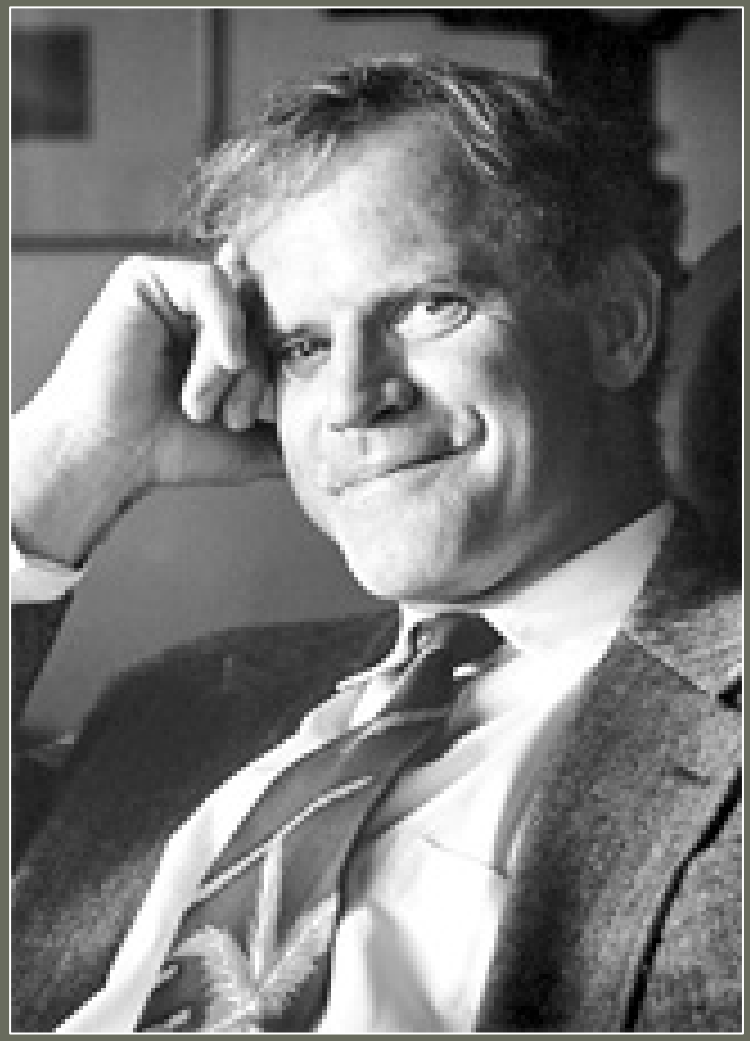
Šedivá je
teorie,
zelený
strom života

Možnosti molekulární diagnostiky v klinické praxi

Centrum molekulární biologie a genové terapie
Interní hematoonkologická klinika
Fakultní nemocnice Brno a MU Brno

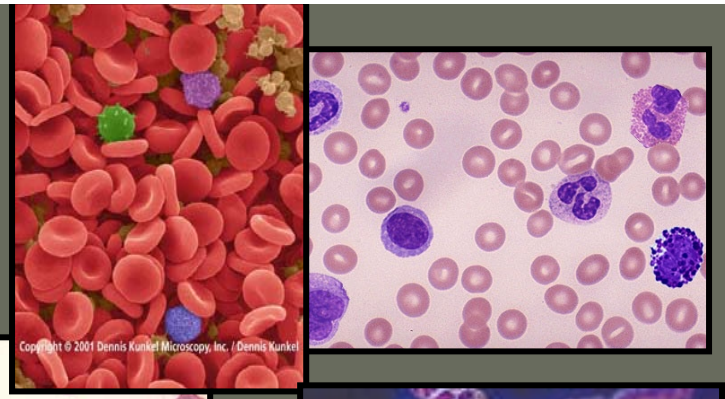
- V kterém roce byla získána Nobelova cena za objev PCR?



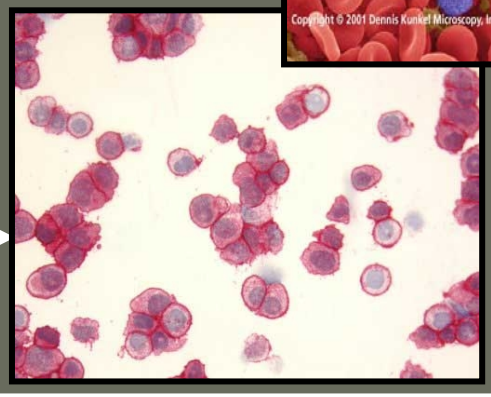
Klinická praxe

Vyšetřovací metody

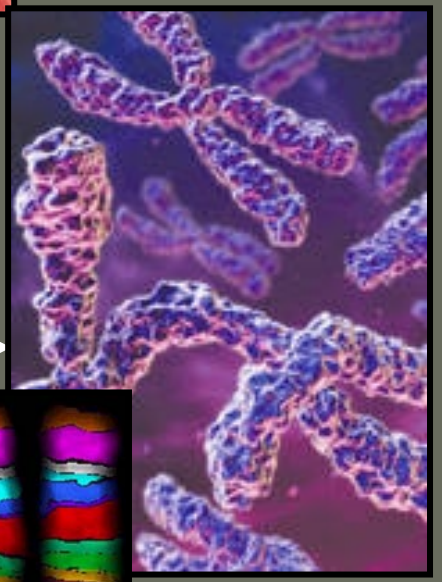
morfologické



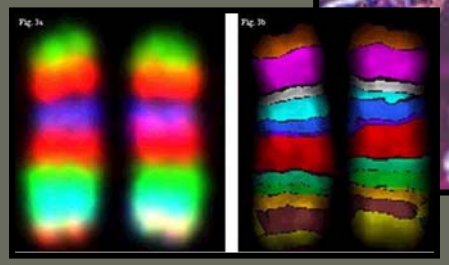
imunologické



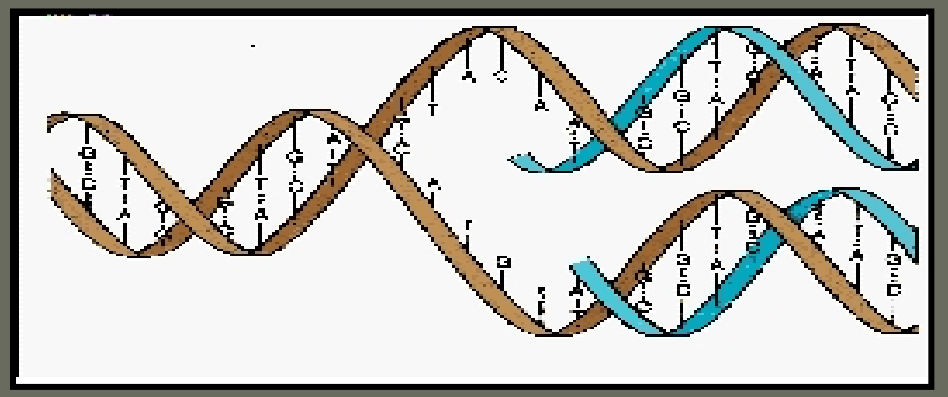
cytogenetické



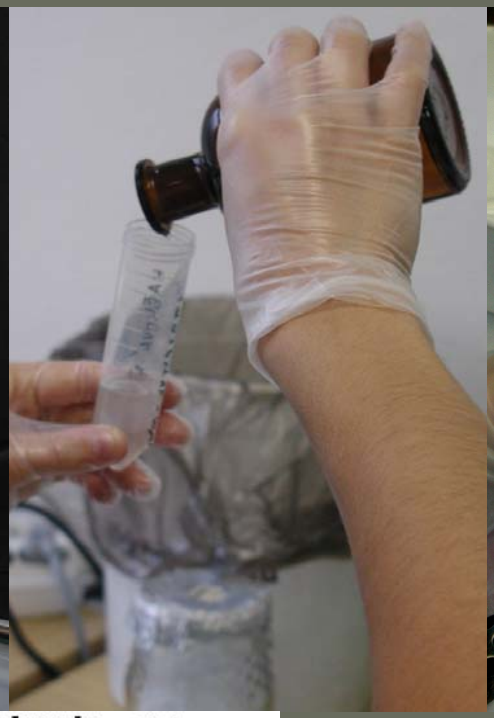
molekulárně cytogenetické

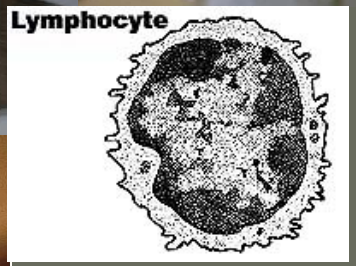


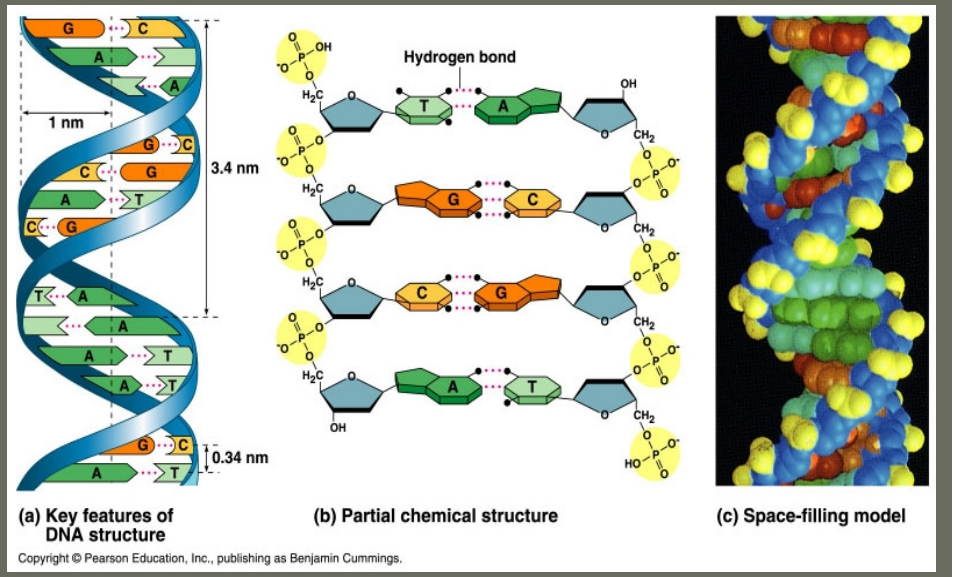
molekulárně genetické




1
2
3
START



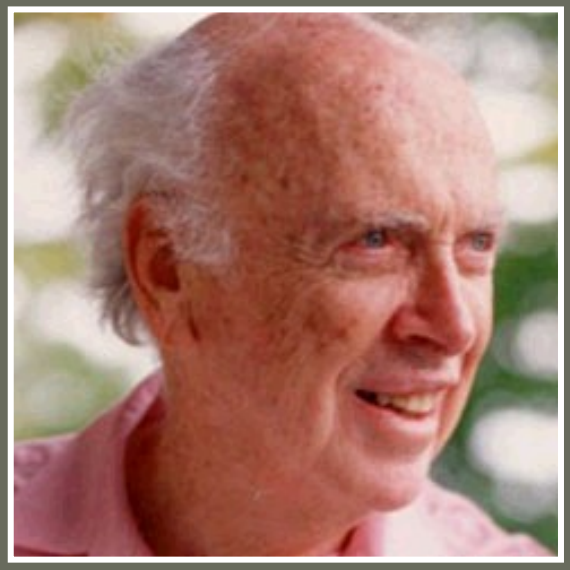

Lymphocyte


1 nm
3.4 nm
0.34 nm
Hydrogen bond
(a) Key features of DNA structure
(b) Partial chemical structure
(c) Space-filling model
Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.

Nobel Prize in physiology/medicine in 1962

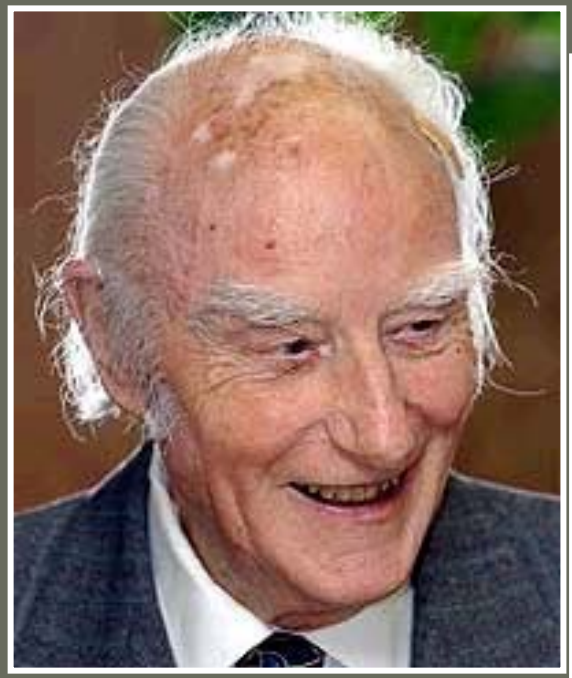
James Dewey Watson

*1928



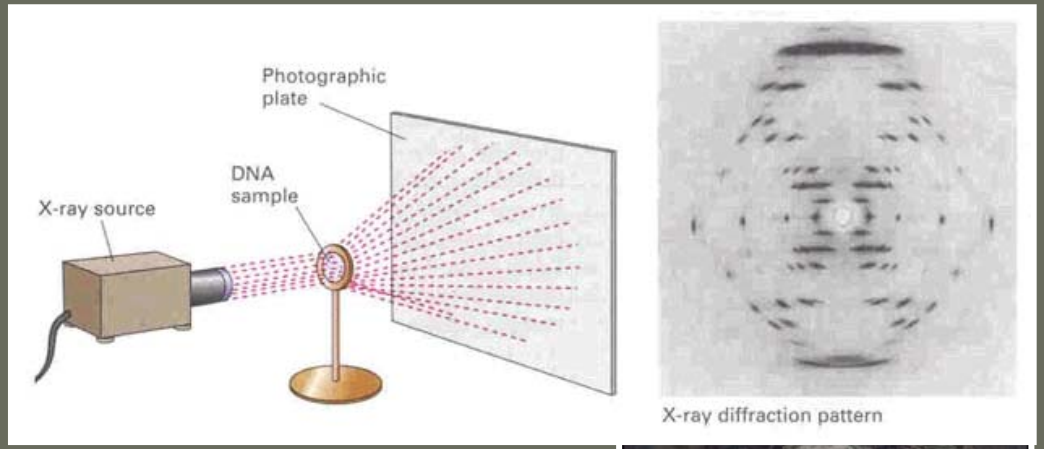
Francis Harry Compton Crick

1916-2004 (88)



Maurice Hugh Frederick Wilkins

1916-2004 (87)



Rosalind Elsie Franklin (1920-1958)

Who (really) discovered the structure of DNA?

In April of 1953, James Watson, Francis Crick and Maurice Wilkins identified the substance of life - the structure of DNA. They later shared a Nobel Prize. Their discovery depended heavily on the work of a woman, chemist **Rosalind Franklin**, whose research was used **without her knowledge or permission**. Watson's memoir of the discovery dismisses Franklin as frumpy, hostile and unimaginative. A later work by a friend casts Franklin as a feminist icon, cheated of recognition.
It was Franklin's photograph of the DNA molecule that sparked a scientific revolution. Wilkins showed Watson the photo, **Watson said, *"My jaw fell open and my pulse began to race „*** *The* photo showed, for the first time, the essential structure of DNA - the double-helix shape, which also indicated its method of replication.

Molekulární diagnostika

- Vrozené genetické choroby - DNA změny vrozené (monogenní onemocnění, chromozomální poruchy, multifaktoriální poruchy)

- Onkologické choroby - DNA změny získané
- Diagnostika patogenů - DNA cizorodá
- Přímá-nepřímá diagnostika

Molekulární diagnostika

- Detekce přenašečů patologické alely
- Prenatální diagnostika
- Stanovení genetického rizika

- Včasné stanovení diagnózy
- Včasné stanovení oportunních patogenů
- Diferenciální diagnostika
- Minimální zbytkové onemocnění
- Včasná detekce relapsu choroby

Molekulární diagnostika

- Specificita a senzitivita
- Rychlost výsledku

a možnost správné a rychlé terapeutické intervence

Podle klasické Omranovy teorie z roku 1971 všechny společnosti procházejí třemi stádii souvisejícími s procesem modernizace:

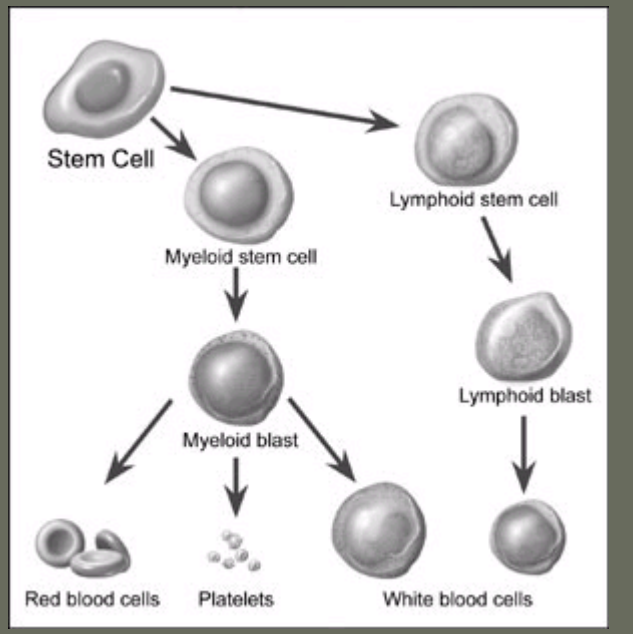
1. Stadium moru a hladomoru
2. Stadium poklesu frekvence výskytu pandemií infekčních nemocí
3. Stadium rozvoje degenerativních a civilizačních chorob (novotvary a nemoci oběhové soustavy)

Omran, A.R.: The epidemiologic transition: a theory of the epidemiology of population change. Milbank memorial Fund Quaterly, 1971, 29

Původ hematoonkologických onemocnění

- Systémová klonální onemocnění, vznik neregulovaným dělením jediné nádorově transformované buňky
- nižší počet genetických změn nutných pro vznik, někdy stačí narušení kontroly buněčného dělení
- změna zahrnující cca 10^{6}-10^{7} bp se může projevit bez viditelné změny morfologie chromozomu

Dělení hematoonkologických onemocnění



A) Podle charakteru

- **Difúzní** (leukémie)
- **Ložisková** (lymfomy)-s nádorovými ložisky v lymfatické tkáni

B) Podle postižené krevní vývojové řady

- **myeloidní prekurzorová buňka (CML nebo AML)**
  -nádorová choroba vlastního krvetvorného systému
- **lymfoidní prekurzorová buňka (CLL nebo ALL nebo lymfomy)**
  -nádorová onemocnění imunitního systému

Hematoonkologická diagnóza

1. Specifické markery pro daný sub/typ
2. Nespecifické (nezávislé) markery

3. Diferenciální diagnostika
4. Stratifikace podle rizika
5. Minimální reziduální choroba

6. Detekce oportunních patogenů

Molekulárně biologické vyšetření oportunních patogenů IHOK FN BRNO

- CMV
- HHV6
- HSV-1,-2
- EBV, VZV
- BK virus, JC virus
- Respirační viry (RSV, PIV1-3, ADV1-7, Flu-A, -B
- Adenoviry
- Detekce mutací vedoucích k rezistenci na léčbu GCV
- Pneumocystis jirovecii
- Mykózy

LEUCOCYTHEMIA,

or

WHITE CELL BLOOD.

A COLLECTION OF THE

PATHOLOGY AND PATHOLOGY OF THE LYMPHATIC GLANDULAR SYSTEM.

BY JOHN HUGHES BENNETT, M.D., F.R.S.E.,

EDIN AND LOND.

LONDON: SIMPKIN, MARSHALL, & CO.

1845

Weißes Blut.

Rudolf Virchow, Berlin

John Hughes Bennett, Edinburgh

P.C. Nowell,
D.A.Hungerford,
University of
Pennsylvania in
Philadelphia

1960

This Week's Citation Classic™

CC/NUMBER 8
FEBRUARY 25, 1985

Nowell P C & Hungerford D A. A minute chromosome in human chronic granulocytic leukemia. *Science* **142**:1497, 1960.
[School of Medicine, University of Pennsylvania, and Institute for Cancer Research, Philadelphia, PA]

This abstract described seven patients (five male, two female) with chronic granulocytic leukemia (CGL) in whom a similar minute chromosome was found in the neoplastic cells in each case. The finding suggested a causal relationship between the chromosome abnormality and CGL. [The *SCI*® indicates that this brief "paper" has been cited in over 510 publications since 1960.]

Peter C. Nowell
Dep[illegible]of Pathology a[illegible]
[illegible] Medicin[illegible]
[illegible]versity [illegible]ennsyl[illegible]
School [illegible]edic[illegible]
[illegible]hiladelp[illegible]PA

De[illegible]mber 21, [illegible]4

cells, and he looked at them. Our first cases, of acute leukemia, were unrewarding. Then Dave spotted a small chromosome in cells from two male patients with chronic granulocytic leukemia (CGL). These findings were published[3] with caution (because the Edinburgh group had found no abnormality in CGL[4]) and with the suggestion that the "minute" chromosome might be an altered Y. Subsequent cases, including women and using an improved "air-drying" technique,[5] led Dave to assign the minute chromosome correctly to the larger pair of G-group autos[illegible]t numbered 21 and later changed, [illegible]on, to no. 22. The additional CGL case[illegible]re being readied for publication when[illegible]. Richards asked us to present [illegible]omething[illegible]a National Academy of Sci[illegible]nces me[illegible] he was organizing at Penn. Our abstr[illegible] published in *Science*, led to [illegible]ide rec[illegible]ion (and citation) of the first [illegible]sistent chromosome abnor[illegible]sia. The Edinburgh group [illegible]sted the name "Philadel[illegible]some."

[illegible]de was frustrating. Cytoge[illegible]CGL proved of some diag[illegible]nostic value and provided [illegible] clonal evolution in tumor [illegible] helping to explain clinical [illegible]n. But since other consis[illegible] changes in neoplasia were [illegible]nificance in tumorigenesis [illegible]nd the term "epiphenome[illegible] used. With the advent of [illegible]nding techniques in the [illegible]lecular genetic techniques [illegible]tory changed dramatically. [illegible]ogenetic alterations were [illegible]any tumors and are now [illegible]ful for exploring oncogene [illegible]romosome studies remain [illegible]oking at genomic changes, [illegible]nise of the Ph chromosome [illegible]of specific genomic alter[illegible]in human tumorigenesis is [illegible] fruition.

A Minute Chromosome[illegible]an Chronic Granulocytic Leukemia

In seven cases thus far investigated (five males, two females), a minute chromosome has been observed replacing one of the four smallest autosomes in the chromosome complement of cells of chronic granulocytic leukemia cultured from peripheral blood. No abnormality was observed in the cells of four cases of *acute* granulocytic leukemia in adults or of six cases of acute leukemia in children. There have been several recent reports of chromosome abnormalities in a number of cases of human leukemia [including two of the seven cases reported here: Nowell and Hungerford, *J. Natl. Cancer Inst.* 25, 85 (1960)], but no series has appeared in which there was a consistent change typical of a particular type of leukemia.

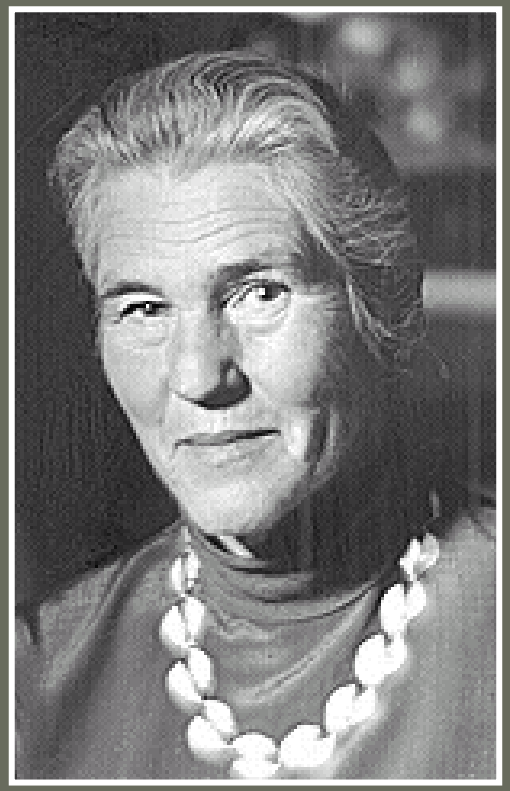
Cells of the five new cases were obtained from peripheral blood (and bone marrow in one instance), grown in culture for 24–72 hours, and processed for cytological examination by a recently developed air-drying technique (Moorhead, *et al., Exptl. Cell Research*, in press). The patients varied from asymptomatic untreated cases to extensively treated cases of several years duration in terminal myeloblastic crisis. All seven individuals showed a similar minute chromosome, and none showed any other frequent or regular chromosome change. In most of the cases, cells with normal chromosomes were also observed. Thus, the minute is not a part of the normal chromosome constitution of such individuals.

The findings suggest a causal relationship between the chromosome abnormality observed and chronic granulocytic leukemia.

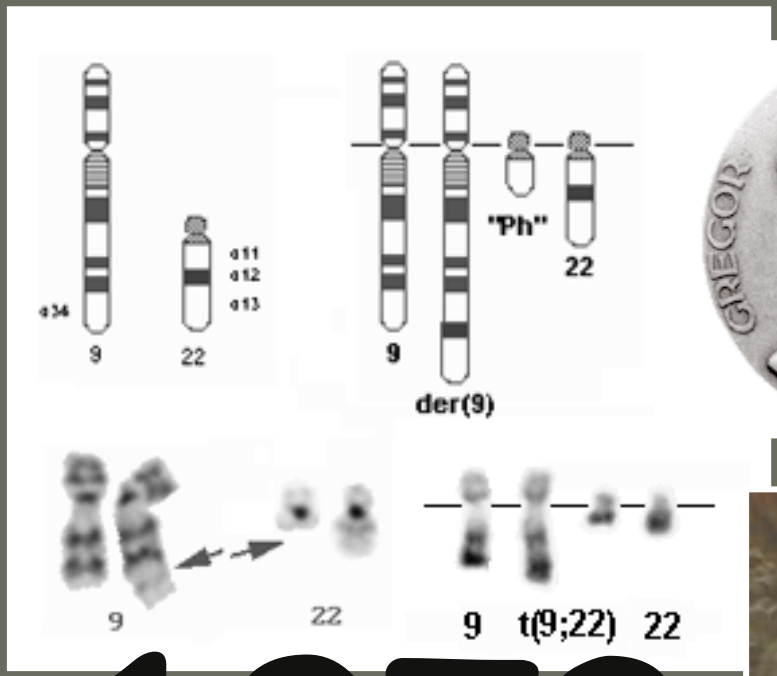
PETER C. NOWELL
School of Medicine,
University of Pennsylvania
DAVID A. HUNGERFORD
Institute for Cancer Research

[illegible], 9:116-27, 1955. (Cited 70 times.)
[illegible]ading the chromosomes of cells in
[illegible]an leukocytes.
[illegible]uman leukaemia.
[illegible]osome preparations of leukocytes
[illegible]also: **Moorhead P S.**
[illegible]ements and oncogenesis.

Nowell & Hungerford, 1960 Science 132.1497



*1925, University of Chicago



1973

Janet D. Rowley : A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining. Nature, 243, 290-293, 1973

1998 Albert Lasker Clinical Medicine Research Prize,
1999 National Medal of Science at a White House,
2003 Mendel Medal Villanova University

Chronická myeloidní leukémie-CML

1845 první dokumentovaný klinický popis choroby — 115

1960 publikace Ph chromozomu u CML pacientů

1970 izolace nového myšího onkogenního viru Abelson

1973 odhalení reciproké translokace t(9;22) jako příčiny Ph chromozomu

1980-1983 klonování v-Abl onkogenu, c-Abl protoonkogenu, jeho lokalizace na chromozomu 9 a demonstrace, že Abl kóduje protein-tyrozin kinázu

1986 klonování BCR-ABL cDNA z CML buněk — 15

1996 imatinib mesylate inhibuje BCR-ABL tyrozin kinázu a CML buněčný růst in vitro

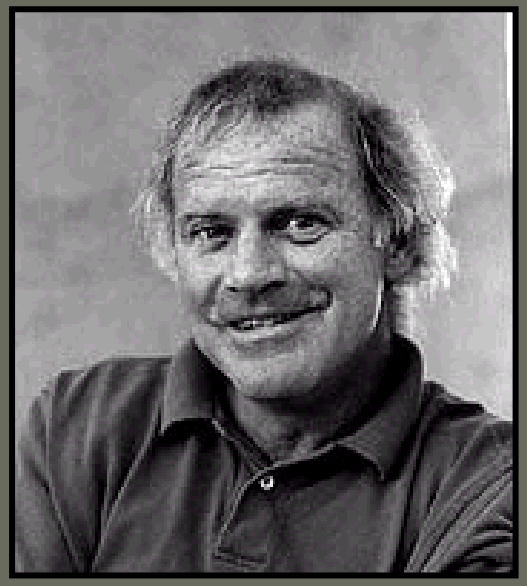
2001 imatinib (Glivec) jako selektivní inhibitor je potvrzen v terapii CML

Metody molekulární diagnostiky

1. PCR
2. PCR
3. PCR

1993

PTC200 (MJR,USA,cca 250000 Kč)



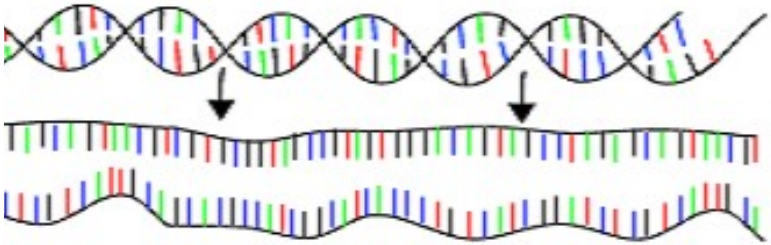
1983 K. Mullis

1985 publikace

1993 Nobelova cena za chemii

PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



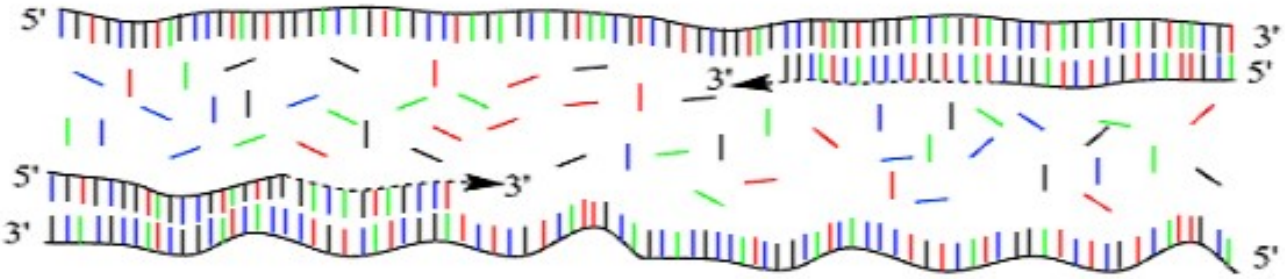
Step 1 : denaturation

1 minut 94 °C

Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



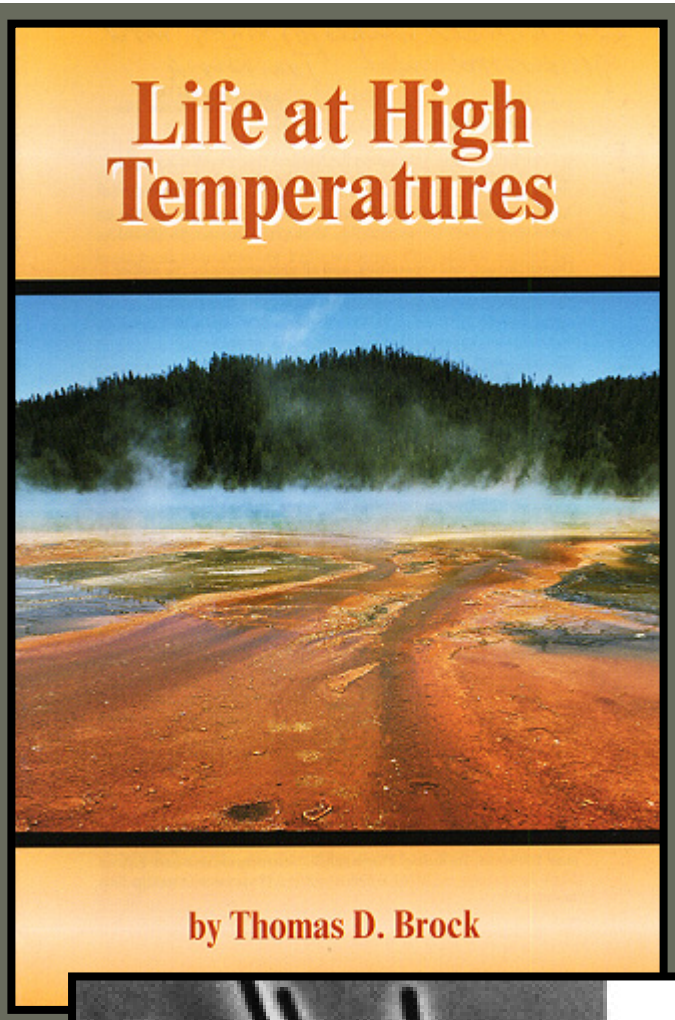
Step 3 : extension

2 minutes 72 °C

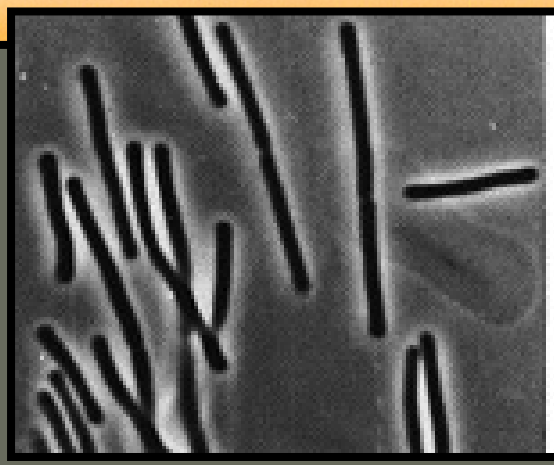
only dNTP's

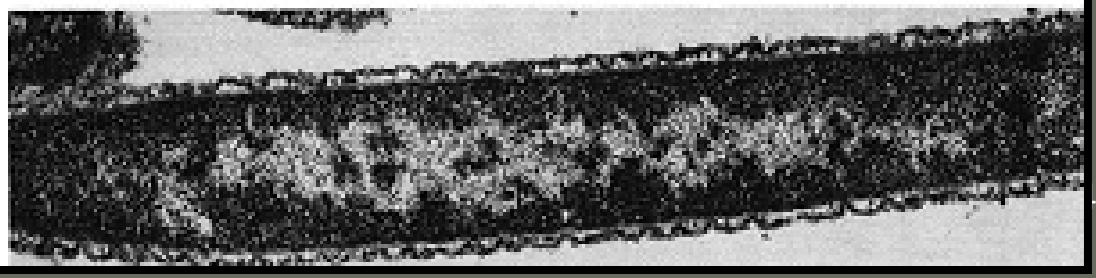
(Andy Vierstraete 1999)

1st cycle 2^2=4 copies **35th cycle 2^{36} = 68 billion copies**


Life at High Temperatures
by Thomas D. Brock


Yellowstone River (1964)





RESEARCH ARTICLE

Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia

Randall K. Saiki, Stephen Scharf, Fred Faloona, Kary B. Mullis
Glenn T. Horn, Henry A. Erlich, Norman Arnheim

Abstract. *Two new methods were used to establish a rapid and highly sensitive prenatal diagnostic test for sickle cell anemia. The first involves the primer-mediated enzymatic amplification of specific β-globin target sequences in genomic DNA, resulting in the exponential increase (220,000 times) of target DNA copies. In the second technique, the presence of the β^A and β^S alleles is determined by restriction endonuclease digestion of an end-labeled oligonucleotide probe hybridized in solution to the amplified β-globin sequences. The β-globin genotype can be determined in less than 1 day on samples containing significantly less than 1 microgram of genomic DNA.*

Recent advances in recombinant DNA technology have made possible the molecular analysis and prenatal diagnosis of several human genetic diseases. Fetal DNA obtained by aminocentesis or chorionic villus sampling can be analyzed by restriction enzyme digestion, with subsequent electrophoresis, Southern transfer, and specific hybridization to cloned gene or oligonucleotide probes. With polymorphic DNA markers linked genetically to a specific disease locus, segregation analysis must be carried out with restriction fragment length polymorphisms (RFLP's) found to be informative by examining DNA from family members (*1*, *2*).

Many of the hemoglobinopathies, however, can be detected by more direct methods in which analysis of the fetus alone is sufficient for diagnosis. For example, the diagnosis of hydrops fetalis (homozygous α-thalassemia) can be made by documenting the absence of any α-globin genes by hybridization with an α-globin probe (*3*–*5*). Homozygosity for certain β-thalassemia alleles can be determined in Southern transfer experiments by using oligonucleotide probes that form stable duplexes with the normal β-globin gene sequence but form unstable hybrids with specific mutants (*6*, *7*).

Sickle cell anemia can also be diagnosed by direct analysis of fetal DNA. This disease results from homozygosity of the sickle-cell allele (β^S) at the β-globin gene locus. The S allele differs from the wild-type allele (β^A) by substitution of an A in the wild-type to a T at the second position of the sixth codon of the β chain gene, resulting in the replacement of a glutamic acid by a valine in the expressed protein. For the prenatal diagnosis of sickle cell anemia, DNA obtained by amniocentesis or chorionic villus sampling can be treated with a restriction endonuclease (for example, Dde I and Mst II) that recognizes a sequence altered by the β^S mutation (*8*–*11*). This generates β^A- and β^S-specific restriction fragments that can be resolved by Southern transfer and hybridization with a β-globin probe.

We have developed a procedure for the detection of the sickle cell mutation that is very rapid and is at least two orders of magnitude more sensitive than standard Southern blotting. There are two special features to this protocol. The first is a method for amplifying specific β-globin DNA sequences with the use of oligonucleotide primers and DNA polymerase (*12*). The second is the analysis of the β-globin genotype by solution hybridization of the amplified DNA with a specific oligonucleotide probe and subsequent digestion with a restriction endonuclease (*13*). These two techniques increase the speed and sensitivity, and lessen the complexity of prenatal diagnosis for sickle cell anemia; they may also be generally applicable to the diagnosis of other genetic diseases and in the use of DNA probes for infectious disease diagnosis.

Sequence amplification by polymerase chain reaction. We use a two-step procedure for determining the β-globin genotype of human genomic DNA samples. First, a small portion of the β-globin gene sequence spanning the polymorphic Dde I restriction site diagnostic of the β^A allele is amplified. Next, the presence or absence of the Dde I restriction site in the amplified DNA sample is determined by solution hybridization with an end-labeled complementary oligomer followed by restriction endonuclease digestion, electrophoresis, and autoradiography.

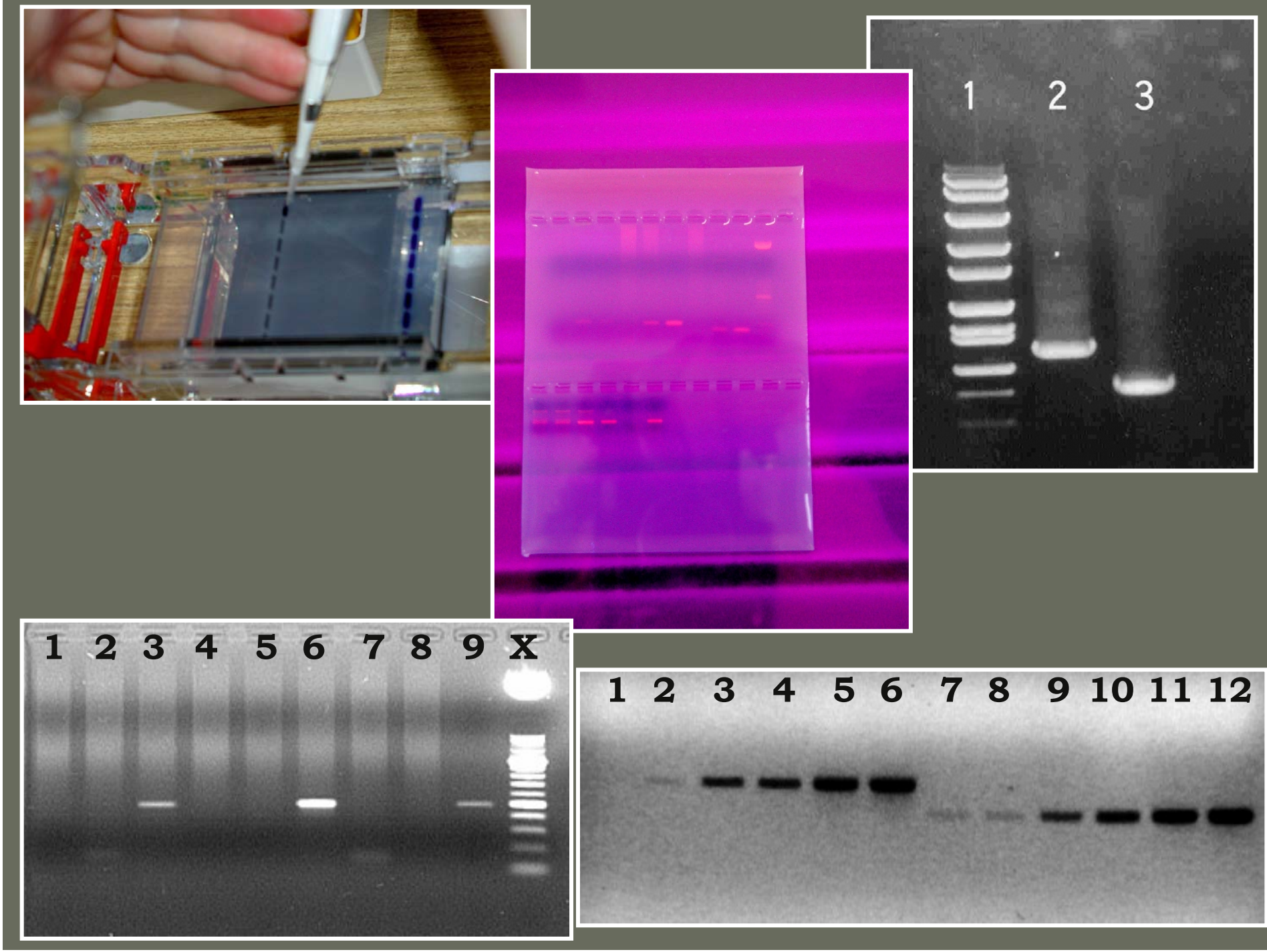
The β-globin gene segment was amplified by the polymerase chain reaction (PCR) procedure of Mullis and Faloona (*12*) in which we used two 20-base oligonucleotide primers that flank the region to be amplified. One primer, PC04, is complementary to the (+)-strand and the other, PC03, is complementary to the (−)-strand (Fig. 1). The annealing of PC04 to the (+)-strand of denatured genomic DNA followed by extension with the Klenow fragment of *Escherichia coli* DNA polymerase I and deoxynucleotide triphosphates results in the synthesis of a (−)-strand fragment containing the target sequence. At the same time, a similar reaction occurs with PC03, creating a new (+)-strand. Since these newly synthesized DNA strands are themselves template for the PCR primers, repeated cycles of denaturation, primer annealing, and extension result in the exponential accumulation of the 110–base pair region defined by the primers.

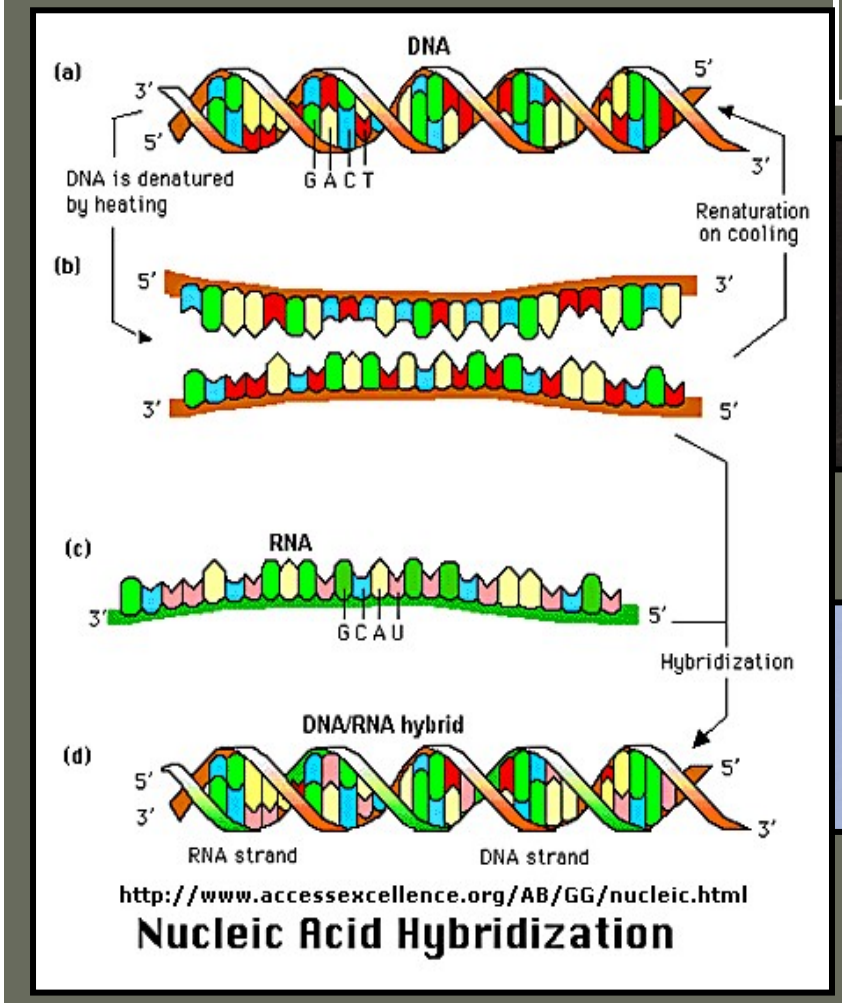
An example of the degree of specific gene amplification achieved by the PCR method is shown in Fig. 2A. Samples of DNA (1 μg) were amplified for 20 cycles and a fraction of each sample, equivalent to 36 ng of the original DNA, was subjected to alkaline gel electrophoresis and transferred to a nylon filter. The filter was then hybridized with a ^{32}P-labeled 40-base oligonucleotide probe, RS06, which is complementary to the target sequence (Fig. 1A) but not to the PCR primers. The results, after a 2-hour autoradiographic exposure, show that a fragment hybridizing with the RS06 probe

The authors are in the Department of Human Genetics, Cetus Corporation, 1400 Fifty-Third Street, Emeryville, California 94608. The present address for N.A. is Department of Biological Sciences, University of Southern California, Los Angeles 90089-0371.

Science, 230 (4732): 1350-1354, 1985

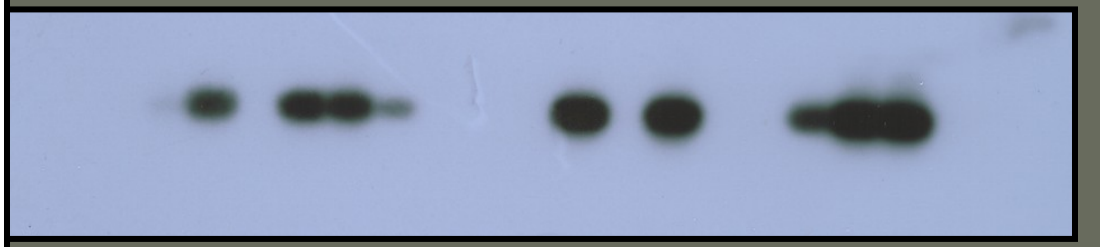
TaqDNA Polymerase, 1989


1 2 3
1 2 3 4 5 6 7 8 9 X
1 2 3 4 5 6 7 8 9 10 11 12

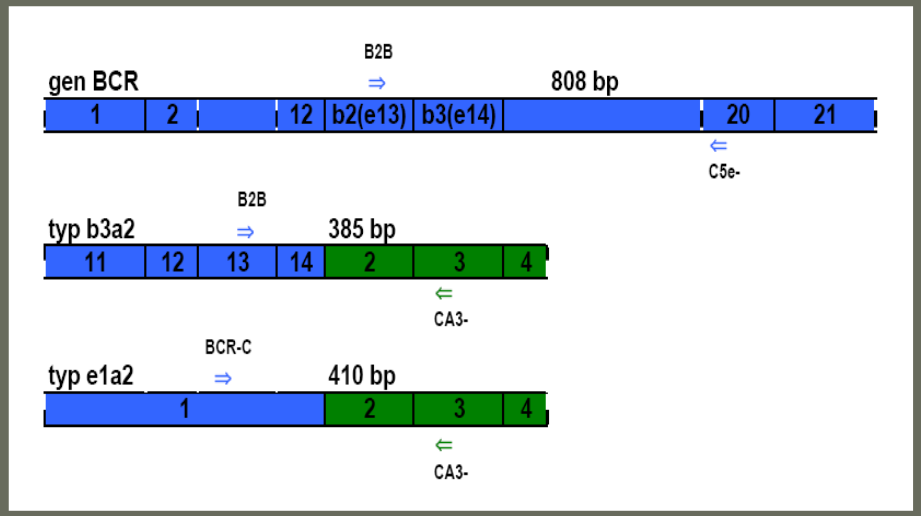


Ethidium bromide-stained agarose gel

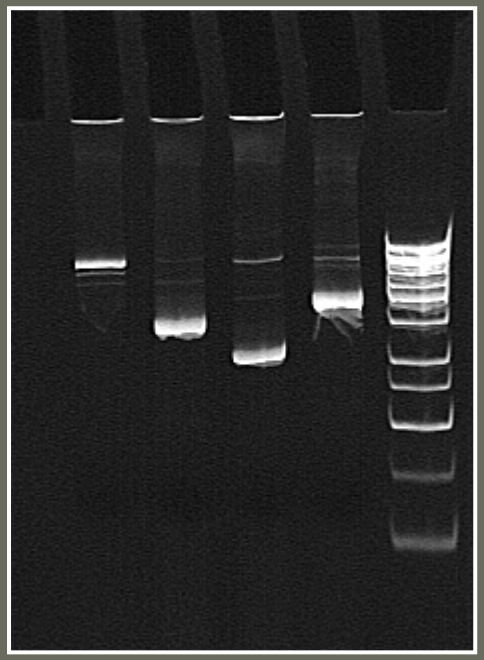
^{32}P probe

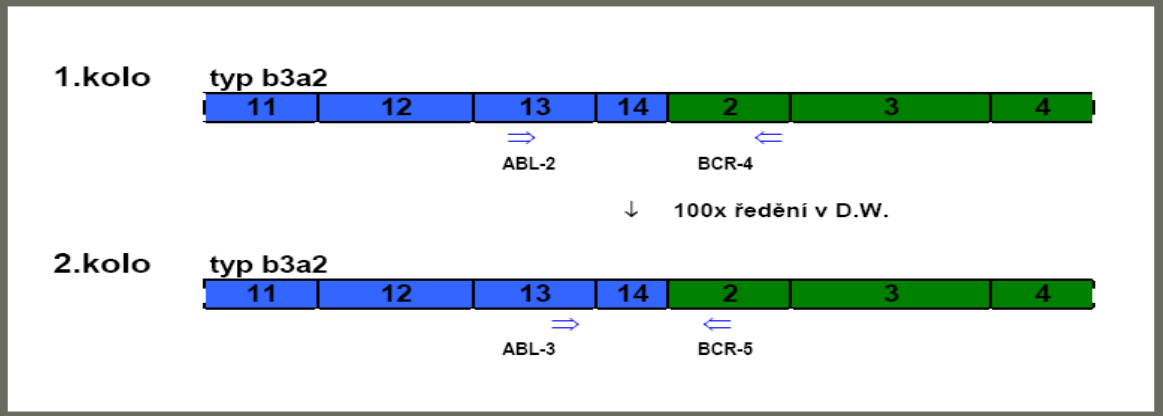


Hybridizace se značenými oligonukleotidovými sondami

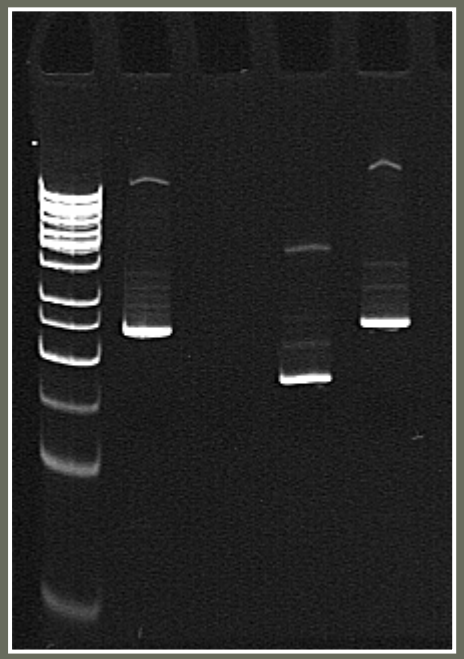


Multiplex-PCR

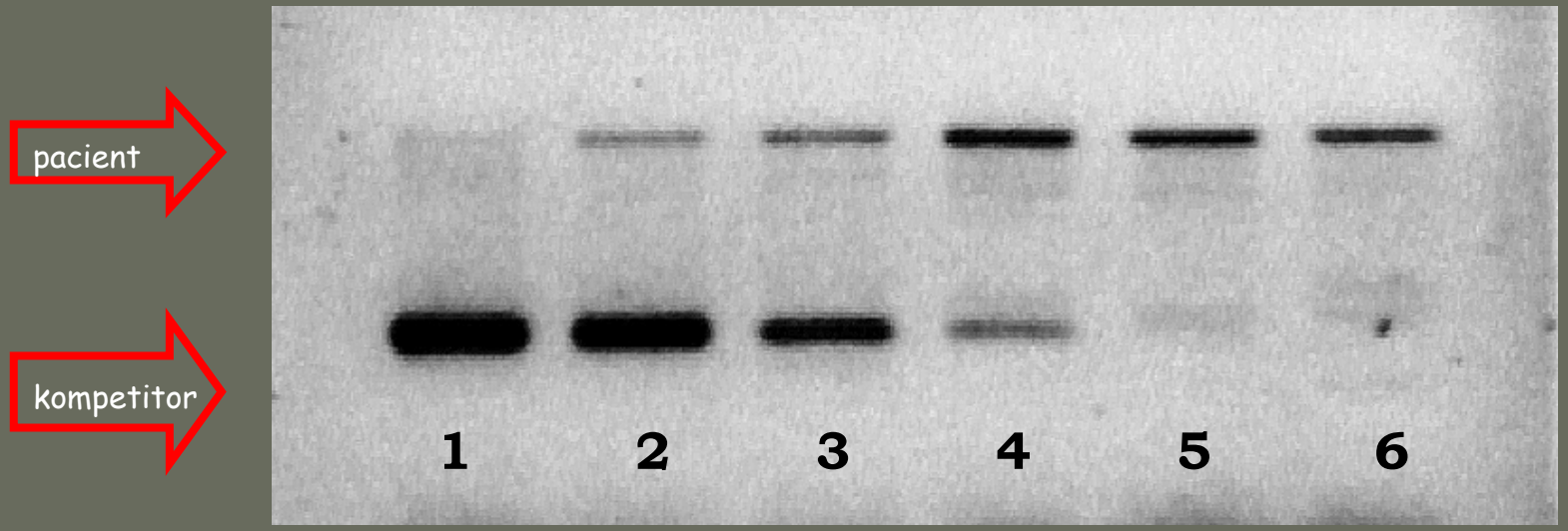




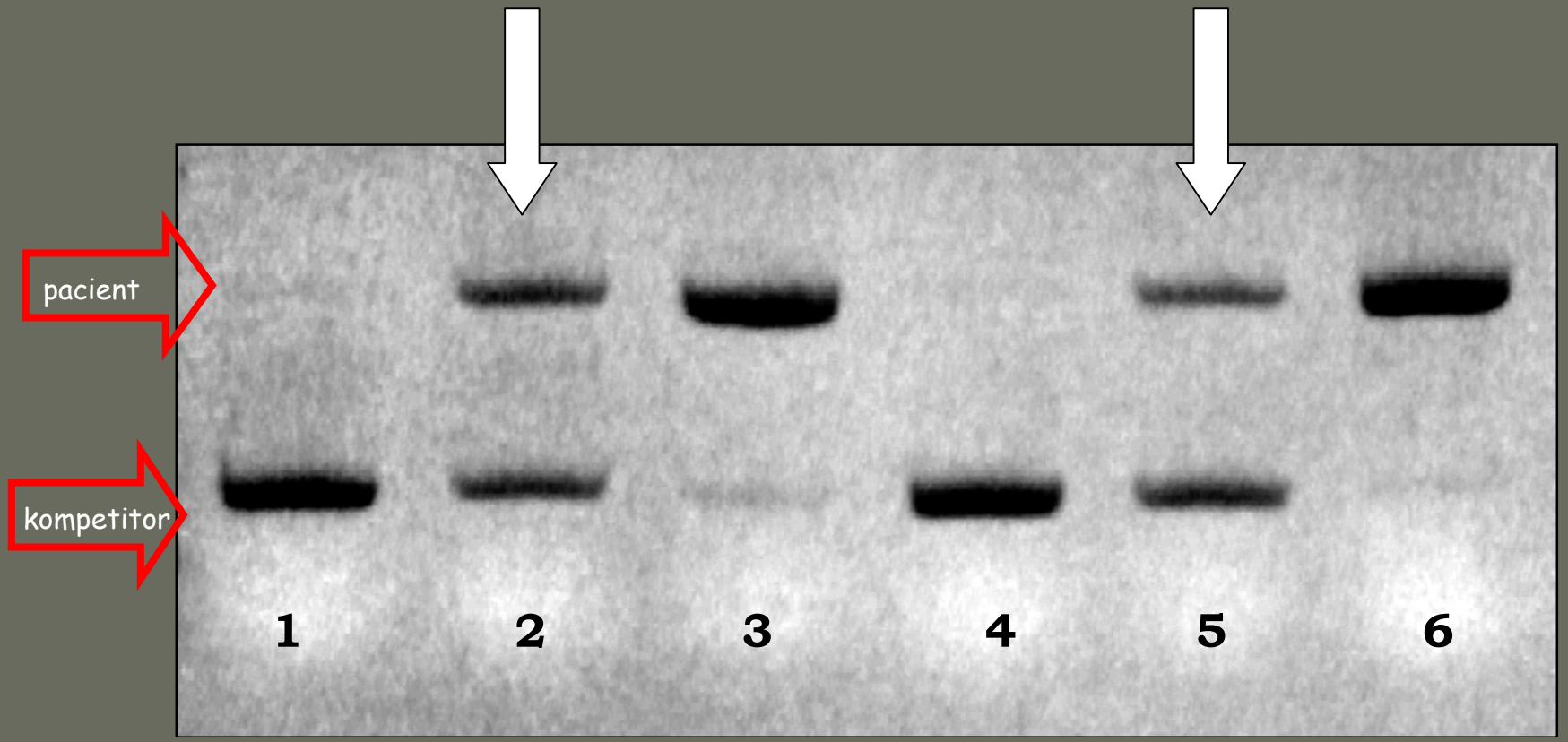
Dvoukolová nested-PCR



Titrační ředění kompetitorového templátu



Kompetitivní PCR a vyznačení bodů ekvivalence



1: Biotechnology (N Y). 1992 Apr;10(4):413-7.

Simultaneous amplification and detection of specific DNA sequences.

Higuchi R, **Dollinger G**, **Walsh PS**, **Griffith R**.
Roche Molecular Systems, Inc., Emeryville, CA 94608.

We have enhanced the polymerase chain reaction (PCR) such that specific DNA sequences can be detected without opening the reaction tube. This enhancement requires the addition of ethidium bromide (EtBr) to a PCR. Since the fluorescence of EtBr increases in the presence of double-stranded (ds) DNA an increase in fluorescence in such a PCR indicates a positive amplification, which can be easily monitored externally. In fact, amplification can be continuously monitored in order to follow its progress. The ability to simultaneously amplify specific DNA sequences and detect the product of the amplification both simplifies and improves PCR and may facilitate its automation and more widespread use in the clinic or in other situations requiring high sample throughput.

1: Biotechnology (N Y). 1993 Sep;11(9):1026-30. Related Articles, Links

Kinetic PCR analysis: real-time monitoring of DNA amplification reactions.

Higuchi R, **Fockler C**, **Dollinger G**, **Watson R**.
Roche Molecular Systems, Inc., Alameda, CA 94501.

We describe a simple, quantitative assay for any amplifiable DNA sequence that uses a video camera to monitor multiple polymerase chain reactions (PCRs) simultaneously over the course of thermocycling. The video camera detects the accumulation of double-stranded DNA (dsDNA) in each PCR using the increase in the fluorescence of ethidium bromide (EtBr) that results from its binding duplex DNA. The kinetics of fluorescence accumulation during thermocycling are directly related to the starting number of DNA copies. The fewer cycles necessary to produce a detectable fluorescence, the greater the number of target sequences. Results obtained with this approach indicate that a kinetic approach to PCR analysis can quantitate DNA sensitively, selectively and over a large dynamic range. This approach also provides a means of determining the effect of different reaction conditions on the efficacy of the amplification and so can provide insight into fundamental PCR processes.

1: Nucleic Acids Res. 1993 Aug 11;21(16):3761-6. Related Articles, Links

Allelic discrimination by nick-translation PCR with fluorogenic probes.

Lee LG, **Connell CR**, **Bloch W**.
Applied Biosystems, Division of Perkin-Elmer, Foster City, CA 94404.

Nick-translation PCR was performed with fluorogenic probes. Two probes were used: one complementary to a sequence containing the F508 codon of the normal human cystic fibrosis (CF) gene (wt DNA) and one complementary to a sequence containing the delta F508 three base pair deletion (mut DNA). Each probe contained a unique and spectrally resolvable fluorescent indicator dye at the 5' end and a common quencher dye attached to the seventh nucleotide from the 5' end. The F508/delta F508 site was located between the indicator and quencher. The probes were added at the start of a PCR containing mut DNA, wt DNA or heterozygous DNA and were degraded during thermal cycling. Although both probes were degraded, each probe generated fluorescence from its indicator dye only when the sequence between the indicator and quencher dyes was perfectly complementary to target. The identify of the target DNA could be determined from the post-PCR fluorescence emission spectrum.

ABI PRISM 7700 (Perkin Elmer/ABI) první dostupný RQ-PCR systém s laserem /1996

Light Cycler (Roche Molecular Biochemicals) extrémní rychlost, kapiláry

5700SDS (Perkin Elmer/ABI) halogen, CCD /1998

...

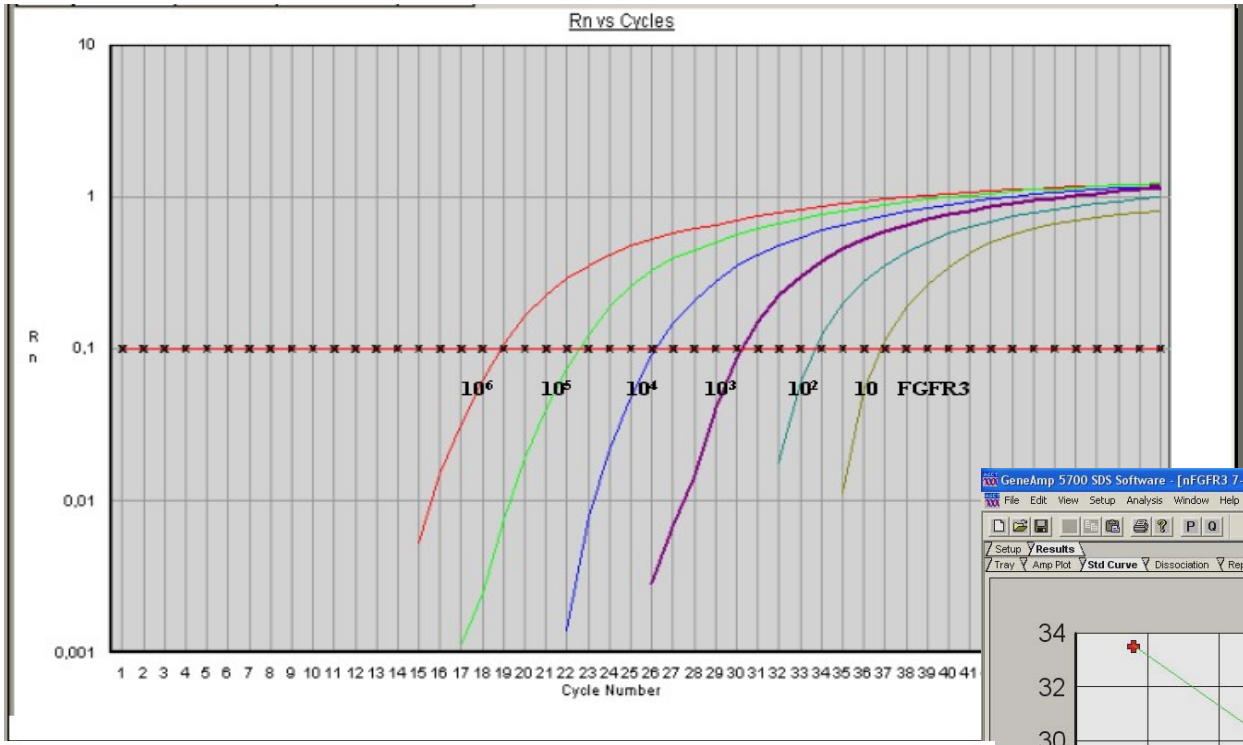
LightCycler (Roche)

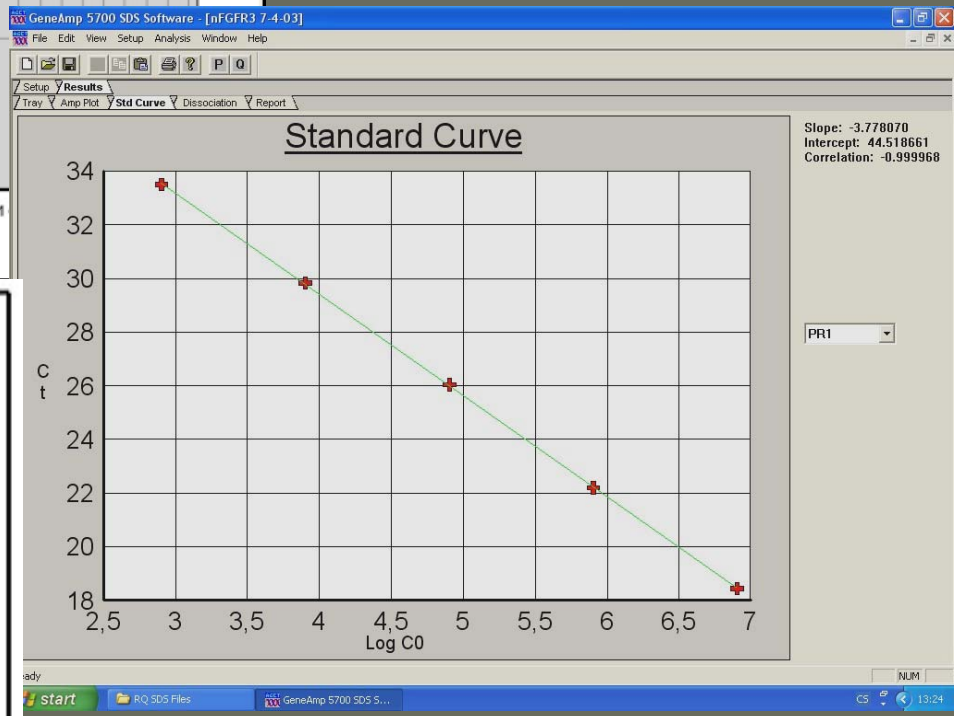
RotorGene 6000
(CorbettResearch, QIAGENE)

SDS 5700 (Applied Biosystems)

7300 System (Applied Biosystems)

RotorGene 3000 (CorbettResearch,QIAGENE)


Rn vs Cycles
Rn
10
1
0,1
0,01
0,001
10⁶ 10⁵ 10⁴ 10³ 10² 10 FGFR3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
Cycle Number


GeneAmp 5700 SDS Software - [nFGFR3 7-4-03]
File Edit View Setup Analysis Window Help
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Tray Amp Plot Std Curve Dissociation Report
Standard Curve
Slope: -3.778070
Intercept: 44.518661
Correlation: -0.999968
PR1
Ct
34 32 30 28 26 24 22 20 18
2,5 3 3,5 4 4,5 5 5,5 6 6,5 7
Log C0
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RQ SDS Files
GeneAmp 5700 SDS S...
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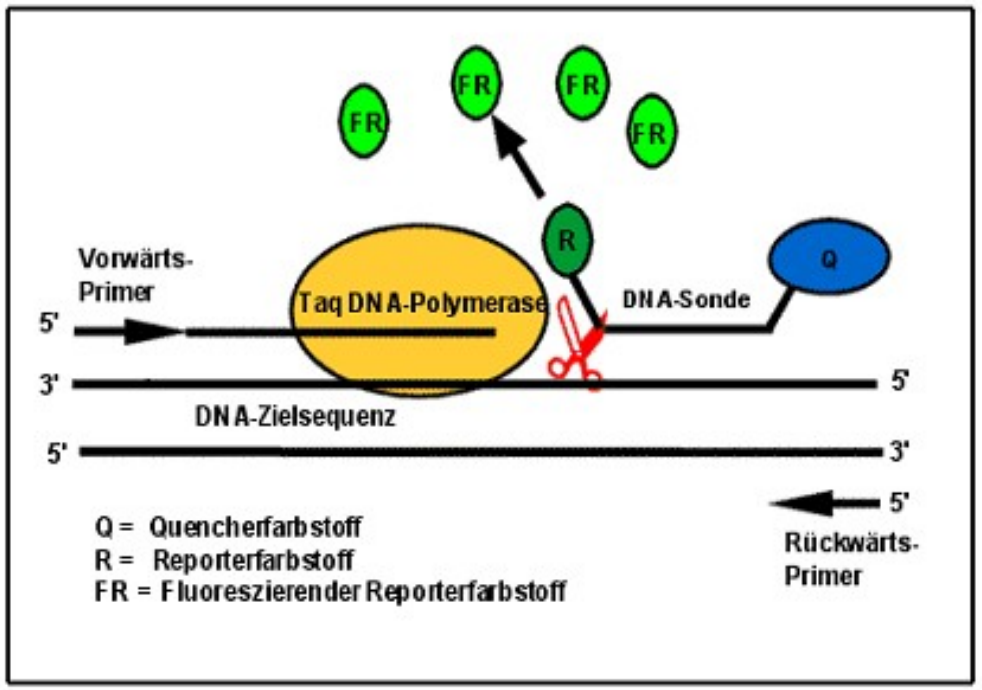
FR
FR
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R
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Vorwärts-
Primer
Taq DNA-Polymerase
DNA-Sonde
5'
3'
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DNA-Zielsequenz
5'
3'
5'
Q = Quencherfarbstoff
R = Reporterfarbstoff
FR = Fluoreszierender Reporterfarbstoff
Rückwärts-
Primer

Leukemia (2003) 17, 2318–2357

www.nature.com/leu

LEADING ARTICLE

Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual dis detection in leukemia – A Europe Against Cancer Program

J Gabert[1,17,18,19], E Beillard[1,17], VHJ van der Velden[2], W Bi[3], D Grimwa ani
JM Cayuela[8], H Cavé[9], F Pane[10], JLE Aerts[11], D De Micheli[12], X Thirion[,5],
M Malec[16], G Saglio[12] and JJM van Dongen[2]

[1]Department of Hematology Biology, Institut Paoli Calmettes, France; [2]D niv
Medical Center Rotterdam, Rotterdam, The Netherlands; [3]Applied Biosys f
Haematology, Division of Medical and Molecular Genetics, Guy's, King' sity
London Hospitals, London, UK; [5]Department of Immuno-haematology, A rk;
[6]Department of Medical Sciences, Uppsala University Hospital, Uppsala, n E
San Gerardo Hospital, Monza, Italy; [8]Central Laboratory of Hematology a ris,
[9]Department of Genetic Biochemistry, Hôpital Robert Debré, Paris, Fran t of
Biochemistry & Medical Biotechnology, University Hospital 'Federico II', I , U
Hospital, Leuven, Belgium; [12]Clinical and Biological Science, University bas
Torino, Italy; [13]Department of Medical Information, University Hôpital S nor
Université de la Méditerranée, Marseille, France; [14]Department of Hemato anc
[15]Biogenetics Laboratory, Children's University Hospital, Giessen, Germa olir
Stockholm, Sweden

Detection of minimal residual disease (MRD) has proven to provide independent prognostic information for treatment stratification in several types of leukemias such as childhood acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML) and acute promyelocytc leukemia. This report focuses on the accurate quantitative measurement of fusion gene (FG)

(EAC) program. Four phases were scheduled: (1) trai optimization, (3) sensitivity testing and (4) patient testing. During our program, three quality control rou large series of coded RNA samples were performed inc balanced randomized assay, which enabled final valic the EAC primer and probe sets. The expression level of

Control Gene Standards:

Product	Reference Name
CGRS-01	ABL
CGRS-02	B2M
CGRS-03	GUS
CGRS-04	TBP

Fusion Gene Standards:

Product	Reference Name
FGRS-01	AML1–ETO
FGRS-02	CBFB-MYH11 A
FGRS-03	CBFB-MYH11 D
FGRS-04	CBFB-MYH11 E
FGRS-05	PML-RARA bcr1
FGRS-06	PML-RARA bcr2
FGRS-07	PML-RARA bcr3
FGRS-08	E2A-PBX1
FGRS-09	BCR-ABL e1a2 m-bcr
FGRS-10	BCR-ABL b3a2 M-bcr
FGRS-11	TEL-AML 1 e4e11
FGRS-12	SIL-TAL
FGRS-13	MLL-AF4 e10e4 = RS411 type
FGRS-14	MLL-AF4 e9e5 = MV411 type
FGRS-15	MLL-AF4 e11e5 = ALL-PO type
FGRS-16	MLL-AF9 type A
FGRS-17	MLL-AF9 type B
FGRS-18	MLL-AF6
FGRS-19	MLL-DUP
FGRS-20 *New*	MLL-ENL ex9
FGRS-21 *New*	MLL-ENL ex10
FGRS-22 *New*	MLL-ENL ex11
FGRS-23 *New*	MLL-AF9 ex9
FGRS-24 *New*	MLL-ELL ex9
FGRS-25 *New*	MLL-ELL ex10
FGRS-26 *New*	MLL-AF1p ex11

Bibliography

1. J. Gabert et al. Standardization and quality control studies of "real-time" quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) of fusion gene transcripts for minimal residual disease detection in leukemia - A Europe Against Cancer Program. In Press, Leukemia.

2. E. Bellard et al. Evaluation of candidate control genes for diagnosis

Výhody RQ-PCR ve srovnání s end-point PCR

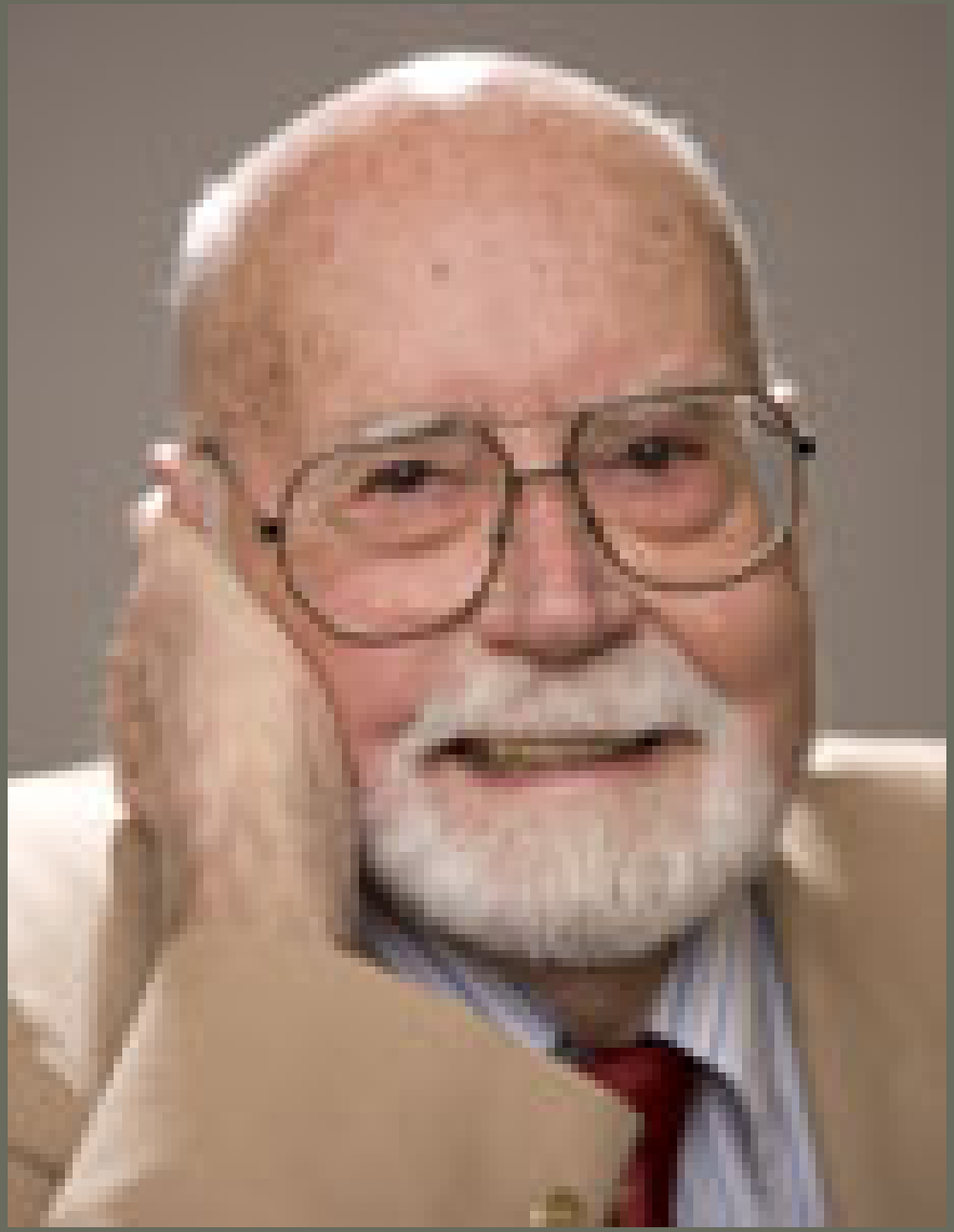
* omezení nespecifické amplifikace (sondy)
* amplifikace je průběžně monitorována během reakce
* mizí post-PCR manipulace s amplikony (snížení nebezpečí kontaminace následné PCR)
* rychlost (30 min-2 hod)
* široký dynamický rozsah 10-10 ^{10} kopií, menší spotřeba testovaného materiálu (RNA),
* vyšší specifita, senzitivita a reprodukovatelnost
* cena reakce srovnatelná

Nevýhody RQ-PCR ve srovnání s end-point PCR

† vysoká pořizovací cena přístroje (stále ještě)

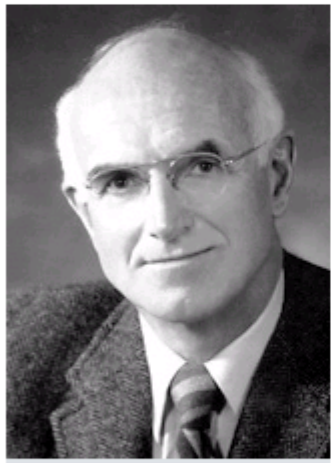
† vyšší nároky na technickou zručnost a interpretaci výsledků

† problémy s RNA kontaminovanou gDNA



The Nobel Prize in Physiology or Medicine 1990

"for their discoveries concerning organ and cell transplantation in the treatment of human disease"



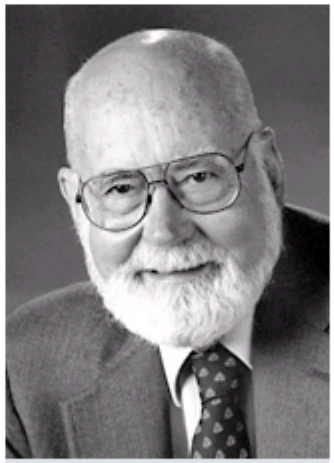
Joseph E. Murray

◐ 1/2 of the prize

USA

Brigham and Women's Hospital
Boston, MA, USA

b. 1919



E. Donnall Thomas

◐ 1/2 of the prize

USA

Fred Hutchinson Cancer Research Center
Seattle, WA, USA

b. 1920

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Medicine

Prev. year Next year

The Nobel Prize in Physiology or Medicine 1990

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Presentation Speech

Joseph E. Murray

Autobiography
Nobel Lecture
Interview
Banquet Speech

E. Donnall Thomas

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Nobel Lecture
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2008

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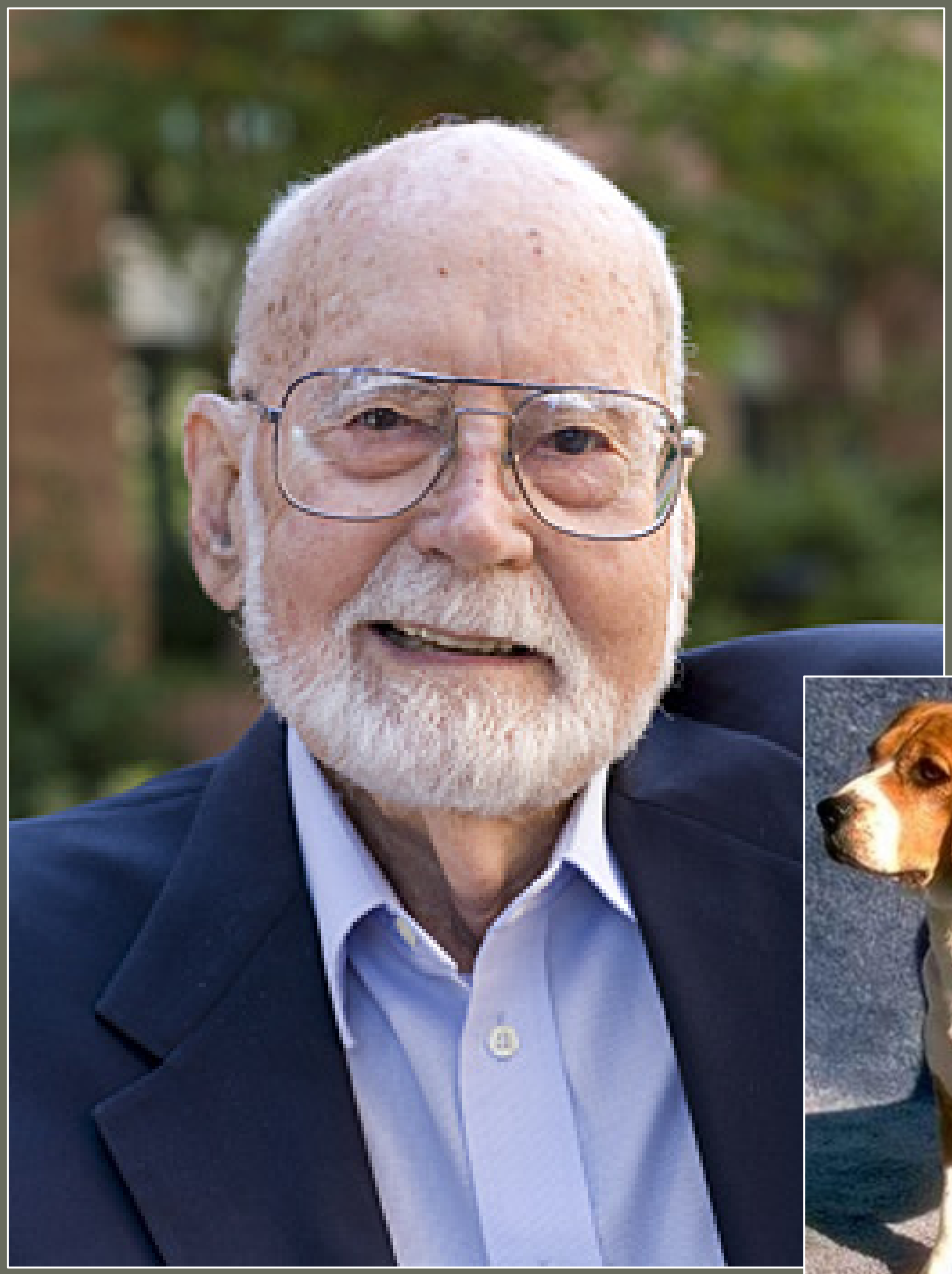
In the face of a skeptical medical community, Dr. E. Donnall Thomas began infusing patients with donor bone marrow in 1955.



Blood 1959; 14: 720-736

Homografts of Bone Marrow in Dogs After Lethal Total-Body Radiation

By E. Donnall Thomas, Charles A. Ashley, Harry L. Lochte, Jr., Alfred Jaretzki III, Otto D. Sahler and Joseph W. Ferrebee

LIFE-SAVING HOMOGRAFTS of bone marrow after lethal whole-body irradiation usually succeed in rodents[1] but fail in dogs.[2,3] Recently we reported a successful and life-saving homograft of marrow after 1,200 roentgens of total-body irradiation in a beagle.[4] Success was attributed to the following procedures: (1) use of a donor and a recipient with a close genetic relationship (litter-mates), (2) use of a supralethal dose of irradiation (1200 r over a three day period), and (3) splenectomy and administration of ACTH to the recipient prior to irradiation. It was felt that splenectomy, ACTH and the supralethal dose of x-ray were necessary to inhibit vigorous immune reactions that normally make homotransplantation impossible in the canine. The intensity of host-donor reactions may have been further reduced by the use of a female litter-mate as the marrow donor.[5] Studies with skin grafts have indicated an occasional relative histo-compatibility between litter-mates in this species.[6]

The present report describes studies on 27 dogs. An attempt is made to evaluate the relative importance of the procedures listed above. In addition a follow-up account is given of the subsequent clinical course and autopsy findings in the dog previously reported (dog 1, table 1).

Methods

The animals studied were beagles, 6 weeks to 18 months old, relatively pure-bred but not inbred. Twenty-five were males; two were females (17 and 18, table 1). The dogs were purchased from outlying farms and confined in a local veterinary kennel 10 to 14 days before use. Dogs to be irradiated were treated for worms with Vermiplex.* Immunization against distemper and hepatitis was attempted by administration of a formalin-killed virus preparation (5 ml. of Virogen D-H*). Irradiation was given with a General Electric Maximar Unit, operating at 250 kv. and 10 Ma. with a half-value layer of 2.2 mm. of copper, and a target-source distance of 100 cm. The conditions used to achieve uniform whole-body irradiation were previously described.[4] A dose of 400 r measured in air was given on the first day. Dog 14 received 200 r on the second day. All other dogs received 400 r on the second day. In the case of animals receiving more than 800 r an additional

From the Mary Imogene Bassett Hospital (affiliated with Columbia University), Cooperstown, N. Y.

Supported in part by a research grant (C-2643) from the United States Public Health Service and by the Atomic Energy Commission, Contract AT(30-1)-2005.

A portion of this material was presented to the meeting of the American Society for Clinical Investigation, Atlantic City, May 4, 1958 and to the meeting of the American College of Physicians, Atlantic City, May 1, 1958.

Submitted Aug. 4, 1958; accepted for publication Nov. 18, 1958.

*Pitman-Moore Company, Indianapolis, Ind.

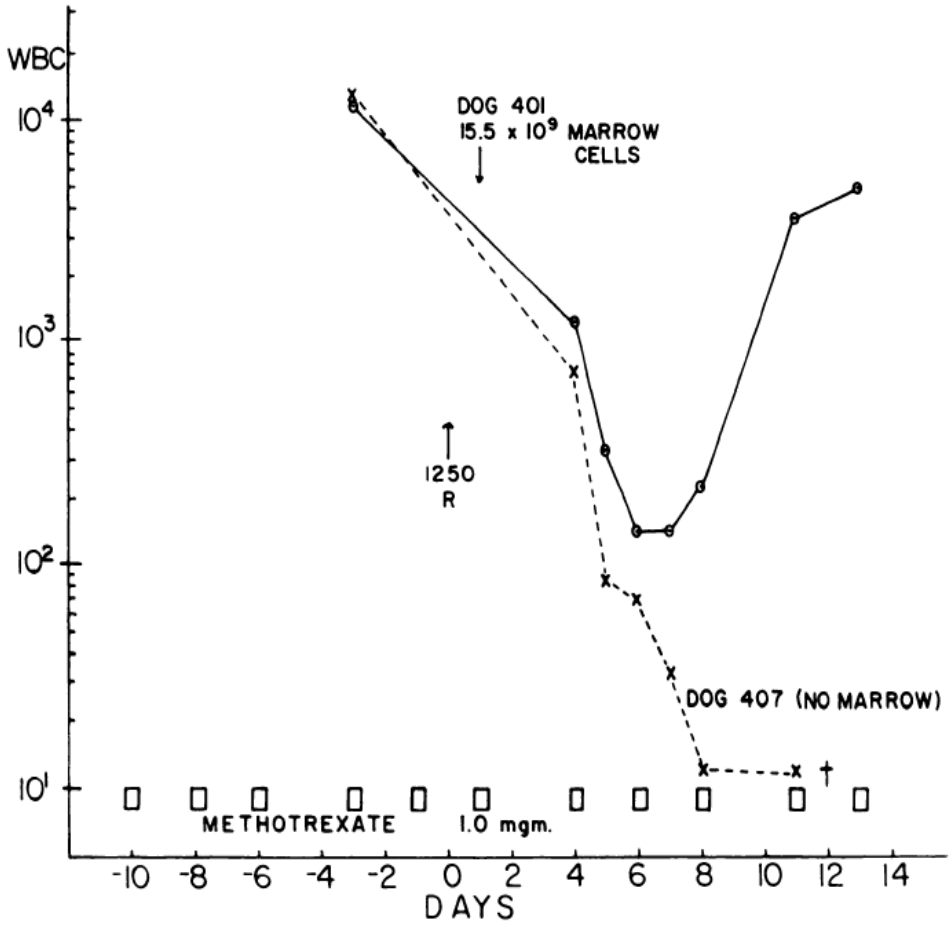
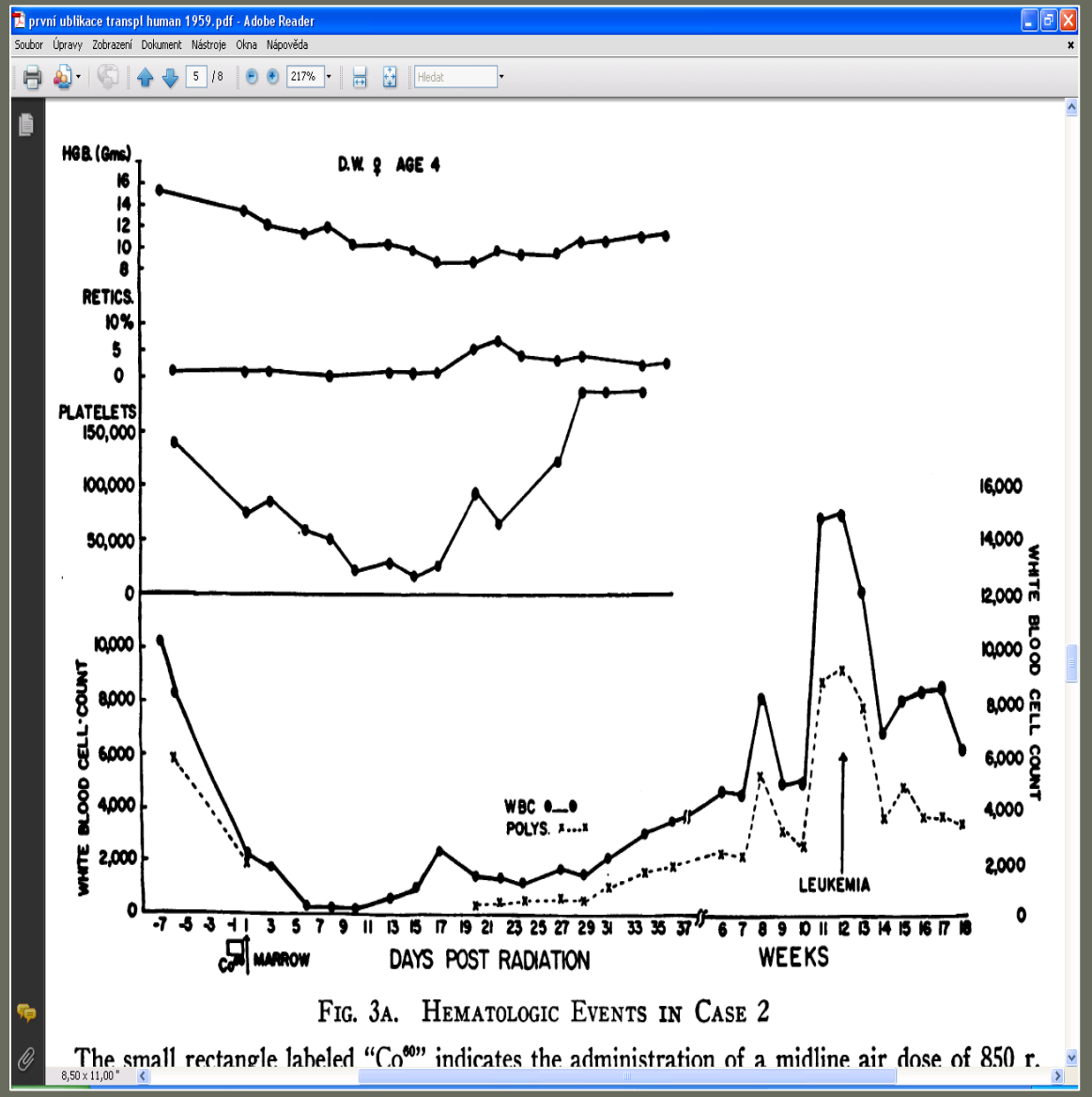


Fig. 1.—White blood cell counts and Methotrexate administration in two dogs irradiated with 1250 r. Dog 401 received homologous marrow on the day after radiation and was alive and well 201 days later. Dog 407 received no marrow and died on the 12th day.

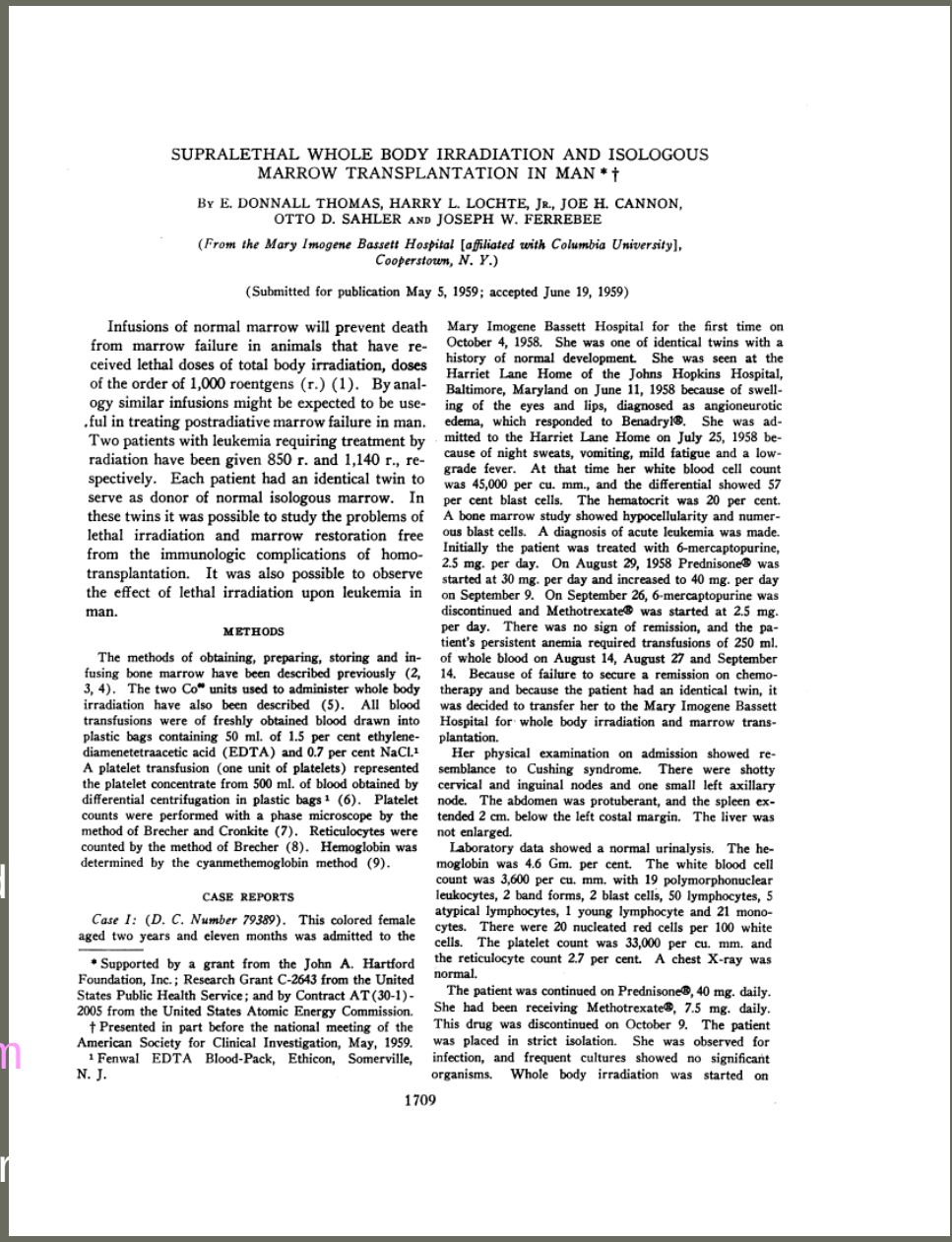
and the marrow is cellular at autopsy. It is obvious that a successful transplant

In the fall of 1955, Thomas and colleagues began infusing patients with donor bone marrow harvested from fetal and adult cadavers, from ribs removed at surgery and from hipbones using an aspiration needle. The collected marrow was passed repeatedly through a stainless-steel screen and broken into a smooth cellular suspension. Fat was removed using centrifugation. The marrow cells were suspended in tissue-culture fluid and serum. Unused cells were frozen in glycerol and stored at -80 C.



J Clin Invest 1959; 1709-1716

1958 On October 7 the marrow donor was taken and under general anesthesia 25 marrow aspirations were performed on the sternum, tibiae and anterior and posterior iliac crests. This marrow was transferred immediately to Hanks' solution containing heparin and passed through stainless steel screens. The marrow was frozen in 15% glycerol and stored at – 80oC. On October 12 this marrow was thawed, deglycerolized and administered to the irradiated recipient 3-year-old girl with end-stage leukemia This time, a large dose of radiation and marrow from twin resulted in a successful transplant. The patient did well for six months until her leukemia returned.



SUPRALETHAL WHOLE BODY IRRADIATION AND ISOLOGOUS MARROW TRANSPLANTATION IN MAN * †

By E. DONNALL THOMAS, HARRY L. LOCHTE, JR., JOE H. CANNON, OTTO D. SAHLER AND JOSEPH W. FERREBEE

(From the Mary Imogene Bassett Hospital [affiliated with Columbia University], Cooperstown, N. Y.)

(Submitted for publication May 5, 1959; accepted June 19, 1959)

Infusions of normal marrow will prevent death from marrow failure in animals that have received lethal doses of total body irradiation, doses of the order of 1,000 roentgens (r.) (1). By analogy similar infusions might be expected to be useful in treating postradiative marrow failure in man. Two patients with leukemia requiring treatment by radiation have been given 850 r. and 1,140 r., respectively. Each patient had an identical twin to serve as donor of normal isologous marrow. In these twins it was possible to study the problems of lethal irradiation and marrow restoration free from the immunologic complications of homotransplantation. It was also possible to observe the effect of lethal irradiation upon leukemia in man.

METHODS

The methods of obtaining, preparing, storing and infusing bone marrow have been described previously (2, 3, 4). The two Co^{60} units used to administer whole body irradiation have also been described (5). All blood transfusions were of freshly obtained blood drawn into plastic bags containing 50 ml. of 1.5 per cent ethylenediaminetetraacetic acid (EDTA) and 0.7 per cent NaCl.[1] A platelet transfusion (one unit of platelets) represented the platelet concentrate from 500 ml. of blood obtained by differential centrifugation in plastic bags[1] (6). Platelet counts were performed with a phase microscope by the method of Brecher and Cronkite (7). Reticulocytes were counted by the method of Brecher (8). Hemoglobin was determined by the cyanmethemoglobin method (9).

CASE REPORTS

Case 1: (D. C. Number 79389). This colored female aged two years and eleven months was admitted to the Mary Imogene Bassett Hospital for the first time on October 4, 1958. She was one of identical twins with a history of normal development. She was seen at the Harriet Lane Home of the Johns Hopkins Hospital, Baltimore, Maryland on June 11, 1958 because of swelling of the eyes and lips, diagnosed as angioneurotic edema, which responded to Benadryl®. She was admitted to the Harriet Lane Home on July 25, 1958 because of night sweats, vomiting, mild fatigue and a low-grade fever. At that time her white blood cell count was 45,000 per cu. mm., and the differential showed 57 per cent blast cells. The hematocrit was 20 per cent. A bone marrow study showed hypocellularity and numerous blast cells. A diagnosis of acute leukemia was made. Initially the patient was treated with 6-mercaptopurine, 2.5 mg. per day. On August 29, 1958 Prednisone® was started at 30 mg. per day and increased to 40 mg. per day on September 9. On September 26, 6-mercaptopurine was discontinued and Methotrexate® was started at 2.5 mg. per day. There was no sign of remission, and the patient's persistent anemia required transfusions of 250 ml. of whole blood on August 14, August 27 and September 14. Because of failure to secure a remission on chemotherapy and because the patient had an identical twin, it was decided to transfer her to the Mary Imogene Bassett Hospital for whole body irradiation and marrow transplantation.

Her physical examination on admission showed resemblance to Cushing syndrome. There were shotty cervical and inguinal nodes and one small left axillary node. The abdomen was protuberant, and the spleen extended 2 cm. below the left costal margin. The liver was not enlarged.

Laboratory data showed a normal urinalysis. The hemoglobin was 4.6 Gm. per cent. The white blood cell count was 3,600 per cu. mm. with 19 polymorphonuclear leukocytes, 2 band forms, 2 blast cells, 50 lymphocytes, 5 atypical lymphocytes, 1 young lymphocyte and 21 monocytes. There were 20 nucleated red cells per 100 white cells. The platelet count was 33,000 per cu. mm. and the reticulocyte count 2.7 per cent. A chest X-ray was normal.

The patient was continued on Prednisone®, 40 mg. daily. She had been receiving Methotrexate®, 7.5 mg. daily. This drug was discontinued on October 9. The patient was placed in strict isolation. She was observed for infection, and frequent cultures showed no significant organisms. Whole body irradiation was started on

* Supported by a grant from the John A. Hartford Foundation, Inc.; Research Grant C-2643 from the United States Public Health Service; and by Contract AT(30-1)-2005 from the United States Atomic Energy Commission.

† Presented in part before the national meeting of the American Society for Clinical Investigation, May, 1959.

[1] Fenwal EDTA Blood-Pack, Ethicon, Somerville, N. J.

J Clin Invest 1959; 1709-1716

DOGS AROUND THE WORLD ARE HAPPY ABOUT THE NEWS!

2008

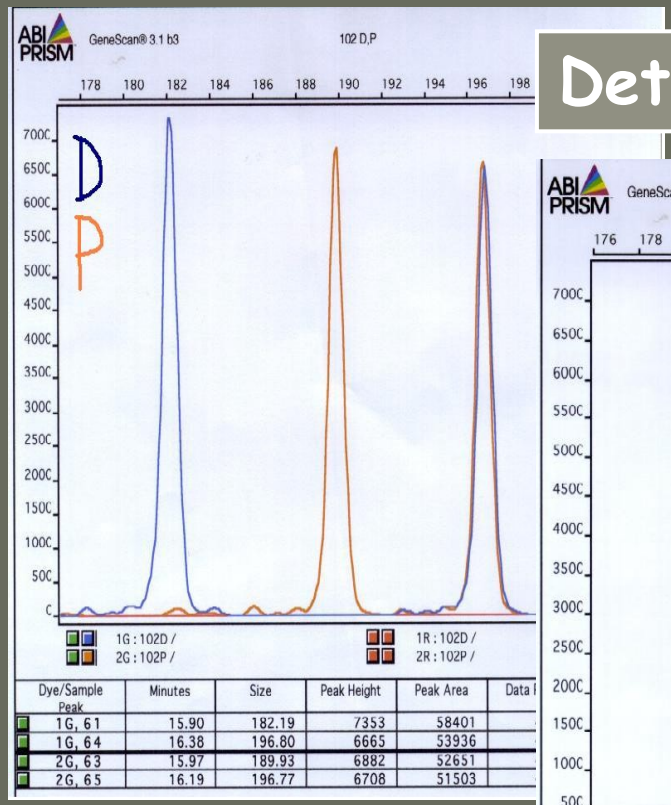
Stem Cells Now Curing Dogs of Leukemia

WSU to offer bone marrow transplants to sick pets

It's a big 'give-back' to dogs for cancer help

The bone marrow or stem cell transplant, a procedure that every year saves tens of thousands of lives and won for the Seattle physician who pioneered it the 1990 Nobel Prize in Medicine, appears poised to come full circle and finally become more widely available to those who first made it all possible.The WSU transplant program, which will be a partnership with a private business based in North Carolina, is intended to make the procedures available to pet owners for about $15,000 to $20,000 per dog. Dogs suffering from lymphoma will be able to receive the same type of medical treatment as their human counterparts, as North Carolina State University becomes the first university in the nation to offer canine bone marrow transplants in a clinical setting. Dr. Steven Suter, assistant professor of oncology in NC State's College of Veterinary Medicine, received three leukophoresis machines donated by the Mayo Clinic in Rochester, Minn. Leukophoresis machines are designed to harvest healthy stem cells from cancer patients. The machines, once used for human patients, are suitable for canine use without modification, as bone marrow therapy protocols for people were originally developed using dogs.

Detekce buněčného chimérismu po HSCT



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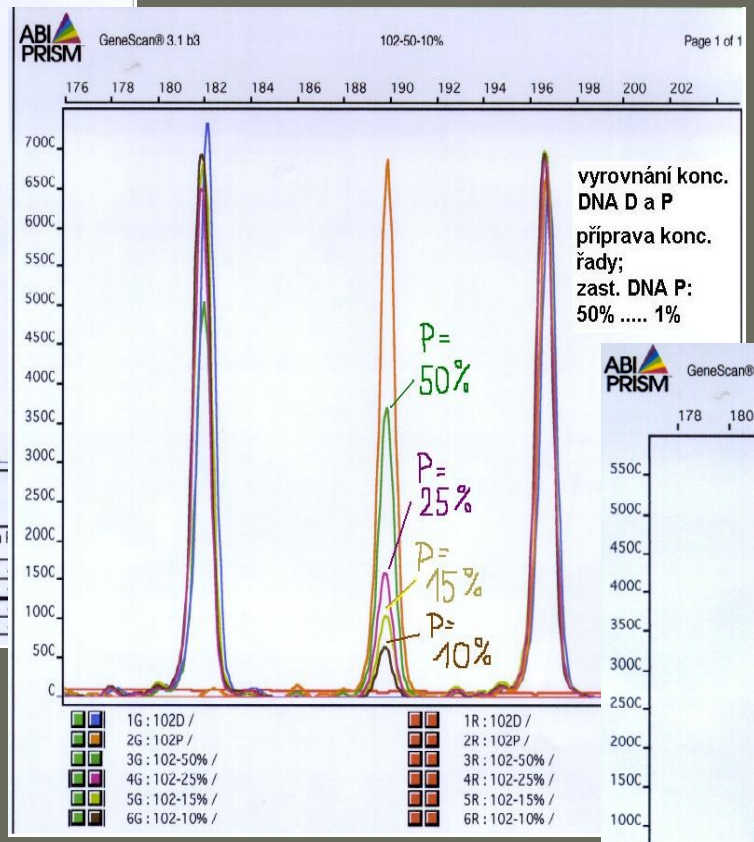
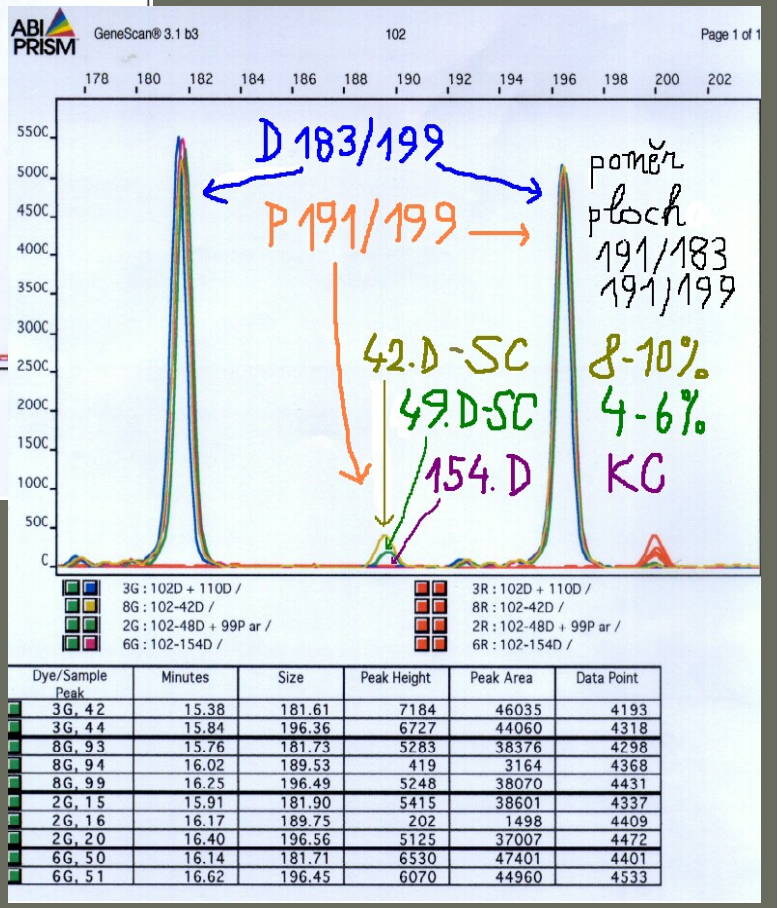
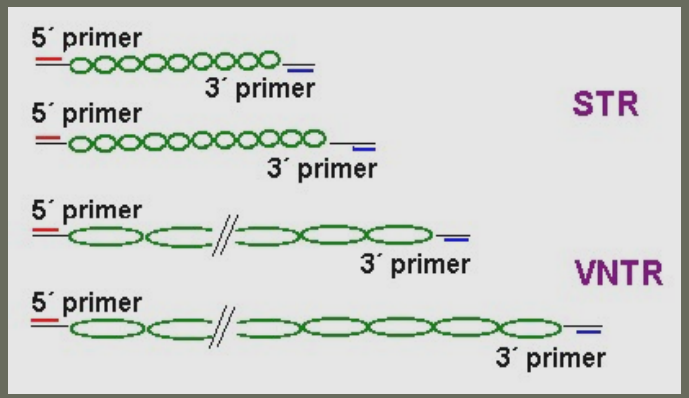
1G, 64	16.38	196.80	6665	53936	
2G, 63	15.97	189.93	6882	52651	
2G, 65	16.19	196.77	6708	51503	

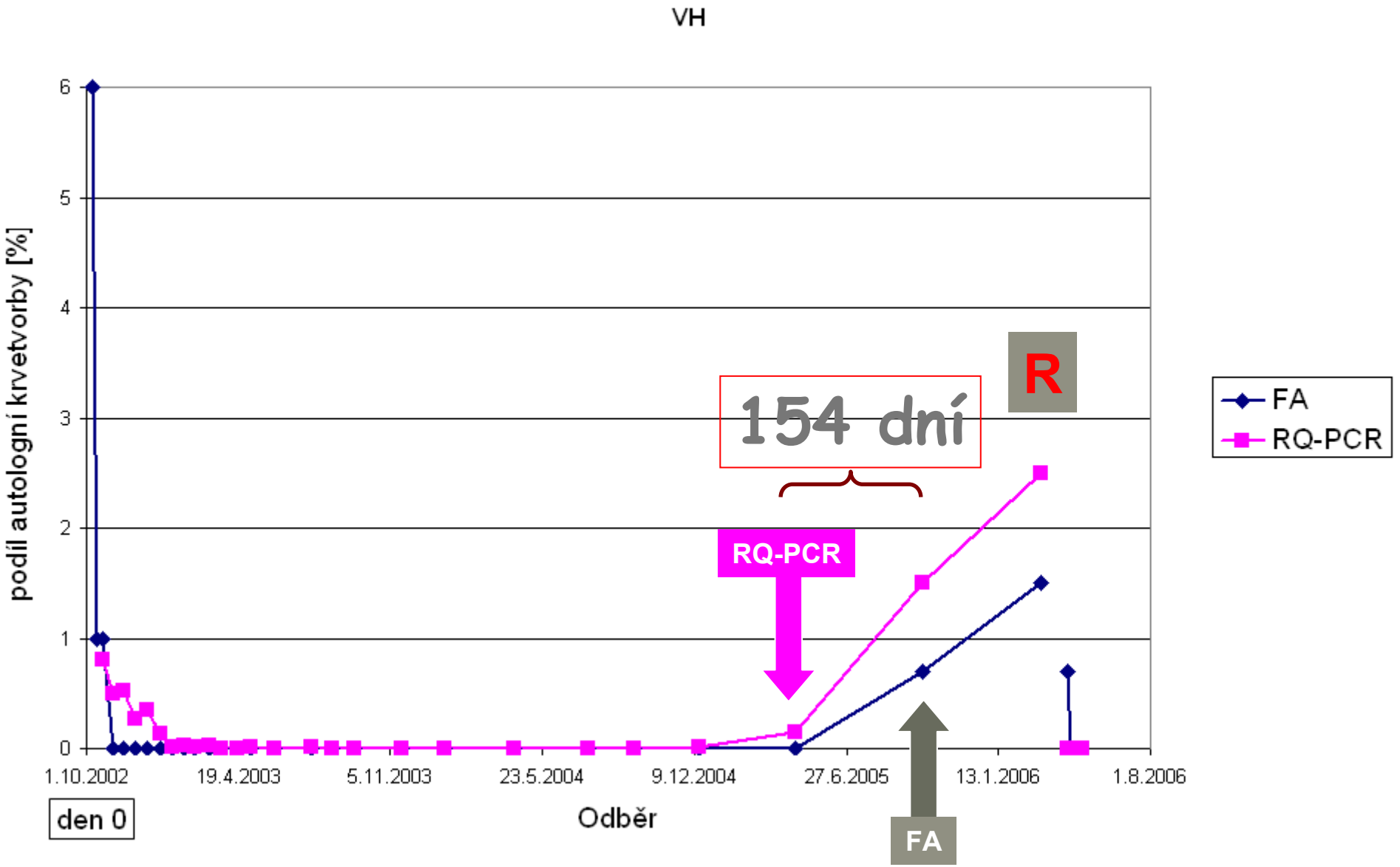


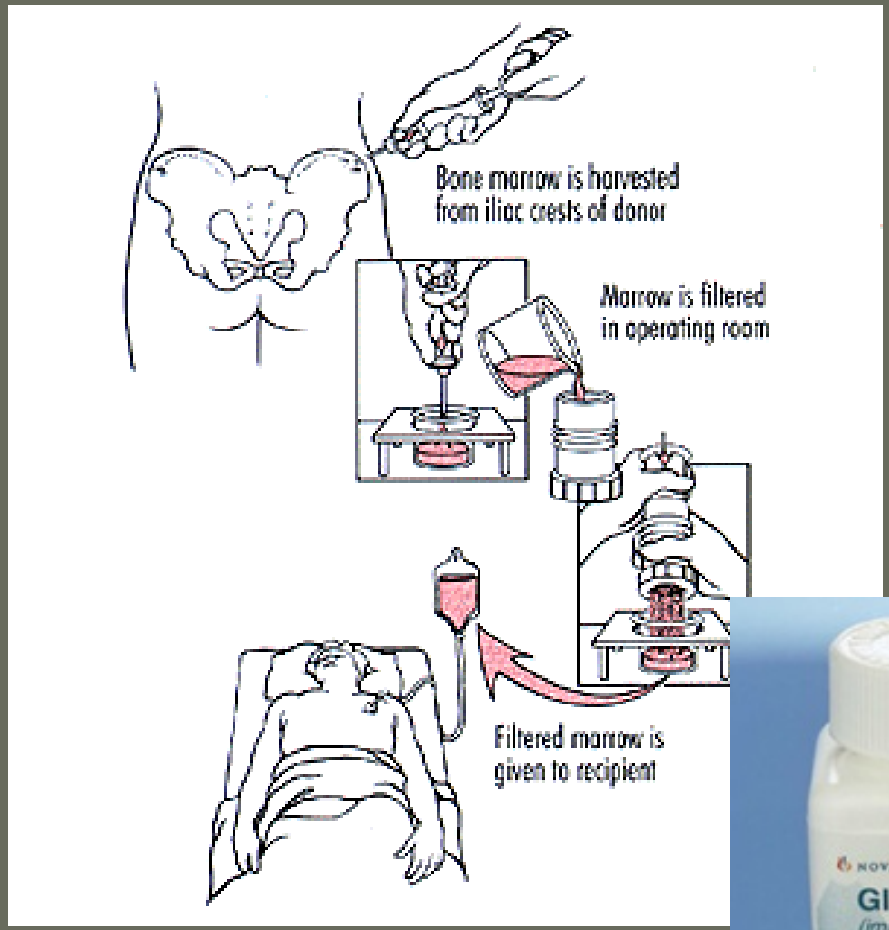


Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
3G, 42	15.38	181.61	7184	46035	4193
3G, 44	15.84	196.36	6727	44060	4318
8G, 93	15.76	181.73	5283	38376	4298
8G, 94	16.02	189.53	419	3164	4368
8G, 99	16.25	196.49	5248	38070	4431
2G, 15	15.91	181.90	5415	38601	4337
2G, 16	16.17	189.75	202	1498	4409
2G, 20	16.40	196.56	5125	37007	4472
6G, 50	16.14	181.71	6530	47401	4401
6G, 51	16.62	196.45	6070	44960	4533



Hematologický relaps-příklad monitorování pacientů po HSCT

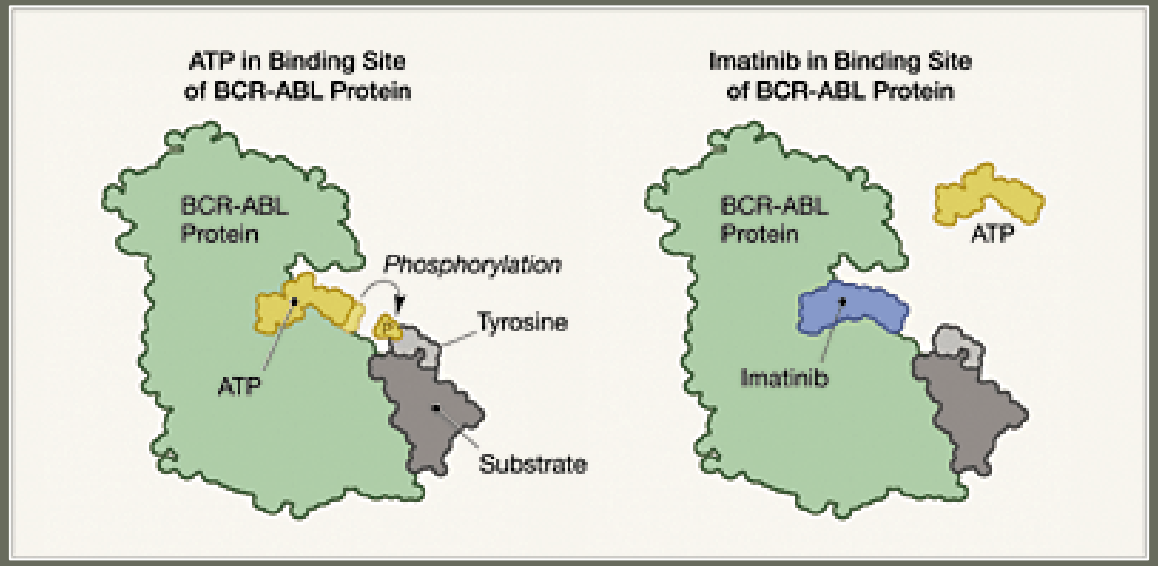




BMT

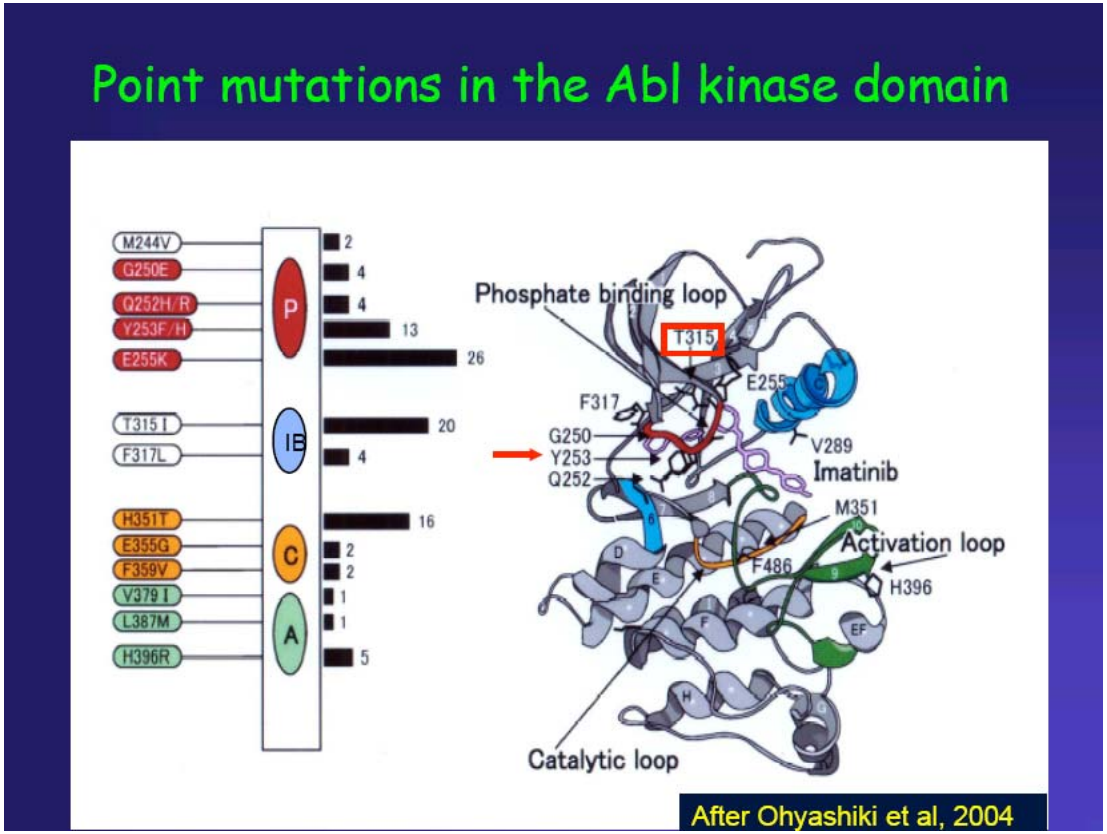
2001

PBSCT



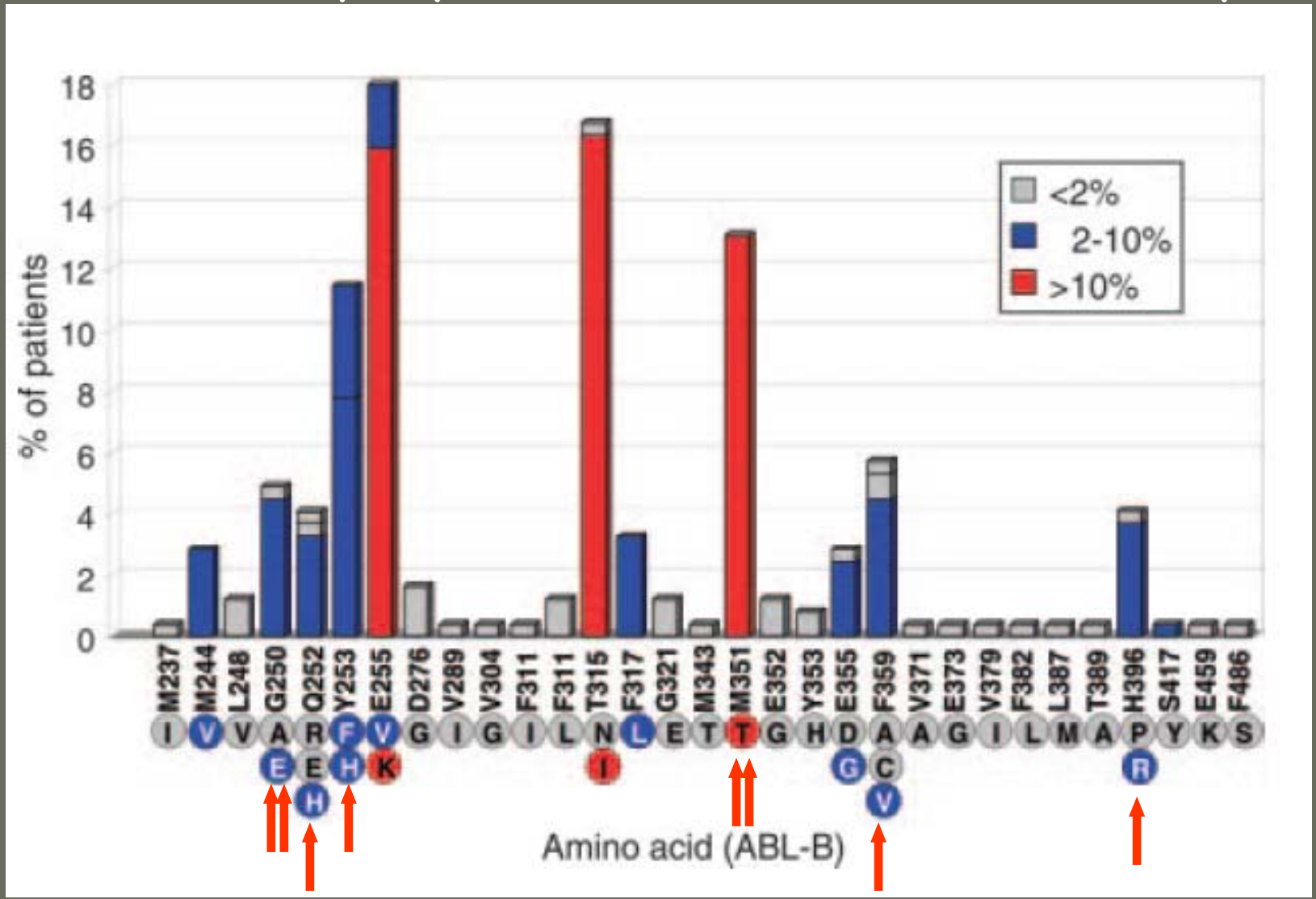
The BCR-ABL fusion protein binds adenosine triphosphate (ATP) and transfers phosphate to tyrosine residues on its substrate, leading to transmission of intracellular signals that promote unregulated cell proliferation and anti-apoptotic effects and alter cell adhesion. Imatinib blocks the ATP binding site.

Point mutations in the Abl kinase domain



Nástup nových terapeutických možností přináší obrovské úspěchy…

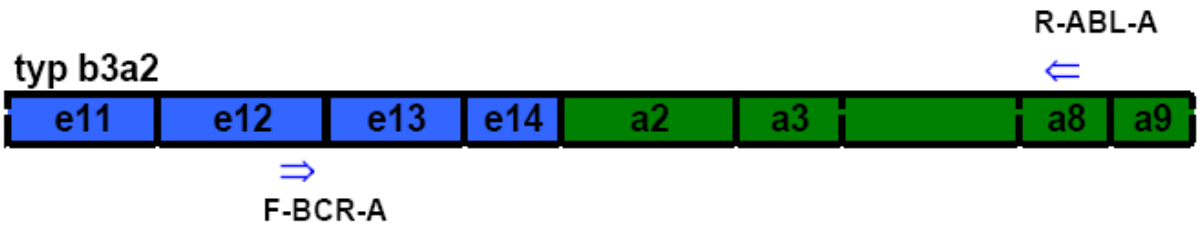
The relative frequency of BCR-ABL mutations at 31 different position



Hughes, T. et al., Blood, 108, 1: 28-37, 2006

Screening of ABL kinase domain mutations

RT-PCR: region ABL rearranged domain in BCR-ABL by Expand High Fidelity Enzyme (Roche) Primers: F-BCR-A (exon e12/e13) x R-ABL-A (exon a8)



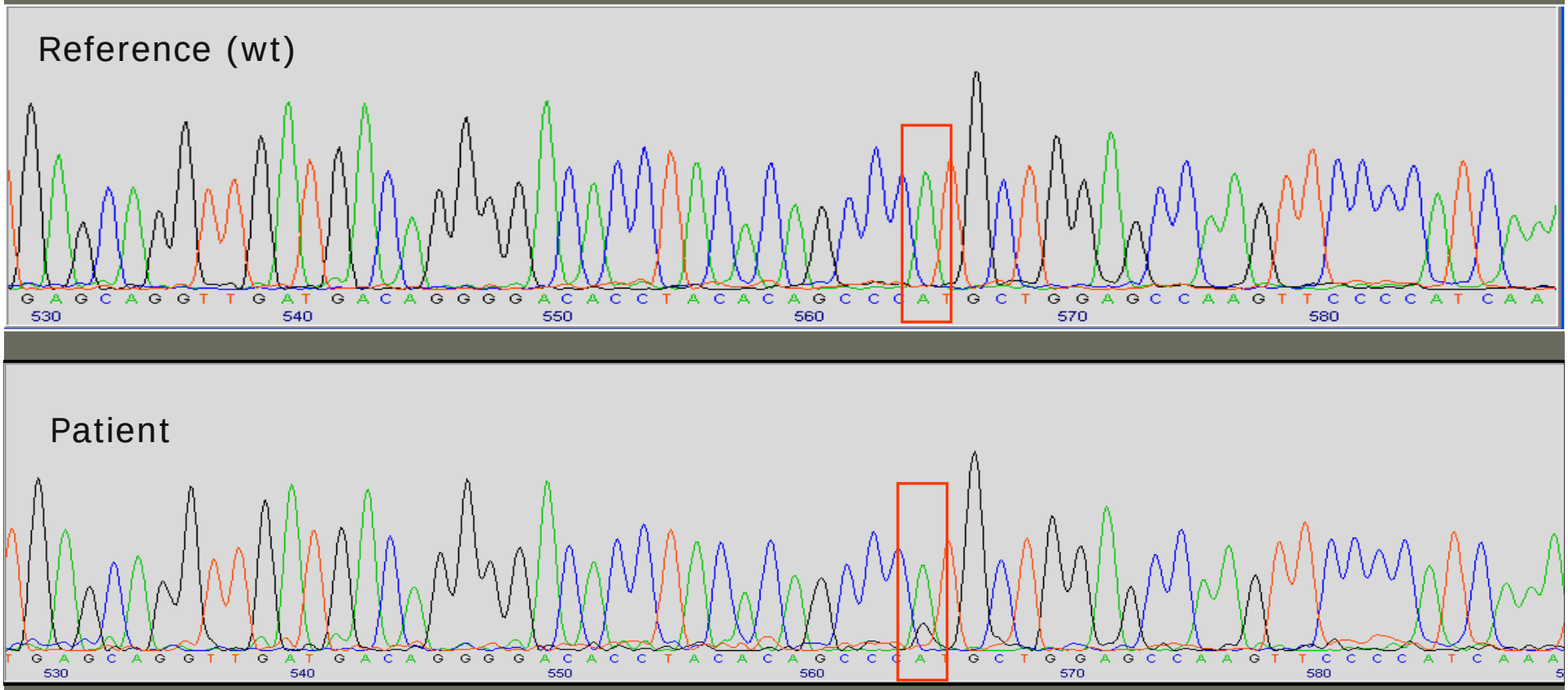
Conventional direct sequencing: by using a BigDye®Terminator v1.1 Seq.kit /AbiPrism 310 System/, exon4-exon8, Primer: ABL-ALT Comparison with the GeneBank mRNA sequence X16416

Primer	5´→ 3´
ABL-ALT*	5´-GCG CAA CAA GCC CAC TGT CTA TGG- 3´
F-BCR-A*	5´-GAG CAG CAG AAG AAG TGT TTC AGA-3´
R-ABL-A	5´-CTC TAG CAG CTC ATA CAC CTG GG-3´

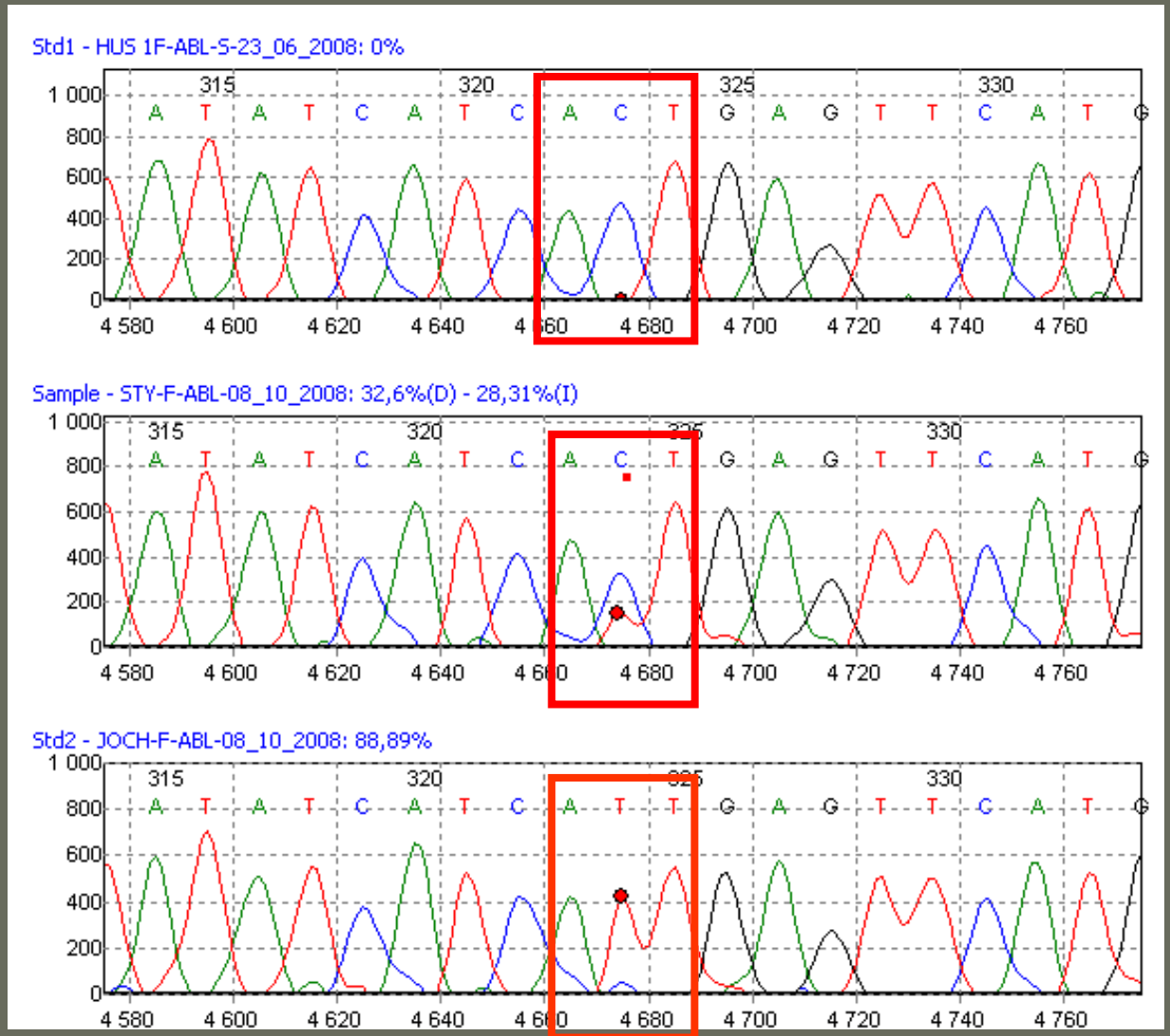
Gorre M.E. et al. 2001 Science*, Soverini S. et al. 2004 Clin.Chem.*

Mutation Surveyor (Softgenetics):

- **Mutation detection**



22/9 PB T315I 32,60%



0%

32,6%

88,9%

Table 2: FDA-approved Indications of Tyrosine Kinase Inhibitors

Gleevec (imatinib mesylate)	• Approved in May 2001 • Treatment of newly diagnosed patients with Philadelphia chromosome positive chronic myeloid leukemia (Ph+CML) in chronic phase (400 mg daily) • Treatment of patients with Ph+CML in blast crisis, accelerated phase, or in chronic phase after failure of interferon-alpha therapy (600 mg daily) • Treatment of pediatric patients with Ph+CML in chronic phase who are newly diagnosed or whose disease has recurred after stem cell transplant or who are resistant to interferon-alpha therapy (260 mg/m^2 daily or 340 mg/m^2 daily) • Treatment of adult patients with relapsed or refractory Ph+ acute lymphoblastic leukemia (600 mg daily) • Treatment of adult patients with myelodysplastic/myeloproliferative diseases (MDS/MPD) associated with PDGFR (platelet-derived growth factor receptor) gene rearrangements (400 mg daily) • Treatment of adult patients with aggressive systemic mastocytosis (ASM) without the D816V c-Kit mutation or with c-Kit mutational status unknown (100 mg daily or 400 mg daily) • Treatment of adult patients with hypereosinophilic syndrome (HES) and/or chronic eosinophilic leukemia (CEL) who have the FIP1L1-PDGFR alpha fusion kinase (mutational analysis or FISH demonstration of CHIC2 allele deletion) and for patients with HES and/or CEL who are FIP1L1-PDGFR alpha fusion kinase negative or unknown (100 mg daily or 400 mg daily) • Treatment of adult patients with unresectable, recurrent and/or metastatic dermatofibrosarcoma protuberans (DFSP) (800 mg daily) • Treatment of patients with Kit (CD117) positive unresectable and/or metastatic malignant gastrointestinal stromal tumors (GIST) (400 mg daily or 800 mg daily)
Sprycel (dasatinib)	• Approved in June 2006 • Treatment of adults with chronic, accelerated, or myeloid or lymphoid blast phase CML with resistance or intolerance to prior therapy including imatinib • Treatment of adults with Ph+ ALL with resistance or intolerance to prior therapy • Chronic phase, 100 mg daily • Advanced phases of disease, 70 mg twice daily
Tasigna (nilotinib)	• Approved in October 2007 • Treatment of chronic phase and accelerated phase Ph+CML in adult patients resistant to or intolerant to prior therapy that included imatinib • Dose, 400 mg twice daily

Acute myeloid leukaemia (AML) and related precursor neoplasms

AML with recurrent genetic abnormalities

AML with gene mutations

AML with myelodysplasia-related changes

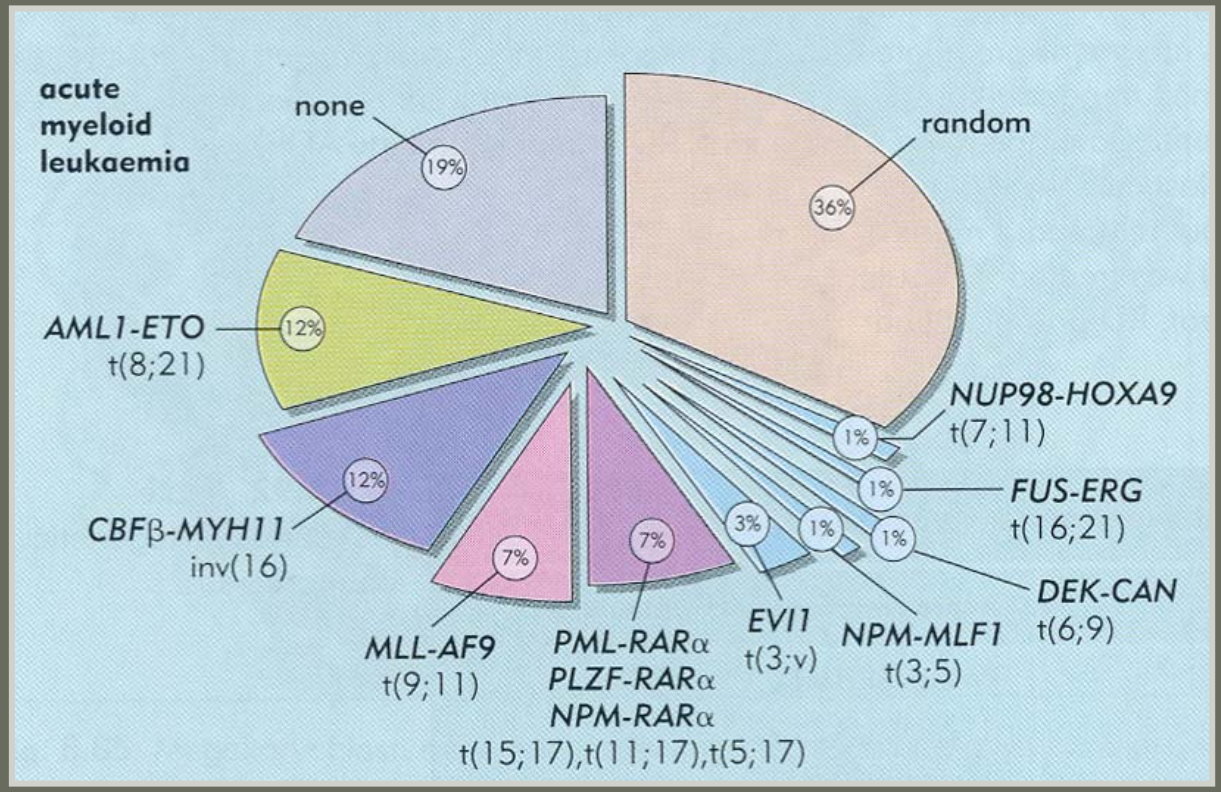
Therapy-related myeloid neoplasms

AML, not otherwise specified

Myeloid sarcoma

Akutní myeloidní leukémie

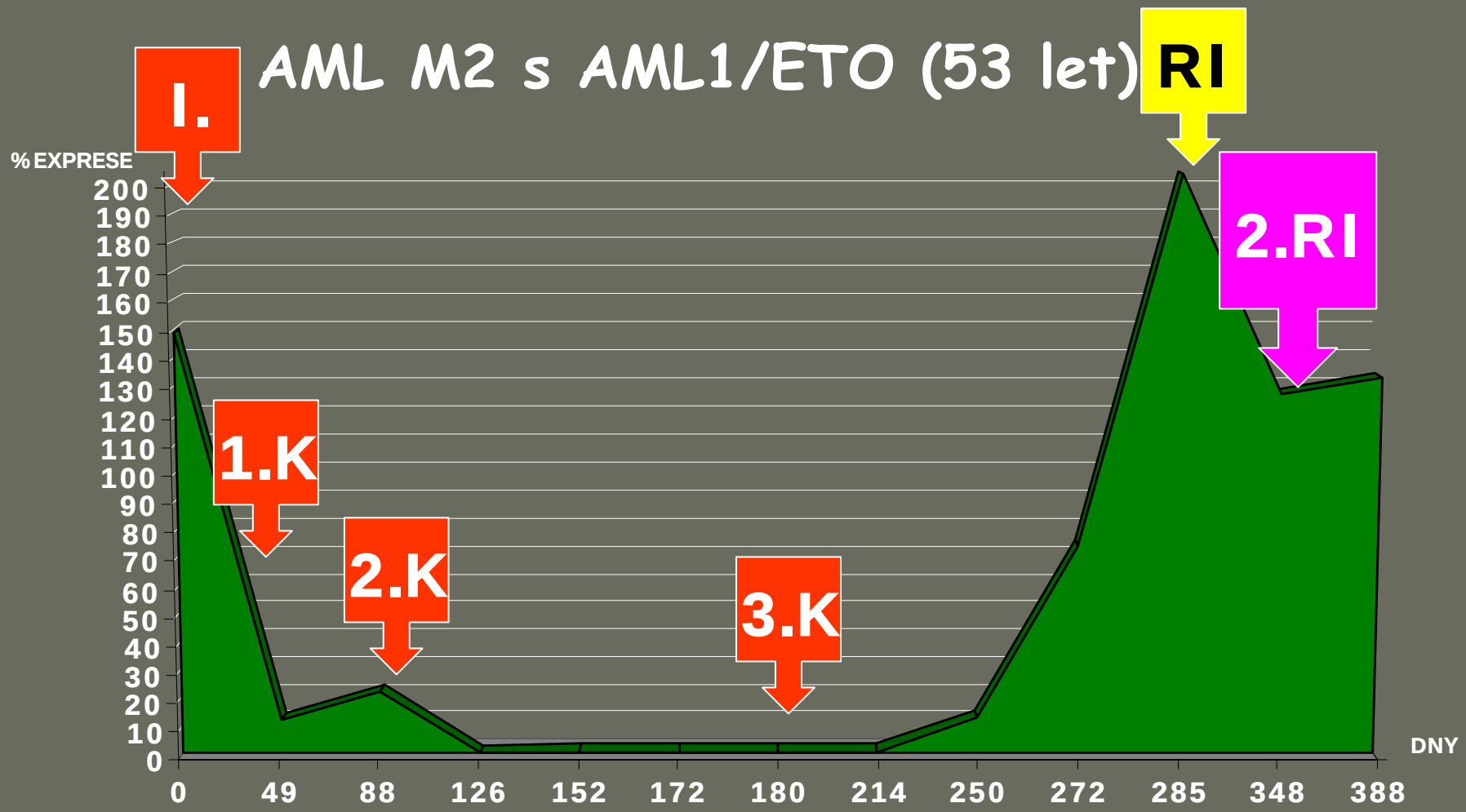
- heterogenní skupina onemocnění
- různé chromozomální aberace
- 40-50% případů AML s molekulárním markerem



Prognostické faktory AML

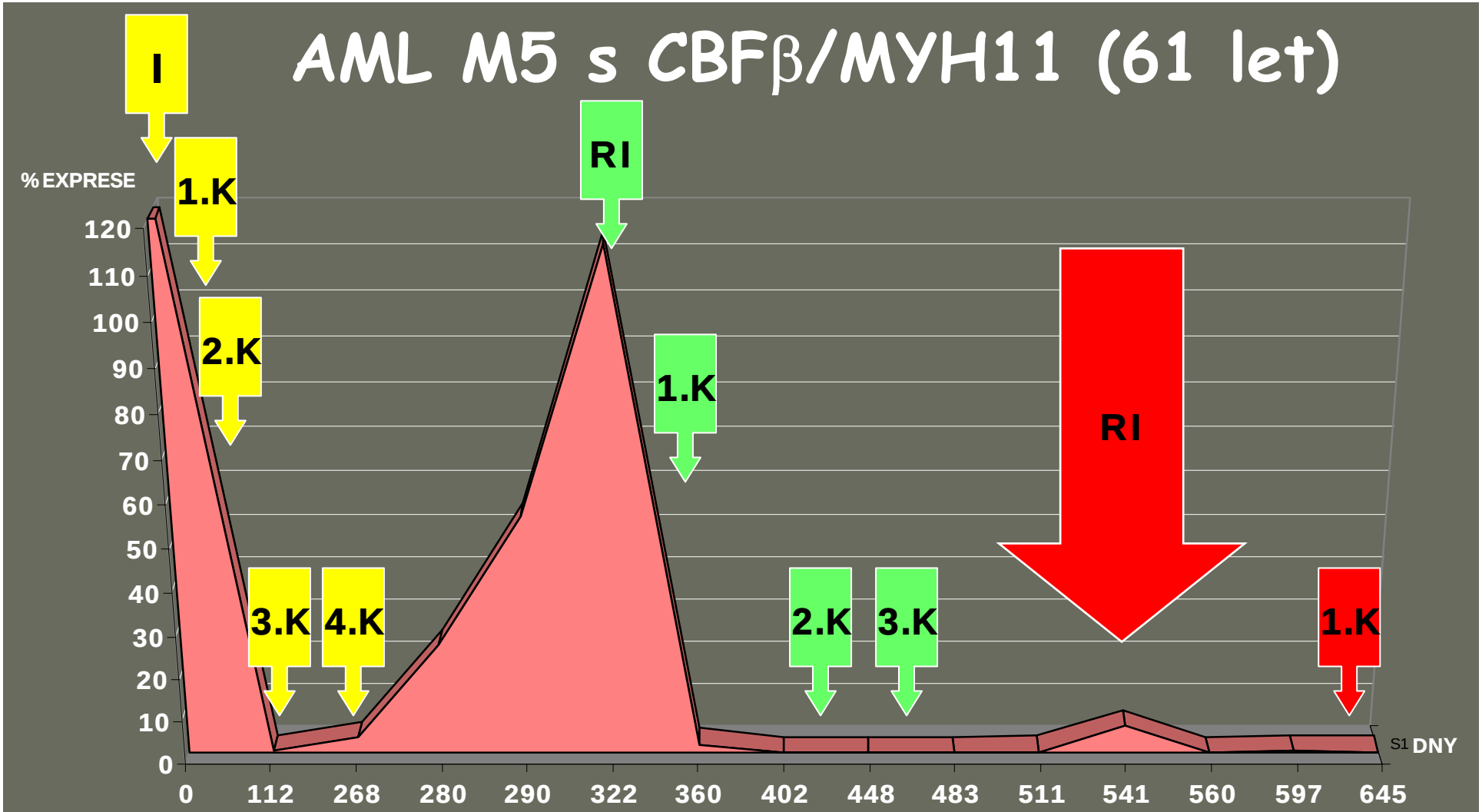
- **Fúzní geny s diagnostickým významem**
  - **PML/RARa** ® dobrá prognóza
  - **AML1/ETO** ® dobrá prognóza
  - **CBFb/MYH** ® dobrá prognóza
  - přestavby **MLL** genu ® špatná prognóza
- **změny genů s prognostickým významem**
  - interní tandemové duplikace **ITD-FLT3** genu
  - bodové mutace aktivační smyčky **FLT3** genu
  - mutace **CEBPa** genu
  - mutace **NPM1** genu

AML M2 s AML1/ETO (53 let)



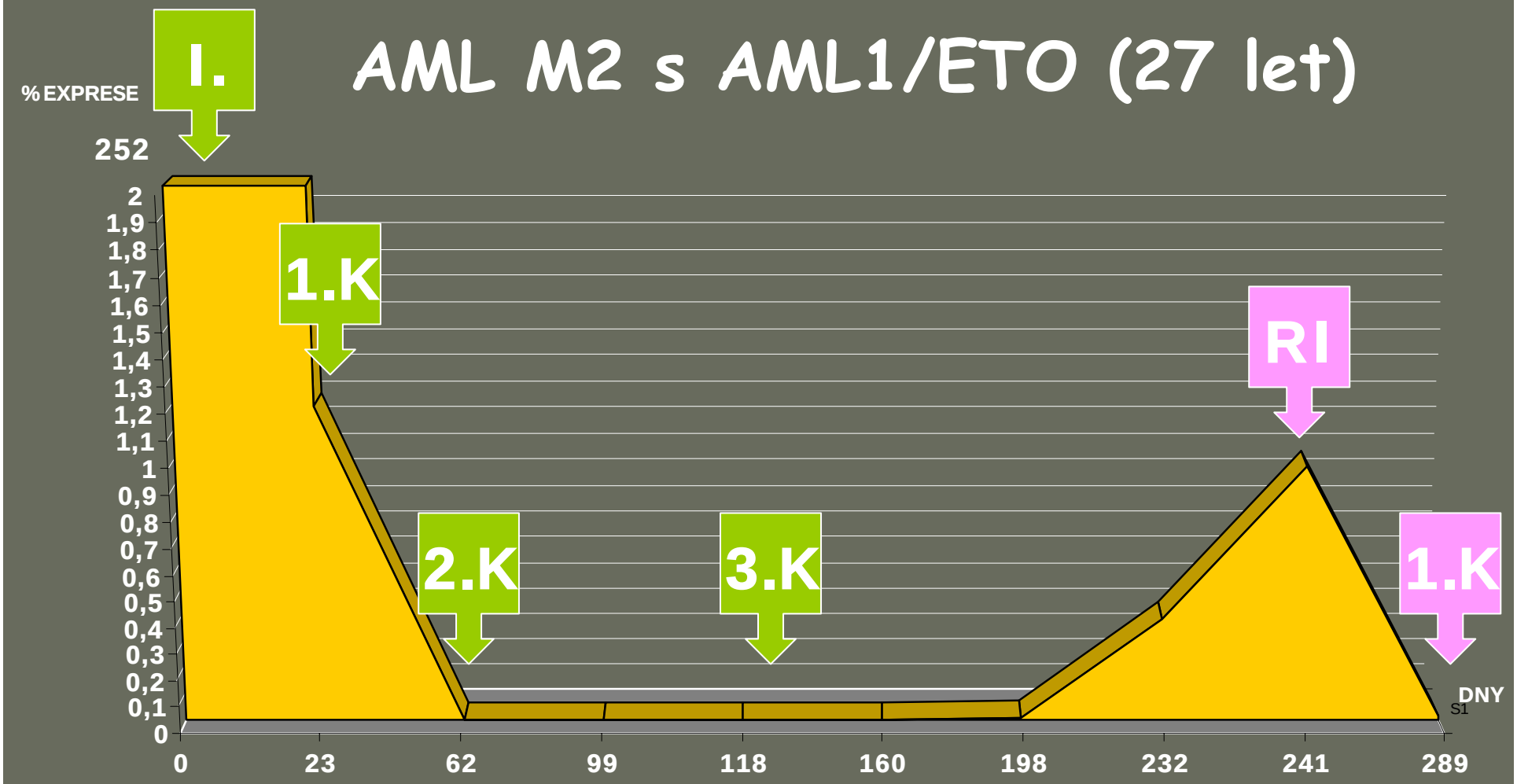
DNY	0	49	88	126	152	172	180	214	250	272	285	348	388
% EXP.- BM	149	11,74	21,77	0,00003	0,03	0,04	0,02	0,09	12,68	74	204	127	133
% EXP.- PB												42	

AML M5 s CBFβ/MYH11 (61 let)



DEN	0	112	268	280	290	322	360	402	448	483	511	541	560	597	645
% EXP.- BM	130	0,18	3,54	25,41	54,92	115	1,97	0	0,025	0,007	0,15	6,3		0,38	0,16
% EXP.- PB										0,05	0,27	6,07	35,96		

AML M2 s AML1/ETO (27 let)



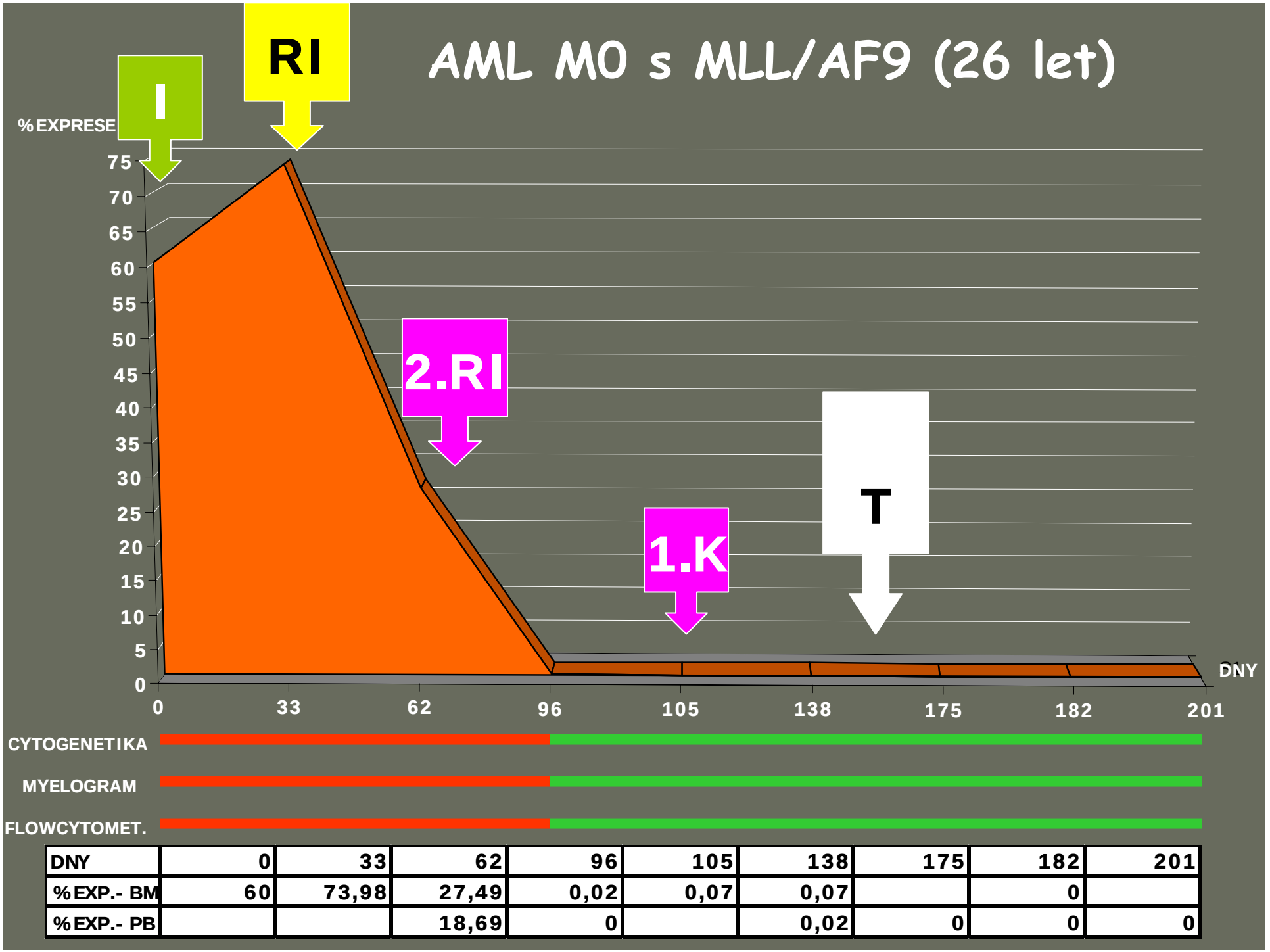
CYTOGENETIKA

MYELOGRAM

FLOWCYTOMET.

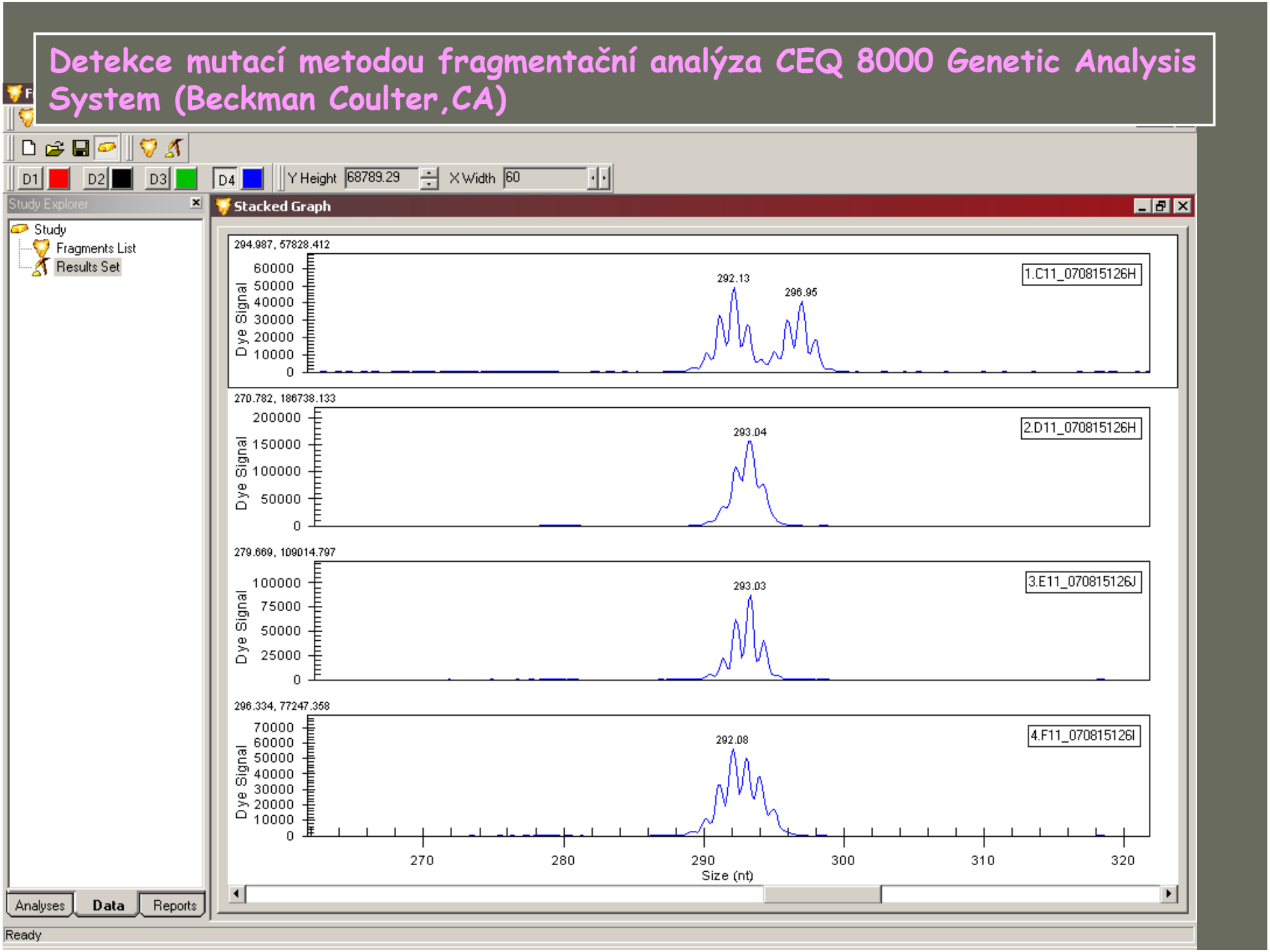
DNY	0	23	62	99	118	160	198	232	241	289
% EXP.- BM	252	1,19	0,0013	0,001	0	0	0,008	0,39	0,97	0,015
% EXP.- PB		0,74			0	0				0,01

AML M0 s MLL/AF9 (26 let)

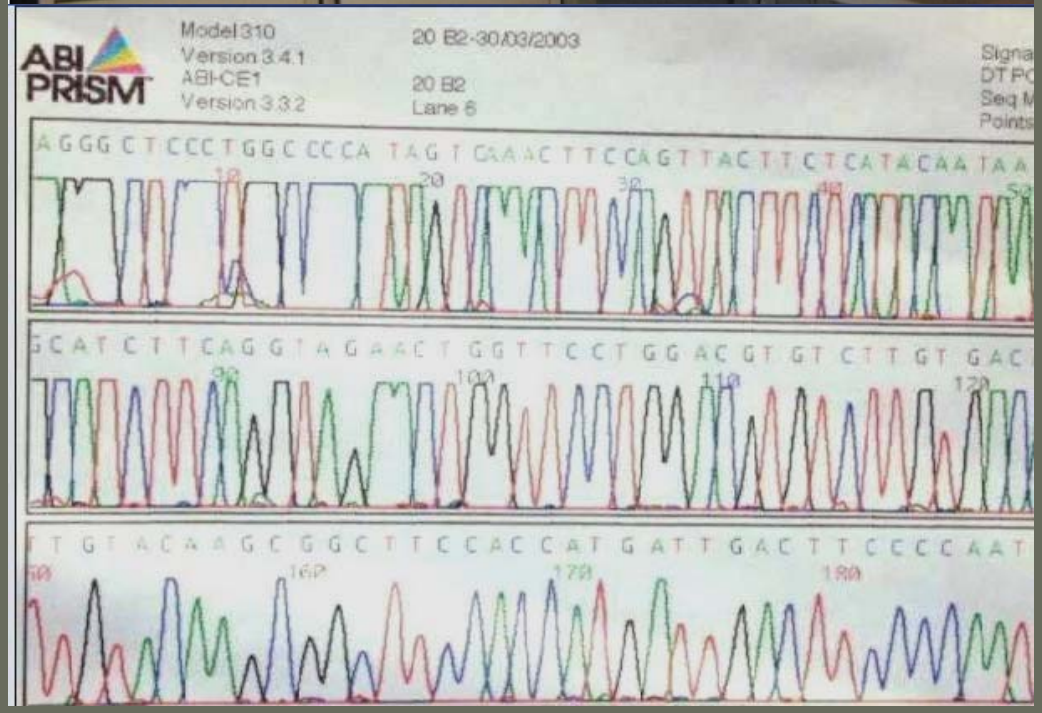


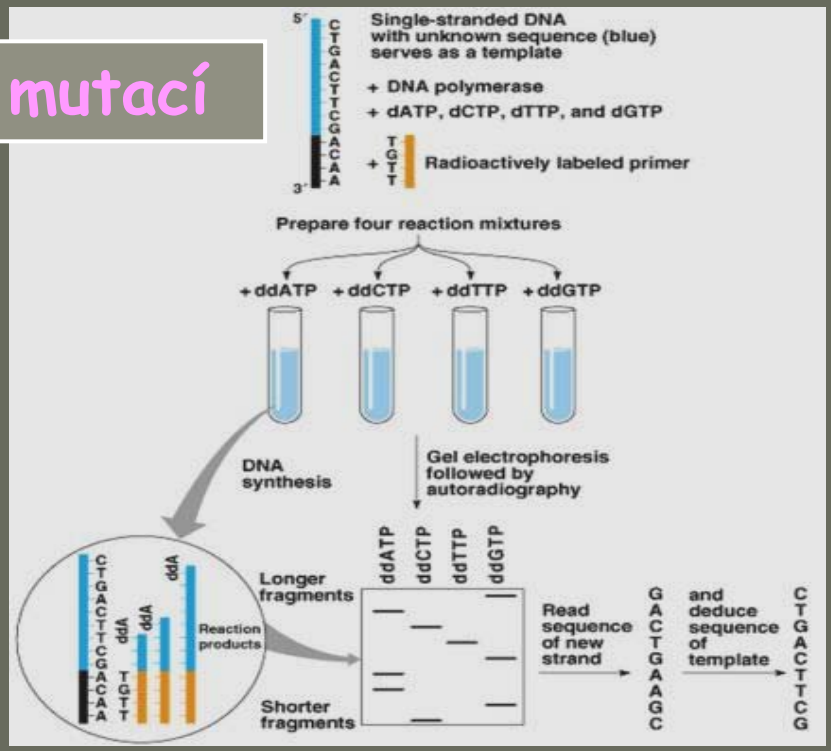
DNY	0	33	62	96	105	138	175	182	201
% EXP.- BM	60	73,98	27,49	0,02	0,07	0,07		0	
% EXP.- PB			18,69	0		0,02	0	0	0

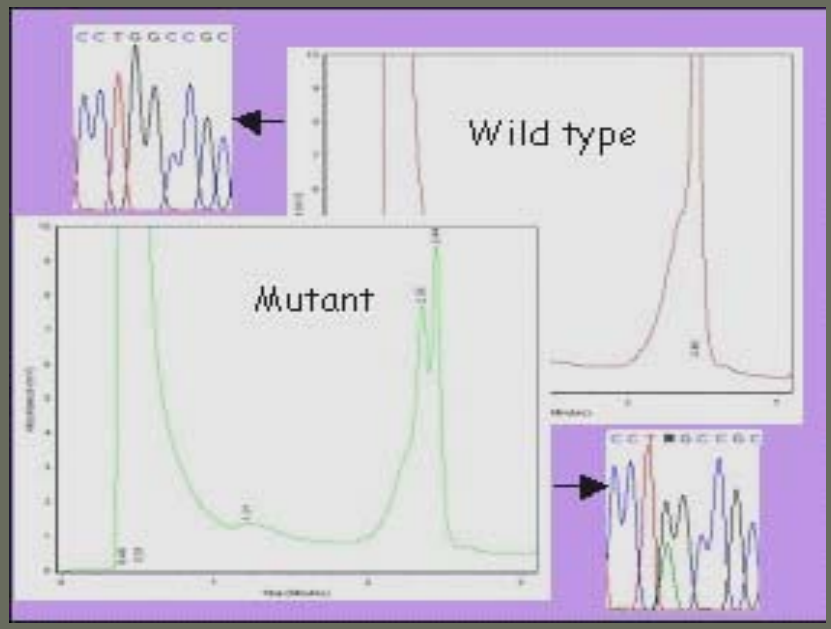
Detekce mutací metodou fragmentační analýza CEQ 8000 Genetic Analysis System (Beckman Coulter,CA)



Detekce bodových mutací

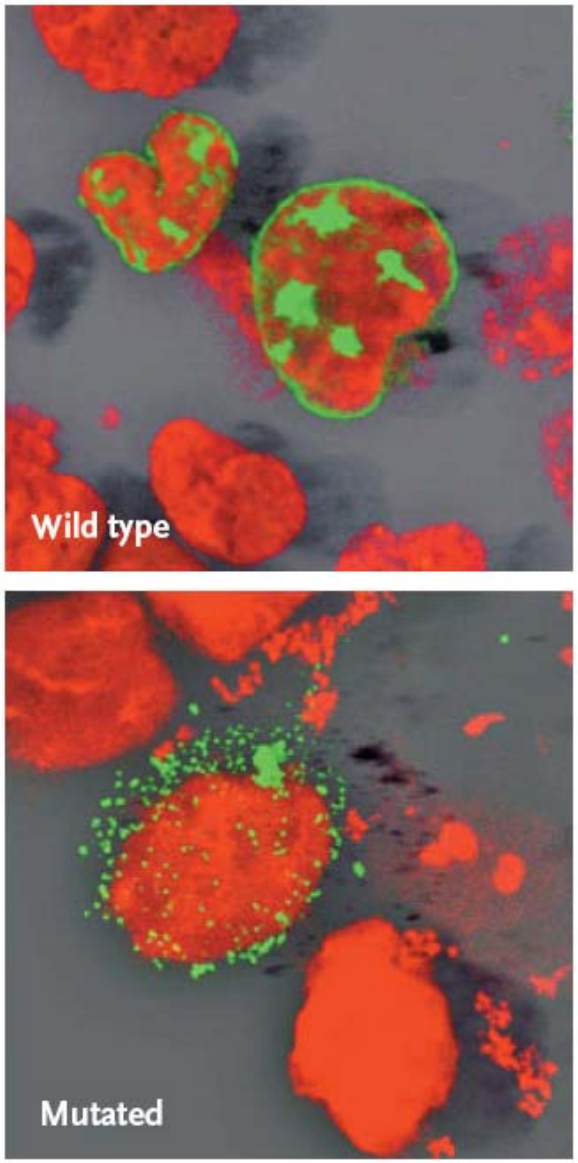




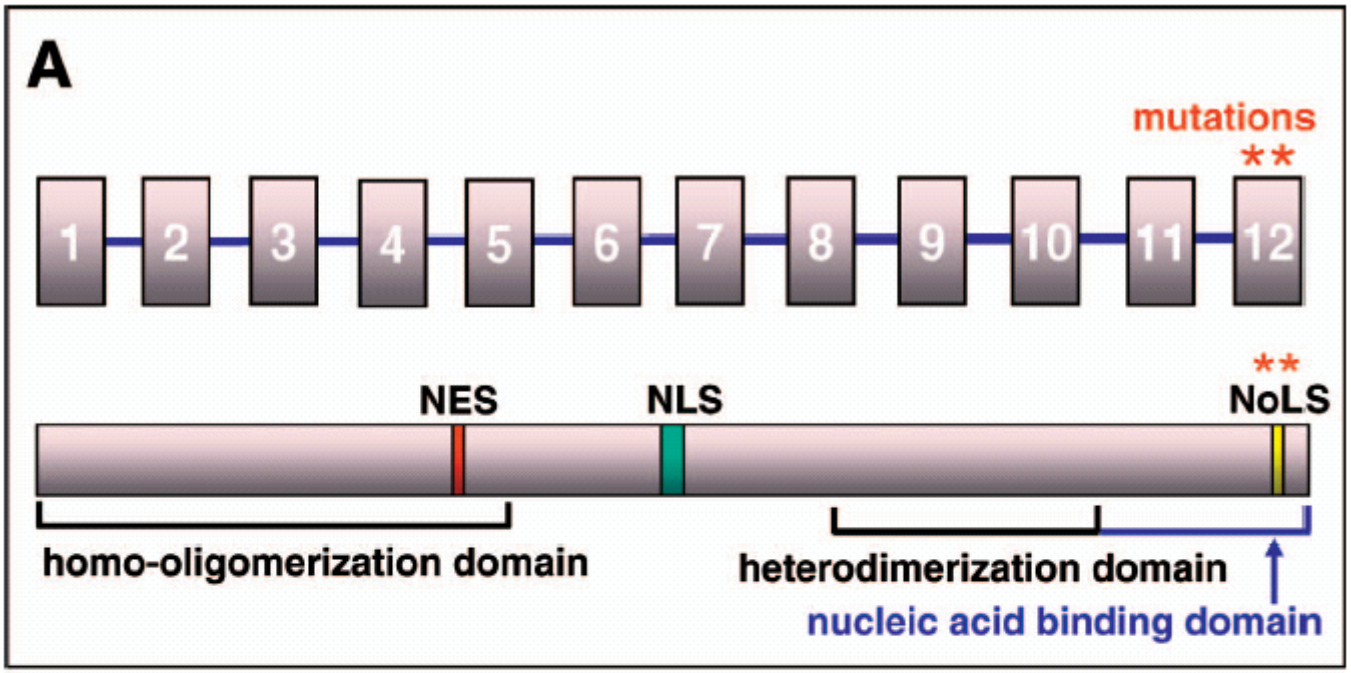


Nucleophosmin (NPM1) , B23, NO38, Numatrin

- In humans, accumulating evidence that NPM is directly implicated in the **pathogenesis of cancer**
- **Over-expressed in solid tumors** of diverse histological origin, is involved in **tumor progression**
- In hematologic malignancies the locus NPM is lost or **translocated** – formation of oncogenic fusion proteins
- **NPM gene contains 12 exons in maps to 5q35**
- C-terminus contains a short aromatic stretch with two tryptophans at positions 288 and 290, which are crucial for NPM binding to the nucleolus (nucleolar localization signal)
- Is more highly expressed in proliferating cells
- Is involved in the apoptotic response to stress and oncogenic stimuli and can modulate the activity and stability of the oncosupressor protein p53
- **Mutations are consistently heterozygous, are restricted to exon 12, except for 2 cases: exon 11(2007) and exon 9(2006), about 50 molecular variants of mutations to date in AML with >95% at nucleotide position 960**
- **Mutation A duplicates a TCTG tetranucleotide at positions 956 to 959 75-80% of adult NPMc+ AML cases**



Falini et al., NEJM, 352, 254-266, 2005

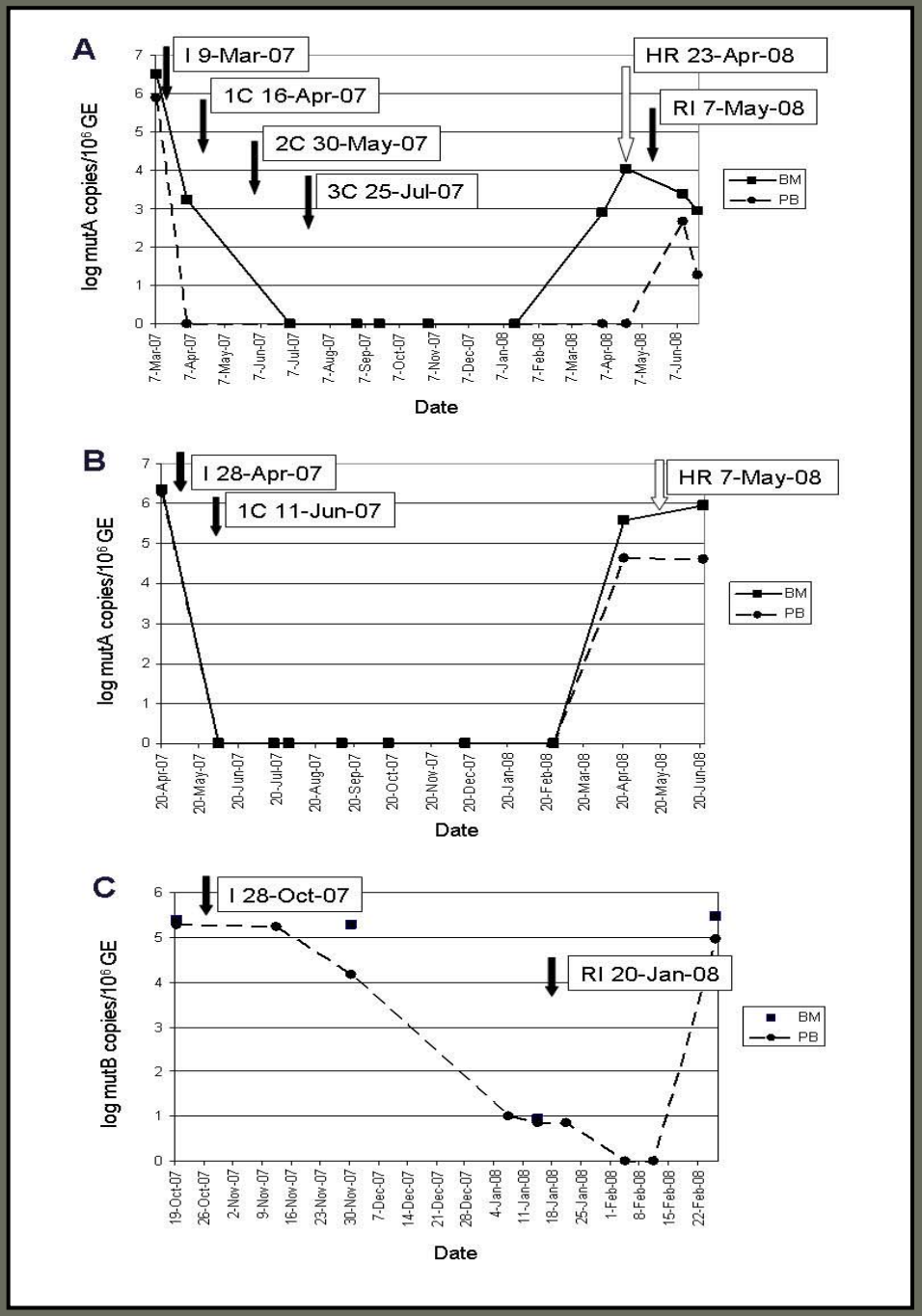


B

type	sequence	protein
Wt	CTCTG.....GCAGT..... GGAGGAAGTCTCTTAACAAAATAG	DLWQWRKSL (wt)
A	CTCTGTCTG GCAGT..... GGAGGAAGTCTCTTAACAAAATAG	DLCLAVEEVSLRK
B	CTCTGCATGGCAGT..... GGAGGAAGTCTCTTAACAAAATA	DLCMAVEEVSLRK
C	CTCTGCGTGGCAGT..... GGAGGAAGTCTCTTAACAAAATAG	DLCVAVEEVSLRK
D	CTCTGCCTGGCAGT..... GGAGGAAGTCTCTTAACAAAATAG	DLCLAVEEVSLRK
E	CTCTG.....GCAGTCTCTTGCCCAAGTCTCTTAACAAAATA	DLWQSLAQVSLRK
F	CTCTG.....GCAGTCCCTGGAGAAAGTCTCTTAACAAAATA	DLWQSLEKVSLRK

Figure 1. *Schematic presentation of the nucleophosmin (NPM1) gene and of normal and mutant NPM protein. A, Schematic representation of NPM1 gene and normal NPM protein. B, Examples of NPM1 gene mutations (types A–F). Inserted nucleotides are in violet. All NPM mutant proteins show mutations in at least one of the tryptophan residues (W) within the nucleolar localization signal domain and share the same last 5 amino acid residues (VSLRK). NES indicates nuclear export signal; NLS, nuclear localization signal; NoLS, nucleolar localization signal; **, site of mutations in exon 12.*

Chen W. et al., Arch Pathol Lab Med, 103, 1687-1692, 2006



The kinetics of changes of mutant signal in two representative patients with mutation A (A,B) and one patient with mutation B (C) during the clinical courses.

The number of NPM1-mutated copies in bone marrow (BM) and peripheral blood (PB) correlated with the treatment and disease status (I: induction chemotherapy; 1-, 2-, 3-C: first, second, and third consolidation chemotherapy; RI: reinduction chemotherapy; HR: hematological relapse, GE: genomic equivalent).

FLT3 (FMS-like tyrosine kinase) STK1 (Stem cell kinase1) flk2 (Fetal liver kinase2)

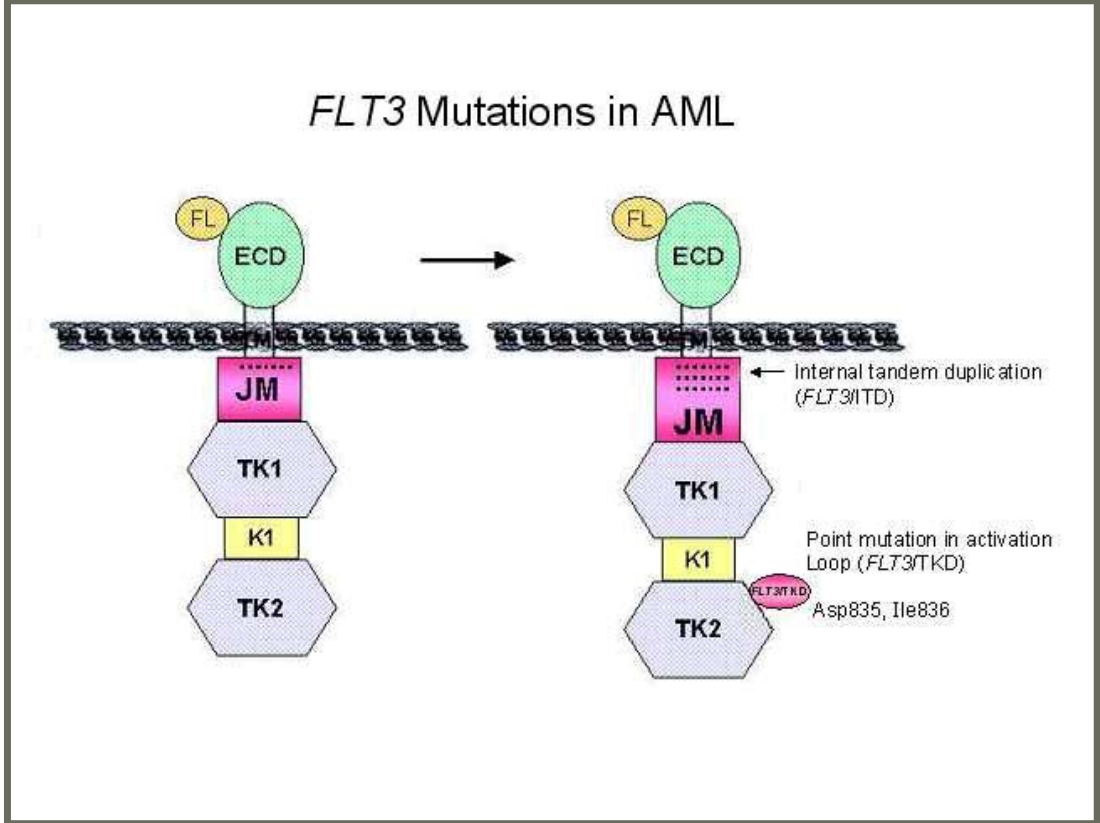
lokalizace 13q12

je členem rodiny receptorových tyrozinových kináz, exprimován na hematopoetických kmenových buňkách a také na buňkách leukemických. Mutace dvou typů popsány:

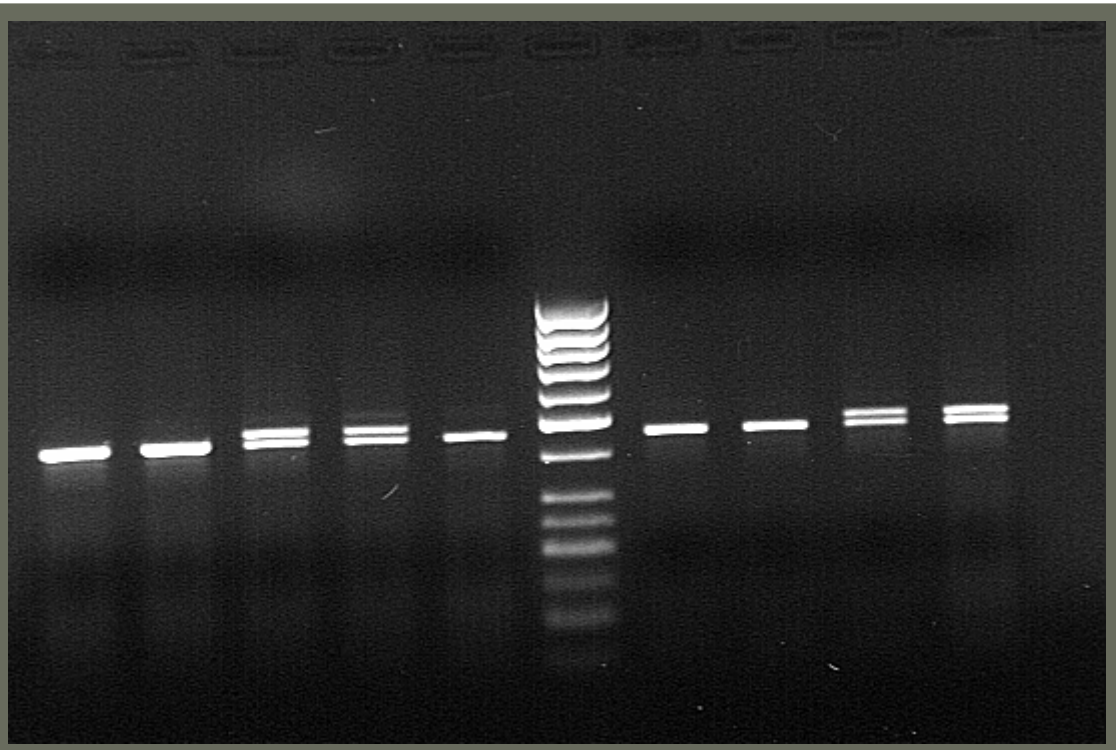
1) délkové v JM doméně (ITD, interní tandemové duplikace části genu) v exonu 14 (příp.15)

2) mutace v katalytické doméně TKD, kde kodóny D835 a I836 jsou kódovány nt GATATC, tvořící restrikční místo pro EcoRV (FLT3/D855)

Mutace jsou detekovány u ~30% AML s normálním karyotypem, představují nepříznivou prognózu často jsou považovány za sekundární aberaci a nejsou stabilní v průběhu follow-up.



RT-PCR detekce interních tandemových duplikací v exonu 12 genu FLT3 (FLT3/ITD)



R5: 5´-TGTCGAGCAGTACTCTAAACATG-3´

12R: 5´-CTTTCAGCATTTTGACGGCAACC-3´, PCR produkt 456 bp

Kiyoi, H. et al., Leukemia, 11, 1447-1452, 1997

Restrikční analýza - Detekce mutací D835 v katalytické doméně TKD v exonu 20 (17) genu FLT3

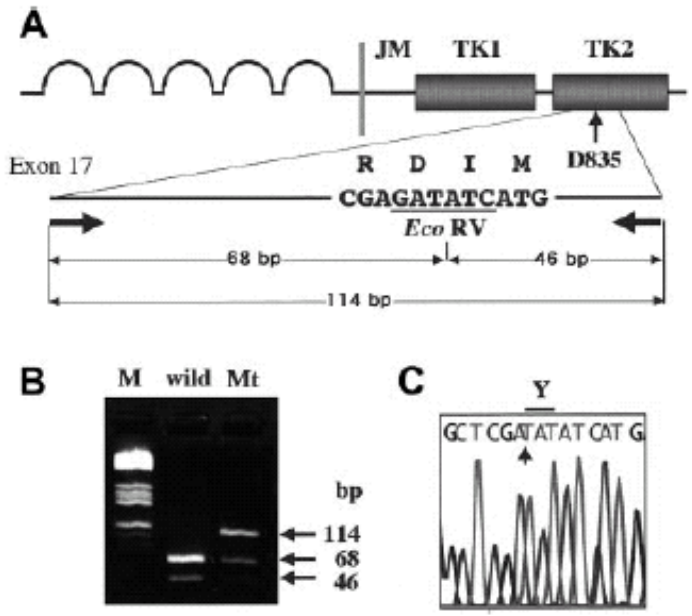
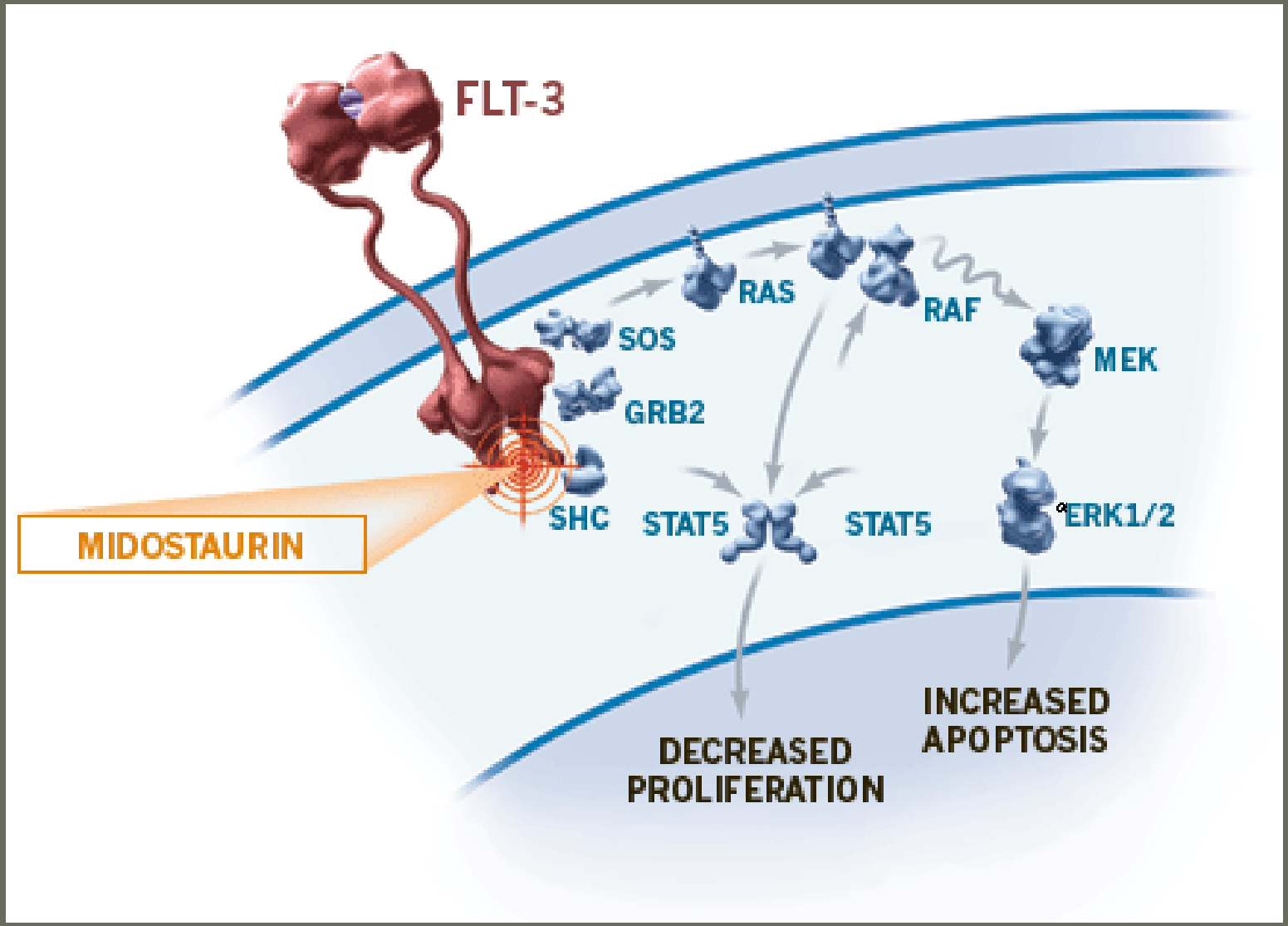


Figure 1. Detection of D835 mutations in the *FLT3* gene. To detect D835 mutations, we amplified exon 17 by PCR, and then digested it with the *Eco*RV endonuclease (A). The amplified products of wild type were digested to 2 bands (68 bp and 46 bp) by the *Eco*RV. When amplified products contained D835 mutations, undigested bands (114 bp) were visualized on agarose gel electrophoresis. M indicates the molecular weight marker (*Hae*III digested pBR332 plasmid DNA) (B). The undigested bands were directly sequenced (C). In this sample, the first nucleotide G of D835 was substituted with T, resulting in an Asp to Tyr amino acid change (D835Y).

Yamamoto, Y., et al., Blood, 97, 2434-2439, 2001

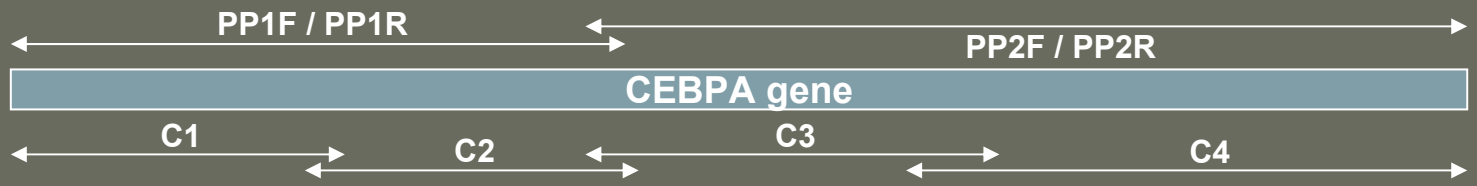


Midostaurin (PKC412A), *N*-benzoyl-staurosporine, potently inhibits protein kinase C alpha (PKC), VEGFR2, KIT, PDGFR and FLT3 tyrosine kinases.
Inhibuje tyrosinovou fosforylaci mutované alely FLT3/ITD i TKD mutace

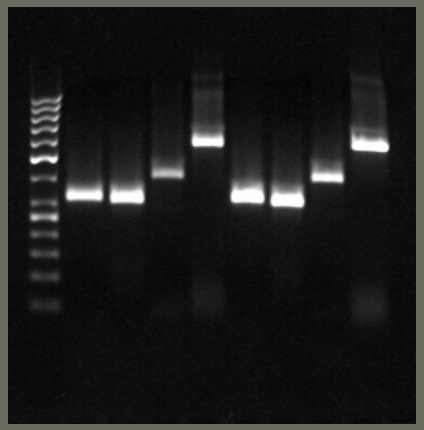
CEBPA gene

- Encodes transcriptional factor (CCAAT/enhancer binding protein alpha) playing crucial role in myeloid differentiation
- Loss of CEBPA function leads to blocking of granulocytic differentiation of AML
- **When mutated** confers favorable prognosis for patients with CN-AML

HRM pre-amplification



- Four independent real-time PCRs performed in **10ul** volume each, selectively amplifying CEBPA gene within four overlapping fragments (PCR products C1-C4)



Reaction mixture C1-C4

- 5ul SensiMix HRM (Quantace, UK)
- 0.4ul EvaGreen Dye (Quantace, UK)
- 2x0.8ul specific primers (10uM)[1]
- 2ul distilled water
- 1ul DNA template

[1]CEBPA primers (Pabst T. et al., Nature genetics 2001, Vol. 27, 263-270)

High Resolution Melt Analysis

Real-time program

- 95°C/10min
- 95°C/15s
- 65°C/40s
- 72°C/20s

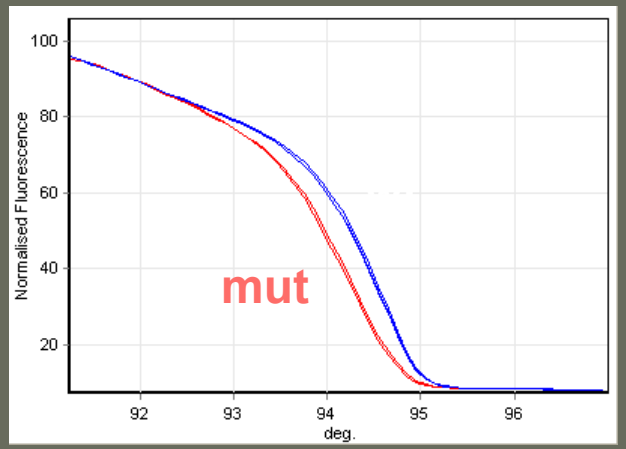
x35

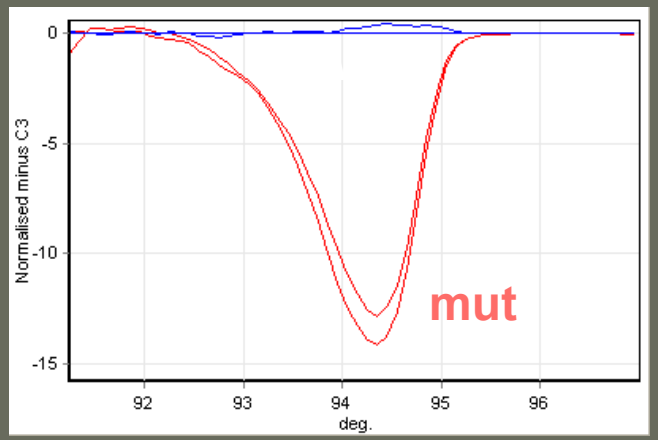
- 53°C/1min
- 87°C/90s
- rising 0.1°C to 97°C/2s

pre-amplification

high resolution melting

HRM results

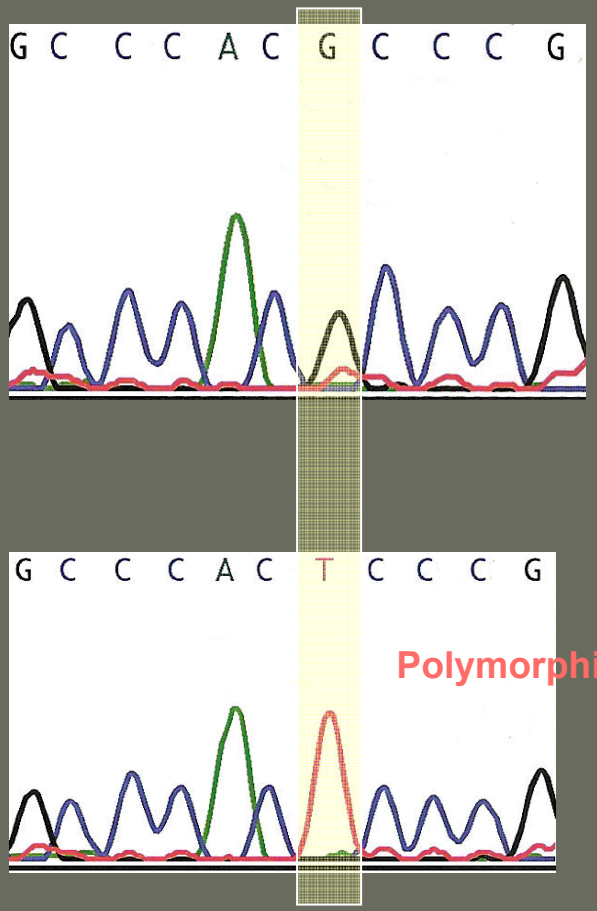




- poorly performed assays must be omitted
- runs with low reproducibility of melting curves must not be included in analysis
- particular wild type sample is set as a reference
- normalized melting graphs and difference graphs are used to distinguish between mutation and wild type samples
- **only mutation positive samples are further sequenced**

Post HRM sequencing

- purification of C1-C4 products
  QIAquick PCR purification Kit (250) (QIAGEN, Germany)
- cycling sequencing reaction
  BigDye Terminator Kit 1.1 (Applied Biosystems)
- final purification
  DyeEx 2.0 Spin Kit 250 (QIAGEN, Germany)
- ABI Prism 310 Genetic Analyzer



Myeloproliferative Neoplasms

Chronic myelogenous leukaemia BCR-ABL positive

Chronic neutrophilic leukaemia

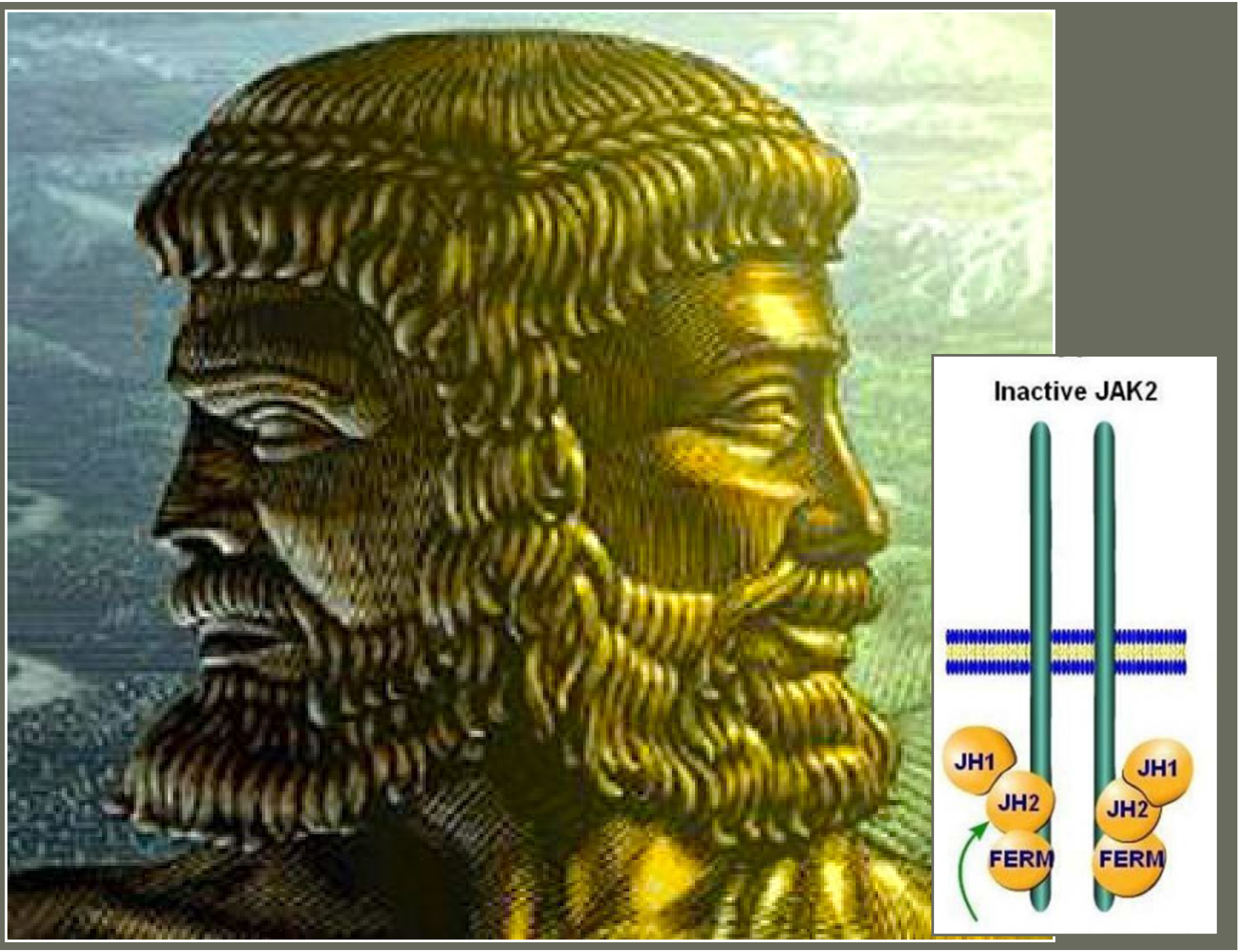
Polycythaemia vera

Primary myelofibrosis

Essential thrombocythaemia

Chronic eosinophilic leukaemia

Mastocytosis


Inactive JAK2
JH1
JH2
FERM
JH1
JH2
FERM

2005 období objevu mutací *JAK2* genu –

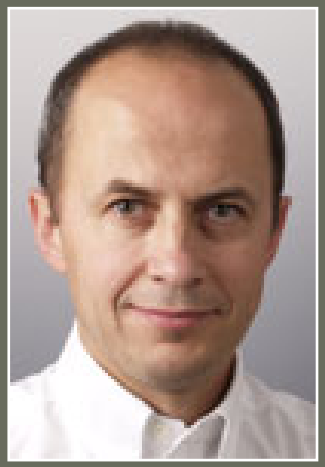
pracoviště **D. Gary Gilliland (Boston), William Vainchenker (INSERM), Radek Skoda (Basel), Anthony Green (Cambridge)** *JAK2*V617F (exon 14)

2006 Gary Gilliland skupina somatické mutace (exon 10) *MPL*W515L a *MPL*W515K u *JAK2*V617F negativních 5% PMF (primárních myelofibróz) a 1% ET

2007 A. Green další 4 mutace v *JAK2* genu (exon 12, delece, inzerce)

Clarifications on the precise pathogenetic role of these mutations as well as their importance as targets for small molecule therapy .

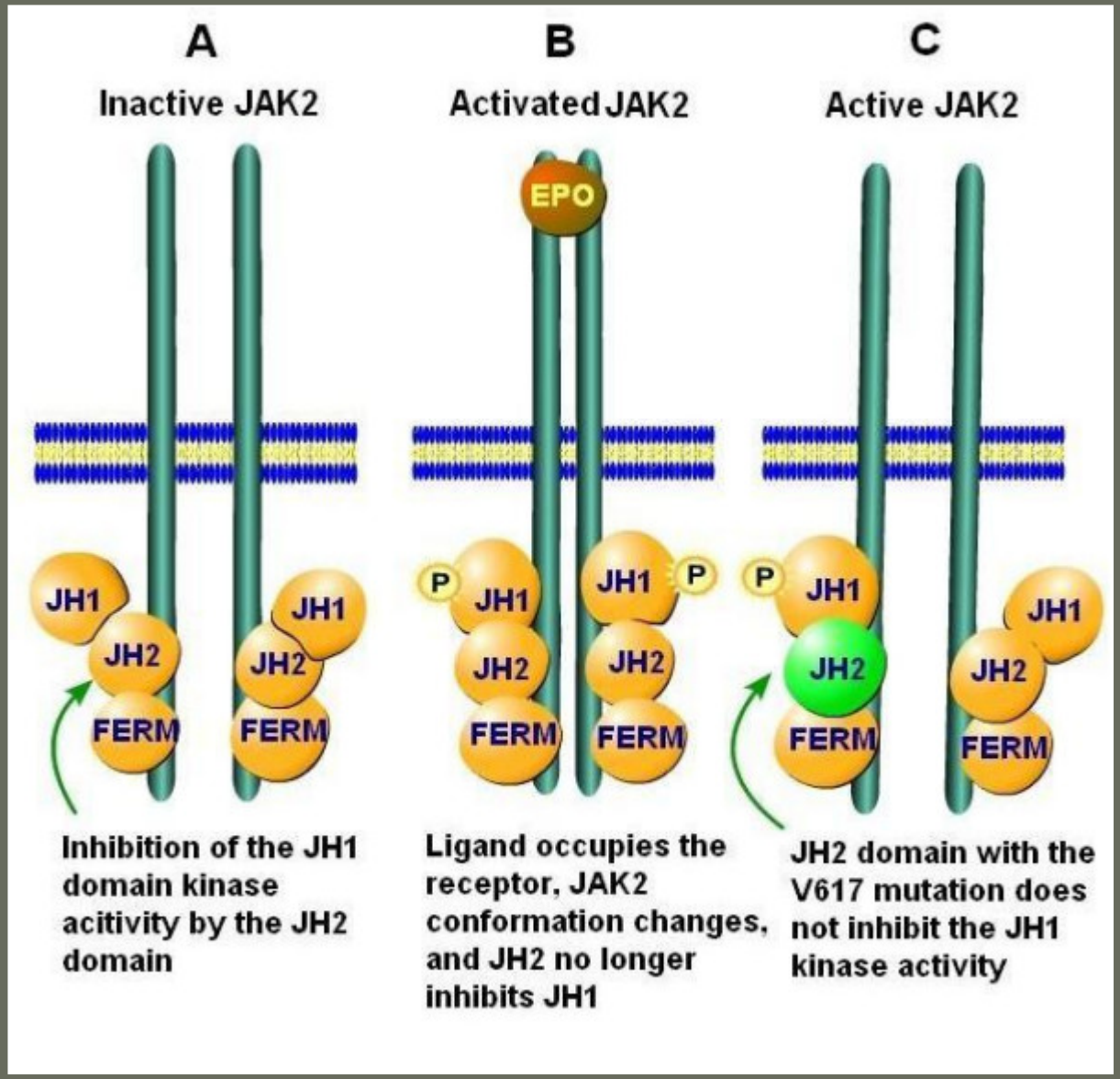





Award winners

25th September 2008: Casino Restaurants

Bern As the chairperson of the five-member jury, **professor Andreas Tobler, M.D., Berne, past president of the Swiss Society for Hematology, presents the HEMATOLOGICAL MALIGNANCIES AWARD 2008 carrying a value of CHF 100.000 to professor of biomedicine Radek Skoda, M.D., Basel. The award was assigned to professor Skoda for a study on the pathogenesis of myeloproliferative disorders entitled "Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice"[1] and published by his research team of Experimental Hematology.**

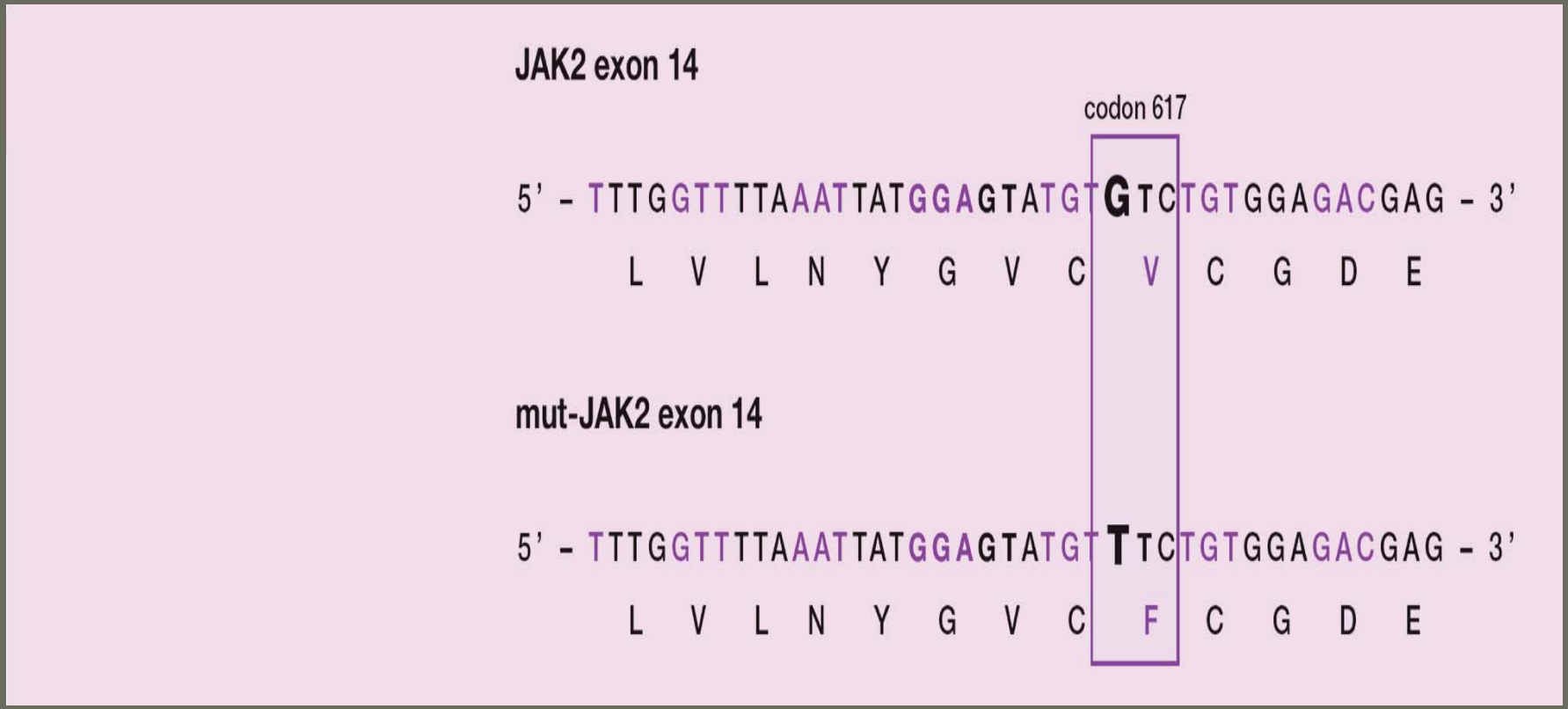


The domains of JAK2 illustrating binding to the receptor and changes consequent to receptor binding and mutation in the JH2 domain. The V617F mutation of the JH2 domain of JAK2 results in constitutive kinase activation. Panel A: When no ligand in bound to the EPO, TPO, G-CSF or GM-CSF receptors, the kinase activity of the JH1 domain is inhibited by the JH2 domain and JAK2 is inactive. Panel B: When EPO binding to it receptor, the two strands of the receptor come closer together, JAK2 changes conformation, the JH1 kinase activity in no longer inhibited by JH2. Panel C: The JAK2 V617F mutation prevents JH2 from inhibiting JH1 and the kinase is active even when no ligand is bound by the receptor.

Bennett and Stroncek Journal of Translational Medicine 2006 4:41 doi:10.1186/1479-5876-4-41

JAK2 mutation site identification

substituce 1849 G>T, exon 14, záměna valinu za fenylalanin v kodónu 617 (V617F)



TCTTTCTTTGAAGCAGCAAGTATGATGAGCAAGCTTTCTCACAAGCATTTGGTTTTAAAT
TATGGAGTATGTGTCTGTGGAGACGAGA

Gen *JAK2* (9p24), substituce 1849 G>T, exon 14, záměna valinu za fenylalanin v kodónu 617 (V617F)

- u 95% pacientů s PV (z toho cca 33% je homozygotních v důsledku LOH při mitotickém crossing-overu) (Pardanani 2008, Leukemia)
- u 50% pacientů s ET a PMF (homozygotní stav je u těchto pacientů vzácný)
- vzácně se mutace může vyskytnout u pacientů s MDS (5%) nebo s CMML (chronic myelomonocytic leukemia) (3%) i u jiných malignit myeloidní řady-RARS-T (ringed sideroblasts associated with marked thrombocytosis), JMML (juvenile myelomonocytic leukemia), aCML (atypical CML), *de novo* AML
- potvrzen i souběžný výskyt V617F mutace s BCR-ABL mutací, přičemž výsledný fenotyp závisí na tom, který buněčný klon získá převahu (Hussein et al. 2008, Leukemia)
- někteří autoři uvádí výskyt V617F mutace i u zdravých jedinců (Sidon et al. 2006, Leukemia), nebo u náhodně vybraných pacientů s jiným než hematologickým onemocněním (Xu et al. 2007, Blood).
- pacient může nést V617F mutaci několik let předtím, než se u něj vyvine myeloproliferativní onemocnění (Bellanné-Chantelot et al. 2008, Leukemia)

Detekce mutace V617F JAK-2

Metoda alelické diskriminace pro *JAK2*^{V617F}

- separace granulocytů z PB
- izolace gDNA
- RQ – PCR s využitím fluorescenčně značených LNA modifikovaných hybridizačních sond (Locked Nucleic Acids)
- vyznačují se 100% alelickou diskriminací obou genotypů
- citlivost detekující 10% příměs granulocytů nesoucích mutantní alelu na pozadí zdravé populace
- fluorescenční značení FAM pro sondu s WT sekvencí a JOE pro sondu s MUT sekvencí

(Pekova, S. et al., Blood, 108, No 11, 313B, 2006
Veselovska et al. Leukemia Research, 32: 369, 2008)

Alelická diskriminace pro detekci *JAK2*^{V617F}

primer F: 5´- GAAGCAGCAAGTATGATGAGCAA – 3

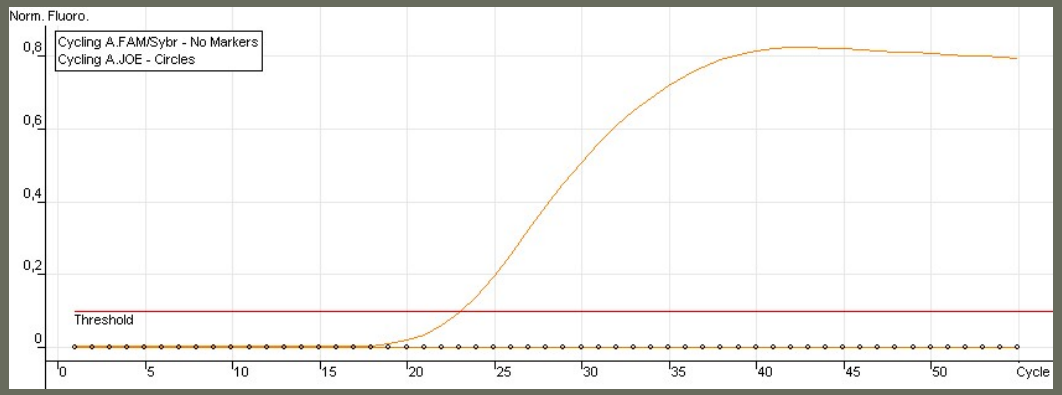
primer R: 5´- ACTGACACCTAGCTGTGATCC – 3´

JAK2 ^{wt} LNA sonda: FAM – tcCacAgaCaCatAc - BHQ1

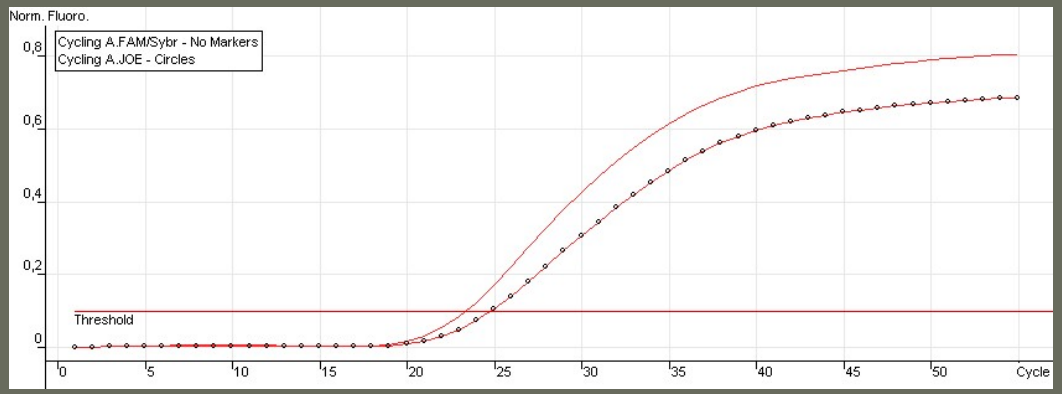
JAK2^{V617F} LNA sonda: HEX – ctcCacAgaAacAtaCtc – BHQ1

RQ – PCR analýza

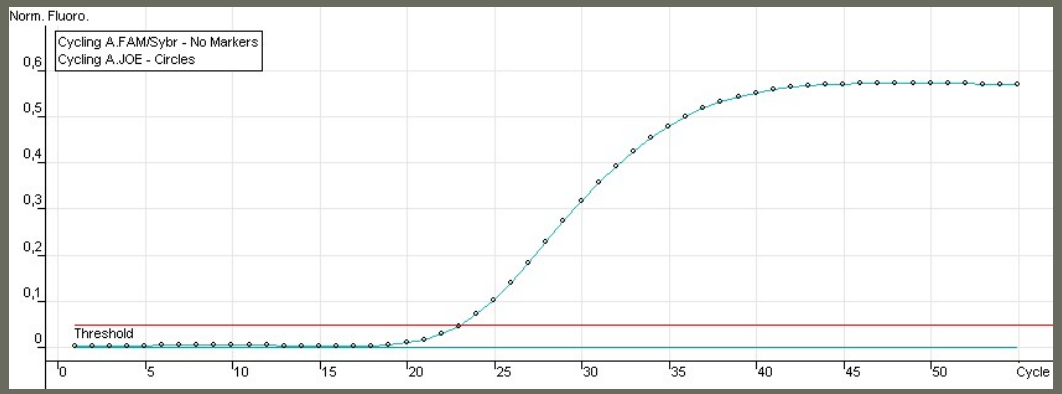
Pacient - wt



Pacient – heterozygot



Pacient – mut. homozygot



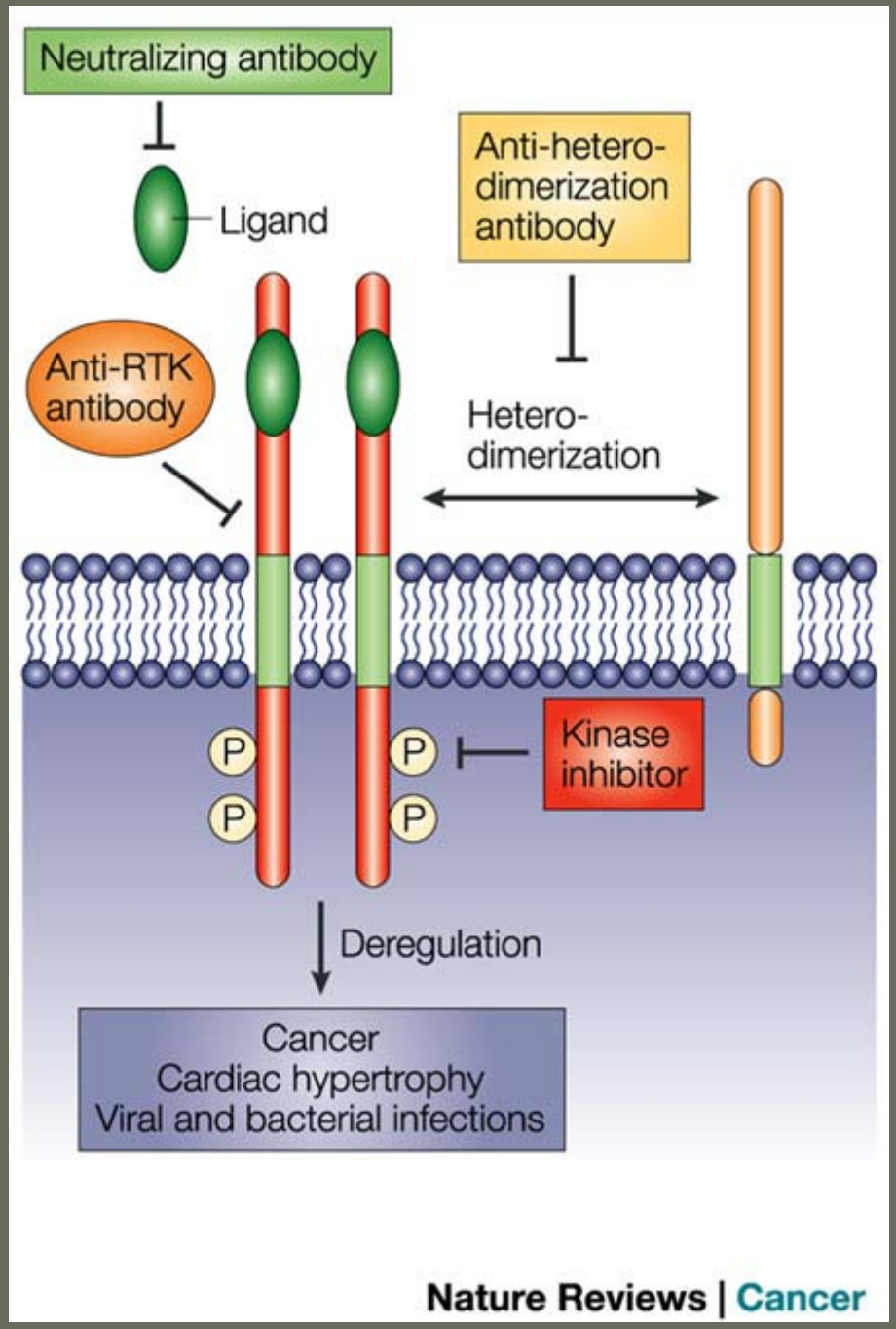
A co dál?

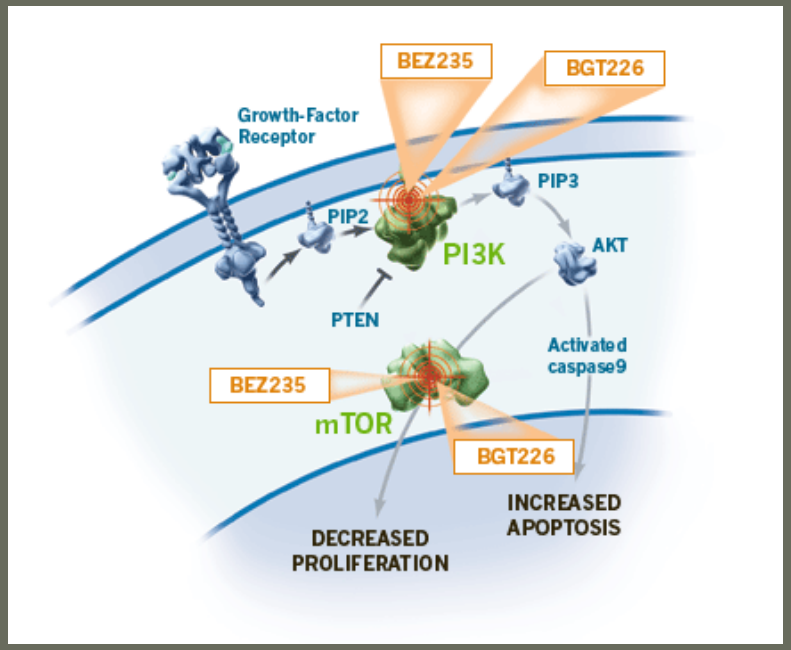
Nové léky na bázi TK inhibitorů první, druhé a další … a další… a další generace

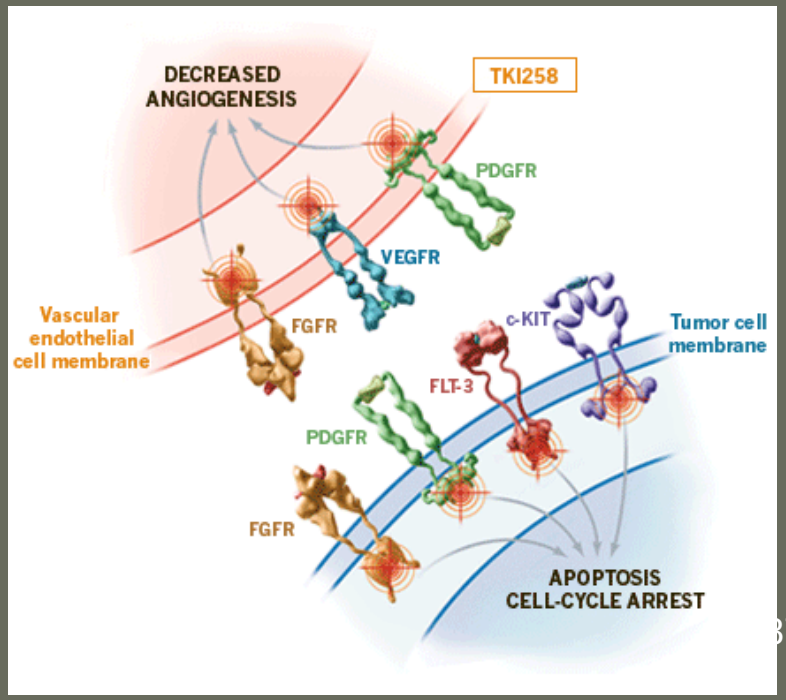
Automatizace extrakce DNA/RNA

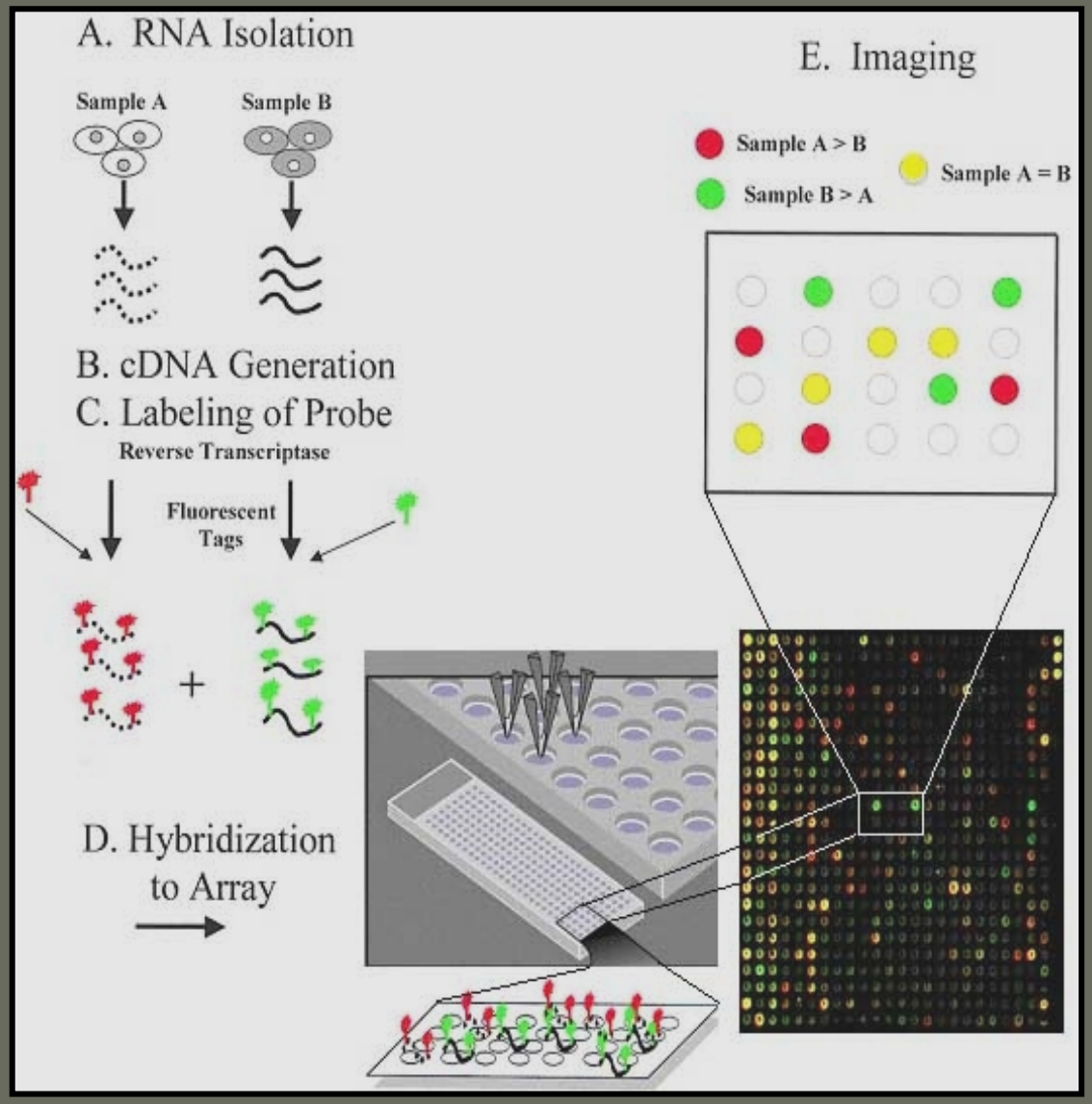
Robotizace míchání reakčních směsí PCR a titrace standardní DNA

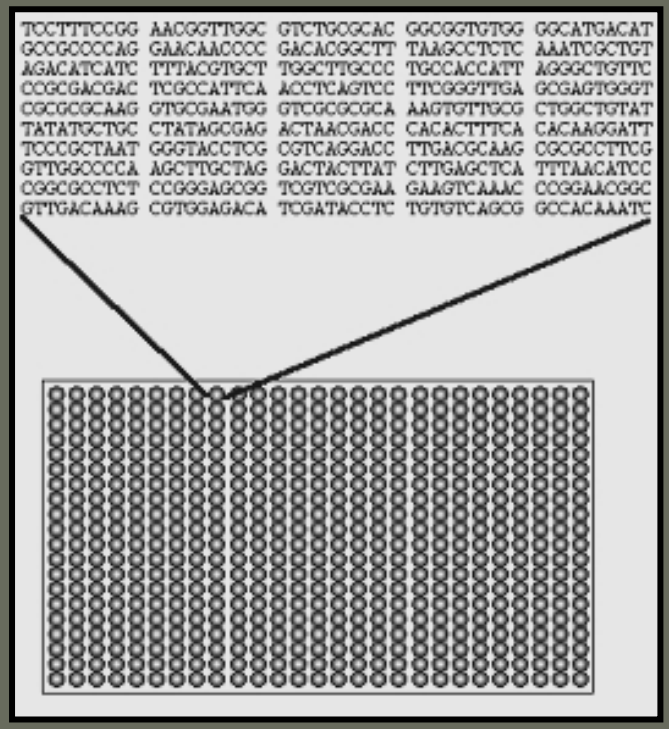
Automatizace PCR vyšetření pacienta na bázi kartridge nebo čipu

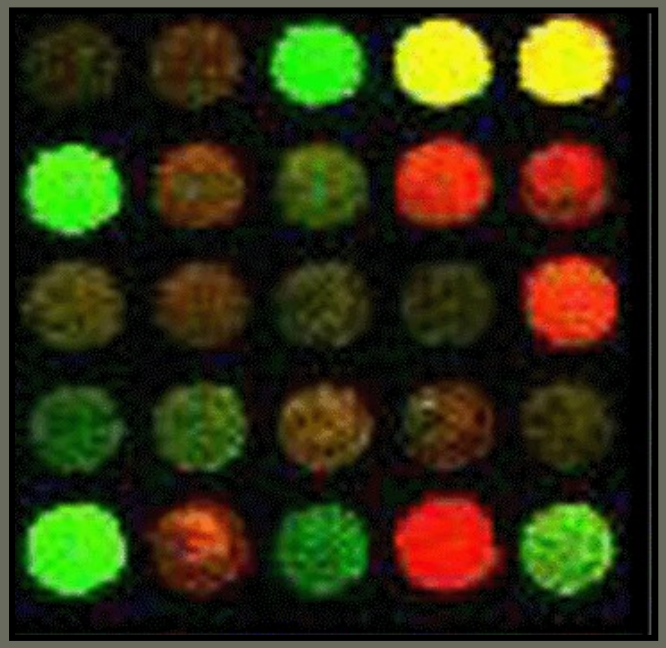

Neutralizing antibody
Ligand
Anti-hetero-dimerization antibody
Anti-RTK antibody
Hetero-dimerization
P
P
P
P
Kinase inhibitor
Deregulation
Cancer
Cardiac hypertrophy
Viral and bacterial infections
Nature Reviews | Cancer


BEZ235
BGT226
Growth-Factor Receptor
PIP3
PIP2
PI3K
AKT
PTEN
Activated caspase9
BEZ235
mTOR
BGT226
INCREASED APOPTOSIS
DECREASED PROLIFERATION

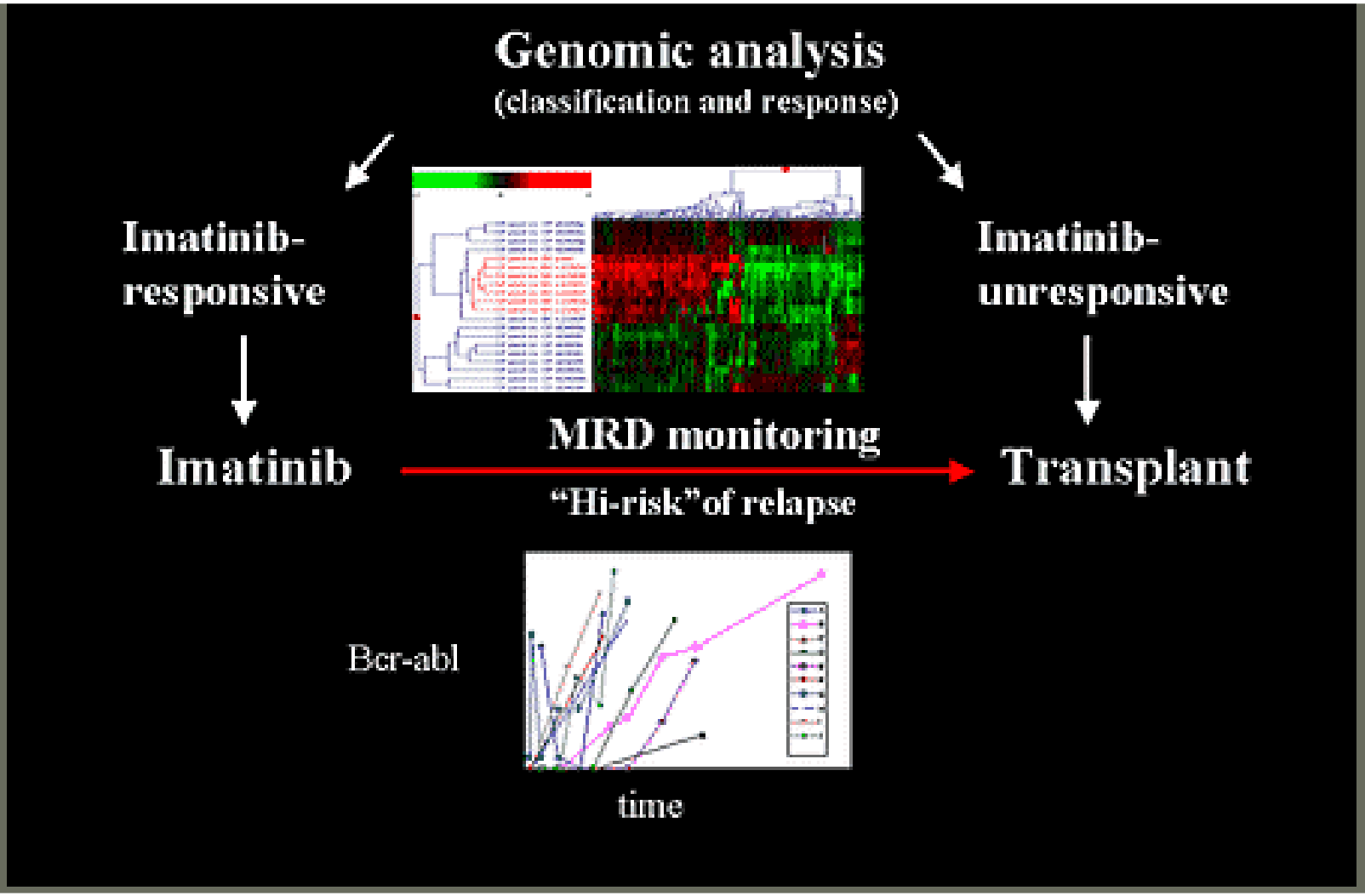

DECREASED ANGIOGENESIS
TKI258
PDGFR
VEGFR
Vascular endothelial cell membrane
FGFR
c-KIT
Tumor cell membrane
FLT-3
PDGFR
FGFR
APOPTOSIS CELL-CYCLE ARREST


A. RNA Isolation
Sample A
Sample B
B. cDNA Generation
C. Labeling of Probe
Reverse Transcriptase
Fluorescent Tags
D. Hybridization to Array
E. Imaging
Sample A > B
Sample B > A
Sample A = B


TCCTTTCCGG AACGGTTGGC GTCTGCGCAC GGCGGTGTGG GGCATGACAT
GCCGCCCCAG GAACAACCCC GACACGGCTT TAAGCCTCTC AAATCGCTGT
AGACATCATC TTTACGTGCT TGGCTTGCCC TGCCACCATT AGGGCTGTTC
CCGCGACGAC TCGCCATTCA ACCTCAGTCC TTCGGGTTGA GCGAGTGGGT
CGCGCGCAAG GTGCGAATGG GTCGCGCGCA AAGTGTTGCG CTGGCTGTAT
TATATGCTGC CTATAGCGAG ACTAACGACC CACACTTTCA CACAAGGATT
TCCCGCTAAT GGGTACCTCG CGTCAGGACC TTGACGCAAG CGCGCCTTCG
GTTGGCCCCA AGCTTGCTAG GACTACTTAT CTTGAGCTCA TTTAACATCC
CGGCGCCTCT CCGGGAGCGG TCGTCGCGAA GAAGTCAAAC CCGGAACGGC
GTTGACAAAG CGTGGAGACA TCGATACCTC TGTGTCAGCG GCCACAAATC



…objevují se nové přístupy v monitorování choroby.

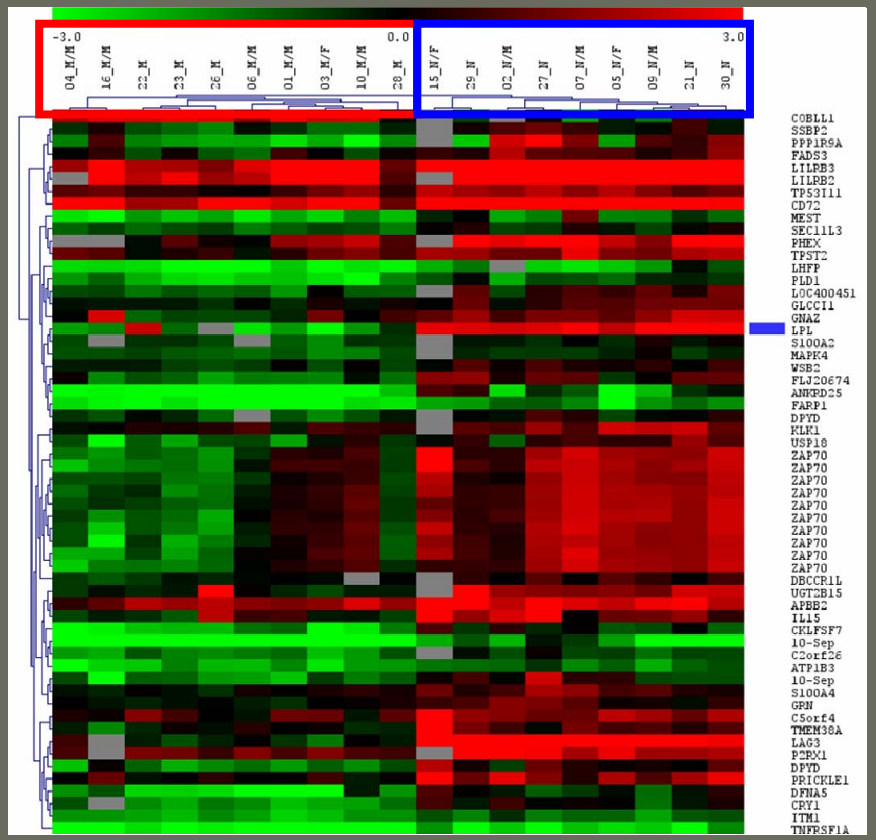


Goldman, J. et al.: Evolution of Management of CML, Tenth International Congress on Hematologic Malignancies

CLL

Oligonukleotidové DNA čipy pro stanovení exprese > 19.000 genů

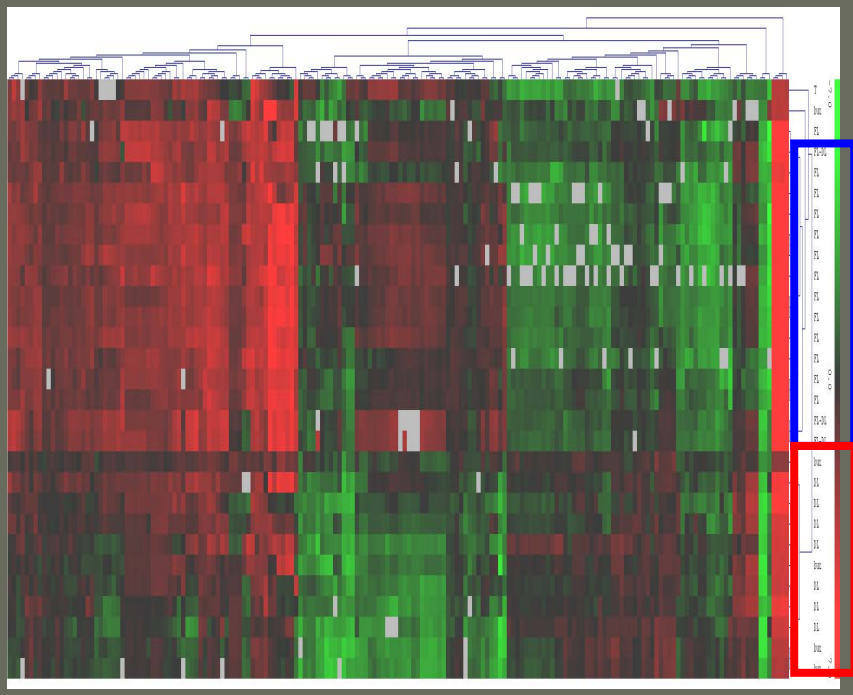
Geny s největšími rozdíly v expresi mezi CLL pacienty s mutovaným a nemutovaným IgVH



LymfoChip

Oligonukleotidové DNA čipy s 15.000 sondami pro 4.000 genů Až 8 pacientů na jednom čipu

Geny s rozdílnou expresí u pacientů s Folikulárním lymfomem a DLBCL nebo Burkittovým lymfomem



Teorie zůstane pouhou teorií, pokud nepřikročíme k činu.