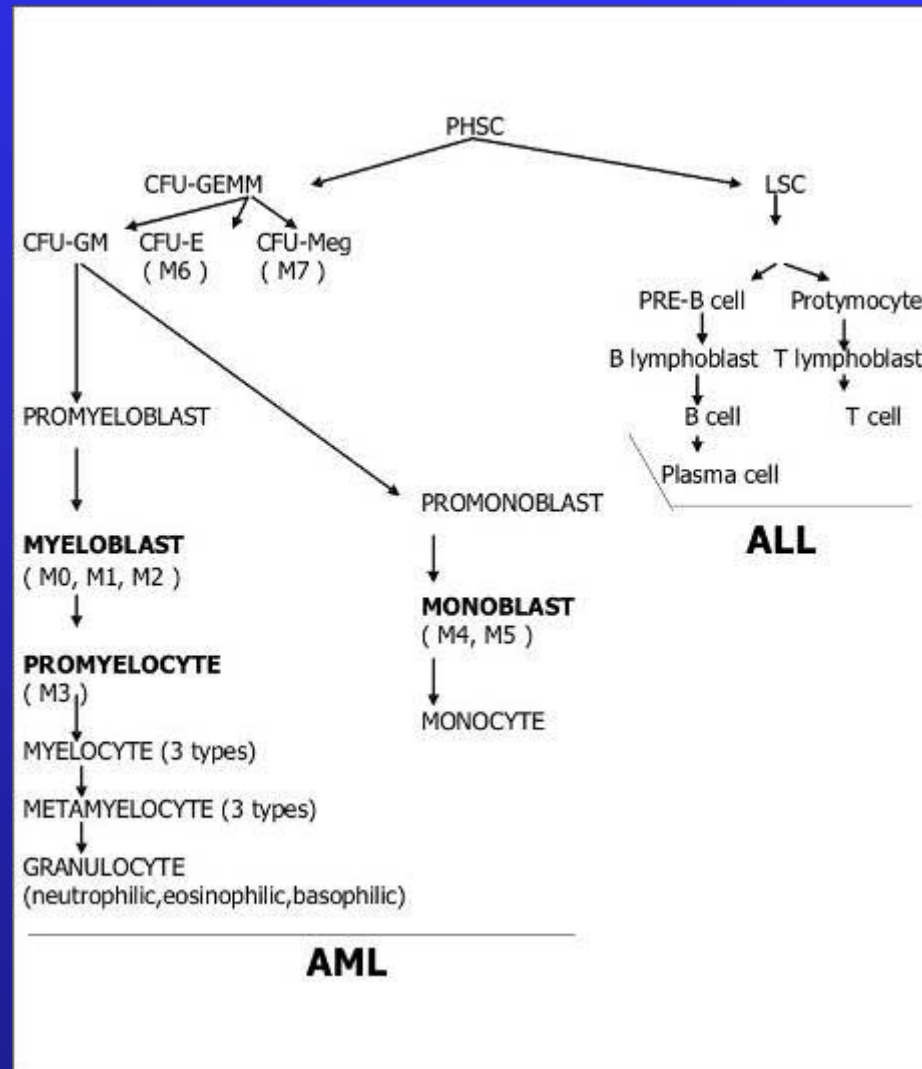
- Molecular genetic markers are changes in DNA and RNA molecules which can cause a disease or influence its course.

Why are molecular genetic markers important to hematologists?

- accurate diagnostics
- prognosis of the disease
- follow-up and early detection of relaps
- choice of the most suitable therapy
- use of molecular-targeted therapeutics

What is Leukemia?

- **Leukemia** is **cancer** of blood-forming tissue such as hematopoietic stem cells in bone marrow.
- Leukemia is characterized by the **rapid proliferation** of abnormal cells which accumulate in the **bone marrow** and interfere with the production of normal blood cells.
- Types of leukemia are grouped by the **type of cell affected** (lymphoid or myeloid) and by the **rate of cell growth** (acute or chronic).
- Leukemia represents the **clonal outgrowth of cells arrested** in early stages of hematopoietic differentiation.



Molecular genetic markers in leukemia

- Breakpoint fusion regions arising from chromosomal translocations
- Rearrangements of the immunoglobulin and T cell receptor genes
- Single gene mutations

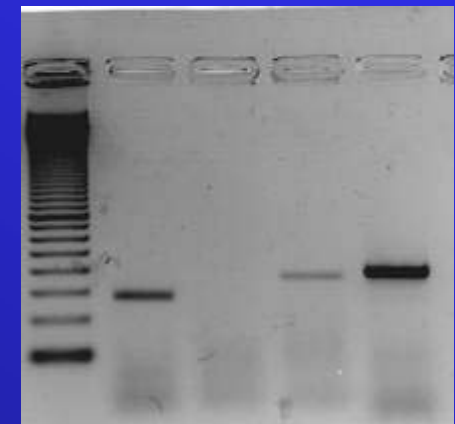
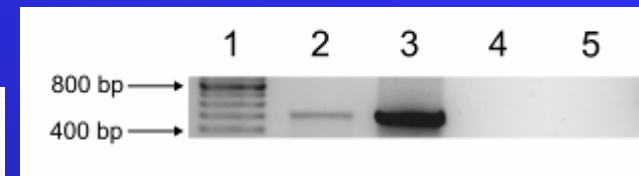
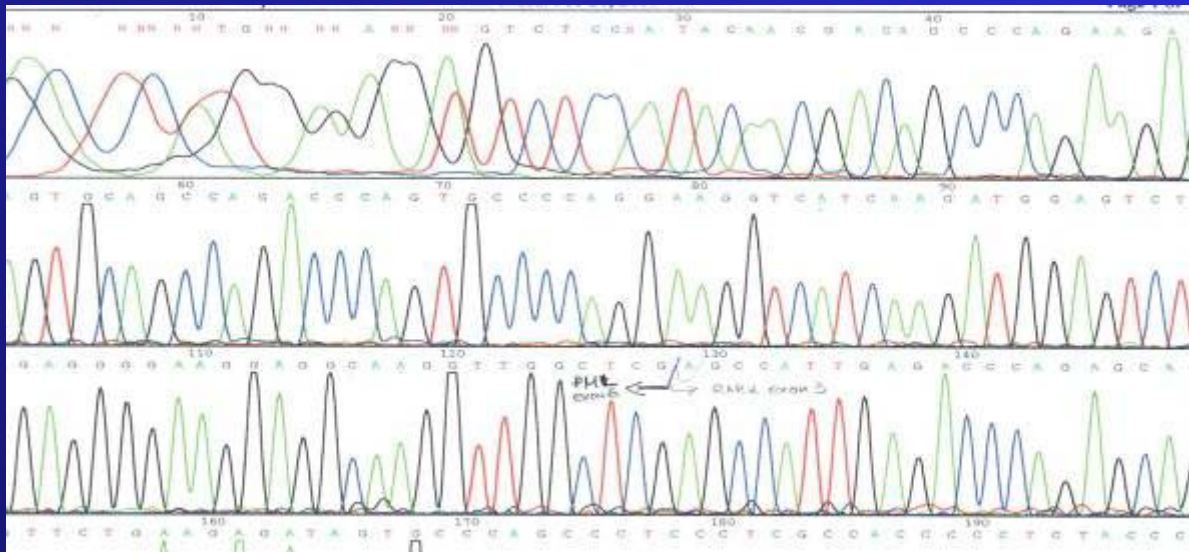
Since year 2003, our Laboratory is involved in molecular diagnostics and follow-up of different genetic aberrations characteristic for different types of leukemias

Chromosomal aberration	Fusion transcript
ALL t(1;19)(q23;p13) t(4;11)(q21;q23) t(9;22)(q34;q11) t(12;21)(p13;q22)	<i>E2A - PBX1</i> <i>MLL - AF4</i> <i>BCR - ABL (p190 and p210)</i> <i>TEL - AML1</i>
AML t(15;17)(q22;q21) t(8;21)(q22;q22) inv(16)(p13;q22)	<i>PML - RARα</i> <i>AML1 - ETO</i> <i>CBFβ - MYH11</i>
CML t(9;22)(q34;q11)	<i>BCR - ABL (p210)</i>

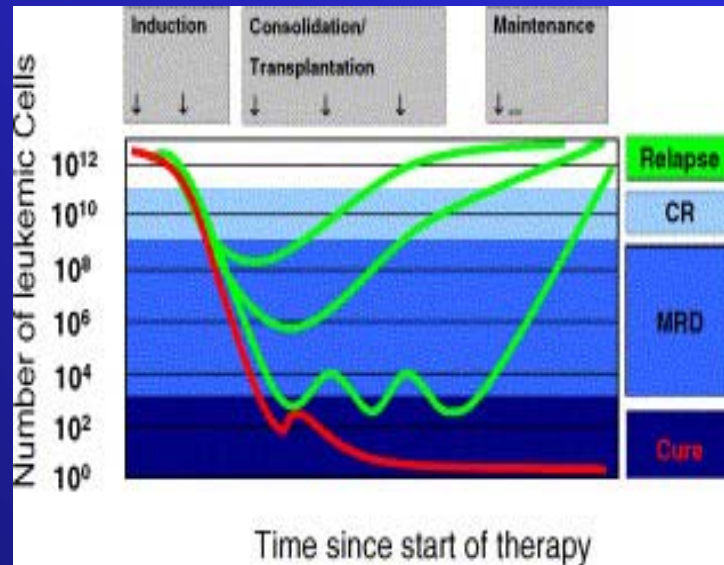
Methodology

Nested reverse-transcriptase PCR (RT PCR) and DNA sequencing analysis

- In most translocations the breakpoints are spread over one or more introns, but fusion genes are transcribed into mRNA molecules detectable by RT PCR analysis. Nested (two step) PCR is used to achieve adequate sensitivity.
- Recommendation of “BIOMED-1” and “Europe Against Cancer” Consortiums



MRD – Minimal Residual Disease



The presence of low number of remaining malignant cells in leukemia patients during and after treatment is referred to as minimal residual disease (MRD).

RT PCR method

Precise, sensitive and rapid method

Sensitivity – 10^{-4} to 10^{-6}

Specific therapy

Modern therapy for leukemia is based on the principle of risk stratification.

The most important laboratory features used to accurately stratify patients is the presence of specific chromosomal translocation within the leukemic blasts.

This stratification includes treatment intensification in patients at high risk of relapse and reduction in low-risk patients, who are being overtreated.

The latest advantage in MRD monitoring

Real-time quantitative PCR

The real breakthrough in modern therapy was the design of specific therapeutics for treating certain types of leukemia hallmarked by the presence of specific markers.

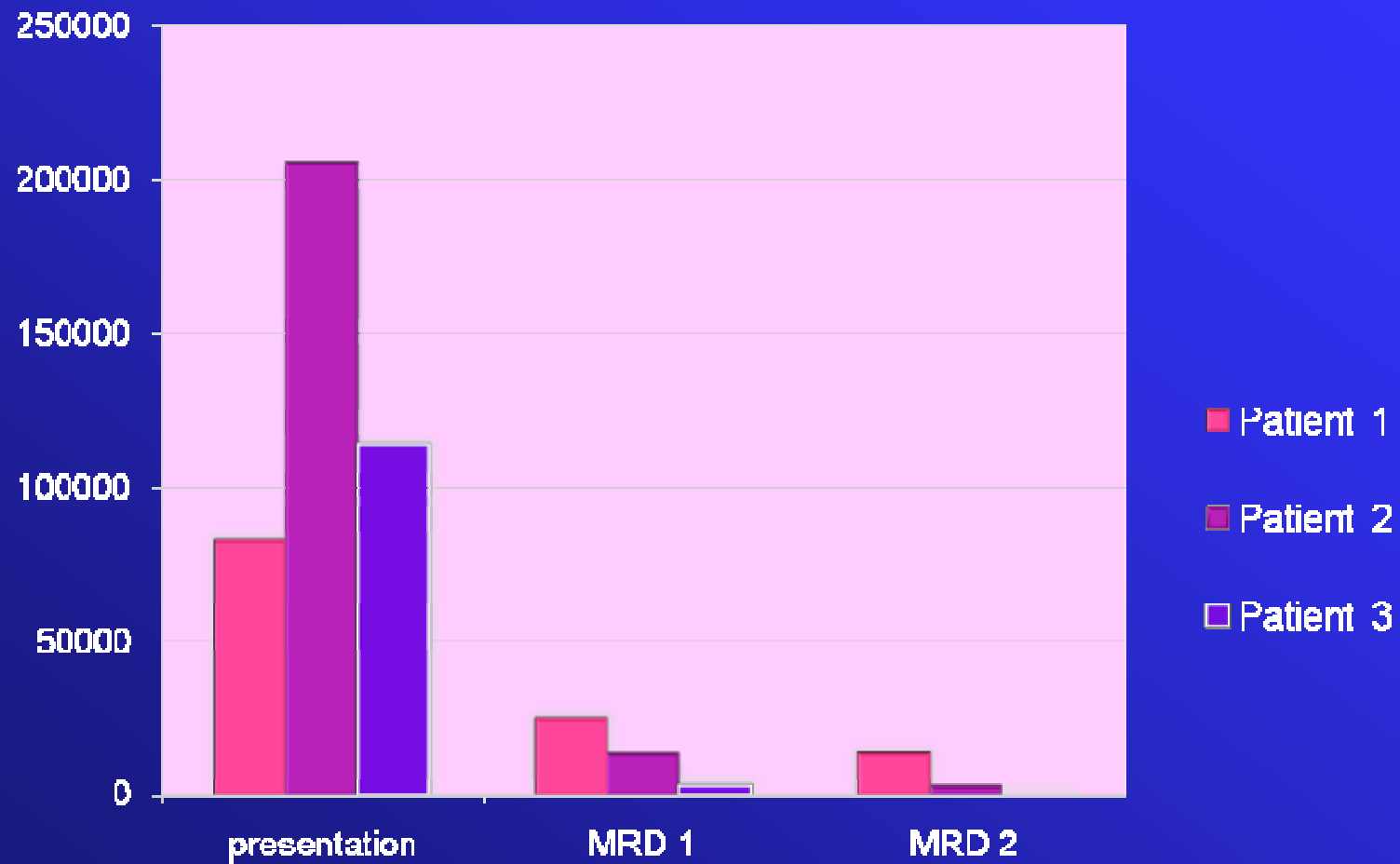
Chromosomal translocations like **t(9;22)** and **t(15;17)** are the first markers used for the designing of specific molecular based therapy

Imatinib (Gleevec) used in the treatment of CML and t(9;22) positive ALL

All-trans retinoic acid (ATRA) used in the treatment of APL (AML-M3) characterised by the presence of t(15;17)

“**Real –time**”**RQ-PCR** is the method of choice in attempt to monitor the treatment response as well as to precisely monitor the rate of eradication malignant cells during the course of the disease.

Absolute quantification of bcr-abl fusion transcript

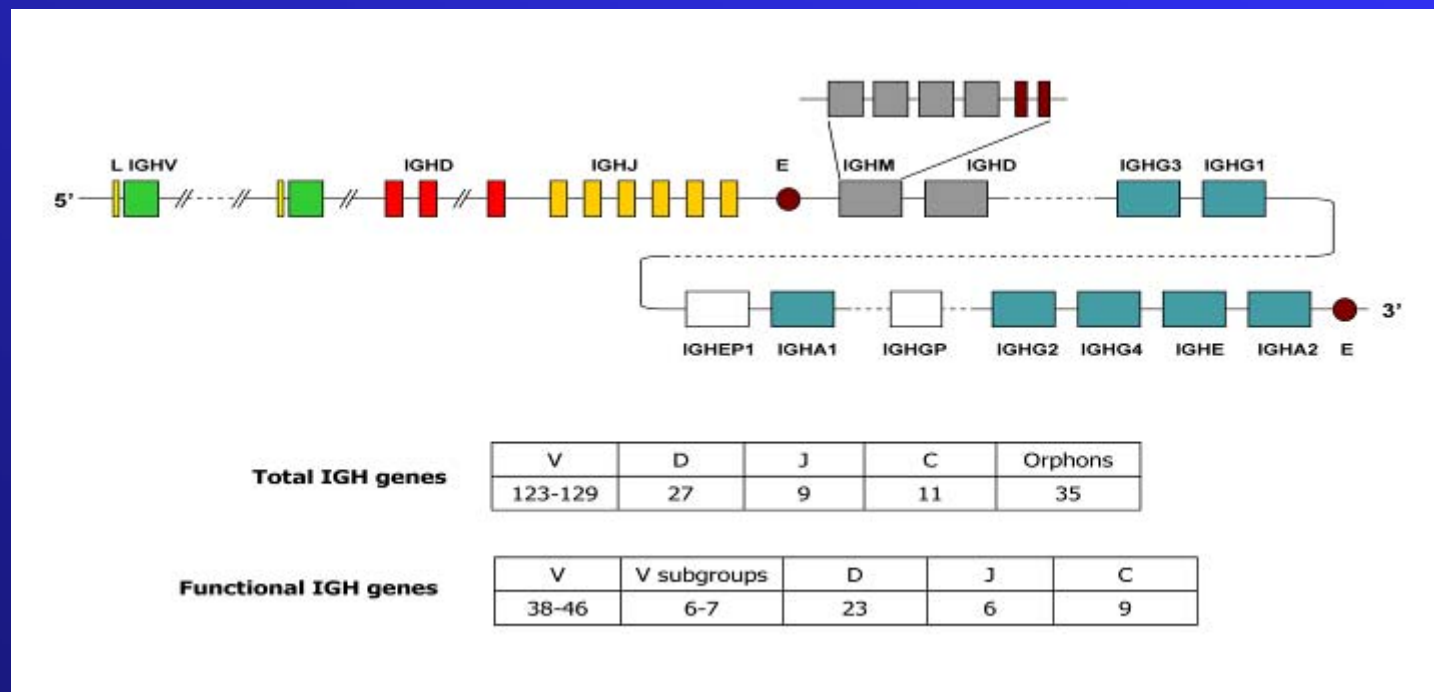


CHRONIC LYMPHOCYTIC LEUKEMIA

- the most frequent type of leukemia in the Western world (30% of all leukemias)
- clonal expansion of mature B lymphocytes
- clinically heterogeneous disorder
extremely variable clinical course with overall survival ranging from months to decades
- new biological prognostic factors -IGHV mutational status

V-D-J REARRANGEMENTS

-B cell receptor – structure determined by specific IGH V-D-J and IGK/IGL V-J rearrangements that occur during B cell differentiation

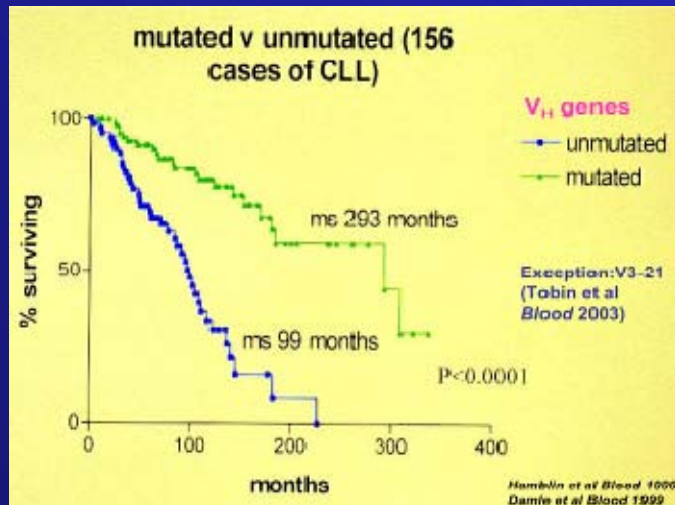


germline
organization
of IGHV
locus

- following antigen encounter, B cells undergo somatic hypermutation (SHM) and class switching process in germinal centre of lymphoid follicles

2 SUBSETS OF CLL

- CLL patients divided into 2 subgroups based on the presence or absence of SHM in IGHV chains (M-CLL and U-CLL)
- cut-off level = 98% of identity with germline sequence
- Set of PCR and sequencing analysis –ERIC recommendation

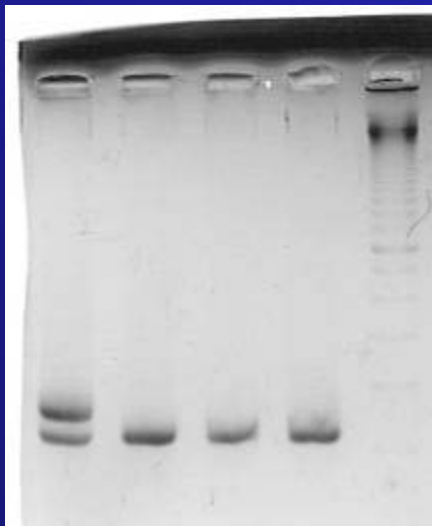


-M-CLL-good prognostic factor
-U-CLL- adverse prognostic factor

- IGHV SHM status can be assessed at any time of the disease

New prognostic molecular genetic markers in hematological malignancies- Single- gene mutations

- FLT3 gene mutations are the most frequent genetic lesion in acute myeloid leukemia (30%) and represent an adverse prognostic factor. The most frequent mutations in the FLT 3 gene are internal tandem duplications (**FLT3/ITD**) and point mutations in FLT 3 tyrosine kinase domen (exon 20).



Case 26 – 24 bp duplication

QFRYESQLQMVTG

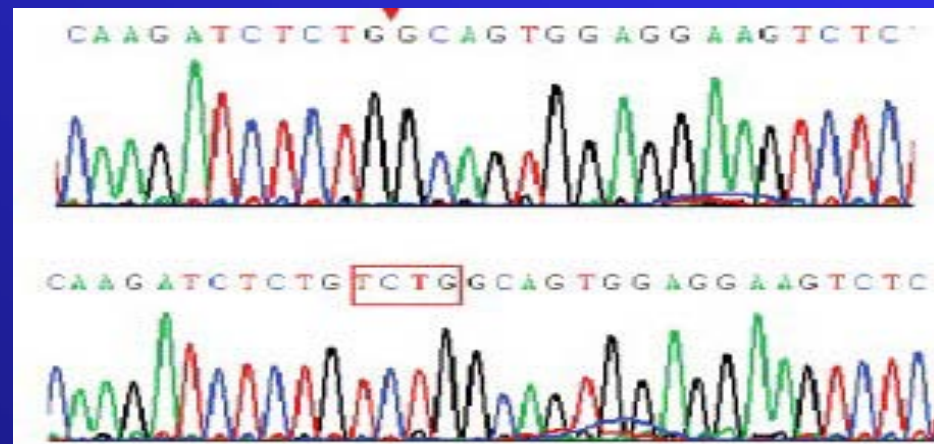
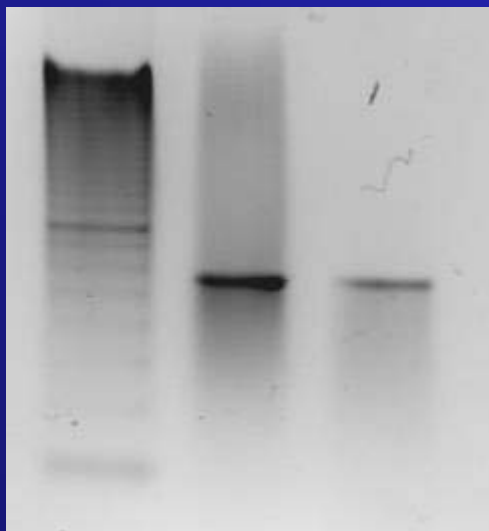
SSDNEYFYVDFREYEYDLKWEFP

SSDNEYFYVDFREYEYDLKWEFPRENLEF



New prognostic molecular genetic markers in hematological malignancies- Single gene mutations

- NPM1 gene mutations are found in 25-35% AML patients. NPM1 mutations are often associated with FLT3 mutations and then represent a good prognostic factor. More than 40 different NPM1 mutations have been identified. NPM1 gene mutations are stable and, therefore, an ideal marker for follow-up of minimal residual disease in AML with normal karyotype.



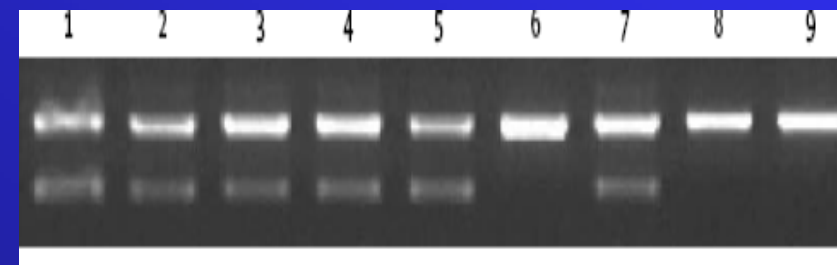
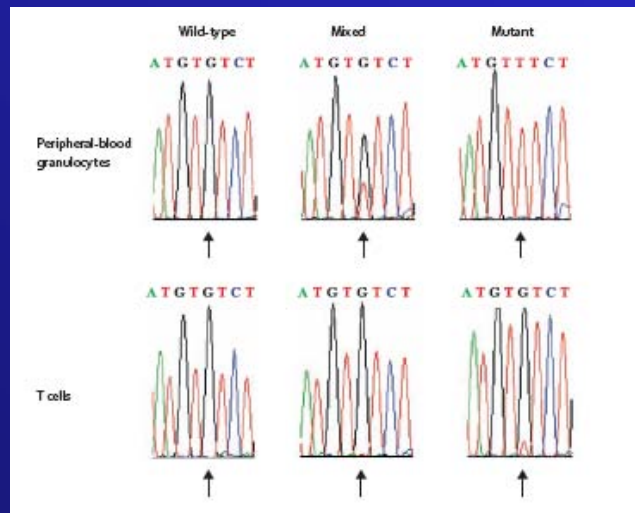
Myeloproliferative diseases

- The **myeloproliferative diseases** or neoplasms (MPDs) are a group of diseases of the bone marrow in which excess cells are produced.
- JAK2 V617F mutation provided evidence to suggest a common pathogenesis for the Philadelphia Chromosome negative MPDs.

Philadelphia Chromosome "positive" Translocation BCR/ABL	Philadelphia Chromosome "negative" JAK2 V617F mutation
<u>Chronic myelogenous leukemia (CML)</u>	<u>Polycythemia vera (PV)</u> 97% <u>Essential thrombocytosis (ET)</u> 57% <u>Myelofibrosis (MF)</u> 50%

Methodology used for detection of JAK 2 V617F mutation

- Direct sequencing of PCR product (exon 14)
- Allele-specific PCR



Commercial application

- More than 20 analysis are routinely performed in the Laboratory for molecular hematology
- Medical institutions that refer their patients to IMGGE:
 - Institute for Hematology, Clinical Center Serbia, Belgrade,
 - Military Medical Academy, Belgrade,
 - Clinical Center Zvezdara, Belgrade,
 - Clinical Center „Dragiša Mišović“, Belgrade,
 - Clinical Center Zemun, Belgrade,
 - University Children Hospital, Belgrade,
 - Mother and Child Healthcare Institute “Dr Vukan Cupic”, Belgrade, as well as
 - Clinical Centres from Novi Sad, Subotica, Niš, Banja Luka, Podgorica and Sarajevo

Basic Science

Leukemogenesis

The pathophysiology of leukemia depends on a combination of more than one of the following factors:

- ❖ Unregulated proliferation of the leukemic stem cell
- ❖ Failure of normal hematopoietic differentiation
- ❖ Inability to undergo spontaneous or chemotherapy-induced programmed cell death (apoptosis).

Leukemia is characterized by a number of genetic defects. These include translocations and single gene mutations involving oncogenes and transcription factors, activation of signal transduction pathways and alterations of growth factors.

Proliferation	Differentiation	Apoptosis
Flt 3 Ras	t(8;21), inv(16), t(15;17),t(9;22)	TRAIL Fas

Regulation of transcription and Gene expression studies

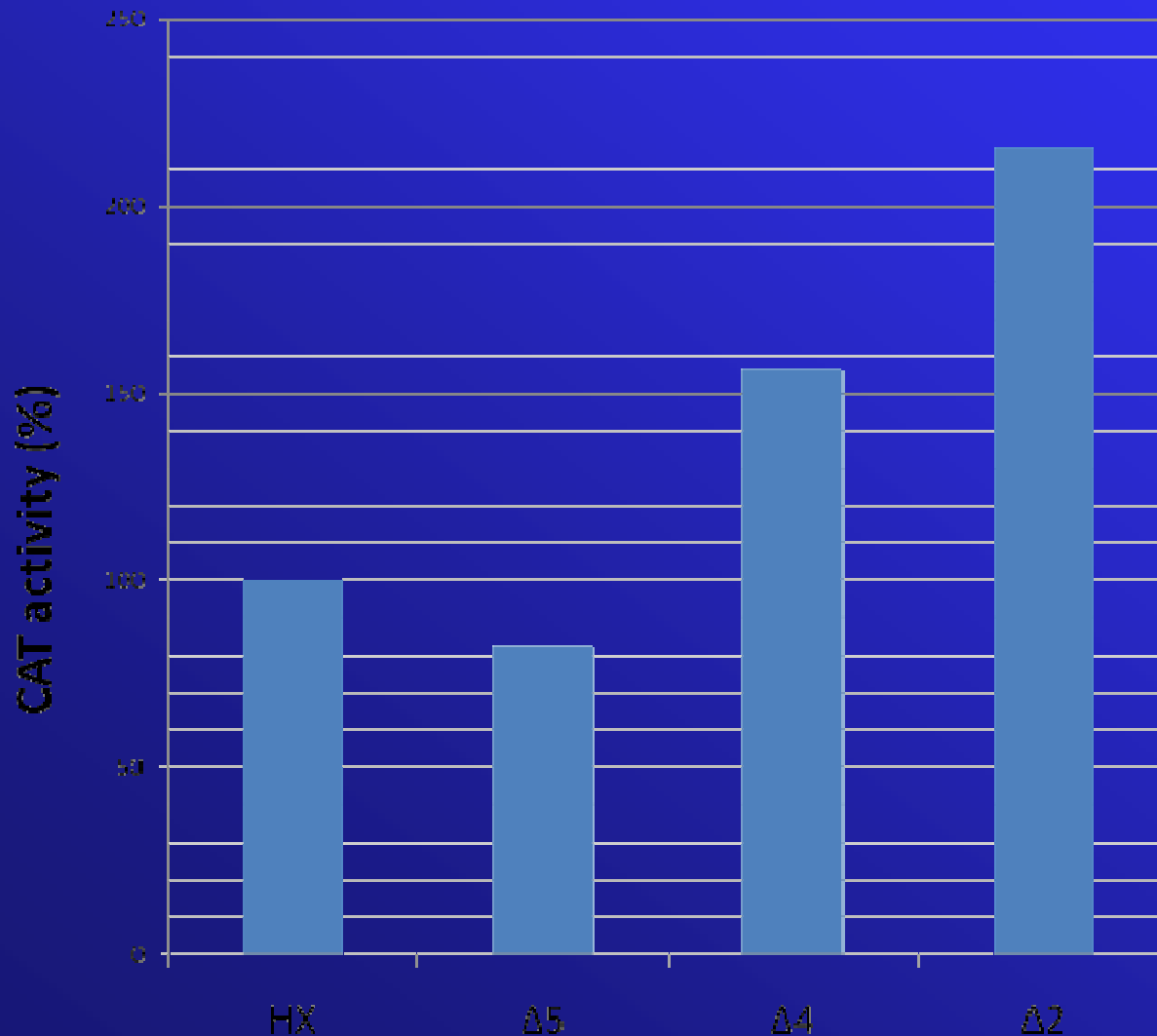
Genes:

FLT3 (proliferation)

Fas R/ Fas L (apoptosis)

BCL2L12 (apoptosis)

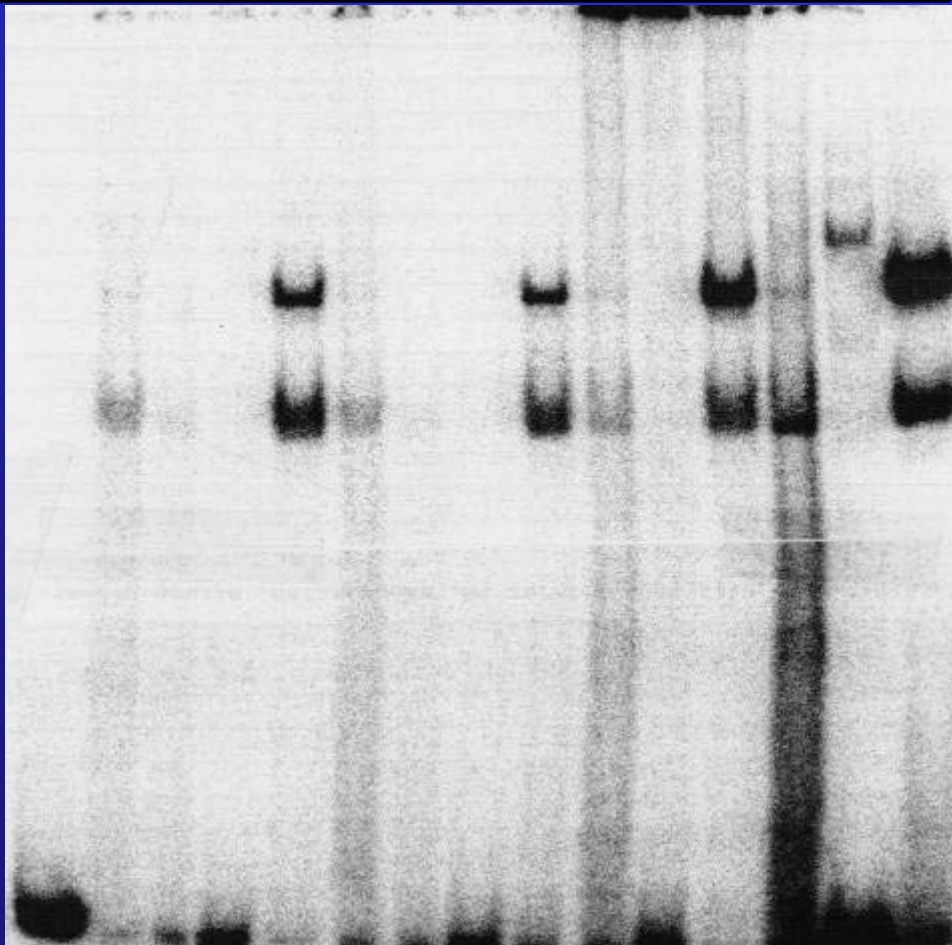
Functional assays



- Deletants ($\Delta 5$, $\Delta 4$ i $\Delta 2$) show increasing CAT activities
- It seems that $\Delta 2$ has higher CAT activity – repressor in deleted part of promoter

Gel shift assay

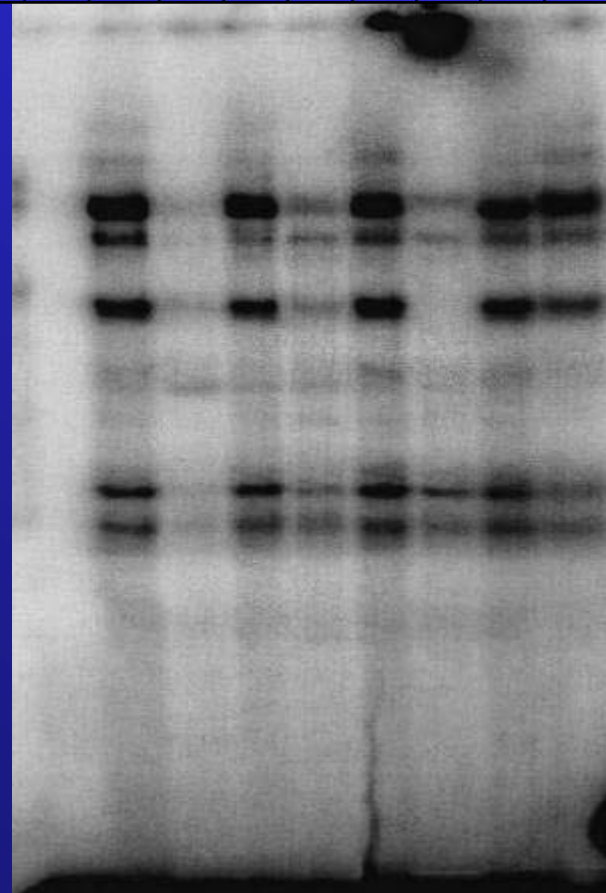
probe	AA				AB				BB				BC			
K562		+	+			+	+			+				+		
K562+ 500 μ M 6MP				+				+			+				+	
HeLa					+				+				+			+
self competitor			+				+									



BC repeats bind nuclear extracts with the highest affinity.

Supershift assay

probe	TPMT VNTR BC repeat							
nuclear extract		K562						
self competitor		+						
-110 competitor (seq. specific)			+					
consensus sequence for Sp1 binding				+				
Sp1 Ab					+			
Sp3 Ab						+		
Sp4 Ab							+	
EKLF Ab								+



← Sp3

← EKLF

← EKLF

Supershift assays confirmed that Sp3 and EKLF transcription factors are involved in transcriptional regulation of TPMT gene.

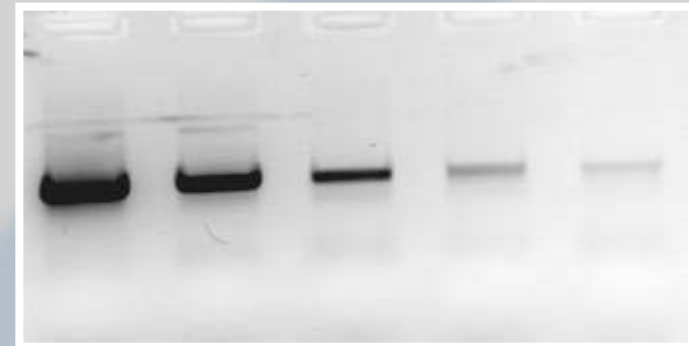
Quantitative competitive RT PCR (qc RT PCR) for FasR

**β globin gene construct with primers
for fas- β globin**

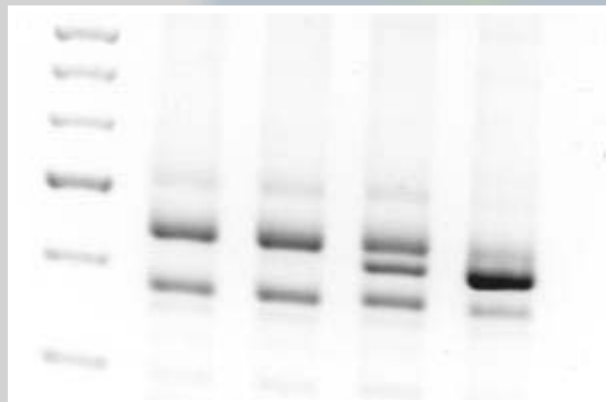
Cloning of PCR product in pUC18

DH5 α E.Coli

10⁻¹ 10⁻² 10⁻³ 10⁻⁴ 10⁻⁵

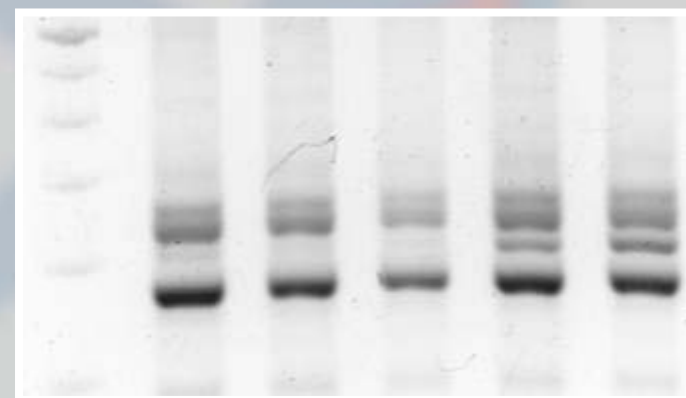


PCR competitor (delutions)



mFasR
sFasR

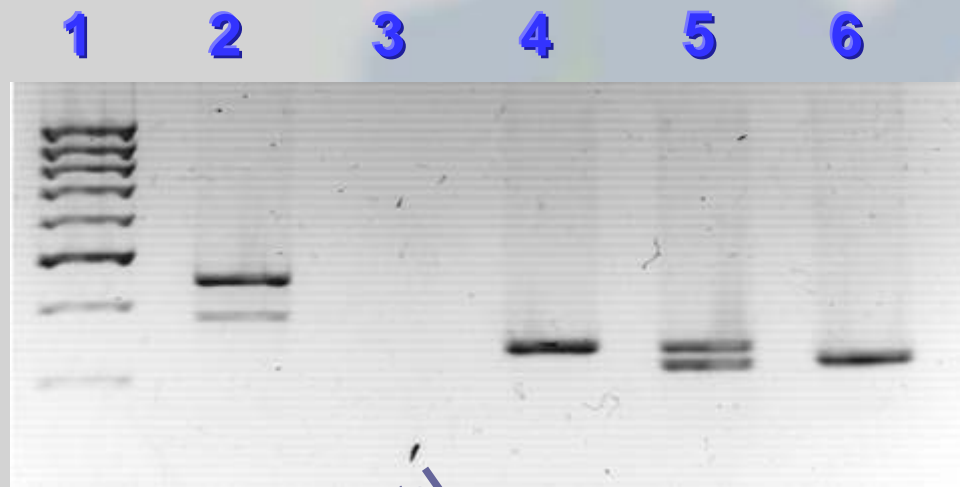
mFasR > sFasR



438 bp
375 bp

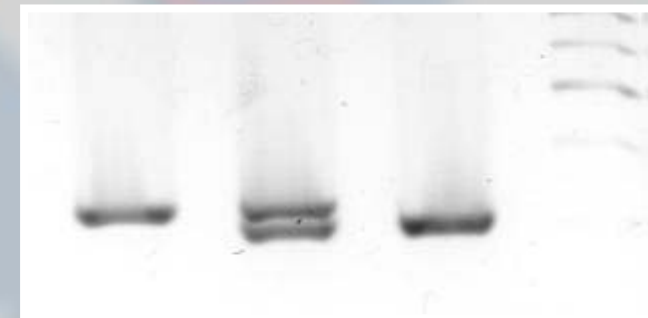
sFasR > mFasR

PCR with internal control
(abl and hpert housekeeping gens)



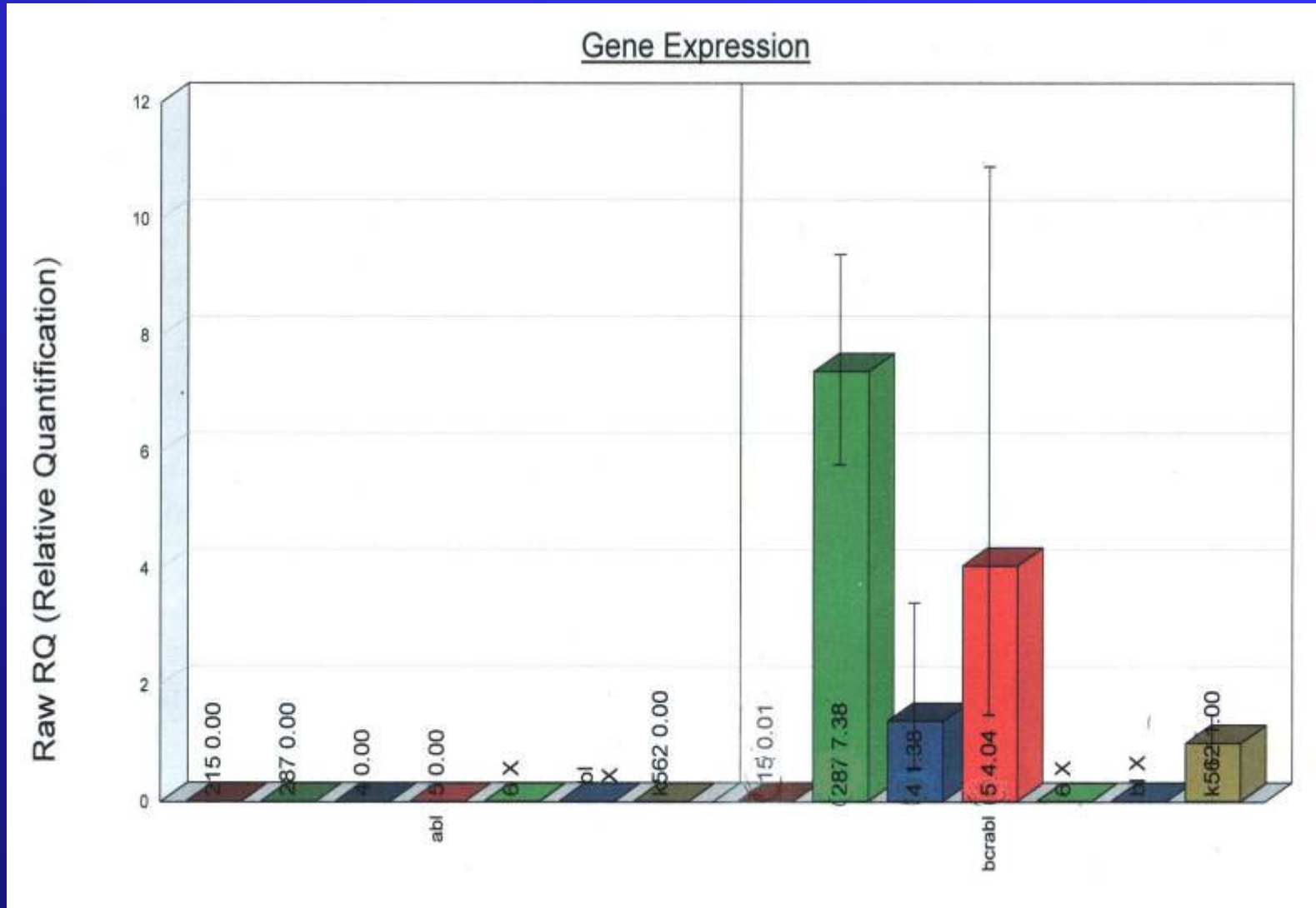
1
2
3
4
5
6

mFas - sFas
mFas - sFas - abl
abl
FasL - abl
FasL



abl
FasL - abl
FasL

Relative quantification by Real Time PCR



Pharmacogenetics

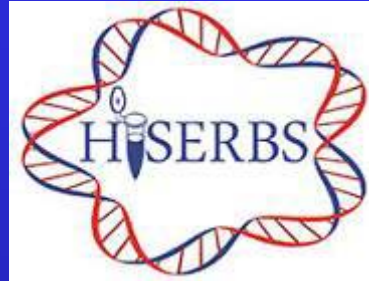
- Thiopurine S-methyltransferase (TPMT) is an enzyme that metabolizes immunosuppressive thiopurine medications, such as 6-mercaptopurine (6-MP). 6-MP is widely used in the treatment of many diseases, including acute leukemia, different types of inflammatory and autoimmune diseases and in transplantation.
- Several SNPs, associated with lower TPMT activity, represent pharmacogenetic markers important for selection of adequate therapy.
- However, genetic variants in the coding region do not correlate with all the changes in the TPMT phenotype.
- Variations in transcriptional regulatory elements could be responsible for some modulations of the TPMT enzyme activity.
- We investigate potentially new pharmacogenetic marker in TPMT gene

Publications

- 5/year (M 21 M22)
- Basic Science (on our own)
- Clinical Studies (collaboration with Serbian medical institutions)
- Interesting Cases (collaboration with Serbian medical institutions)
- Medical phenomena explained at molecular level (collaboration with colleagues from EU Institutions, our EU partners)

Health Improvement in Serbia Through Reinforcement of Biomedical Science and Technology

FP6 HISERBS PROJECT



- Local networking

IMR (Institute for Medical Research)

INEP (Institute for Application of Nuclear Energy)

Serbian Medical Institutions (Serbian LeukemiaNet
SELNET)

HISERBS Project Task

Training and Mobility

- The objectives related to training and mobility were of a particularly high significance for this project.
- Exposing researchers from our Laboratory to the latest developments in technology and science of EU was achieved through technology related training, visits of experts from EU partner institutions and training of researchers in EU partner institutions.

HISERBS EU Partners

- Erasmus University Medical Center
- Faculty of Medicine and Health Sciences
- MGC-Department of Cell Biology and Genetics
- Rotterdam, The Netherlands
- George Patrinos, PhD

- National Center for Scientific Research "Demokritos"
- Department of Biochemistry and Molecular Biology
- University of Athens
- Athens, Greece
- Andreas Scorilas, PhD

- Centro Ricerca Tettamanti
- Clinica Pediatrica Università di Milano-Bicocca
- Ospedale San Gerardo
- Monza (Mi) Italy
- Andrea Biondi, MD PhD

- Department of Haematology
- Imperial College at Hammersmith Hospital
- London, United Kingdom
- David Grimwade, MD, PhD

Collaborative European Projects after HISERBS

- European LeukemiaNet (LSHC-CT-2004-503216 EU, FP6/NoE, 2004-2008, 2008-2011).
- FP6 LIFESCIHEALTH)
 - “Strengthen and develop scientific and technological excellence in research and therapy of leukemia (CML, AML, ALL, CLL, MDS, CMPD) by integration of the leading national leukemia networks and their interdisciplinary partner groups in Europe”
- ENERCA 3 (European Network for Rare and Congenital Anemias) Public Health and Consumer Protection Directorate (DG SANCO).
- 4 FP7 Projects submitted (2 of them passed threshold)

FP6 HISERBS deliverables

- Improvement of clinical practice in Serbia in the field of hematology
- facilitation of integration of researchers into the ERA.

Want to join the ERA?

START NETWORKING

AT ALL LEVELS

Laboratory for Molecular Hematology

Networking by all means



Branka Zukic, PhD student
Maja Stojiljkovic, PhD
Natasa Tosic, MSc
Milena Radmilovic, PhD student
Teodora Karan-Djurasevic, PhD student
Vesna Spasovski, PhD student
Tanja Kostic, PhD

