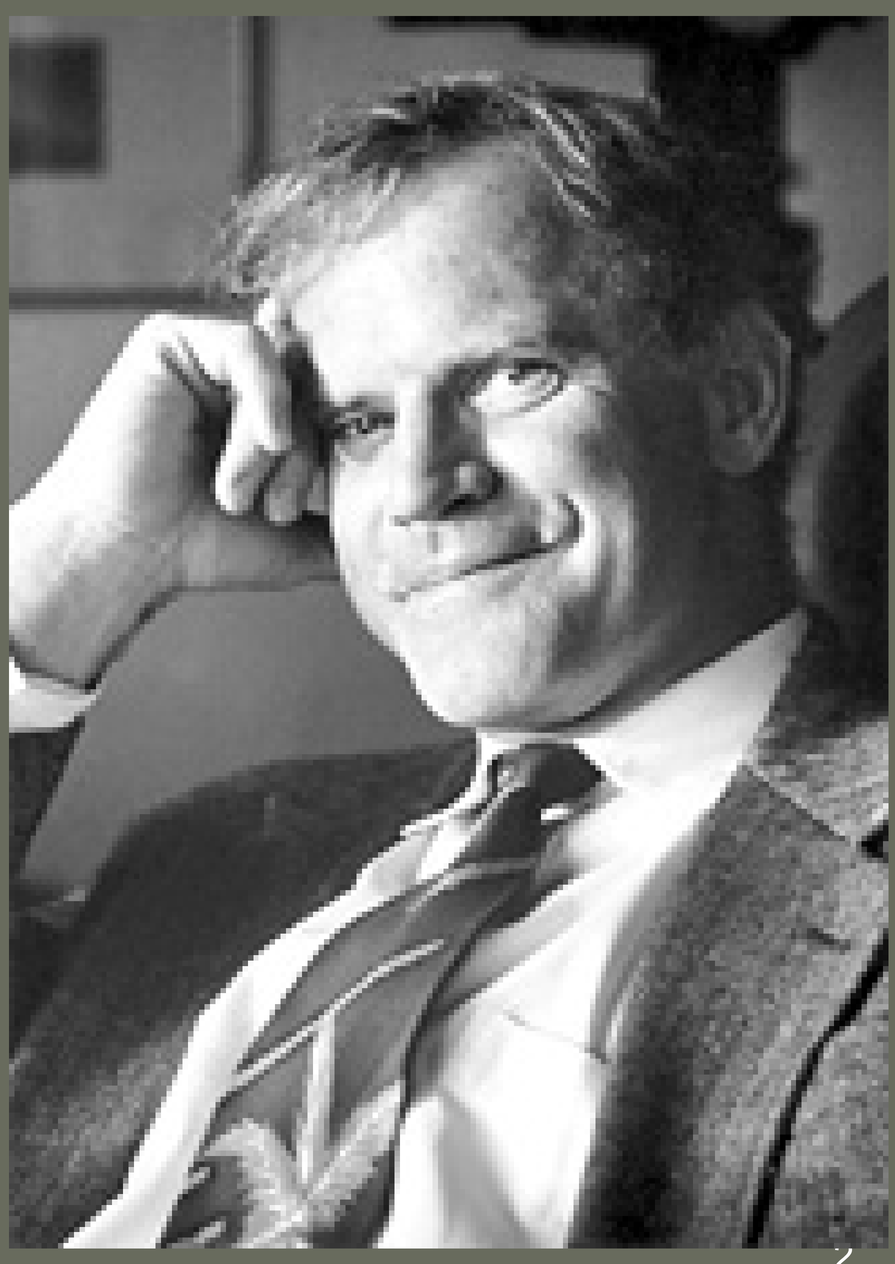
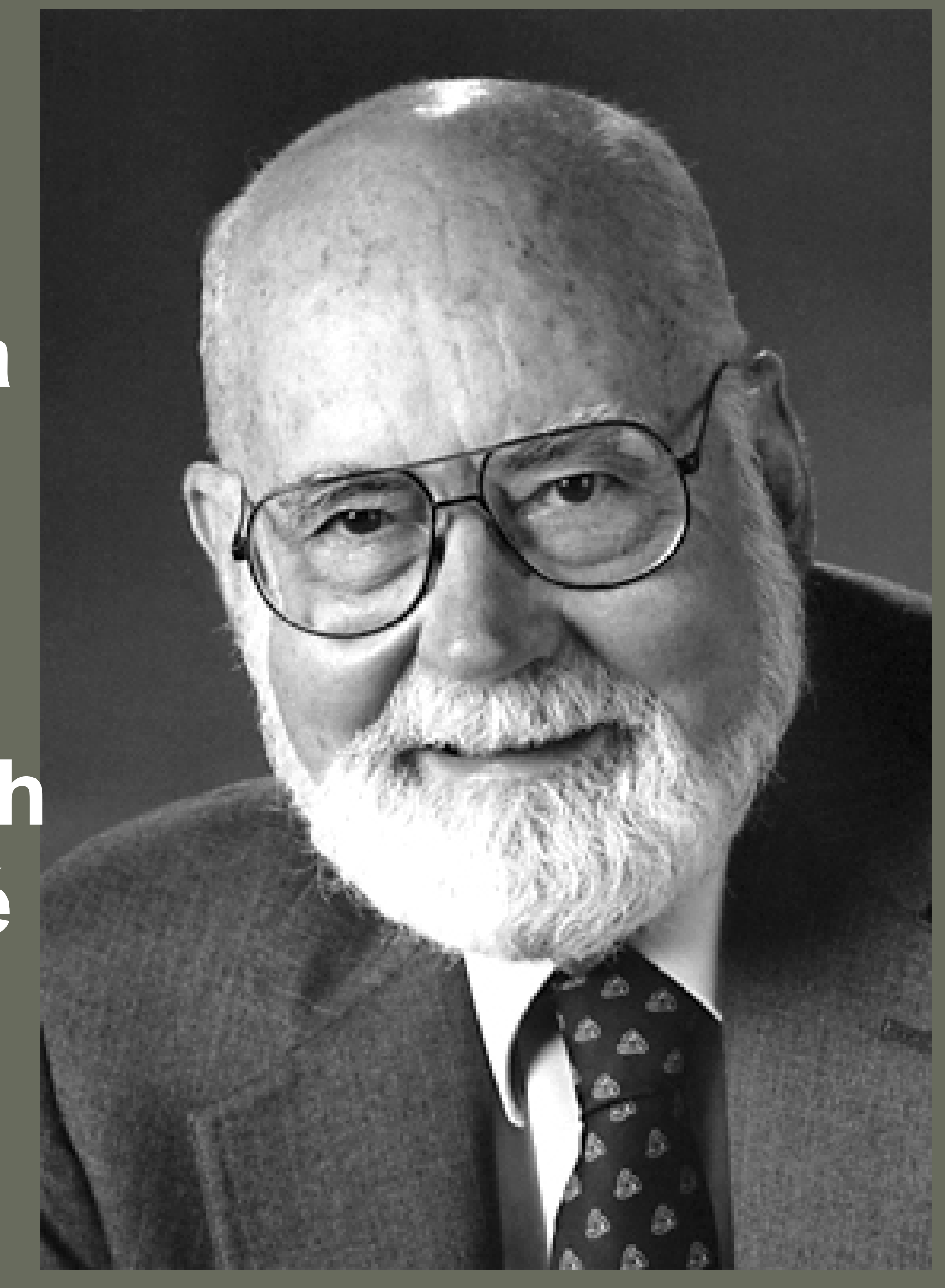
Možnosti molekulární diagnostiky v klinické praxi 2013

Interní hematologická a onkologická klinika
FN Brno a LF MU

1. V kterém roce byla získána Nobelova cena za objev PCR?



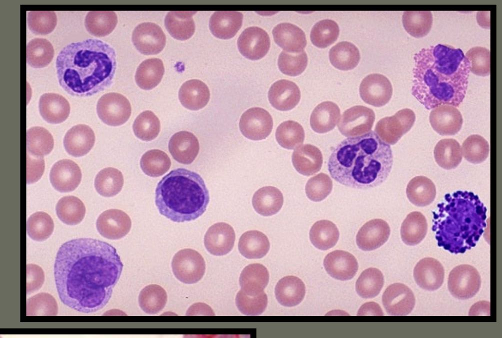
2. **Jaké je celé jméno nositele Nobelovy ceny za příspěvek k zavedení transplantace hematopoetických buněk do klinické medicíny?**



Obsah

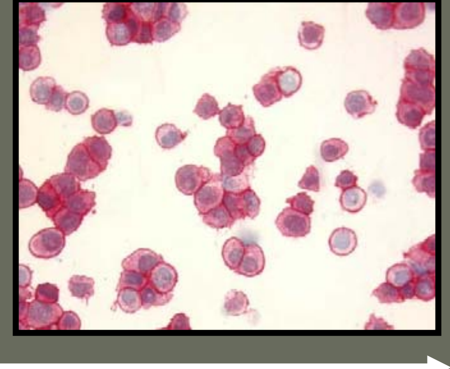
- Co nabízí molekulární diagnostika
- Stádium civilizačních chorob
- Jaké metody a jaké molekulární markery jsou dnes využívány v onkologické praxi
- Jakou informaci molekulární diagnostika poskytuje dnes
- Jakou informaci bychom chtěli získat zítra (jestli to dokážeme)

Diagnostika



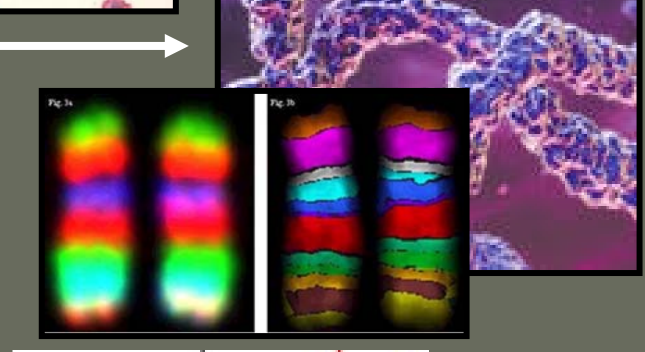
- ❑ Morfologická

(cytologie, histologie, cytochemie)



- ❑ Imunologická

(IF, průtoková cytometrie)



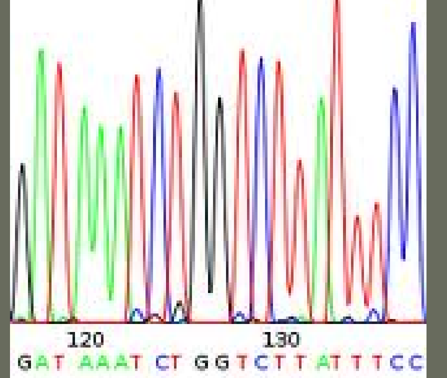
- ❑ Cytogenetická

(početní a strukturní změny)

- ❑ Molekulárně cytogenetická

(FISH, CGH)

- ❑ Molekulárně genetická

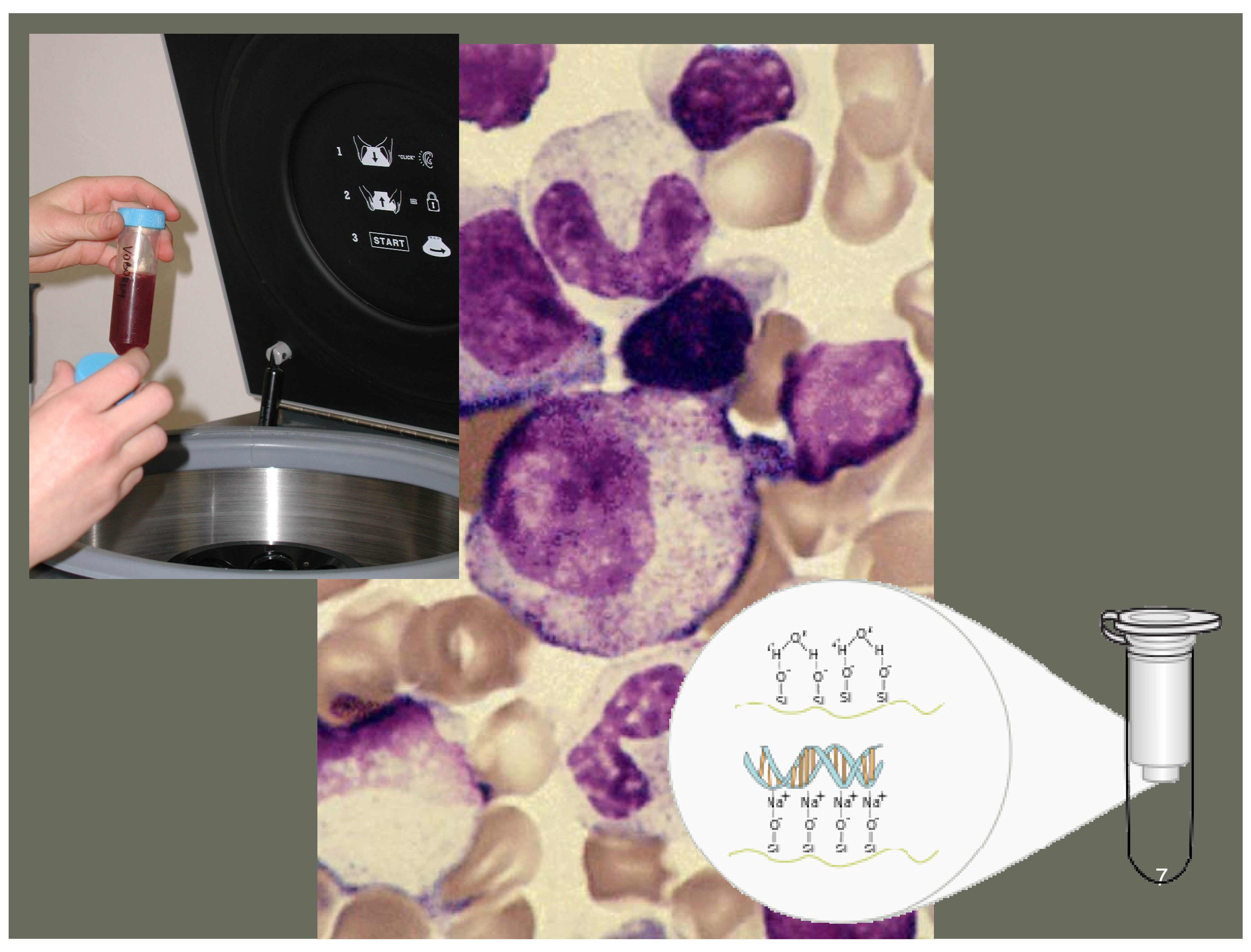


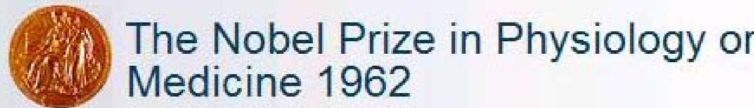
Senzitivita diagnostických metod (CML)

Metoda	**Target**	**Tkáň**	**Senzitivita**
Cytogenetika	Ph	BM	**1%-10%**
FISH	spojení Bcr a Abl	PB/BM	0,2%-5%
Southern	M-BCR	PB/BM	1%-10%
Western	Bcr-Abl protein	PB/BM	0,2%-1%

genetické změny zahrnující 10^6 - 10^7 bp se mohou projevit bez viditelné změny morfologie chromozomu

(n)PCR	**BCR-ABL** mRNA	PB/BM	**-0,00001%**
RQ-PCR	BCR-ABL mRNA	PB/BM	**0,001%-0,0001%**


1
2
3 START
Na+ Na+ Na+ Na+

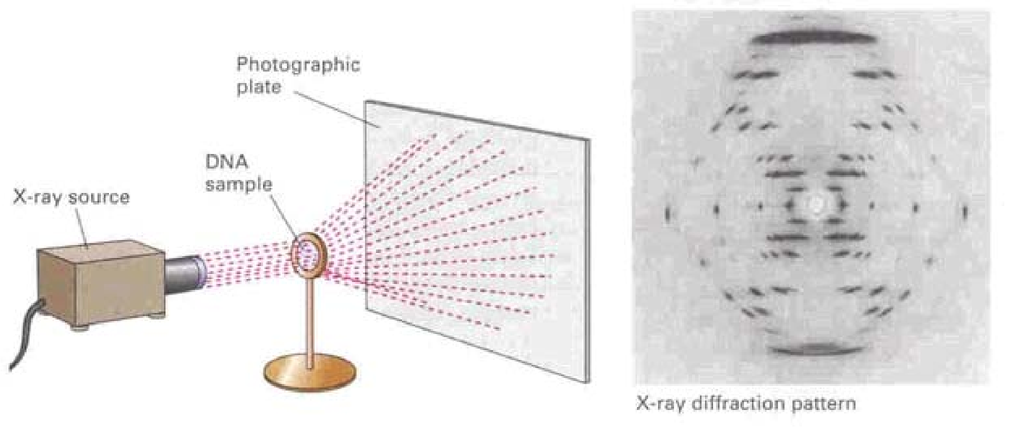


"for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material"

Francis Harry Compton Crick	**James Dewey Watson**	**Maurice Hugh Frederick Wilkins**
1/3 of the prize	1/3 of the prize	1/3 of the prize
United Kingdom	USA	United Kingdom and New Zealand
MRC Laboratory of Molecular Biology Cambridge, United Kingdom	Harvard University Cambridge, MA, USA	London University London, United Kingdom
b. 1916 d. 2004	b. 1928	b. 1916 (in Pongaroa, New Zealand) d. 2004

Titles, data and places given above refer to the time of the award.

Photos: Copyright © The Nobel Foundation



Rosalind Elsie Franklin (1920-1958)

Who (really) discovered the structure of DNA?

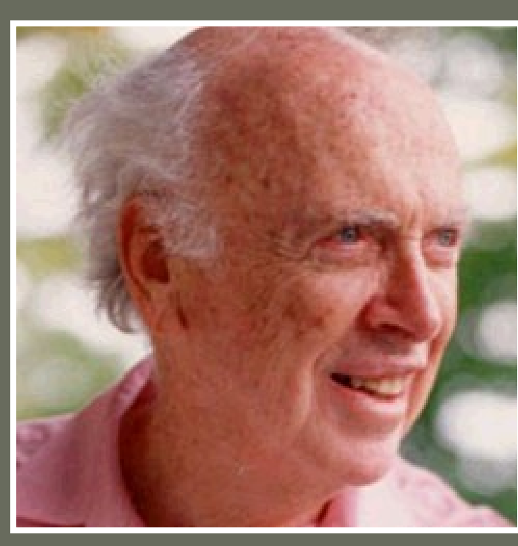
In April of 1953, James Watson, Francis Crick and Maurice Wilkins identified the substance of life - the structure of DNA. They later shared a Nobel Prize. Their discovery depended heavily on the work of a woman, chemist **Rosalind Franklin**, whose research was used **without her knowledge or permission**. 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, fo the first time, the essential structure of DNA - the double-helix shape, which also indicated its method of replication.

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)



Najdete dva rozdíly?
Crick má světlý oblek
a nemá kravatu

Molekulární diagnostika

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

- **DNA změny získané** - onkologie

- **DNA cizorodá** - diagnostika patogenů

Molekulární diagnostika v klinice

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

- Včasné stanovení diagnózy
- Včasné stanovení oportunních patogenů
- Diferenciální diagnostika
- Stratifikace podle rizika
- Minimální reziduální choroba
- Včasná detekce relapsu choroby

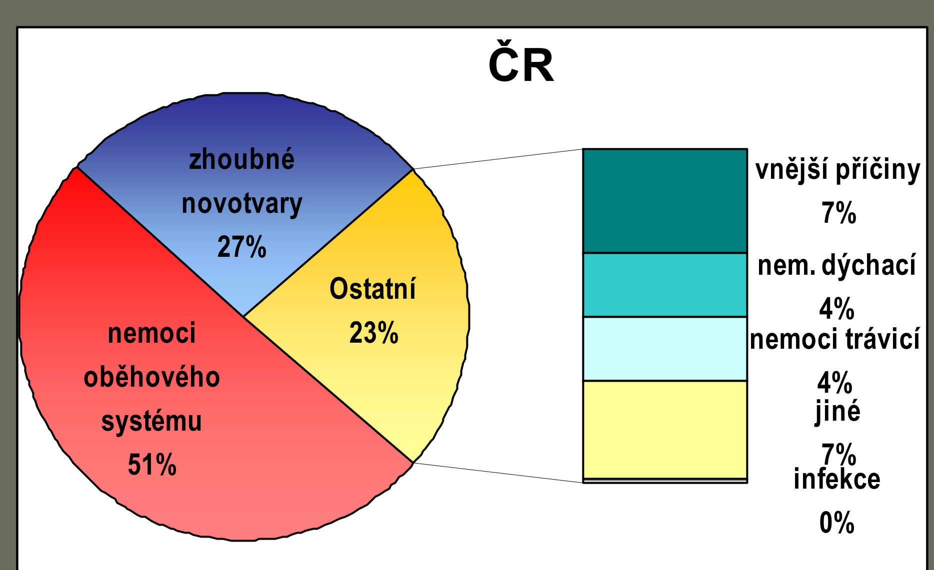
Specificita a senzitivita, rychlost získání výsledku a možnost správné a rychlé terapeutické intervence

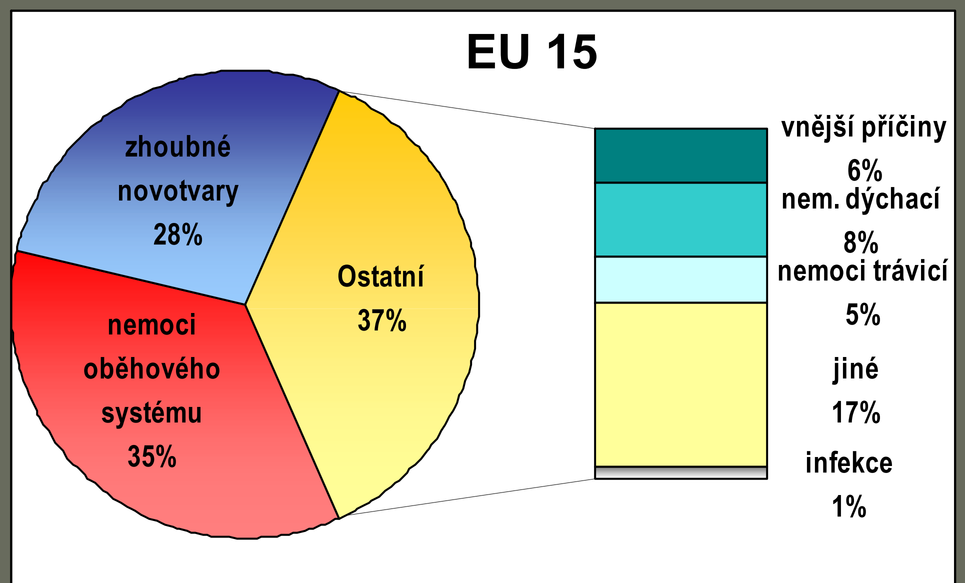
Proč budeme mluvit hlavně o onkologii?

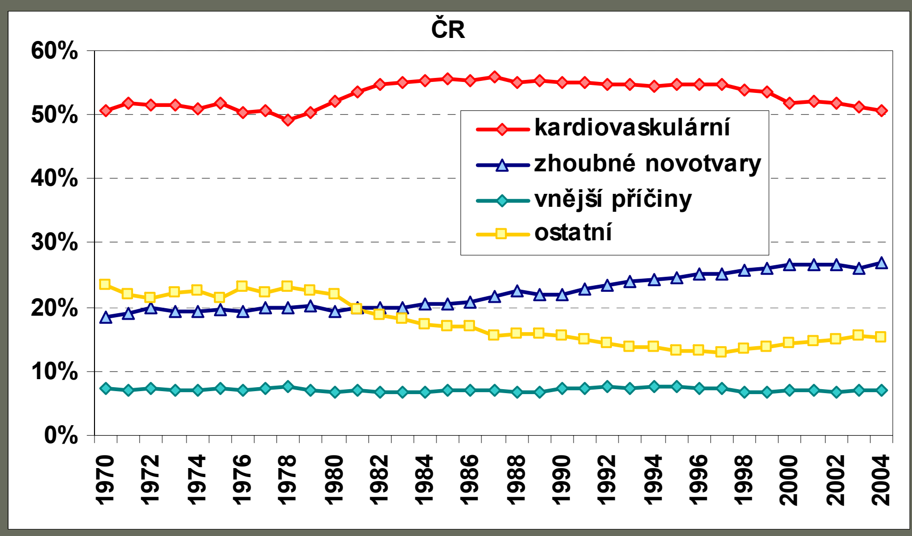
Podle klasické Omranovy teorie z roku 1971 všechny společnosti procházejí třemi stádii souvisejícími s procesem modernizace:

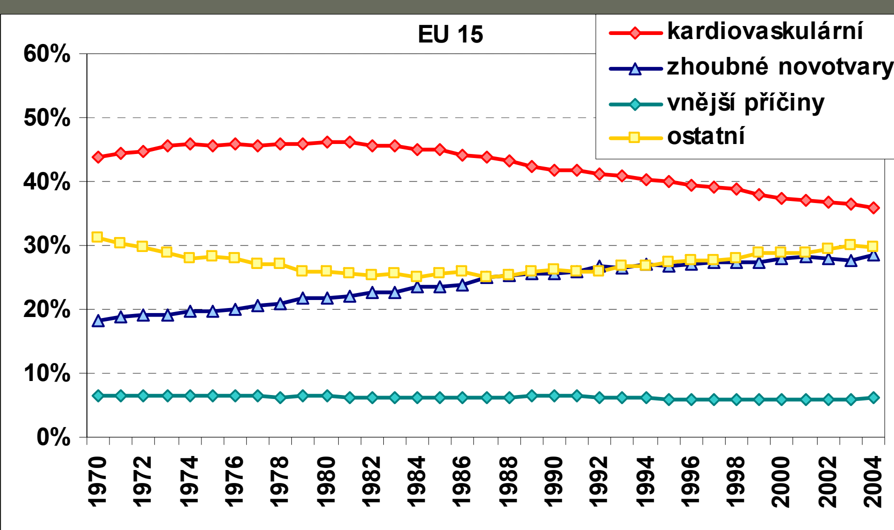
1. Stadium moru a hladomoru
2. Stadium poklesu frekvence výskytu pandemií infekčních nemocí
3. Stadium rozvoje degenerativních a civilizačních chorob (novotvary a nemoci oběhové soustavy)

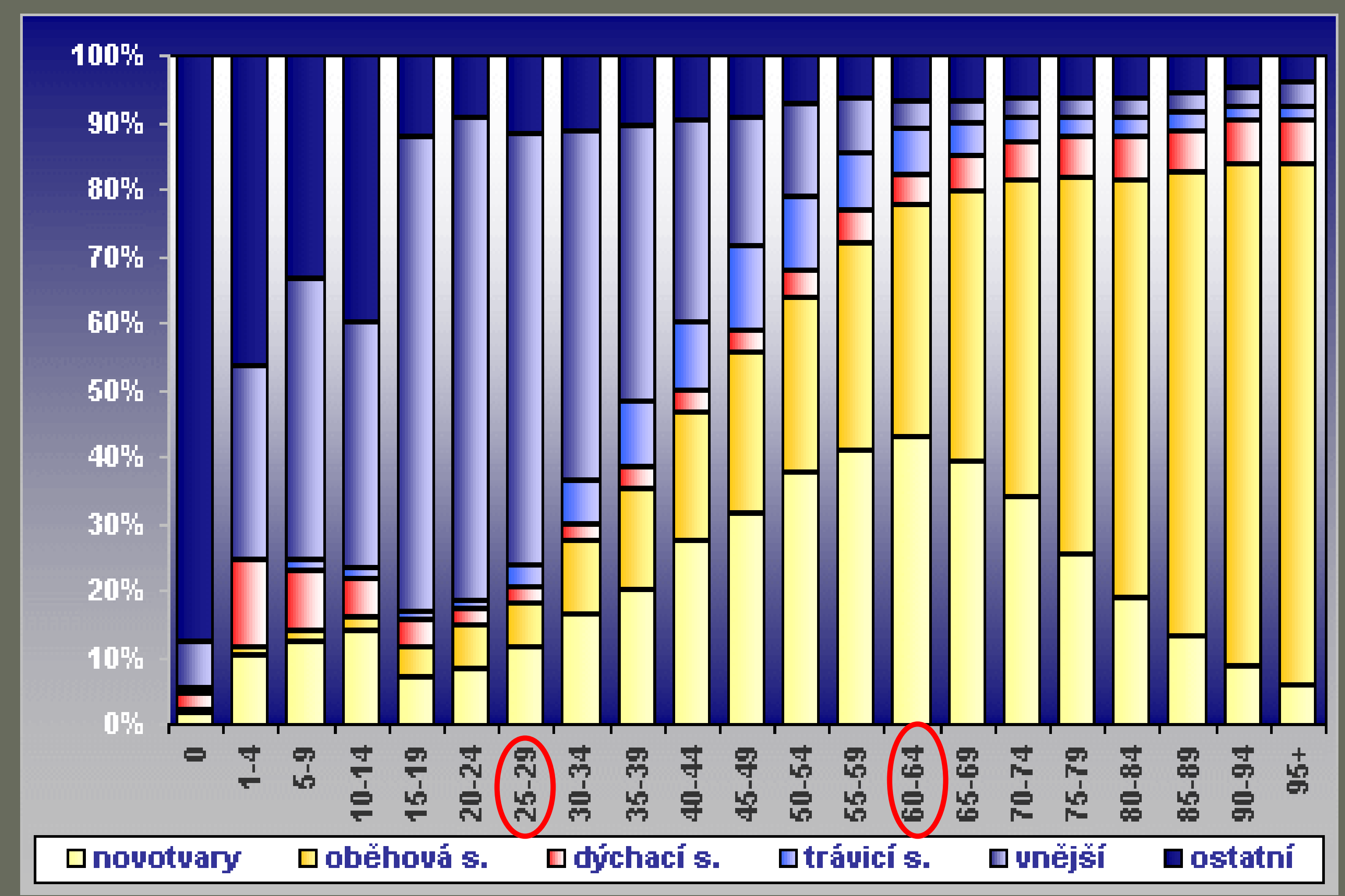
Omran, A.R.: The epidemiologic transition: a theory of the epidemiology of population change. Milbank memorial Fund Quaterly, 1971, 29


ČR
zhoubné novotvary 27%
Ostatní 23%
nemoci oběhového systému 51%
vnější příčiny 7%
nem. dýchací 4%
nemoci trávicí 4%
jiné 7%
infekce 0%


EU 15
zhoubné novotvary 28%
Ostatní 37%
nemoci oběhového systému 35%
vnější příčiny 6%
nem. dýchací 8%
nemoci trávicí 5%
jiné 17%
infekce 1%


ČR
kardiovaskulární
zhoubné novotvary
vnější příčiny
ostatní


EU 15
kardiovaskulární
zhoubné novotvary
vnější příčiny
ostatní



Zemřelí podle vybraných skupin příčin smrti a věku, ČR, 2005

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

Dělení hematoonkologických onemocnění

- **akutní** leukémie (rychle probíhající, jsou neschopné vývoje v plnohodnotnou buňku, mají blok a ztrátu diferenciace)

- **chronické** leukémie (pomalu probíhající, mohou diferencovat v dospělé buňky, ale je u nich postižena apoptóza - přirozeně neumírají).

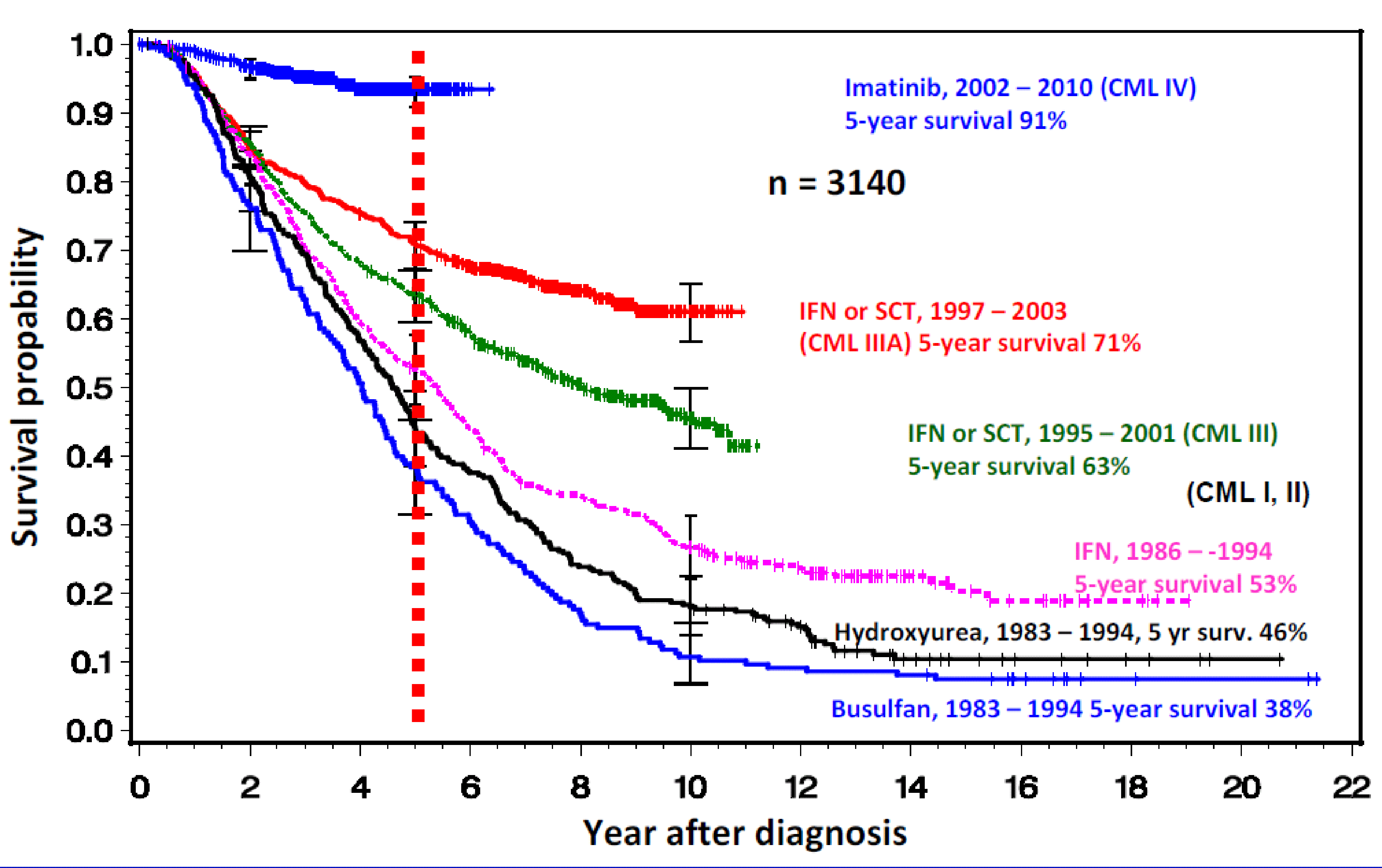
Incidence leukémií na 100 000 lidí/1 rok (evropská standardní populace)

• CML	1-2
• AML, APL	~3
• ALL	~1
• CLL	3-4
• MDS	~3
• CMPD	2-4

Protinádorová terapie

- Chemoterapie (léčiva s cytotoxickým účinkem)
- Transplantace hematopoetických progenitorů
- Hormonální léčba (nádory odvozené od hormonálně dependentní tkáně)
- Diferenciační léčba retinoidy (stimulace diferenciace maligní buňky)
- Bioterapie (hemopoetické růstové faktory, interferony a další cytokiny)
- Radioterapie
- Biologická léčba (MCA)
- Inhibitory konstitutivně aktivovaných tyrozinkináz

Survival of CML 1983 – 2010



Molekulárně biologické vyšetření oportunních patogenů IHOK FN BRNO

- HHV-6,-7
- HSV-1,-2
- EBV, VZV
- BK virus, JC virus
- Respirační viry (RSV, PIV1-3, ADV1-7, Flu-A,-B)
- Adenoviry
- CMV a detekce mutací
- Pneumocystis jirovecii
- Mykózy

Aspergillus fumigatus miluje teplo. Optimálně roste při 37C (tělní teplota).

Máme dostatečně kvalitní metody?

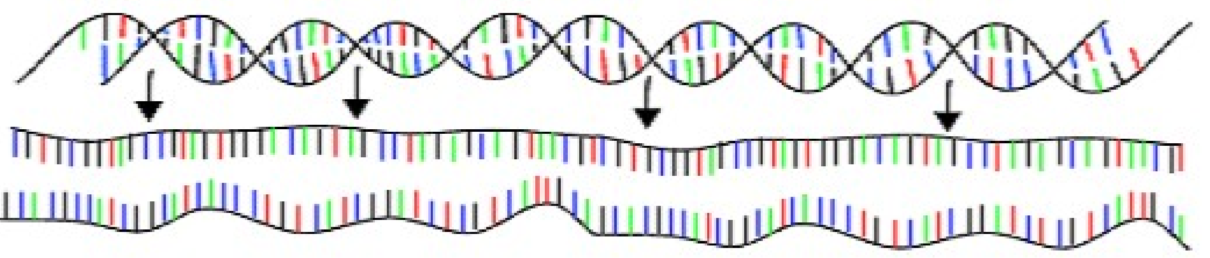
Metody molekulární diagnostiky

1. PCR
2. PCR
3. PCR

1993
1983 K. Mullis
1985 publikace
1993 Nobelova cena za chemii
PTC200 (MJR,USA, 250000 Kč)
DNA Engine
MJ Research
PTC-200

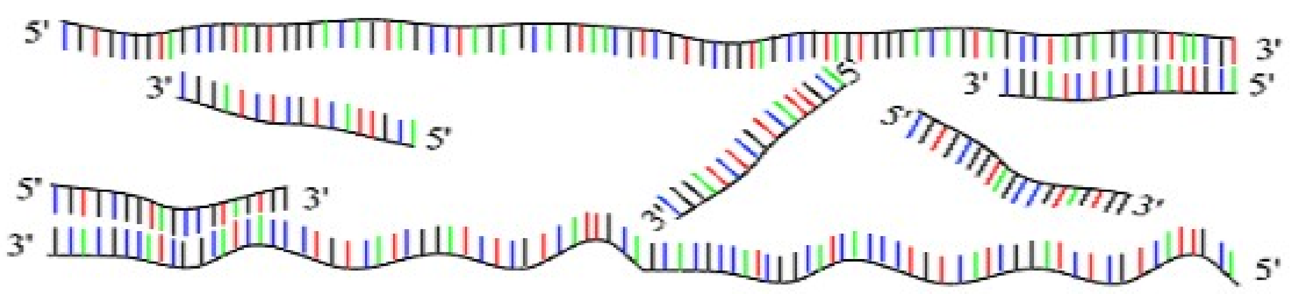
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

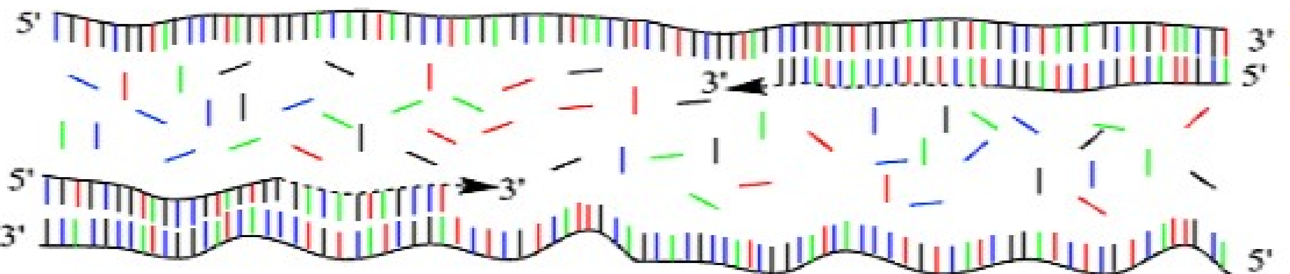
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



Step 3 : extension

2 minutes 72 °C

only dNTP's

(Andy Vierstraete 1999)

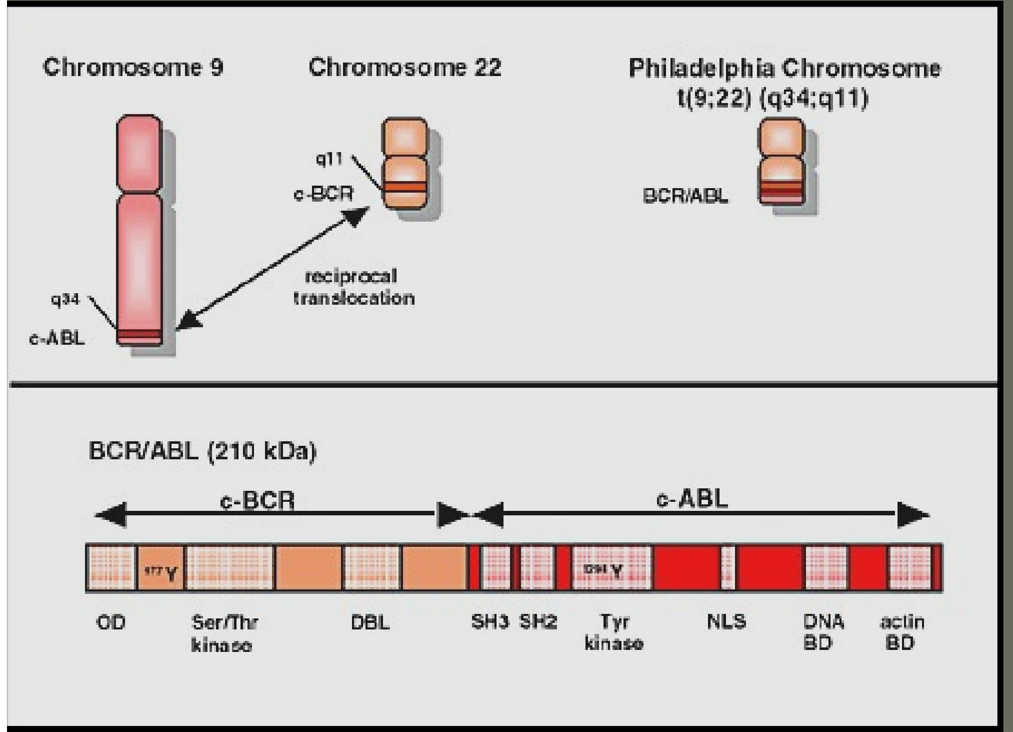
1st cycle 2^2=4 copies **35th cycle 2^{36} = 68 billion copies**

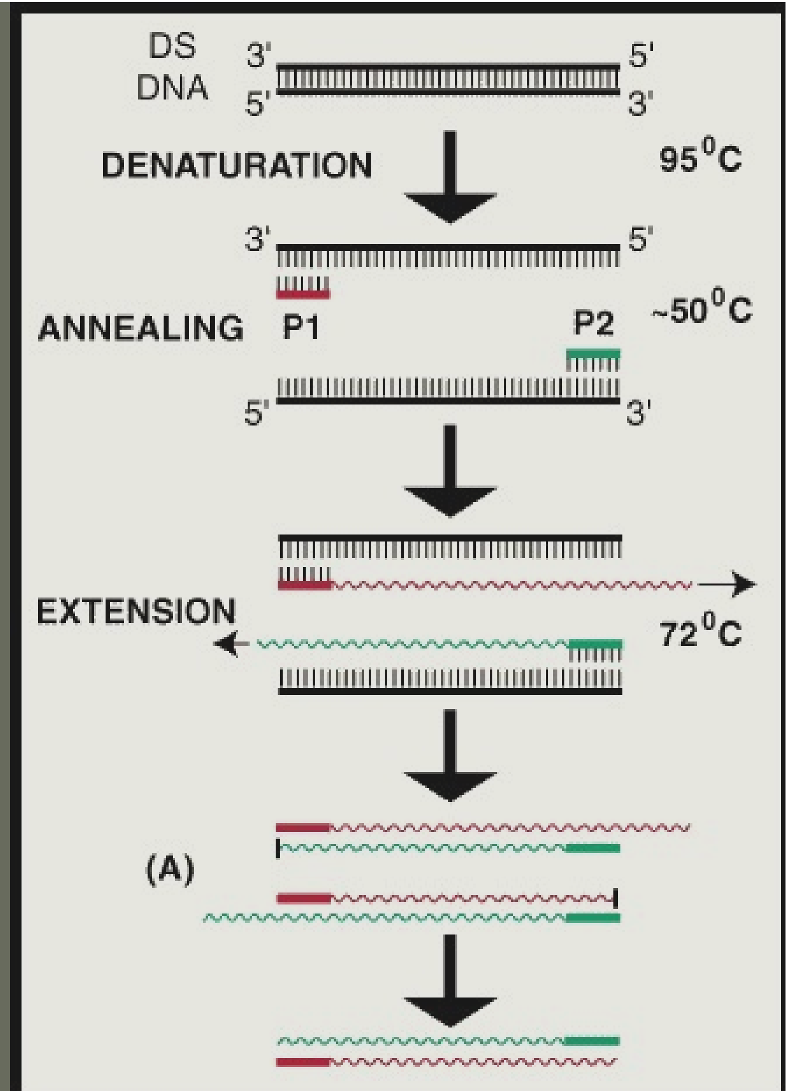
Brock TD and Freeze H (1969). ***"Thermus aquaticus*, a Nonsporulating Extreme Thermophile"**. *J. Bact.* **98** (1): 289–297.

TaqDNA Polymerase, 1989

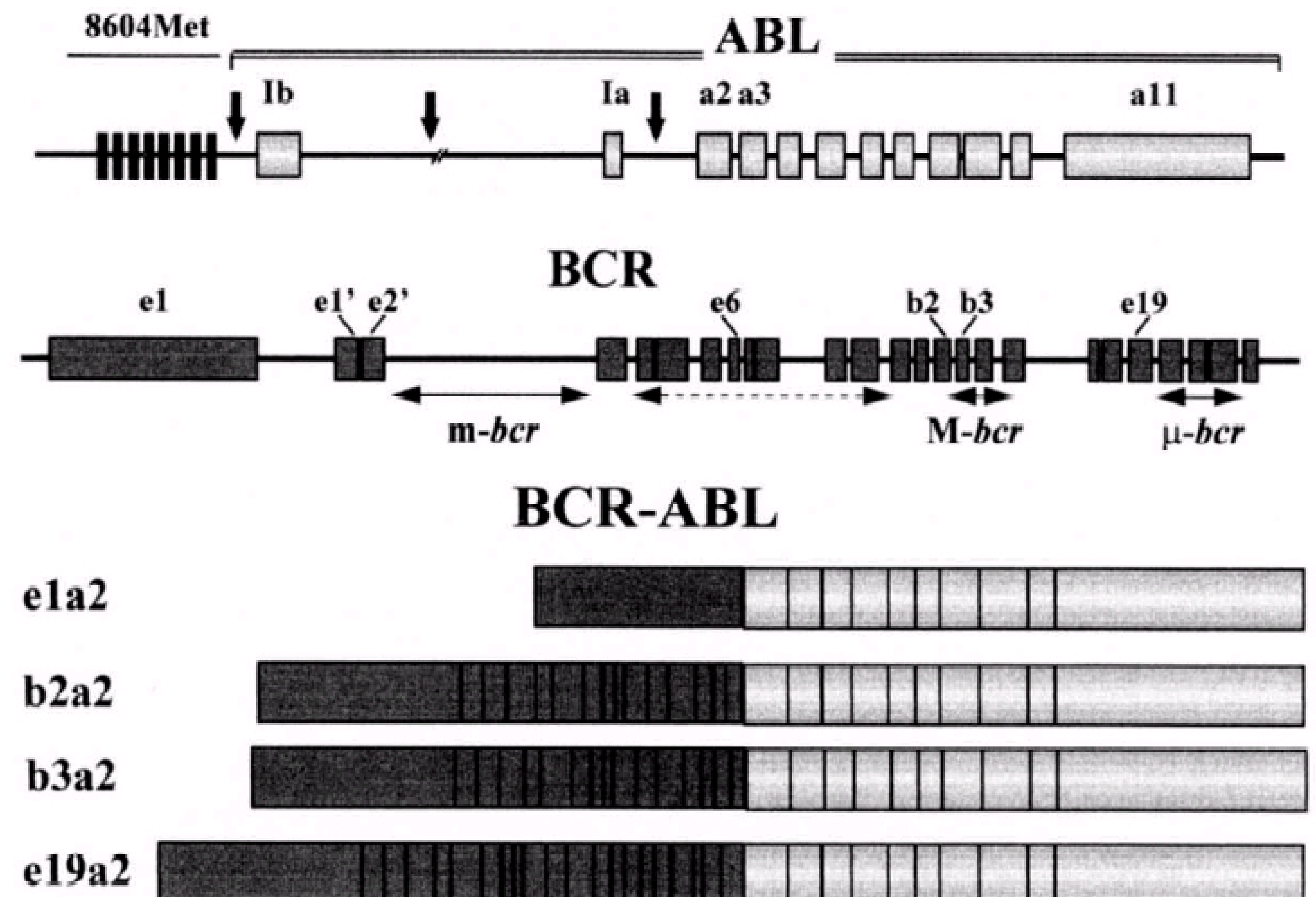
Jaké jsou praktické aplikace PCR v klinické praxi ?

Diagnostické vstupní vyšetření

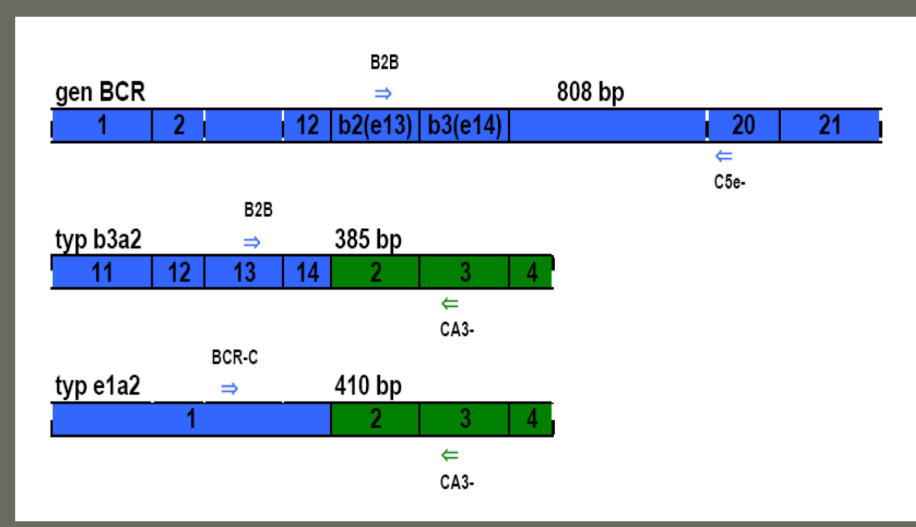




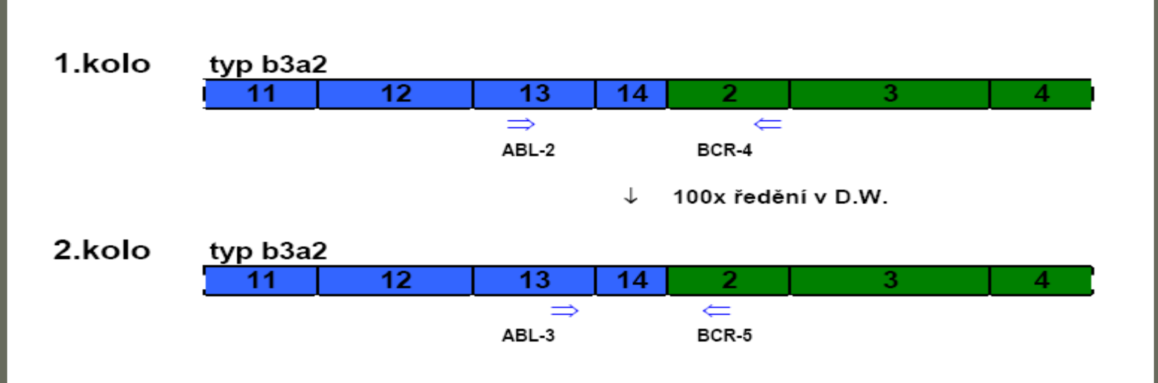
Jednokolová PCR



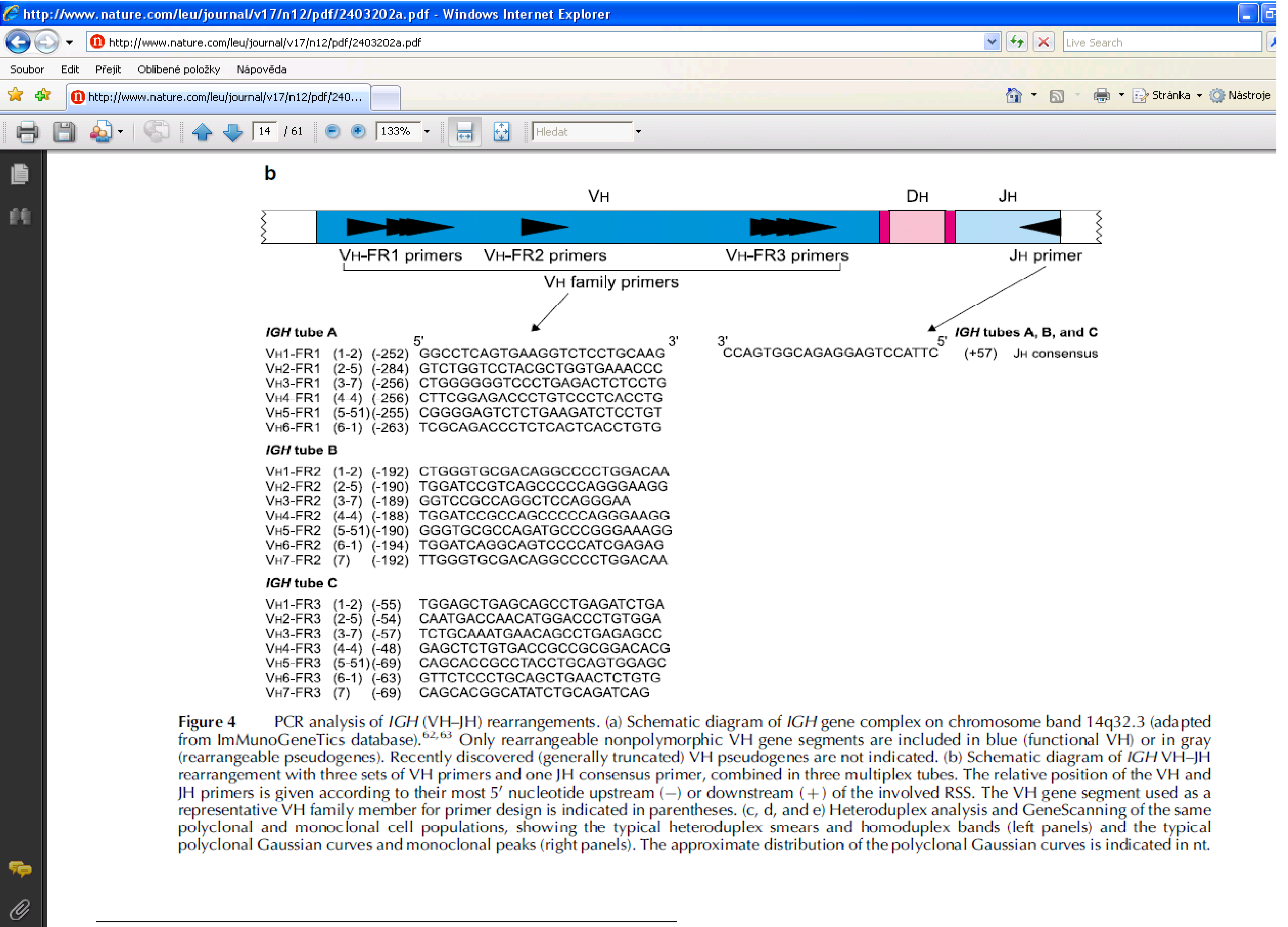
matic representation of the ABL and the BCR genes disrupted in the t(9;22)(q34;q11). Note the 8604Met gene, whic
ıpstream of the ABL locus, and whose function is unknown.[24] Exons are represented by boxes and introns by
s. Breakpoints in ABL, illustrated as vertical arrows, almost invariably occur either upstream of exon Ib, between I
d a2. The BCR gene contains 25 exons, including two putative alternative first (e1′) and second (e2′) exons.[24] The
occur within one of three breakpoint cluster regions (*bcr*), the location and probable extents of which are shown I
l horizontal arrows. In exceptional cases the BCR breakpoints fall between m-*bcr* and M-*bcr*, within the region indi
l dashed-line arrow. The lower half of the figure shows the structure of the various BCR-ABL mRNA transcripts which
with the position of the breakpoint in BCR. Breaks in m-*bcr* give origin to BCR-ABL mRNA molecules with an e1
M-*bcr* occur either between exons b2 (e13) and b3 (e14) or between b3 and b4 (e15), generating fusion transcripts



Multiplex-PCR



Dvoukolová nested-PCR



IGH tube A 5' … 3'

VH1-FR1	(1-2)	(-252)	GGCCTCAGTGAAGGTCTCCTGCAAG
VH2-FR1	(2-5)	(-284)	GTCTGGTCCTACGCTGGTGAAACCC
VH3-FR1	(3-7)	(-256)	CTGGGGGGTCCCTGAGACTCTCCTG
VH4-FR1	(4-4)	(-256)	CTTCGGAGACCCTGTCCCTCACCTG
VH5-FR1	(5-51)	(-255)	CGGGGAGTCTCTGAAGATCTCCTGT
VH6-FR1	(6-1)	(-263)	TCGCAGACCCTCTCACTCACCTGTG

IGH tubes A, B, and C 3' … 5'

CCAGTGGCAGAGGAGTCCATTC	(+57)	JH consensus

IGH tube B

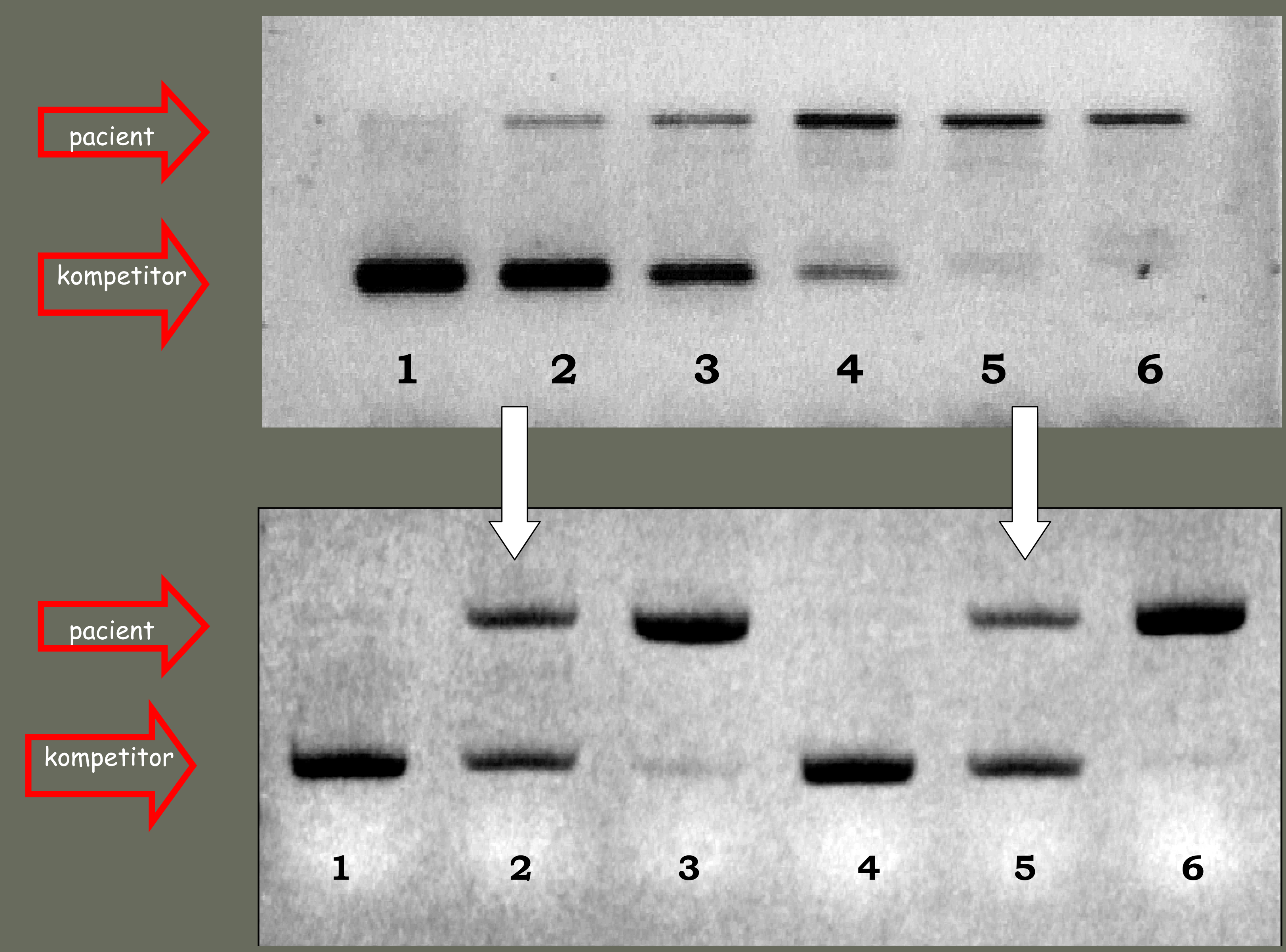
VH1-FR2	(1-2)	(-192)	CTGGGTGCGACAGGCCCCTGGACAA
VH2-FR2	(2-5)	(-190)	TGGATCCGTCAGCCCCCAGGGAAGG
VH3-FR2	(3-7)	(-189)	GGTCCGCCAGGCTCCAGGGAA
VH4-FR2	(4-4)	(-188)	TGGATCCGCCAGCCCCCAGGGAAGG
VH5-FR2	(5-51)	(-190)	GGGTGCGCCAGATGCCCGGGAAAGG
VH6-FR2	(6-1)	(-194)	TGGATCAGGCAGTCCCCATCGAGAG
VH7-FR2	(7)	(-192)	TTGGGTGCGACAGGCCCCTGGACAA

IGH tube C

VH1-FR3	(1-2)	(-55)	TGGAGCTGAGCAGCCTGAGATCTGA
VH2-FR3	(2-5)	(-54)	CAATGACCAACATGGACCCTGTGGA
VH3-FR3	(3-7)	(-57)	TCTGCAAATGAACAGCCTGAGAGCC
VH4-FR3	(4-4)	(-48)	GAGCTCTGTGACCGCCGCGGACACG
VH5-FR3	(5-51)	(-69)	CAGCACCGCCTACCTGCAGTGGAGC
VH6-FR3	(6-1)	(-63)	GTTCTCCCTGCAGCTGAACTCTGTG
VH7-FR3	(7)	(-69)	CAGCACGGCATATCTGCAGATCAG

Figure 4 PCR analysis of *IGH* (VH–JH) rearrangements. (a) Schematic diagram of *IGH* gene complex on chromosome band 14q32.3 (adapted from ImMunoGeneTics database).[62,63] Only rearrangeable nonpolymorphic VH gene segments are included in blue (functional VH) or in gray (rearrangeable pseudogenes). Recently discovered (generally truncated) VH pseudogenes are not indicated. (b) Schematic diagram of *IGH* VH–JH rearrangement with three sets of VH primers and one JH consensus primer, combined in three multiplex tubes. The relative position of the VH and JH primers is given according to their most 5′ nucleotide upstream (−) or downstream (+) of the involved RSS. The VH gene segment used as a representative VH family member for primer design is indicated in parentheses. (c, d, and e) Heteroduplex analysis and GeneScanning of the same polyclonal and monoclonal cell populations, showing the typical heteroduplex smears and homoduplex bands (left panels) and the typical polyclonal Gaussian curves and monoclonal peaks (right panels). The approximate distribution of the polyclonal Gaussian curves is indicated in nt.

Kvantitativní kompetitorová PCR



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

Real-time quantitative PCR (RQ-PCR)

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)

ABI7900HT (Applied Biosystems)

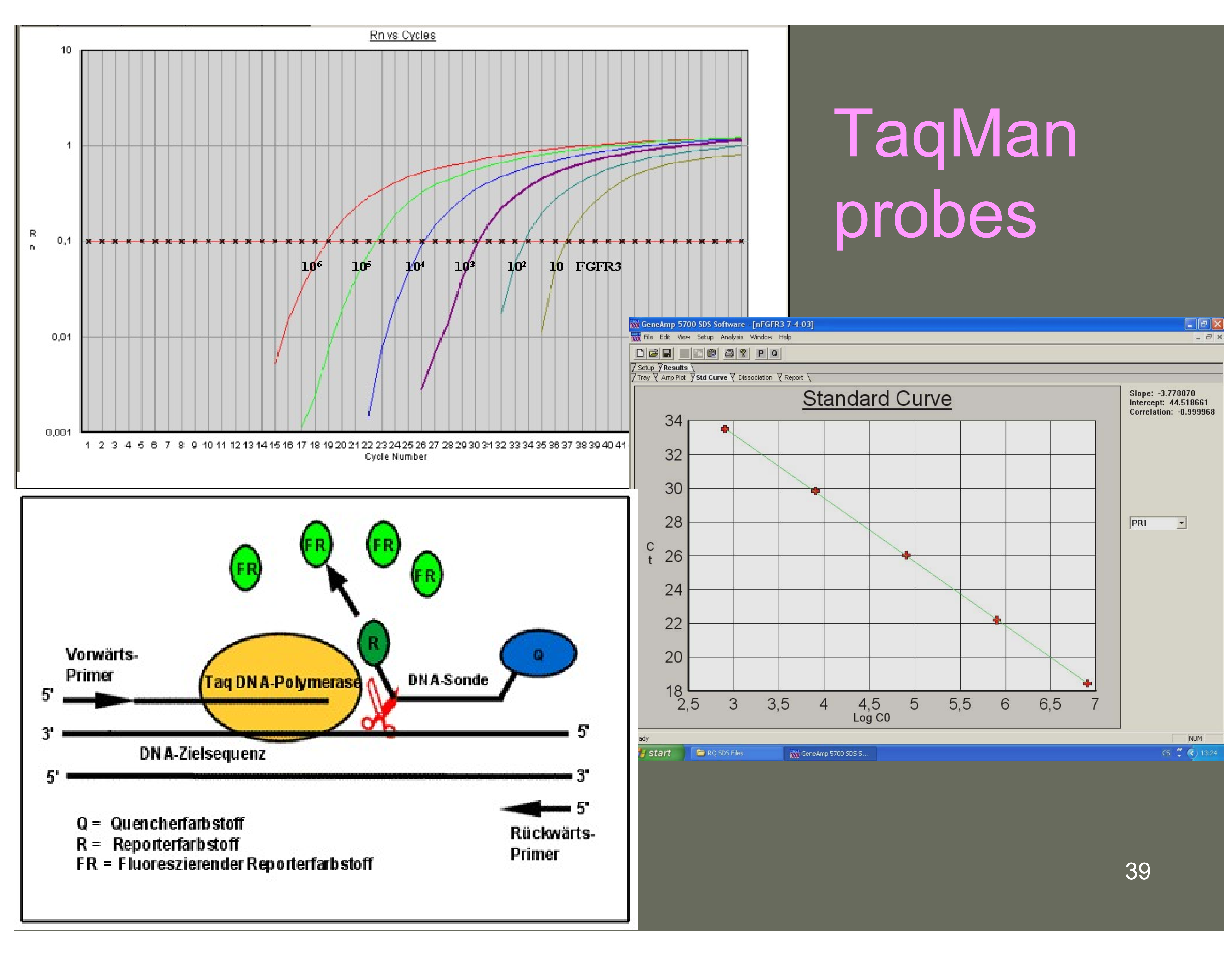
7300 System (Applied Biosystems)

RotorGene 3000 (CorbettResearch,QIAGENE)

Real-time quantitative PCR (RQ-PCR)

a) Interkalační barviva
b) Hybridizační jednoduše značené sondy
c) Hydrolyzační duálně značené sondy

TaqMan probes



Digital PCR is a new approach to nucleic acid detection and quantification, which is a different method of absolute quantification and rare allele detection relative to conventional qPCR, because it directly counts the number of target molecules rather than relying on reference standards or endogenous controls.

Real Time PCR vs Traditional PCR vs Digital PCR at a Glance

	Digital PCR	Real-Time PCR	Traditional PCR
Overview	Measures the fraction of negative replicates to determine absolute copy number.	Measures PCR amplification as it occurs.	Measures the amount of accumulated PCR product at the end of the PCR cycles.
Quantitative?	Yes, the fraction of negative PCR reactions is fit to a Poisson statistical algorithm .	Yes, because data is collected during the exponential growth (log) phase of PCR when the quantity of the PCR product is directly proportional to the amount of template nucleic acid.	No, though comparing the intensity of the amplified band on a gel to standards of a known concentration can give you 'semi-quantitative' results.
Applications	• Absolute Quantification of Viral Load • Absolute Quantification of Nucleic Acid Standards • Absolute Quantification of Next-Gen Sequencing Libraries • Rare Allele Detection • Low-Fold Copy Number Discrimination • Enrichment and Separation of Mixtures	• Quantitation of Gene Expression • Microarray Verification • Quality Control and Assay Validation • Pathogen detection • SNP Genotyping • Copy Number Variation • MicroRNA Analysis • Viral Quantitation • siRNA/RNAi experiments	Amplification of DNA for: • Sequencing • Genotyping • Cloning
Summary	Advantages of Digital PCR: • No need to rely on references or standards Desired precision can be achieved by increasing total number of PCR replicates • Highly tolerant to inhibitors • Capable of analyzing complex mixtures • Unlike traditional qPCR, digital PCR provides a linear response to the number of copies present to allow for small fold change differences to be detected	Adavantages of Real-Time PCR • Increased dynamic range of detection • No post-PCR processing • Detection is capable down to a 2-fold change • Collects data in the exponential growth phase of PCR • An increase in reporter fluorescent signal is directly proportional to the number of amplicons generated • The cleaved probe provides a permanent record amplification of an amplicon	Disadvantages of Traditional PCR • Poor Precision • Low sensitivity • Short dynamic range < 2 logs • Low resolution • Non-Automated • Size-based discrimination only • Results are not expressed as numbers • Ethidium bromide for staining is not very quantitative • Post-PCR processing

Leukemia (2003) 17, 2318–2357
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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's sity
London Hospitals, London, UK; [5]Department of Immuno-haematology, A k;
[6]Department of Medical Sciences, Uppsala University Hospital, Uppsala, n E
San Gerardo Hospital, Monza, Italy; [8]Central Laboratory of Hematology is,
[9]Department of Genetic Biochemistry, Hôpital Robert Debré, Paris, Fran t of
Biochemistry & Medical Biotechnology, University Hospital 'Federico II', 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 nc.
[15]Biogenetics Laboratory, Children's University Hospital, Giessen, Germa olin
Stockholm, Sweden

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European Network

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 rour 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

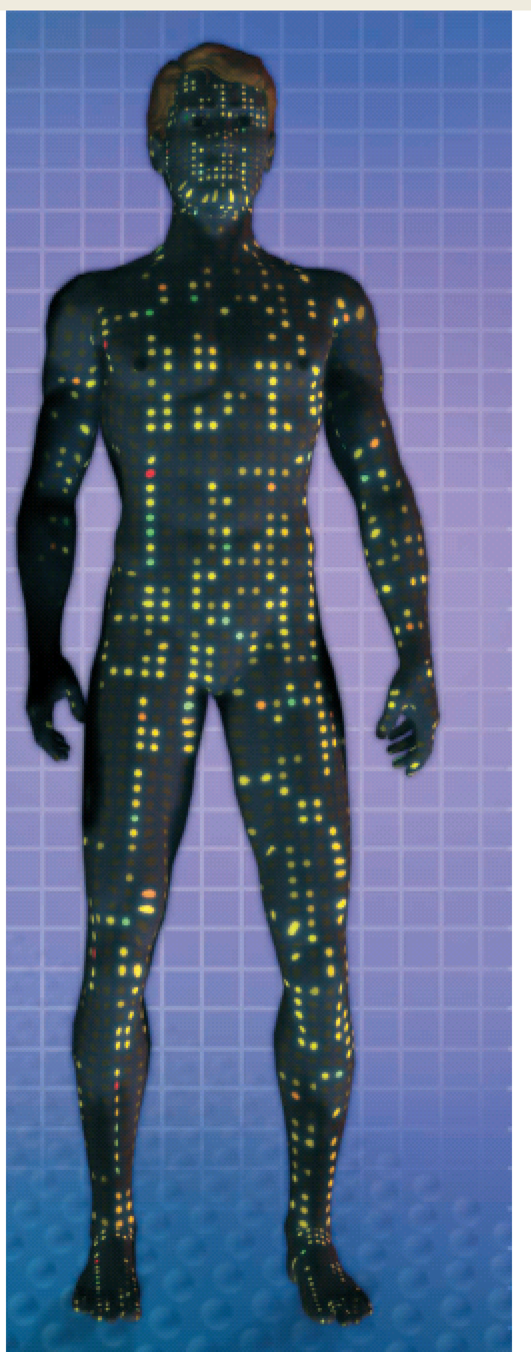
Fusion Gene Standards:

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. Beillard et al. Evaluation of candidate control genes for diagnosis



The European LeukemiaNet is an EU-funded organisation of physicians, scientists and patients with interest in leukemia. It aims to improve the treatment and knowledge about Leukemia in Europe and spread excellence. The website delivers information for physicians, patients (e.g. patient organisations in Europe), ongoing clinical trials and further information about the disease. You can get information about the European LeukemiaNet in various european languages. The European LeukemiaNet is funded by the 6th Framework Program of the European Community.

- Calendar
- ELN-Symposia
- Current Meetings
- The Project
- ELN Structure
- ELN Foundation
- Contact Info
- Information Letter
- email-Newsletter
- Countries
- Translation Leukemia
- Disclaimer

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We comply with the HONcode standard for trustworthy health information: verify here

News

EUTOS – eCRF – Registry
The EUTOS for CML Registry will collect baseline, treatment and outcome data on patients with CML in Europe by implementing and enlarging the current European CML Registry. The Online access to the EUTOS – eCRF – System is available at the EUTOS website.

EUTOS: Abstracts and ppt-slides of CML meetings available
A new section of the EUTOS website has been arranged to offer information about actual and forthcoming meetings.

Health Economic Issues in

Events

Mo 2010/05/17 - Tu 2010/05/18
The management of CML: current treatment paradigms and future perspectives

Th 2010/06/10 - Su 2010/06/13
EHA 2010: 15th Congress of the EHA

Th 2010/09/02 - Su 2010/09/05
The 1st World Congress on Controversies in Hematology (COHEM)
The 1st World Congress on Controversies in Hematology (COHEM) will function as an exclusive forum for international experts···

Dates & Meetings

April 2010 « | »

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12	13	14	15	16	17	18
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May 2010 « | »

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24	25	26	27	28	29	30
31						

RSS

Online Registration

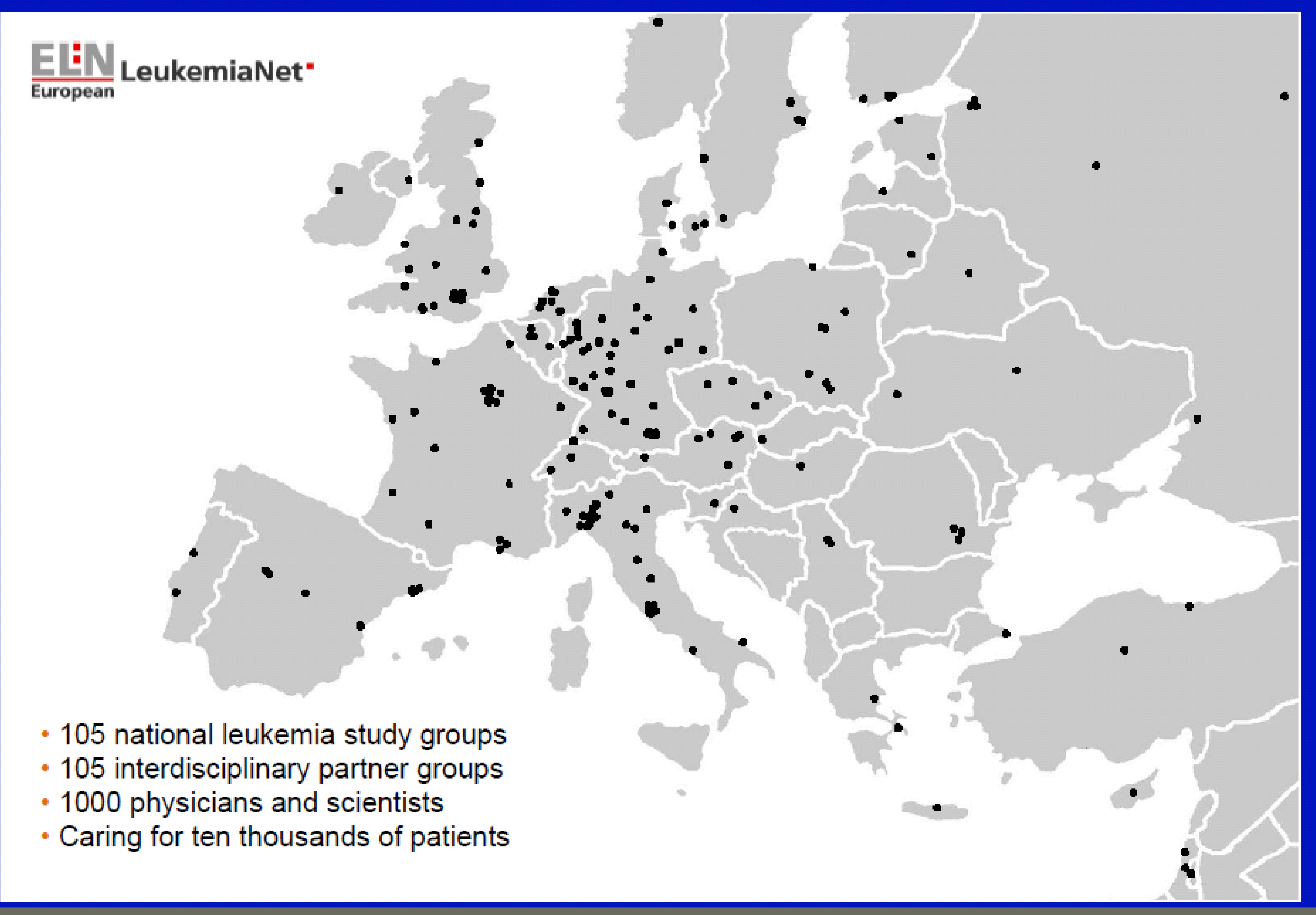
How to join the European LeukemiaNet

European Leukemia Trial Registry

European Leukemia Trial Registry (ELTR)

European Treatment and

European LeukemiaNet
175 centers in 33 countries



Standardization of BCR-ABL quantification in Europe – 2010

Chronická myeloidní leukémie

Chronická myeloidní leukémie

- Incidence 1.09 / 100 000 / rok (Evropská standardní populace)
- Chronická fáze, akcelerovaná fáze, blastický zvrat
- 95% pacientů s typickým molekulárním markerem BCR-ABL
- pomalu probíhající, leukemické buňky mohou dále diferencovat
- porucha apoptózy

LEUCOCYTHEMIA,

WHITE CELL BLOOD,

PHYSIOLOGY AND PATHOLOGY OF THE LYMPHATIC GLANDULAR SYSTEM.

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

LONDON: SIMPKIN, MARSHALL, & CO.

MDCCCLII.

1845

John Hughes Bennett, Edinburgh

Weißes Blut.

Dr. Virchow.

Rudolf Virchow, Berlin

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
Depa... of Pathology
... Medicin...
...versity ...ennsy...
School ...edic...
...iladel... PA
D... ...ber 21, ...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 auto... ...t numbered 21 and later changed, ...on, to no. 22. The additional ...L case... ...re being readied for publica...on when ... Richards asked us to present ...omething ...a National Academy of Sci...nces me... he was organizing at Penn. ...ur abstr... published in *Science*, led to ...ide rec... ...ion (and citation) of the first ...sistent chromosome abnor...sia. The Edinburgh group ...sted the name "Philadel...some."
...de was frustrating. Cytoge... CGL proved of some diag...nostic value and provided ...clonal evolution in tumor ...helping to explain clinical ...n. But since other consis... changes in neoplasia were ...nificance in tumorigenesis ...nd the term "epiphenome... used. With the advent of ...nding techniques in the ...lecular genetic techniques ...tory changed dramatically. ...genetic alterations were ...any tumors and are now ...ful for exploring oncogene ...romosome studies remain ...oking at genomic changes, ...nise of the Ph chromosome of specific genomic alter... in human tumorigenesis is ...fruition.

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

A Minute Chromosome ... ...an Chronic Granulocytic Leukemia

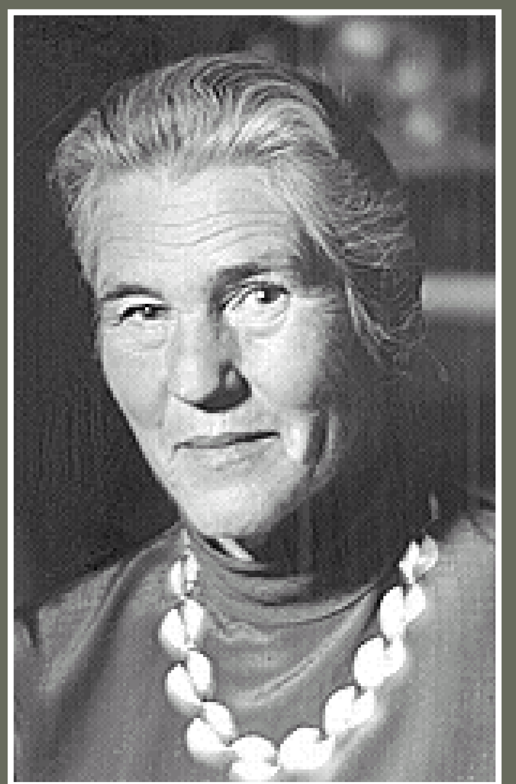
In seven cases thus far investigated (five males, two females), a minute chromosome has been observed replacing one of the four smallest autosomes in the chromosome complement of cells of chronic granulocytic leukemia cultured from peripheral blood. No abnormality was observed in the cells of four cases 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.

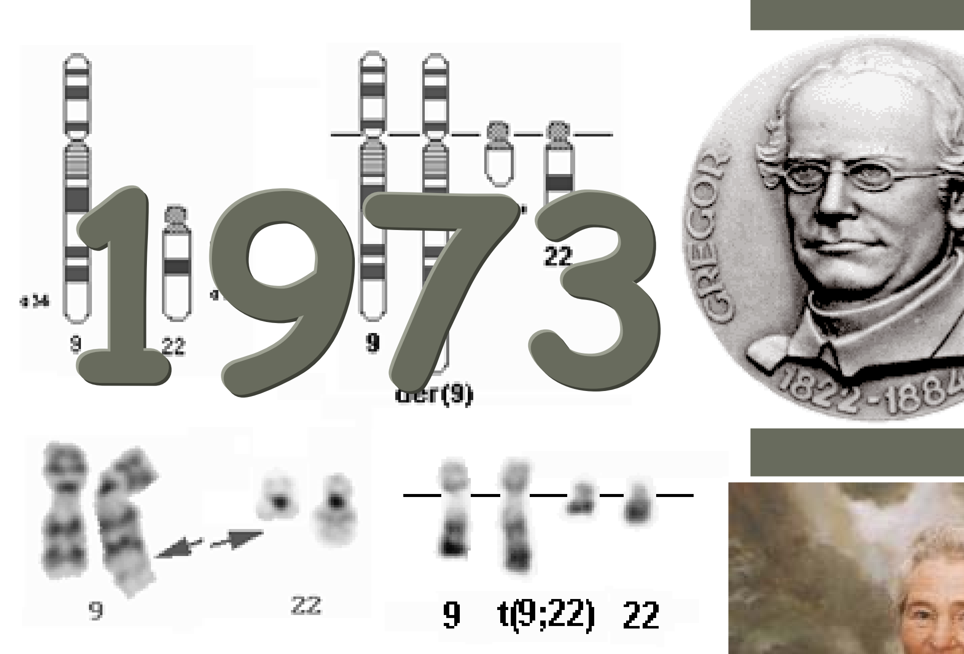
The findings suggest a causal relationship between the chromosome abnormality observed and chronic granulocytic leukemia.

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

Nowell & Hungerford, 1960 Science 132.1497



*1925, University of Chicago



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

1960 publikace Ph chromozomu u CML pacientů

115

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

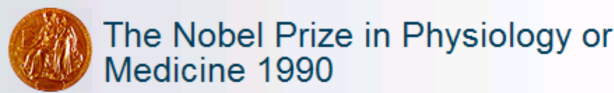
2006 dasatinib (Sprycel)

2007 nilotinib (Tasigna)

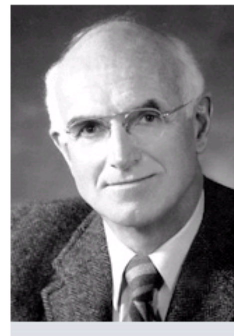
5

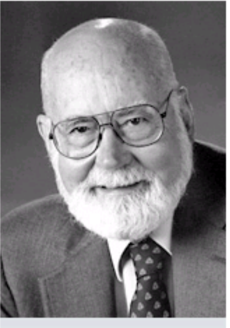
Protinádorová terapie

- **Chemoterapie** (léčiva s cytotoxickým účinkem)
- **Transplantace hematopoetických progenitorů**
- Hormonální léčba (nádory odvozené od hormonálně dependentní tkáně)
- Diferenciační léčba retinoidy (stimulace diferenciace maligní buňky)
- Bioterapie (hemopoetické růstové faktory, interferony a další cytokiny)
- Radioterapie
- Biologická léčba (MCA)
- Inhibitory konstitutivně aktivovaných tyrozinkináz



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





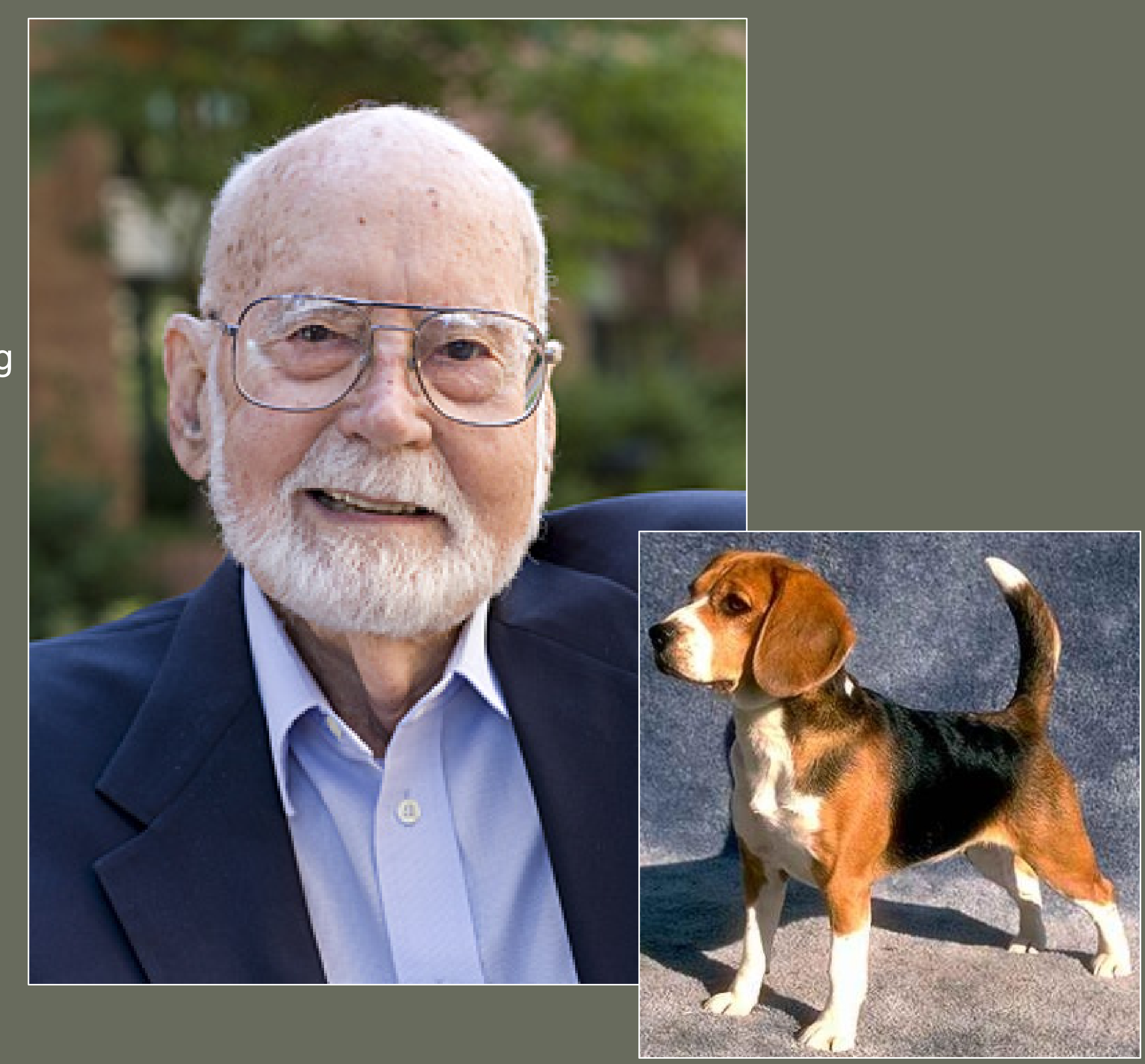
Joseph E. Murray	E. Donnall Thomas
1/2 of the prize	1/2 of the prize
USA	USA
Brigham and Women's Hospital Boston, MA, USA	Fred Hutchinson Cancer Research Center Seattle, WA, USA
b. 1919	b. 1920

Titles, data and places given above refer to the time of the award.
Photos: Copyright © The Nobel Foundation

Edward Donnall Thomas

(1920-2012)

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



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.

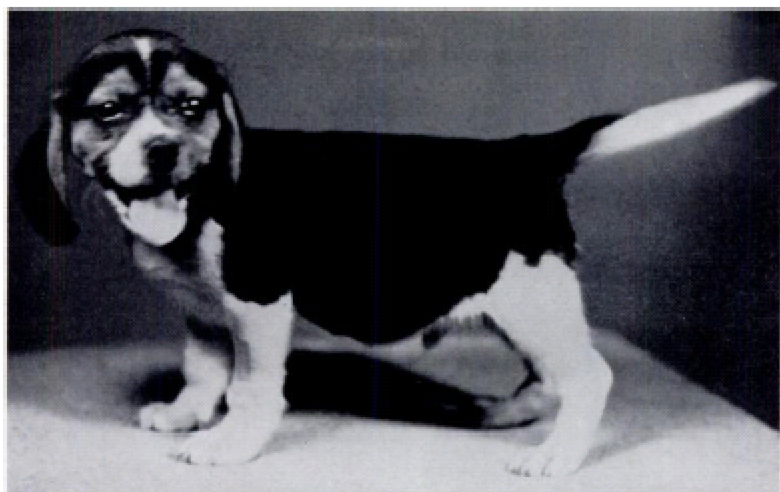


FIG. 16.—Photograph of dog 7 taken thirty days after he had received 800 roentgens of whole-body irradiation and litter-mate marrow.

removed and although operative injury to the blood supply of the pancreas was considered as a possible causal factor, it was believed very unlikely.

Sections of lymph nodes and intestine of dog 1 showed incomplete regeneration of lymphoid tissue, with aggregates of lymphocytes suggesting follicles. There were no true germinal centers. This dog had received ACTH.

Miscellaneous autopsy findings.—Focal necrosis of the liver was seen in 10 of 21 dogs given marrow. In three of these, typical inclusion bodies of hepatitis contagiosa canis were identified in the liver and other organs. The liver lesions in dog 7 have been described. In six other dogs (1, 3, 4, 15, 21, 22) there were multiple foci of necrosis scattered throughout the liver lobules (fig. 17). The areas were small, comprising 10 to 30 liver cells in various stages of degeneration. Some contained fat, whereas others contained "hyaline" material. Varying degrees of nuclear degeneration and necrosis were also present. Some foci contained an infiltration of polymorphonuclear leukocytes. Bacteria were not identified in the lesions. Focal glomerular changes in the kidney of the type seen in generalized infections were not present.

Attempts to correlate liver lesions with other factors were unsuccessful. All animals with lesions were adults except dog 15. There was no correlation with radiation dose (800 r to 1,200 r), or with period of survival 12 to 109 days). Some dogs with lesions had received ACTH; about half had not. Other drugs administered had also been received by dogs that did not show liver necrosis. Half of the dogs with liver lesions had intrapulmonary hemorrhage, but half did not. Spleens had been removed from three.

The liver lesions somewhat resembled those described by Congdon in seven of 57 mice receiving homologous marrow.[11] But the lesions in the dogs were

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.

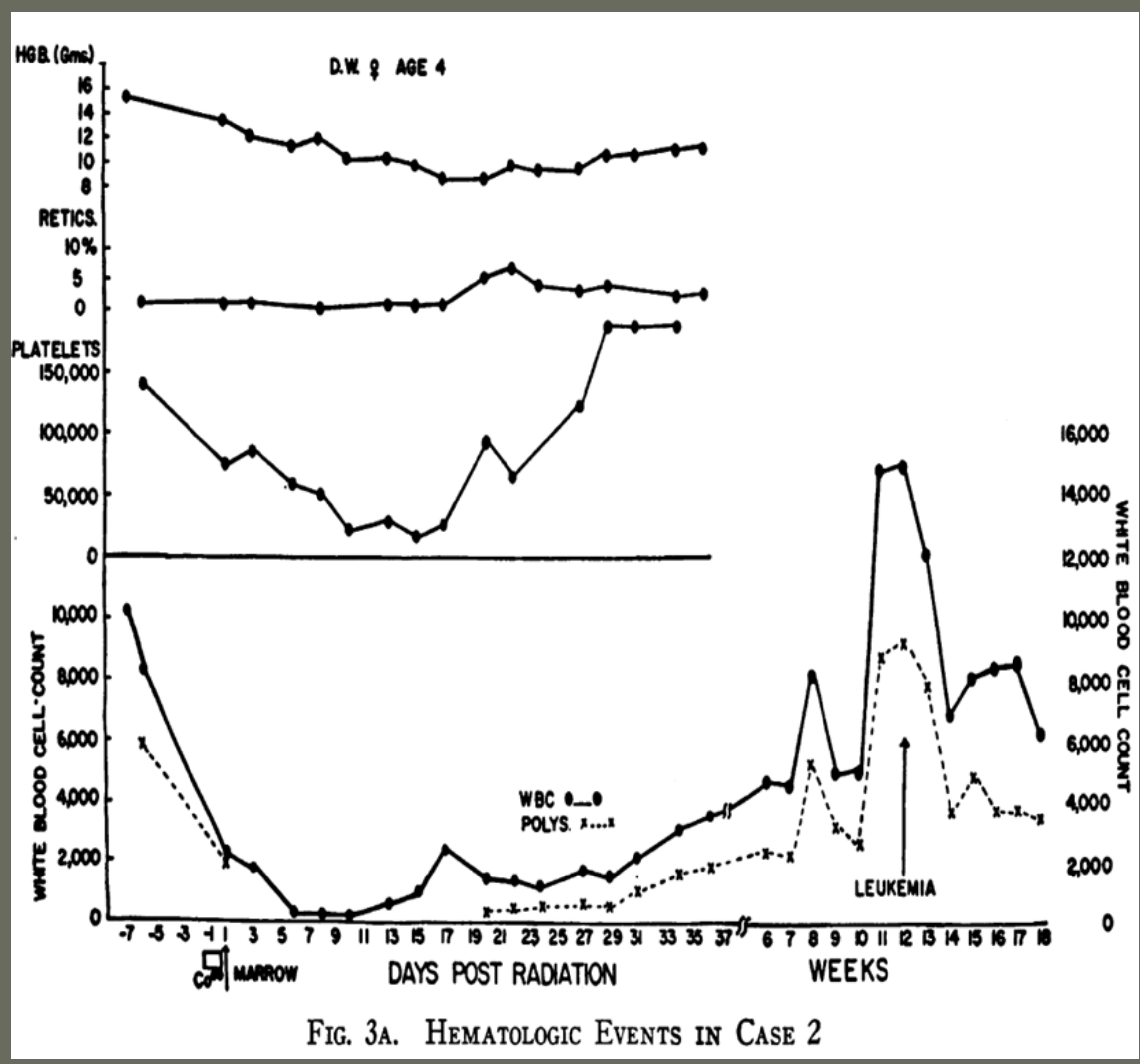


FIG. 3A. HEMATOLOGIC EVENTS IN CASE 2

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 – 80°C. 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 ethylenediamenetetraacetic 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 I: (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.

• Chimérizmus

- výjimečný imunologický jev, kdy buňky z geneticky rozdílných jedinců spoluexistují v jednom těle

- ***Kompletní dárcovský buněčný chimérizmus*** (donor chimerism, DC)
- pokud původní krvetvorba příjemce je kompletně nahrazena dárcovskou krvetvorbou získanou a rozvinutou na základě aktivity přijatého štěpu obvykle spojený s nízkým rizikem relapsu a lepší prognózou

- ***Smíšený chimérizmus*** (mixed chimerism, MC)
- původní krvetvorba příjemce v lymfohematopoetických tkáních koexistuje s krvetvorbou dárce
- přetrvávání příjemcovy hematopoézy zesiluje riziko relapsu,

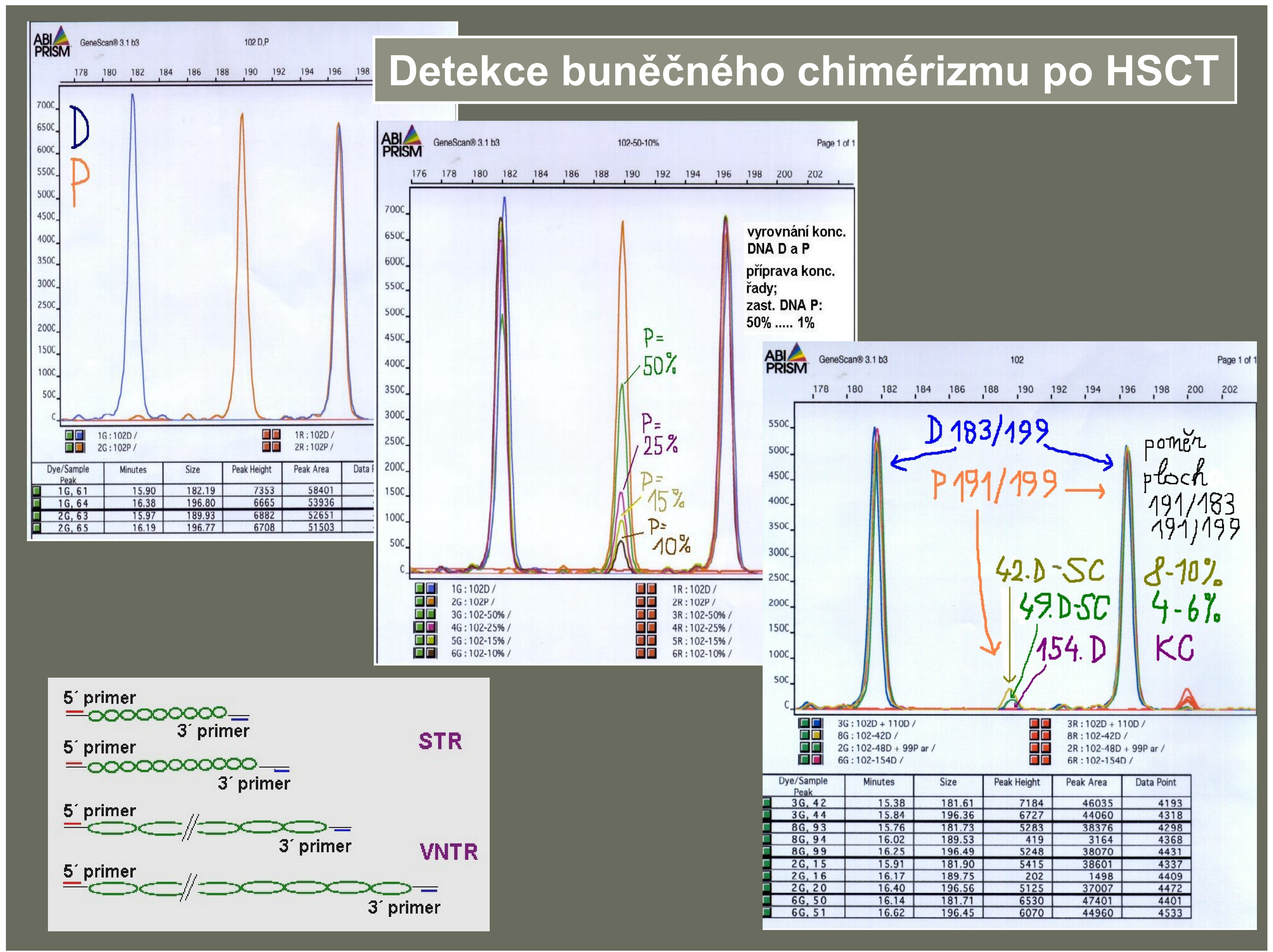
- ***Autologní obnova***

- **Polymorfismy VNTR (Variable Number of Tandem Repeats)** nebo **STR** (***short tandem repeats*** **)**
- polymorfismus mezi dárcem a příjemcem, tzn. znaku jedinečného pro příjemce. Nalezení takového markeru je nezbytné k určení podílu krvetvorby dárce a příjemce po alogenní transplantaci krvetvorných buněk. K analýze pomocí RQ-PCR využíváme existence polymorfismů typu inserce/delece (in/del)
- hodnocení úspěšnosti transplantace, upozorňuje na počátek nové expanze patologického klonu

Fragmentační analýza

- fragmenty DNA syntetizované PCR s fluorescenčně značeným primerem
- v automatickém analyzátoru jsou jednotlivé DNA fragmenty ve velmi tenké kapiláře,naplněné polymerem na bázi PAA, rozděleny dle jejich velikosti a náboje a postupně detekovány laserovým detektorem
- v průběhu jediného běhu pak můžeme současně analyzovat několik různých fragmentů za předpokladu, že se navzájem liší svou délkou nebo jsou označeny různými fluorescenčními barvami. Spolu s každým vzorkem běží interní délkový standard, který umožní přesné určení délky jednotlivých fragmentů.

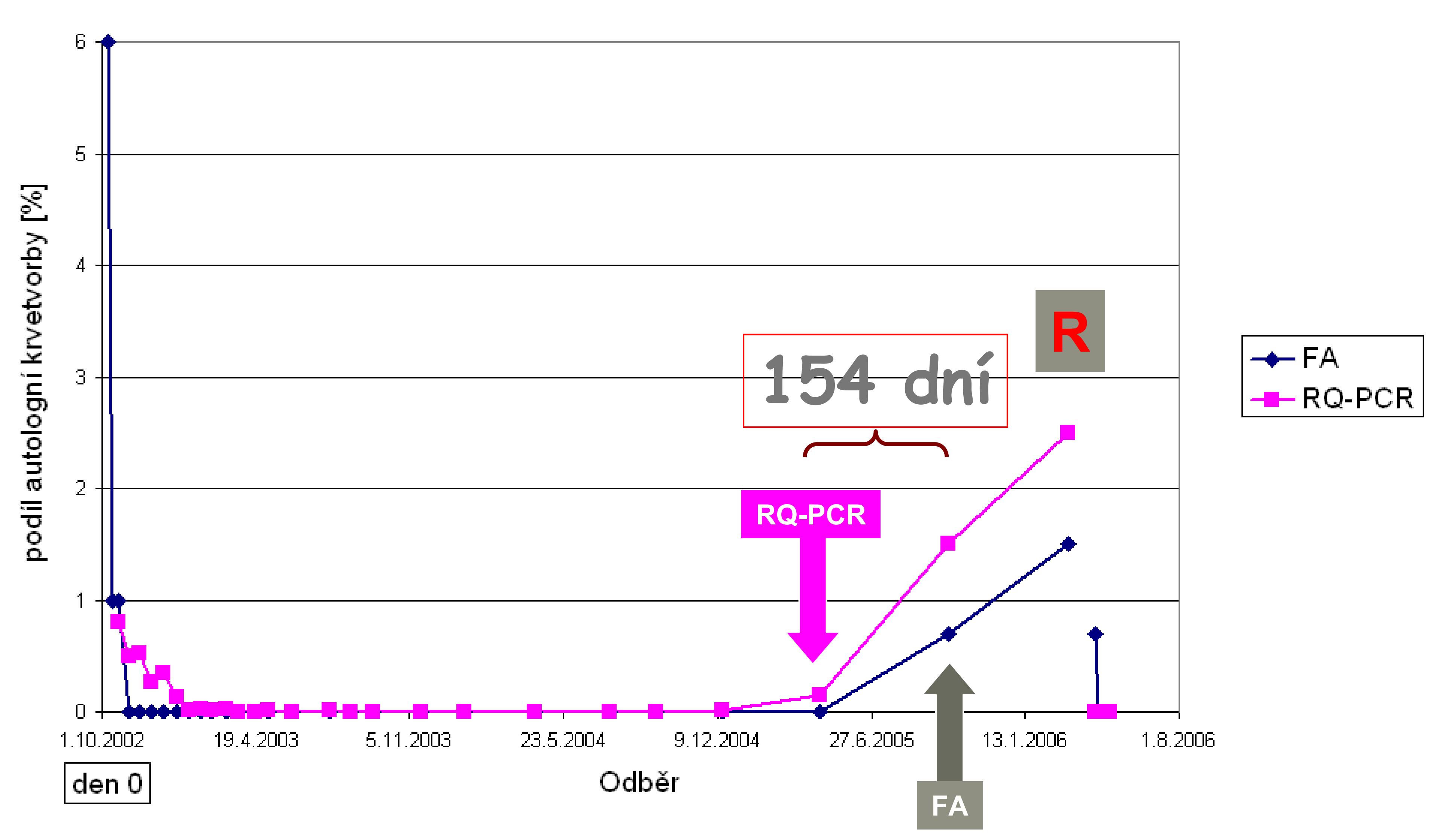
Detekce buněčného chimérizmu po HSCT



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area
1G, 61	15.90	182.19	7353	58401
1G, 64	16.38	196.80	6665	53936
2G, 63	15.97	189.93	6882	52651
2G, 65	16.19	196.77	6708	51503

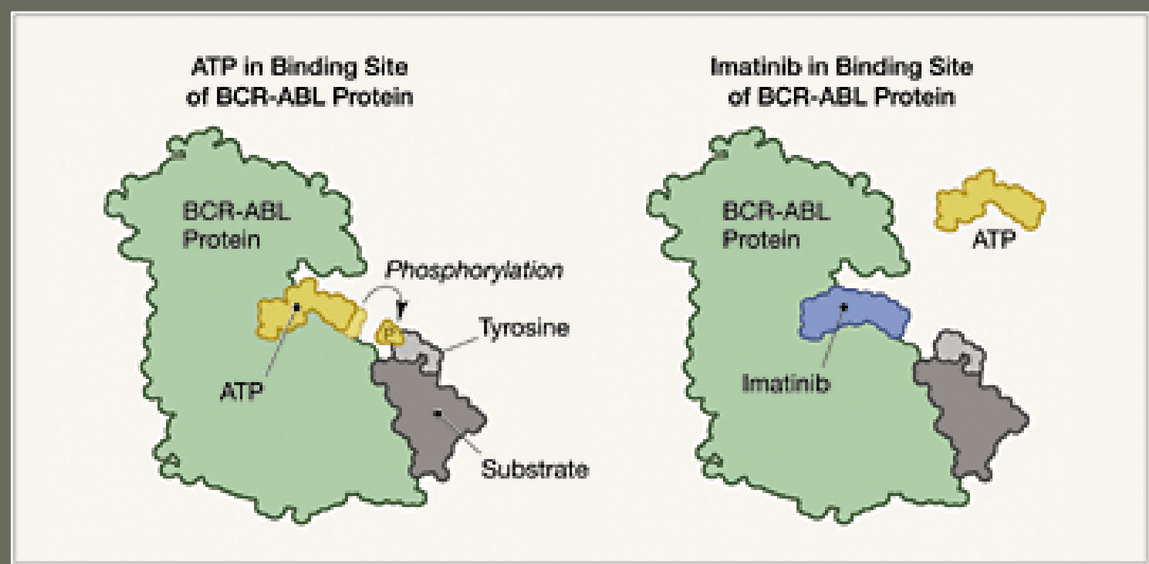
Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
3G, 42	15.38	181.61	7184	46035	4193
3G, 44	15.84	196.36	6727	44060	4318
8G, 93	15.76	181.73	5283	38376	4298
8G, 94	16.02	189.53	419	3164	4368
8G, 99	16.25	196.49	5248	38070	4431
2G, 15	15.91	181.90	5415	38601	4337
2G, 16	16.17	189.75	202	1498	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



Protinádorová terapie

- Chemoterapie (léčiva s cytotoxickým účinkem)
- Transplantace hematopoetických progenitorů
- Hormonální léčba (nádory odvozené od hormonálně dependentní tkáně)
- Diferenciační léčba retinoidy (stimulace diferenciace maligní buňky)
- Bioterapie (hemopoetické růstové faktory, interferony a další cytokiny)
- Radioterapie
- Biologická léčba (MCA)
- **Inhibitory konstitutivně aktivovaných tyrozinkináz**



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.

Imatinib mesylate (STI 571)

Table 2: FDA-approved Indications of Tyrosine Kinase Inhibitors

Drug	Indications
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

FDA accepts New Drug Application for bosutinib for previously treated Ph+ CML

Source: **BioSpace**

Date published: **30/01/2012 15:45**

Summary

by: Nicola Pocock

According to BioSpace, the US FDA has accepted a New Drug Application (NDA) for standard review of bosutinib as a treatment option for adult patients with previously treated Philadelphia chromosome positive (Ph+) chronic myeloid leukaemia (CML). The NDA was based on efficacy and safety data from Study 200, a single-arm study of bosutinib in over 500 patients with previously treated Ph+ CML, including patients resistant or intolerant to imatinib as well as patients who were previously treated with dasatinib or nilotinib.

Gone to the dogs? New therapies for the treatment of dog cancer

The convergence of Pfizer and AB Science in the tyrosine kinase inhibitor market is an interesting conundrum.

The market for tyrosine kinase inhibitors (TKI) is one that I keep an active interest in having worked in new product development and marketing at Novartis Oncology bringing Gleevec to market in CML and GIST.

I was, therefore, fascinated to read that AB Science, a fifty person, privately held company based in Paris, launched the first targeted cancer therapy for dogs in June this year. Masivet (masitinib) is a TKI with approval by the European Medicines Agency (EMEA) for the treatment of non-resectable grade 2 or 3 mast cell tumors (mastocytoma) in dogs. Up to 20 to 25 percent of skin tumors in dogs and cats are mast cell tumors

In clinical trials, Masivet increased the survival time of dogs by 300 days compared to placebo, and after two years the survival rate was 2.5 times that of dogs receiving placebo. The drug also demonstrated increased progression free survival. Masitinib is currently under review by the FDA veterinary division for approval in the U.S..

c-kit receptor

Mechanismus rezistence k TKIs

- **Rezistence je multifaktoriální proces**

BCR-ABL kinase domain mutations

TKI resistance (imatinib)

BCR-ABL overexpression

Src kinase overexpression

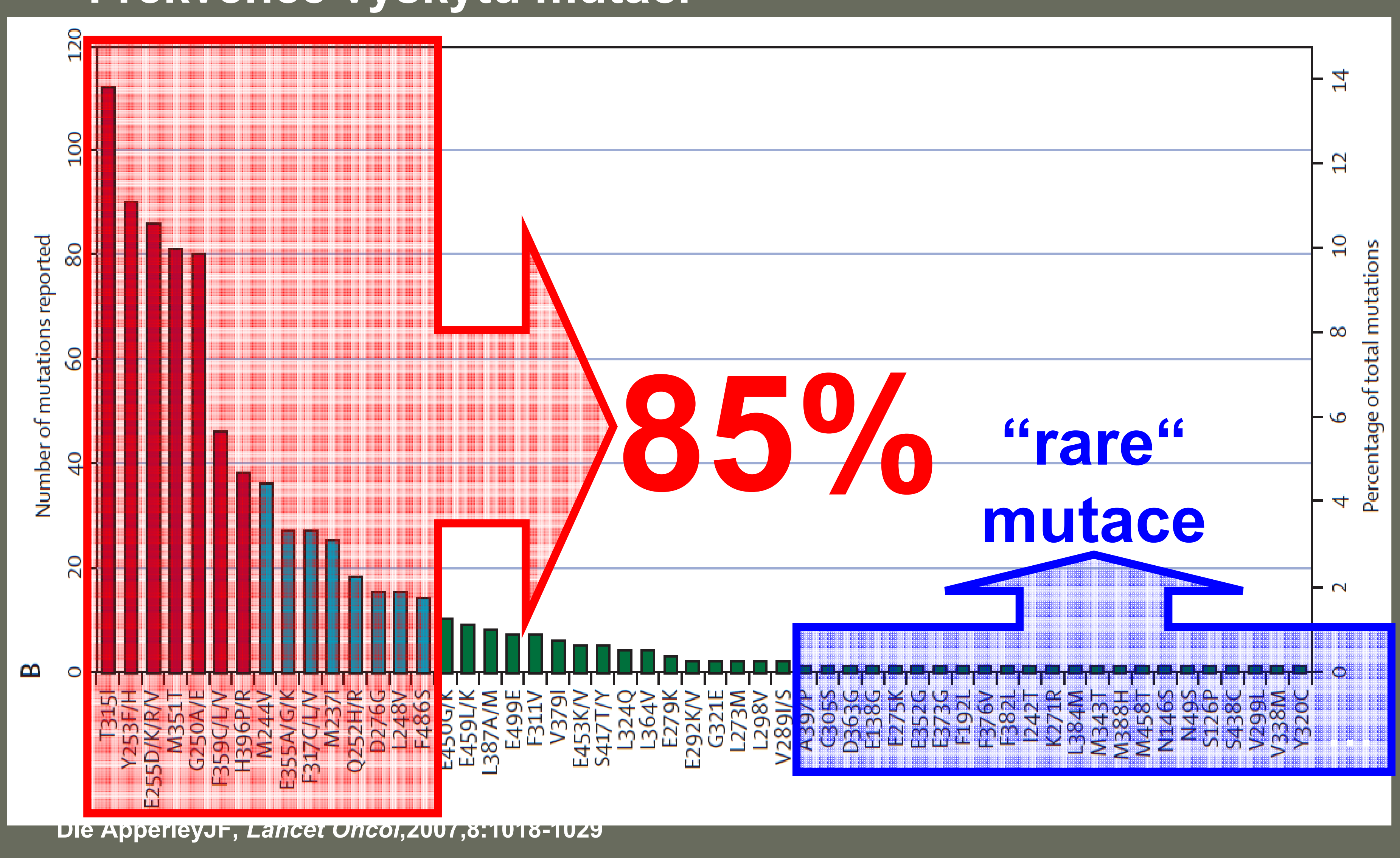
Altered expression/activity of imatinib transporters (*hOCT1, ABCB-1*)

Leukemic stem cell resistance
(activation of β-katenin pathway, loss of p14ARF)

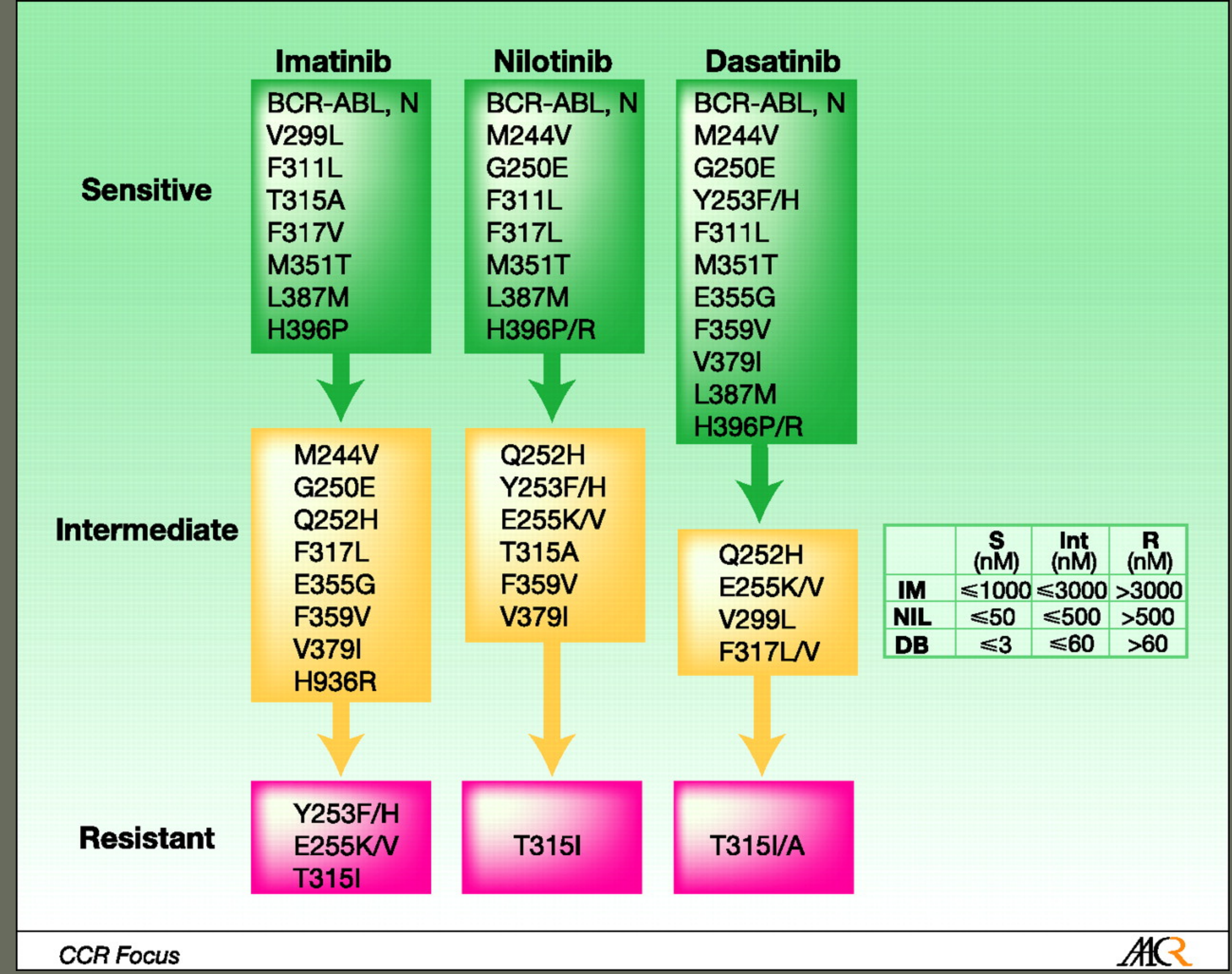
Adapted from Martinelli et al., *Haematologica* (2008);2:8-12

Mutace v kinázové doméně *BCR-ABL*

- **Frekvence výskytu mutací**



Dle ApperleyJF, *Lancet Oncol*,2007,8:1018-1029



Milojkovic D , Apperley J Clin Cancer Res 2009;15:7519-7527

AACR Clinical Cancer Research

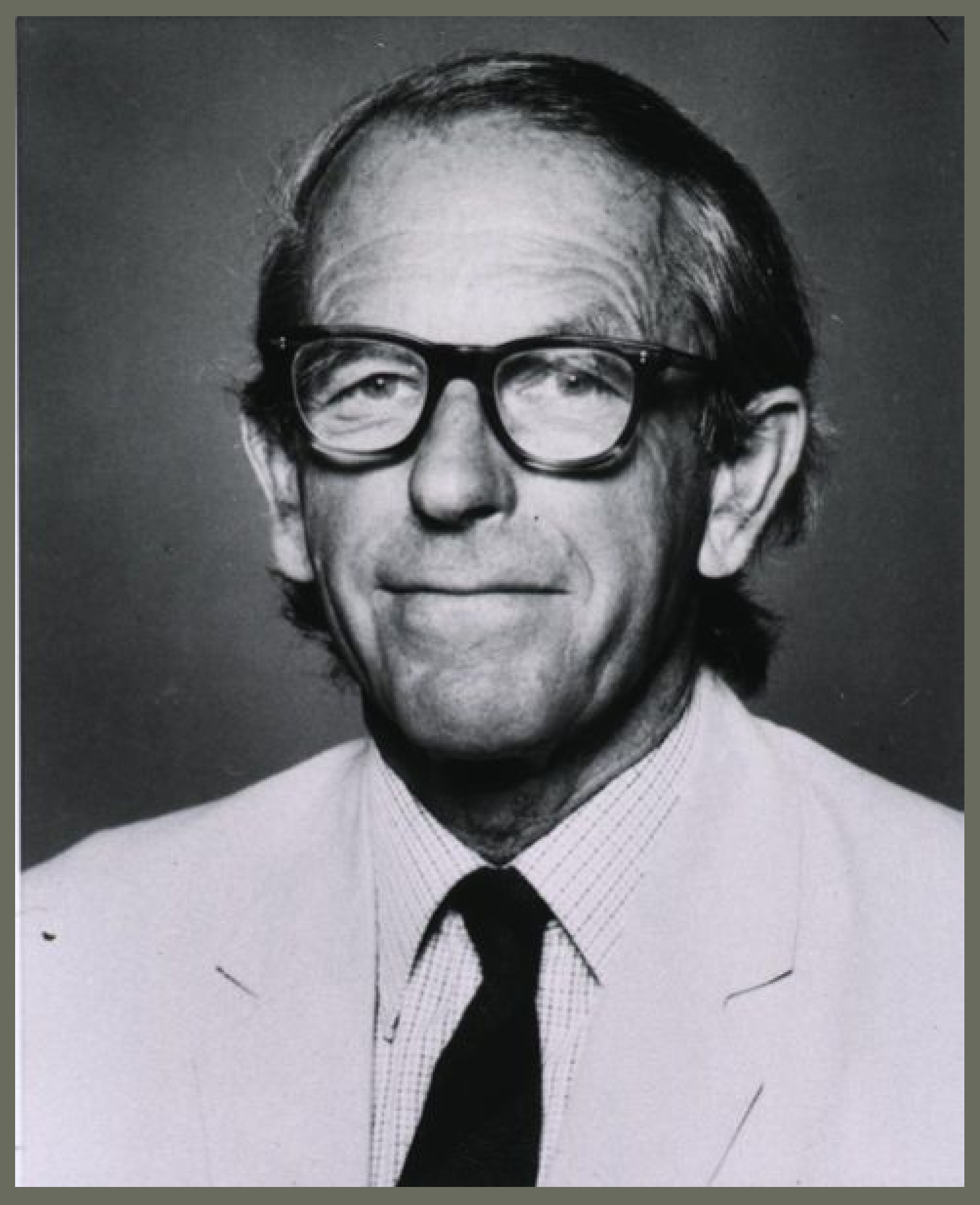
Metody detekce mutací v KD *BCR-ABL*

Methods	Sensitivity (%)	Quantitative	Availability
Direct sequencing	15-25	No	+++
Subcloning and sequencing	5-10	Yes	+
D-HPLC	0.1 - 10	No	++
Pyrosequencing	5	Semi-quantitative	++
Double-gradient denaturing electrophoresis	5	No	+
High Resolution Melting analysis	5	No	+
Multiplex SNP and mass spectrometry	1.3-5	Yes	+
Fluorescence PCR and PNA clamping	0.2	Semi-quantitative	+
TaqMan-based RQ-PCR	1 – 0.01	Yes	+
Polymerase colony assay	0.01	Yes	+
Nanofluidic plattform	0.01	Yes	+
Allele-specific oligonucleotide PCR	0.01	Yes	++

sensitivita

Sekvenace DNA

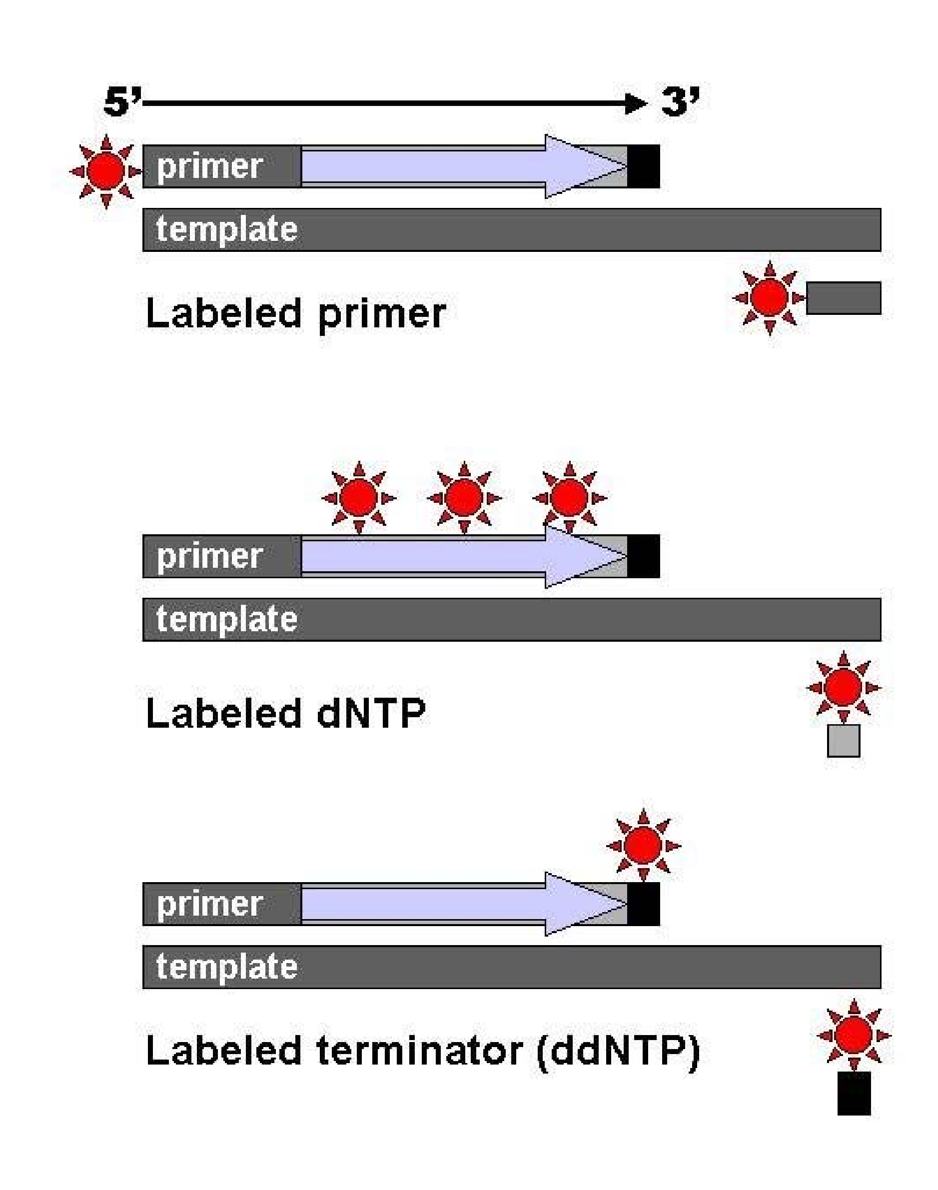
Počátky sekvenačních metod sahají do roku 1977, kdy byly téměř současně uvedeny dvě metody, Sangerova a Maxamova-Gilbertova. Sangerova metoda se během let ukázala jako efektivnější a až donedávna byla v laboratořích téměř výlučně používána. S pokrokem vědy se ale zvedají nároky i na sekvenační metody, zpracovávají se větší objemy dat a je třeba, aby sekvenace byla nejen přesná, ale i levná a rychlá. Proto se vedle klasické Sangerovy metody začínají objevovat „metody nové generace“ (metody pyrosekvenace od 1996, SOLiD, Solexa a další)

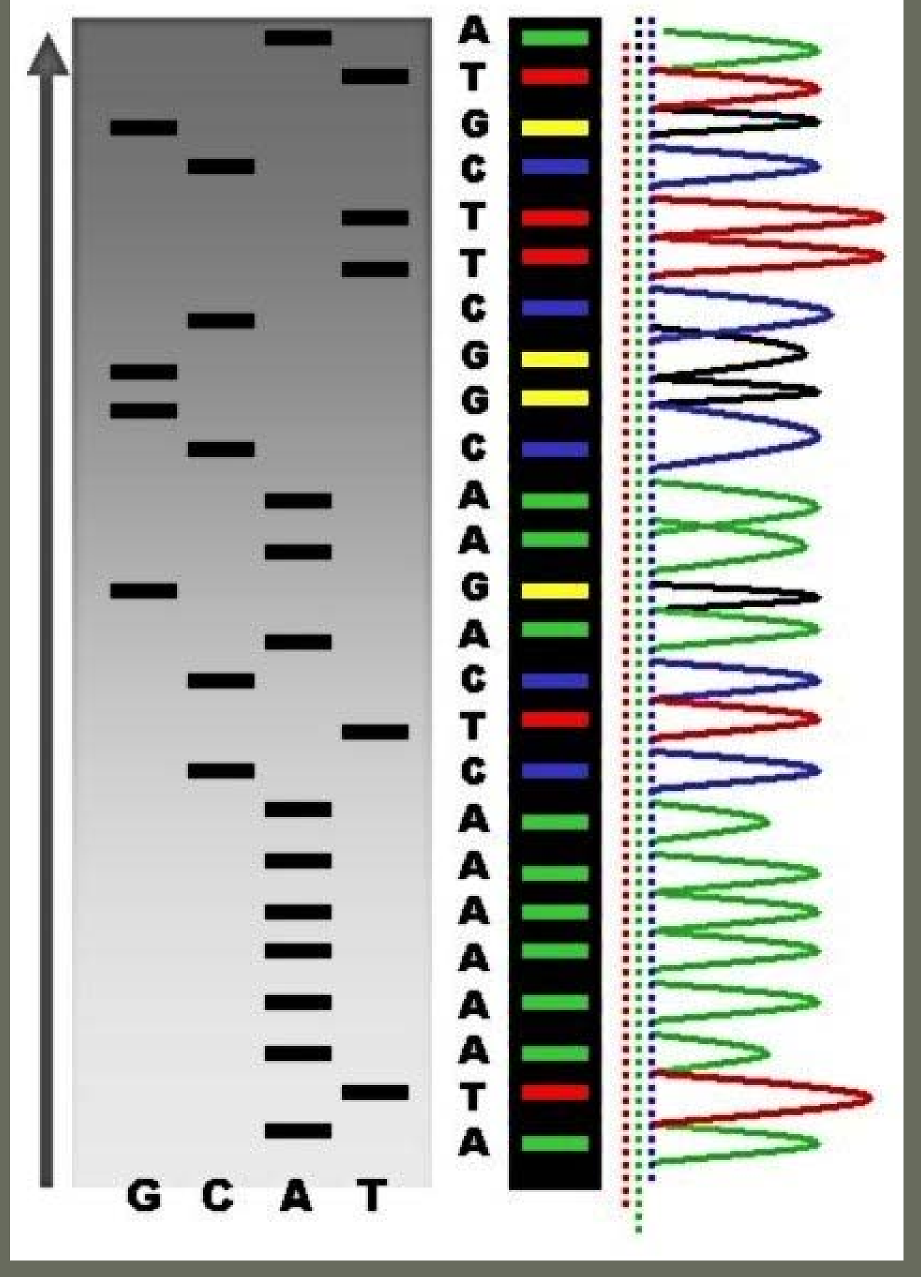


Frederick Sanger

Sekvenace DNA

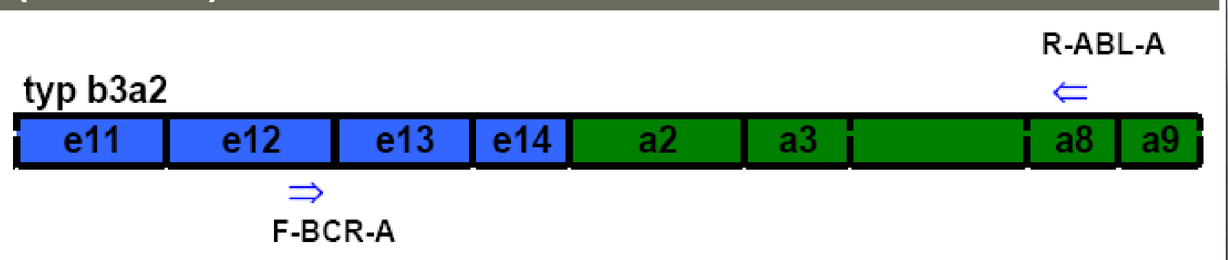
- **chemická** (Maxamovo-Gilbertovo sekvenování) - fragmenty DNA terminálně značené pomocí 32P - vystaveny působení chemických činidel, štěpících specificky v určitém místě molekuly DNA
- **enzymová** (Sangerovo sekvenování) - automatické sekvenování
- První část - standardní PCR probíhá se dvěma primery v jedné reakci. V druhé části probíhá vlastní asymetrická PCR, kdy je v jedné reakci použit vždy jen jeden primer. Dochází tak k amplifikaci jen jednoho ze dvou řetězců.
- V reakční směsi jsou spolu s dNTPs použity i jejich fluorescenčně značené analogy **dideoxyribonukleozidtrifosfáty** (ddNTPs) - koncové inhibitory elongace→různě dlouhé značené fragmenty DNA.
- fluorescenčně značené produkty jsou následně elektroforeticky rozděleny v kapiláře a detekovány pomocí laserového detektoru. Laserem excitované fluorescenční fluorochromy emitují záření různých vlnových délek
- Pořadí nukleotidů je automaticky odečítáno detektorem
- Sekvence je následně komplexně analyzována speciálním softwarovým programem.


5'
3'
primer
template
Labeled primer
primer
template
Labeled dNTP
primer
template
Labeled terminator (ddNTP)


A
T
G
C
T
T
C
G
G
C
A
A
G
A
C
T
C
A
A
A
A
A
A
T
A
G C A T

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)

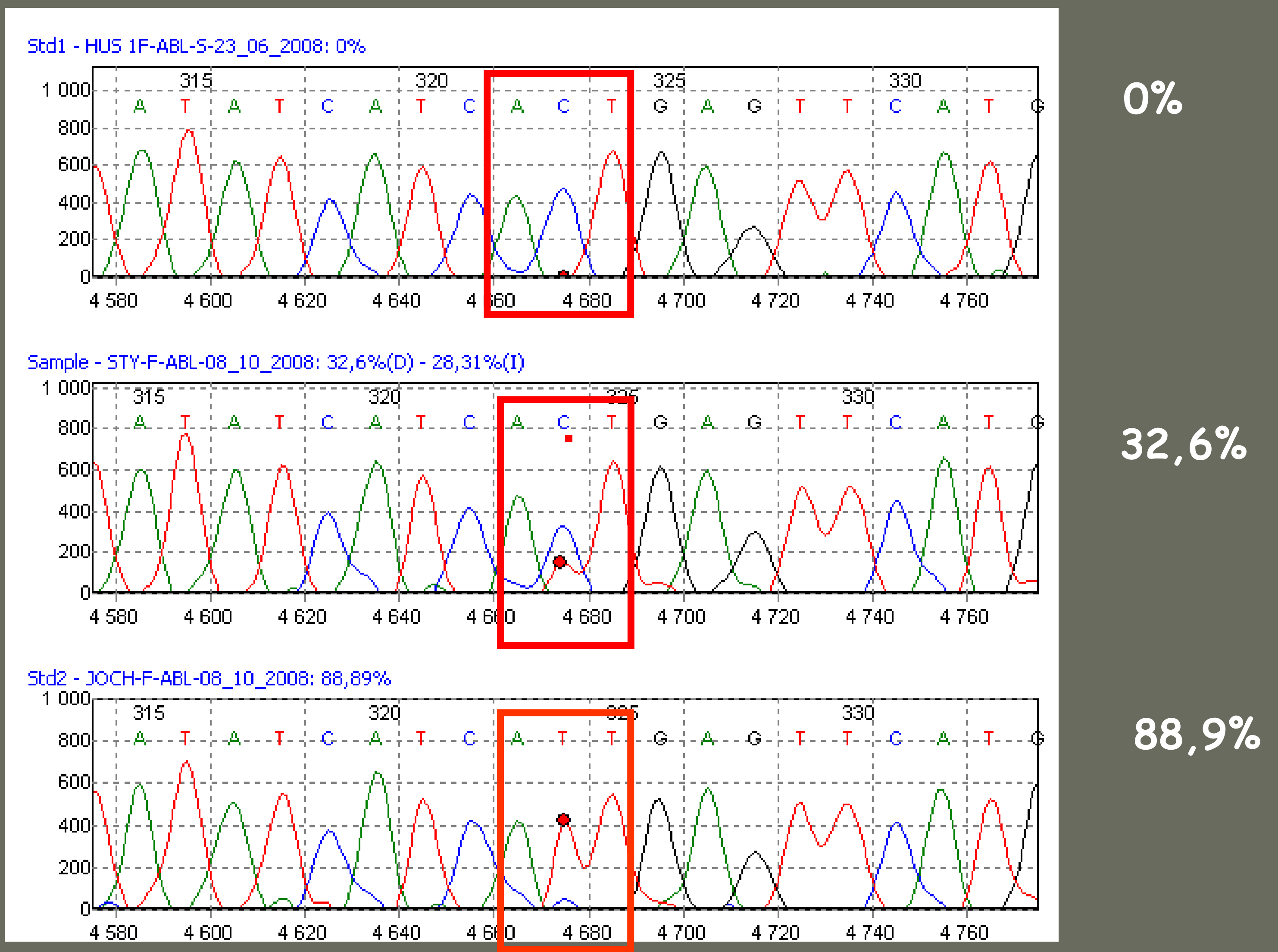


Conventional direct sequencing: by using a BigDye®Terminator v1.1 Seq.kit /AbiPrism 310 System/, exon4-exon8, Primer: ABL-ALT Comparison with the GeneBank mRNA sequence X16416

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.*

22/9 PB T315I 32,60%



0%

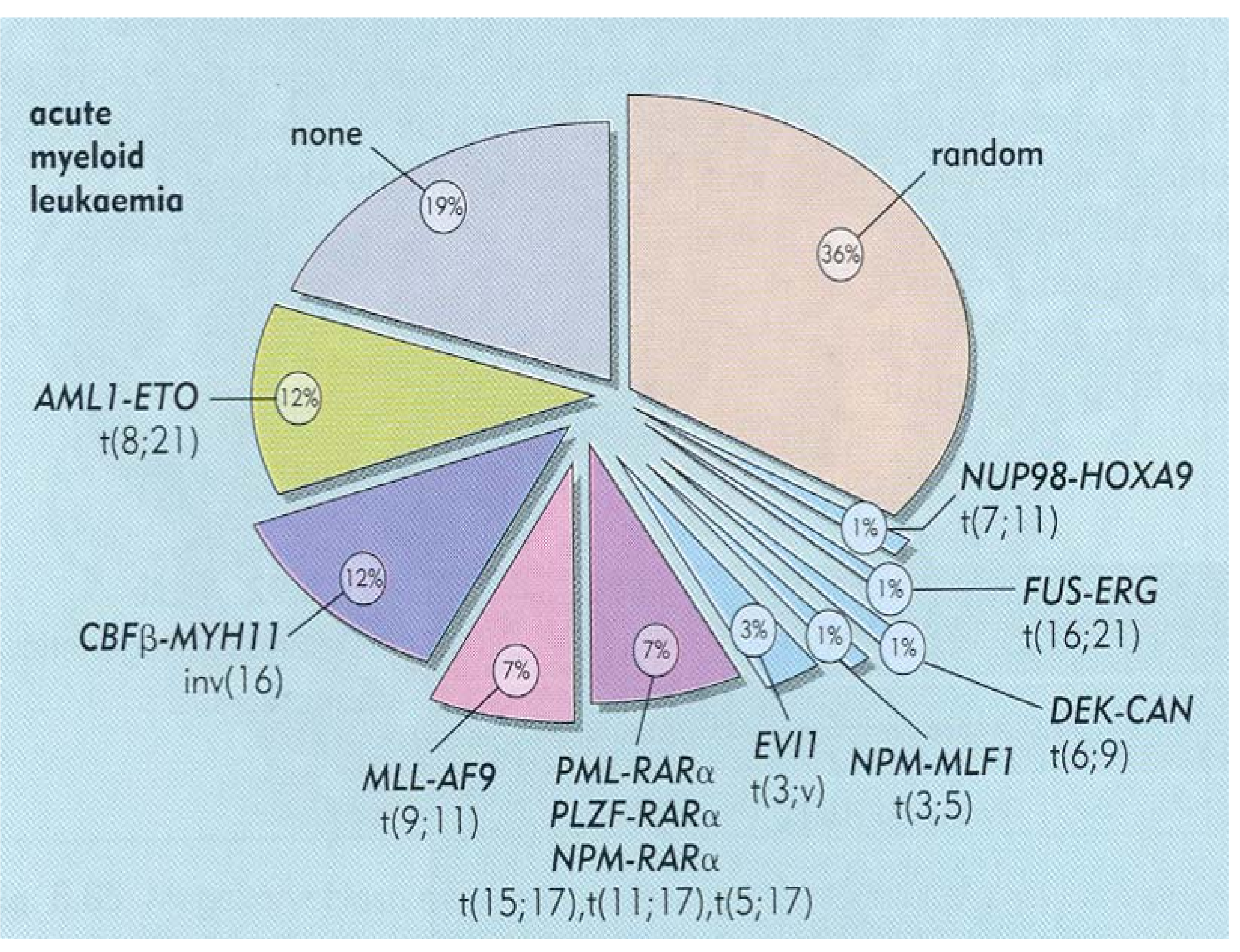
32,6%

88,9%

Akutní myeloidní leukémie

Akutní myeloidní leukémie

- **Incidence 3 / 100 000 / rok** (Evropská standardní populace)
- **heterogenní skupina onemocnění**
- **získané chromozomální aberace v hematopoetických progenitorech**
- **40-50% případů AML s molekulárním markerem**
- **prudká manifestace, porucha vyzrávání a porucha diferenciace**



acute
myeloid
leukaemia
none
19%
random
36%
AML1-ETO
12%
t(8;21)
CBFβ-MYH11
12%
inv(16)
MLL-AF9
7%
t(9;11)
PML-RARα
PLZF-RARα
NPM-RARα
7%
t(15;17),t(11;17),t(5;17)
EVI1
3%
t(3;v)
NPM-MLF1
1%
t(3;5)
NUP98-HOXA9
1%
t(7;11)
FUS-ERG
1%
t(16;21)
DEK-CAN
1%
t(6;9)

AML s rekurentními genetickými abnormalitami

1) AML s t(8;21)(q22;q22) gen *RUNX1/RUNXT1 (AML1/ETO)*

2) AML s inv(16)(p13.1q22) nebo t(16;16)(p13.1;q22) gen *CBFB/MYH11*

3) APL s t(15;17)(q22;q12) gen *PML/RARa (bcr1, bcr2, bcr3)*

4) Aberace genu *MLL/** *(*partneři AF4, AF6, AF9, ELL, ENL, …)*

Nespecifické prognostické markery

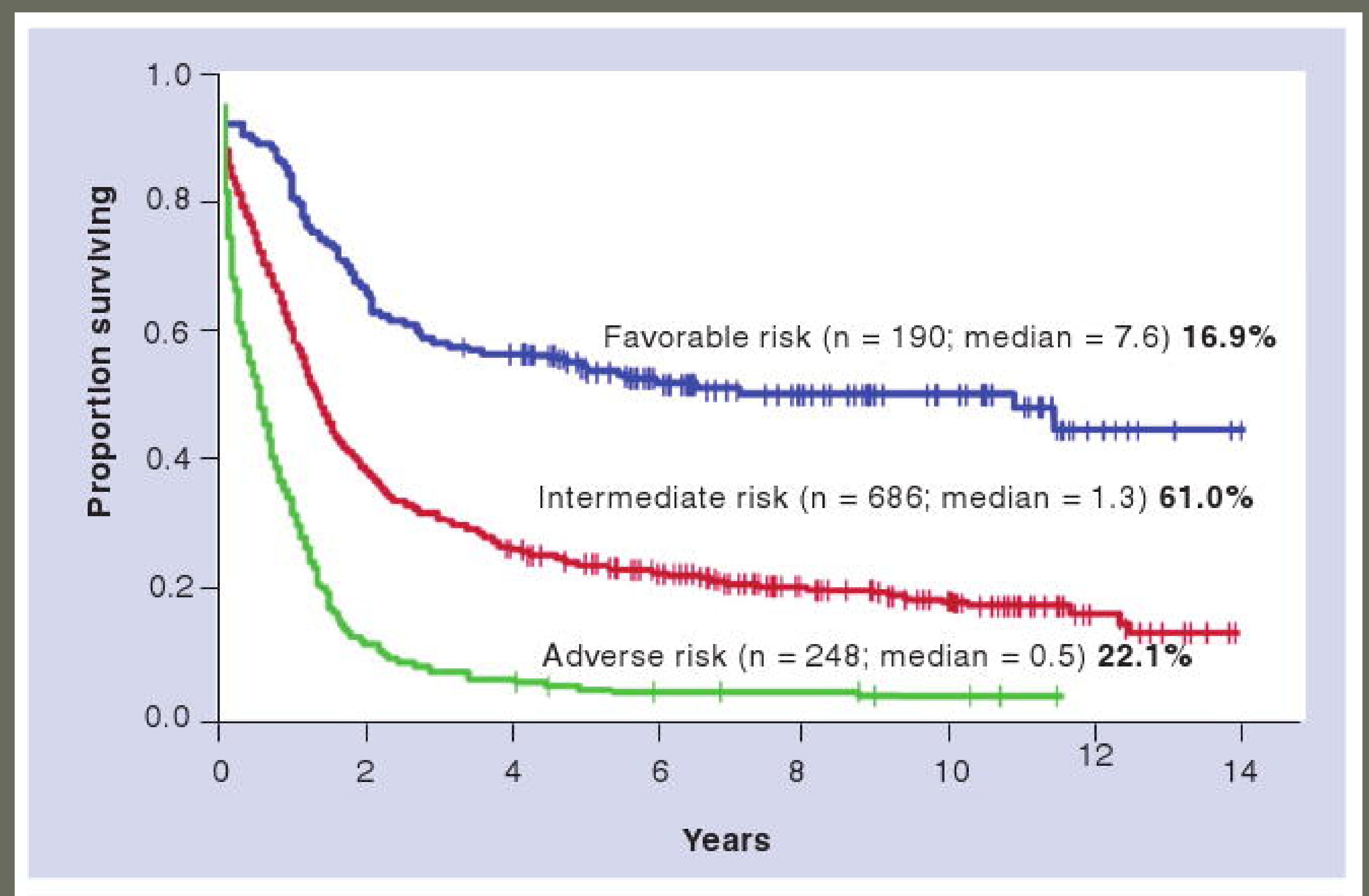
1) Mutace v genu *NPM1*

2) Mutace v genu *CEBPA*

3) Mutace v genu *FLT3* (*FLT3*-ITD nebo *FLT3*-TKD)

4) Mutace v genu *IDH1* a *IDH2*

5) Parciální tandemové duplikace v genu *MLL* (*MLL*-PTD)

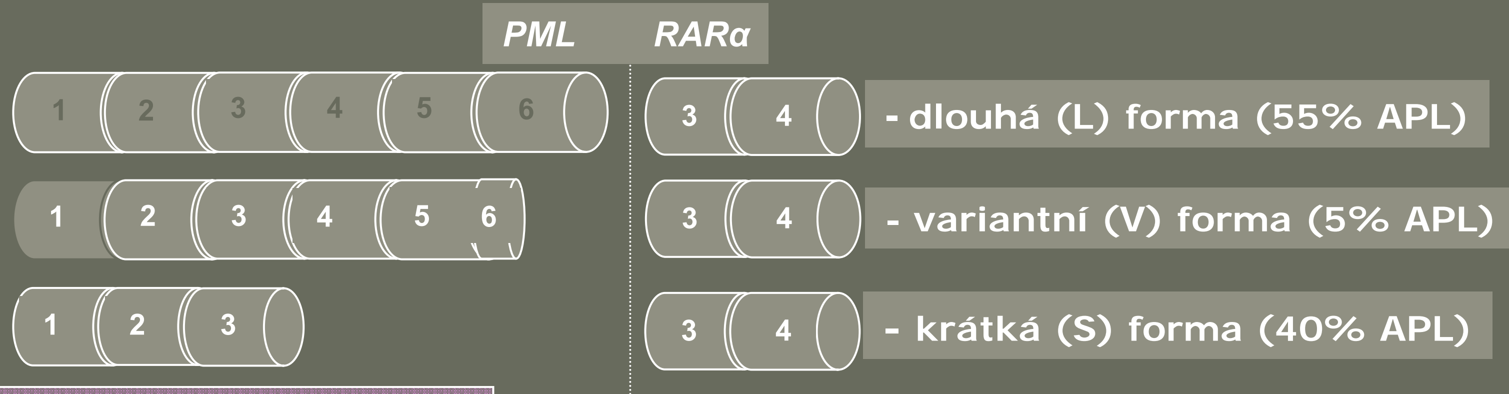


Walter MJ et al. Next-generation sequencing of cancer genomes: back to the Future. *Per Med*. 2009 November 1; 6(6): 653. doi:10.2217/pme.09.52.

gen *PML/RARa*

15 t(15;17) 17

- typicky výskyt u akutní promyelocytární leukémie (APL) (dle FAB – AML M3)
- t(15;17)(q22;q12) – detekována u více jak 99% všech APL
- *PML – Promyelocytic Leukemia* gene
- *RARa – Retinoic Acid Receptor alpha* gene
- podle zlomu v genu PML rozlišujeme tři základní typy transkriptů
- pro MRN je nutná specifikace zlomu



Frekvence

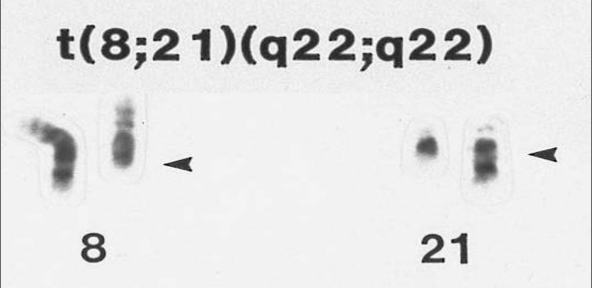
- 10% případů AML
- prognóza – příznivá

Protinádorová terapie

- Chemoterapie (léčiva s cytotoxickým účinkem)
- Transplantace hematopoetických progenitorů
- Hormonální léčba (nádory odvozené od hormonálně dependentní tkáně)
- **Diferenciační léčba retinoidy (stimulace diferenciace maligní buňky)**
- Bioterapie (hemopoetické růstové faktory, interferony a další cytokiny)
- Radioterapie
- Biologická léčba (MCA)
- Inhibitory konstitutivně aktivovaných tyrozinkináz

gen *RUNX1/RUNX1T1* *(AML1/ETO)*

- t(8;21)(q22;q22)



- *RUNX1* - Runt-Related Transcription Factor 1 (9 exonů)
- *RUNX1T1* – Runt-Related Transcription Factor 1; translated to 1 (13 exonů)

Fúzní gen

- typický je zlom za exonem 5 genu *RUNX1* a před exonem 2 genu *RUNX1T1* → protein obsahuje prvních 177 amk z RUNX1 a plnou délku RUNX1T1 proteinu

Frekvence

- 5% případů AML - typický výskyt u AML M2 (dle FAB) – až 40% AML M2
- prognóza – příznivá

gen *CBFB/MYH11*

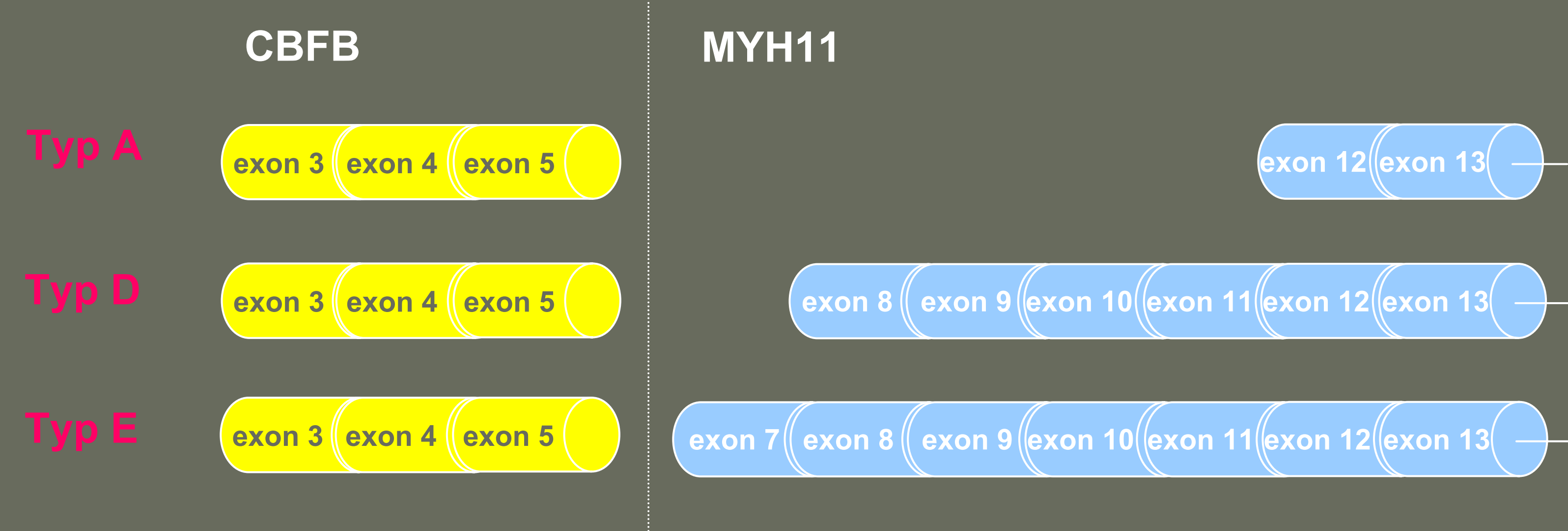
- inv.(16)(p13.1q22) nebo méně často t(16;16)(p13.1;q22)
- *CBFB - Core Binding Factor b subunit* gene
- *MYH11 – Myosin Heavy Chain 11* gene

Frekvence

5-10% AML, subtyp AML M4, příznivá prognóza

Fúzní gen

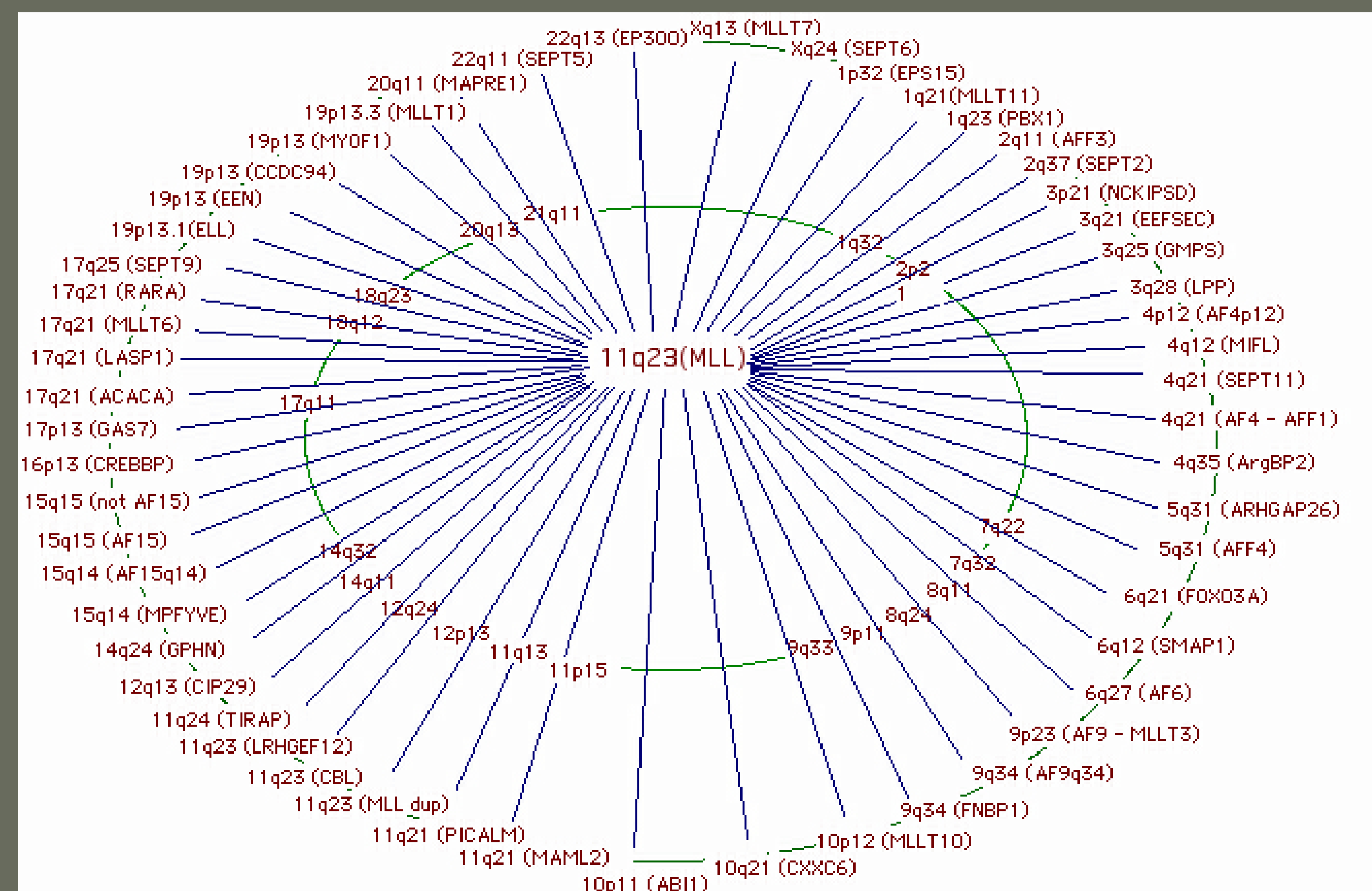
- typy transkriptů podle místa zlomu v genu *MYH11*, nutná specifikace zlomu
  85% pozitivních pacientů nese typ A
  5% pacientů nese typ D nebo E, ostatní typy jsou sporadické



gen *MLL/* (ALL-1, HRX, HTRX)*

MLL – mixed lineage leukemia **gene**

- **lokalizace zlomu v 11q23**
- **jedním z mechanizmů iniciujících zlom je inhibice enzymu topoizomerázy II (primární a sekundární leukémie)**
- **37 exonů, protein 3969 AMK**
- **tzv. promiskuitní gen – množství (80) potenciálních partnerů pro tvorbu FG**



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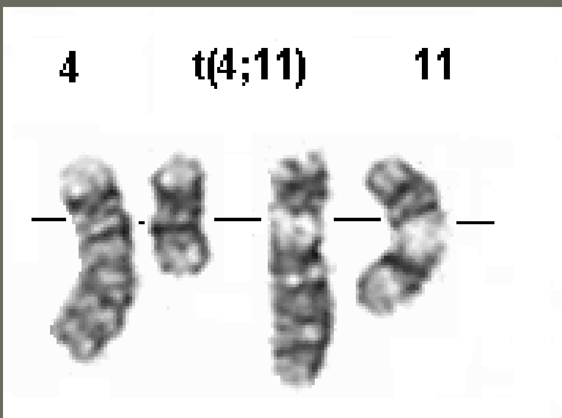
Partneři genu *MLL*

gen *AF4* → *MLL/AF4* t(4;11) – nejčastější translokace detekovaná u pacientů s ALL (3-8%)
gen *AF6* → *MLL/AF6* t(6;11)
gen *AF9* → *MLL/AF9* t(9;11) – 2% dospělých AML
gen *ENL* → *MLL/ENL* t(11;19)
gen *ELL* → *MLL/ELL* t(11;19)



- prognóza – nepříznivá

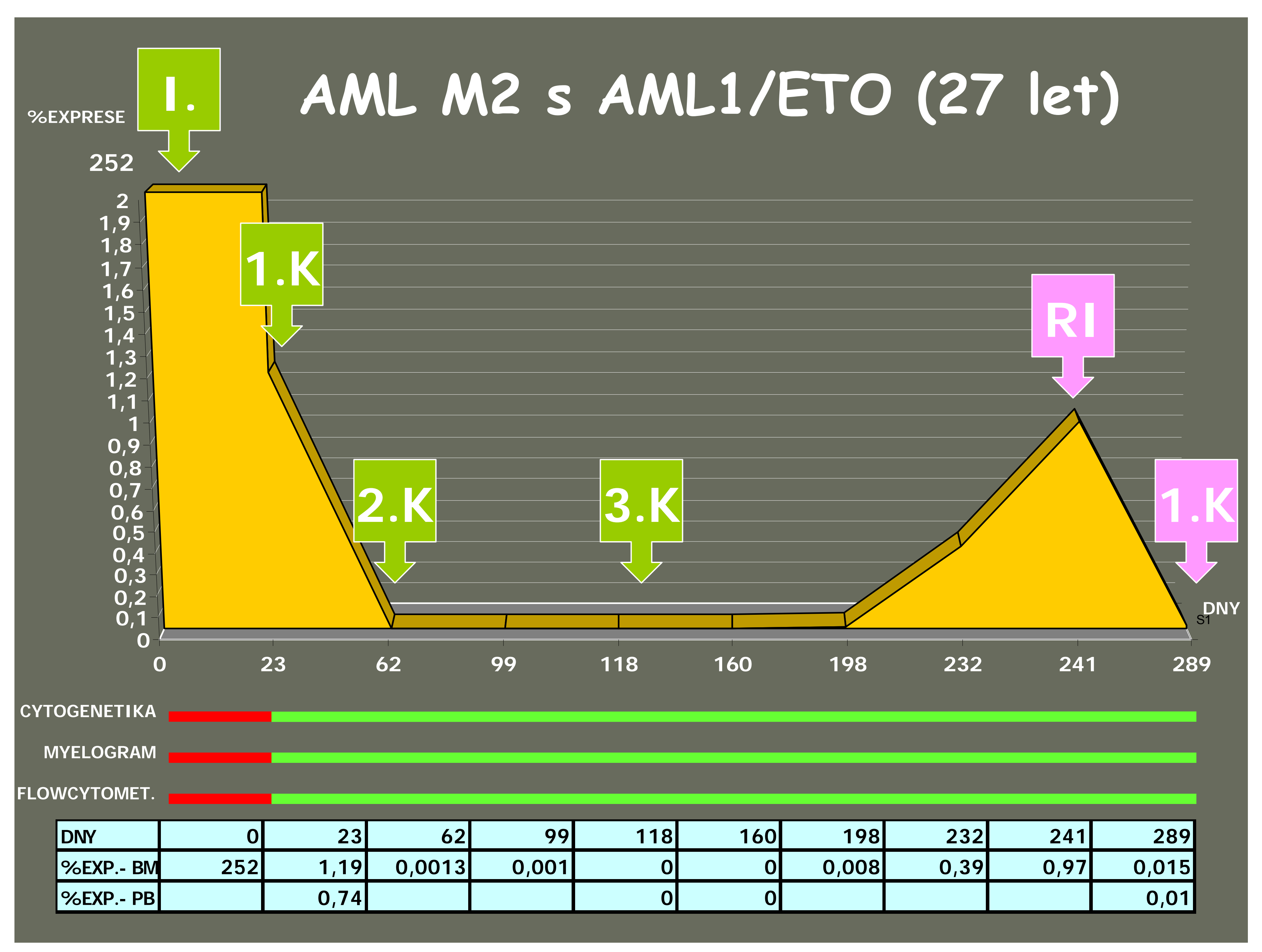
Detekce

Diagnóza

- multiplexová PCR reakce pro 6 nejčastějších fúzních genů
- při pozitivitě sada samostatných PCR reakcí vždy s jednou sadou primerů pro konkrétní FG
- pro ověření + identifikaci přesného zlomového místa - sekvenace

Sledování MRN

- míra exprese konkrétního FG je sledována metodou real-time PCR na úrovni mRNA (reverzní transkripce předchází RQ-PCR)



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

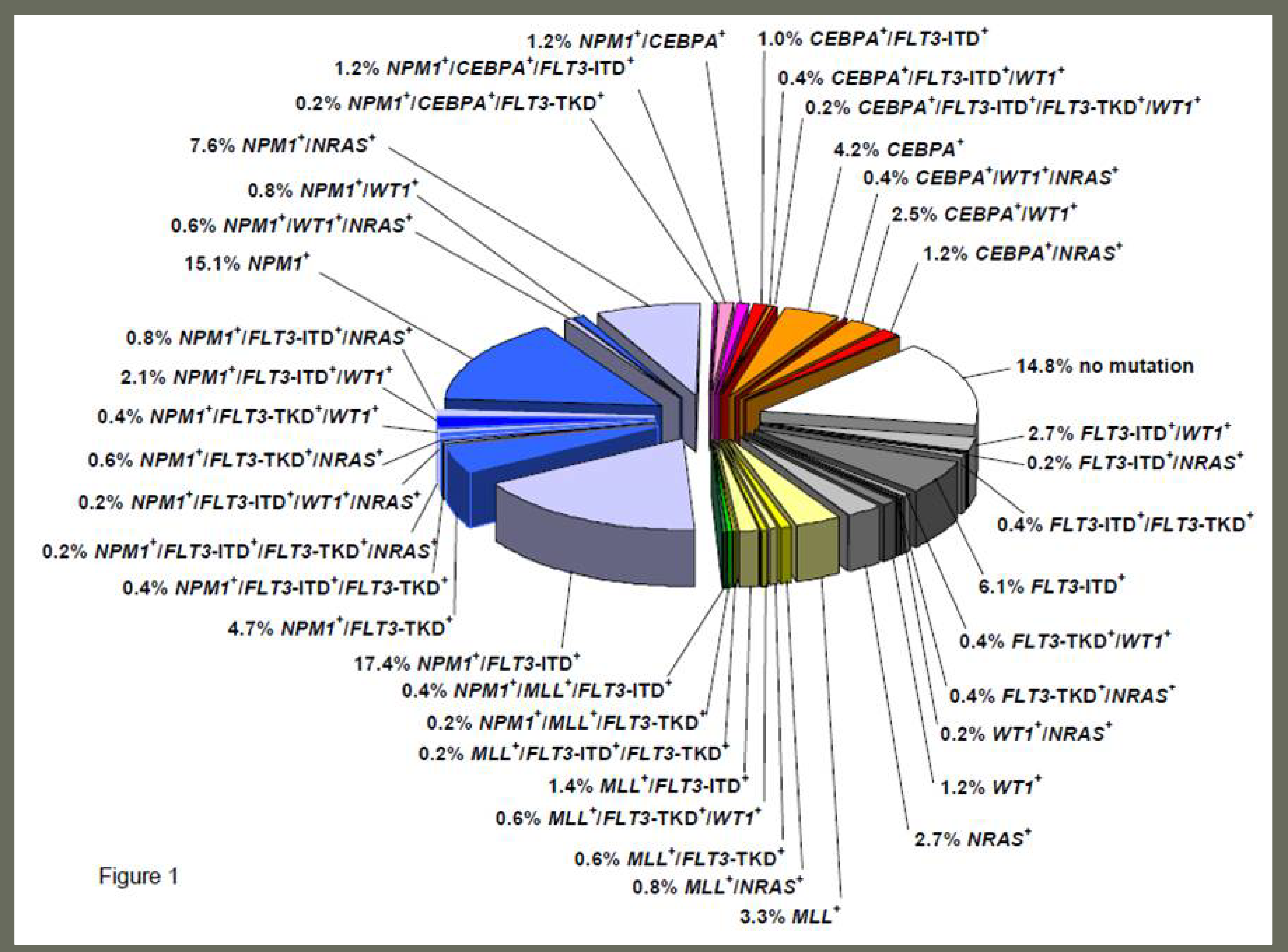


Figure 1

FLT 3

Gen

- mutován až u 20-30 % NK-AML
- lokalizace: 13q12
- velikost: 97,3 kb, 24 exonů

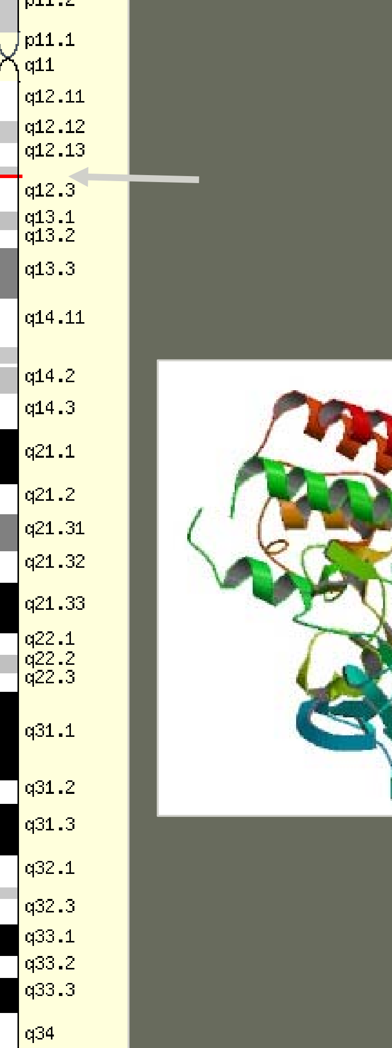
Protein

- 993 amk (112,8 kDa)
- tyrozin kinázový receptor tř. III.
- regulace hematopoézy

Mutace

- nejvýznamnější FLT3-ITD
- *in frame* mutace (exon 14,15, velikost 3 až 400 bp)
- bodové mutace (D385, exon 20)
- převážně heterozygotní

Prognóza: nepříznivá



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

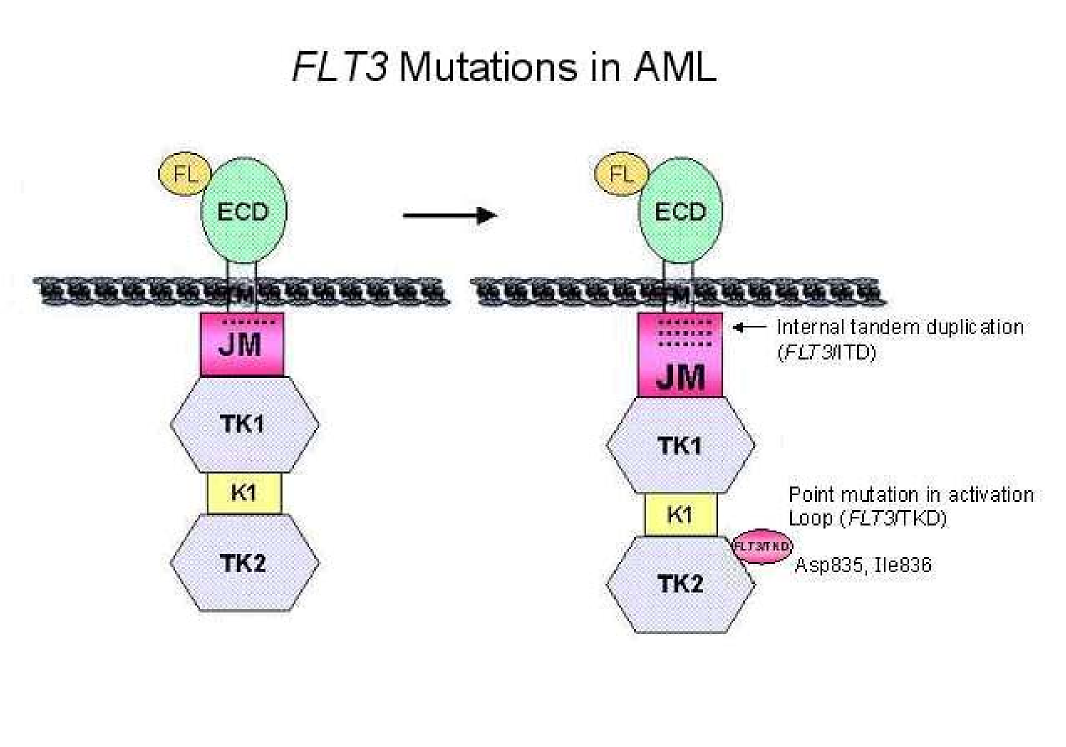
lokalizace 13q12

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/D835)

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.



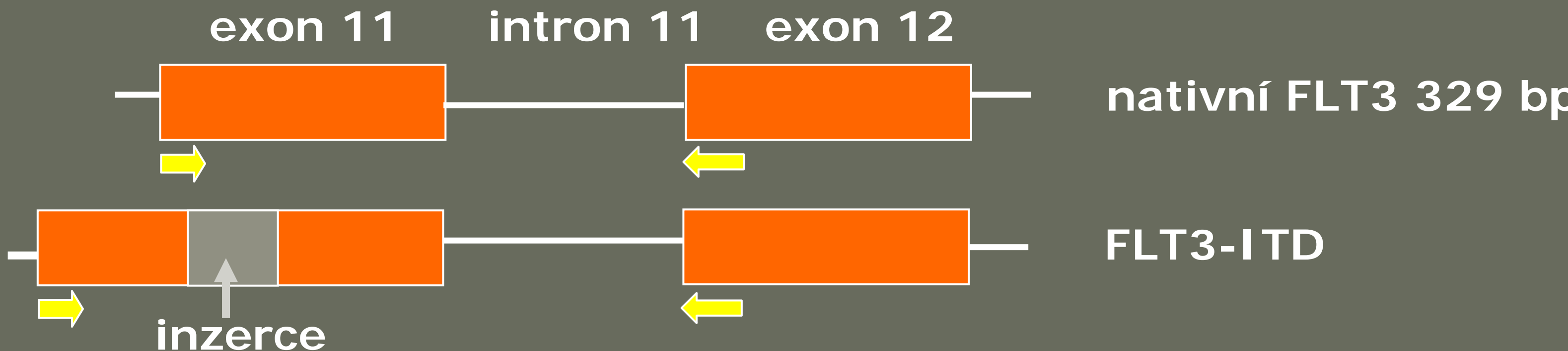
FLT3/ITD

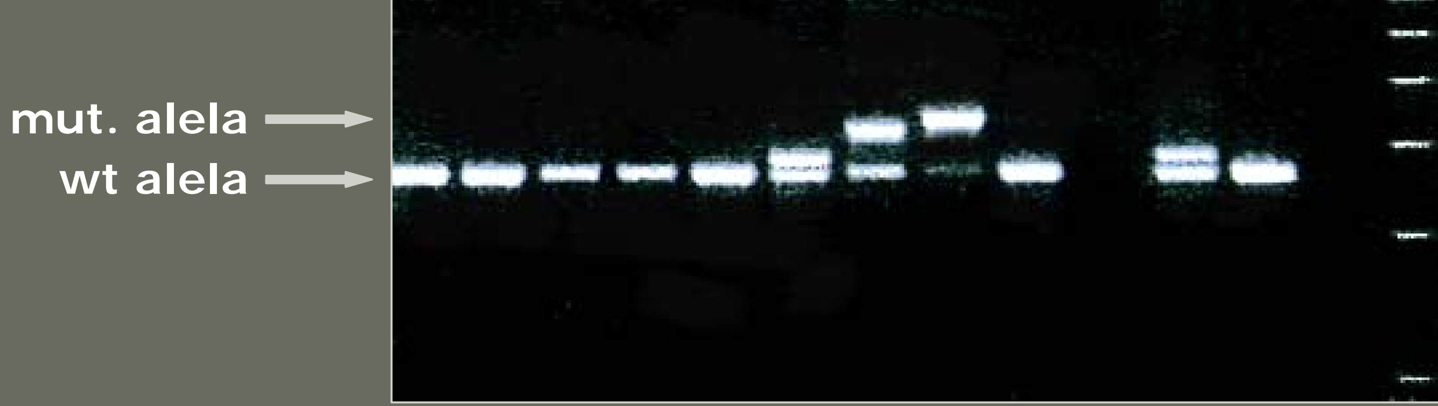
FLT/D835

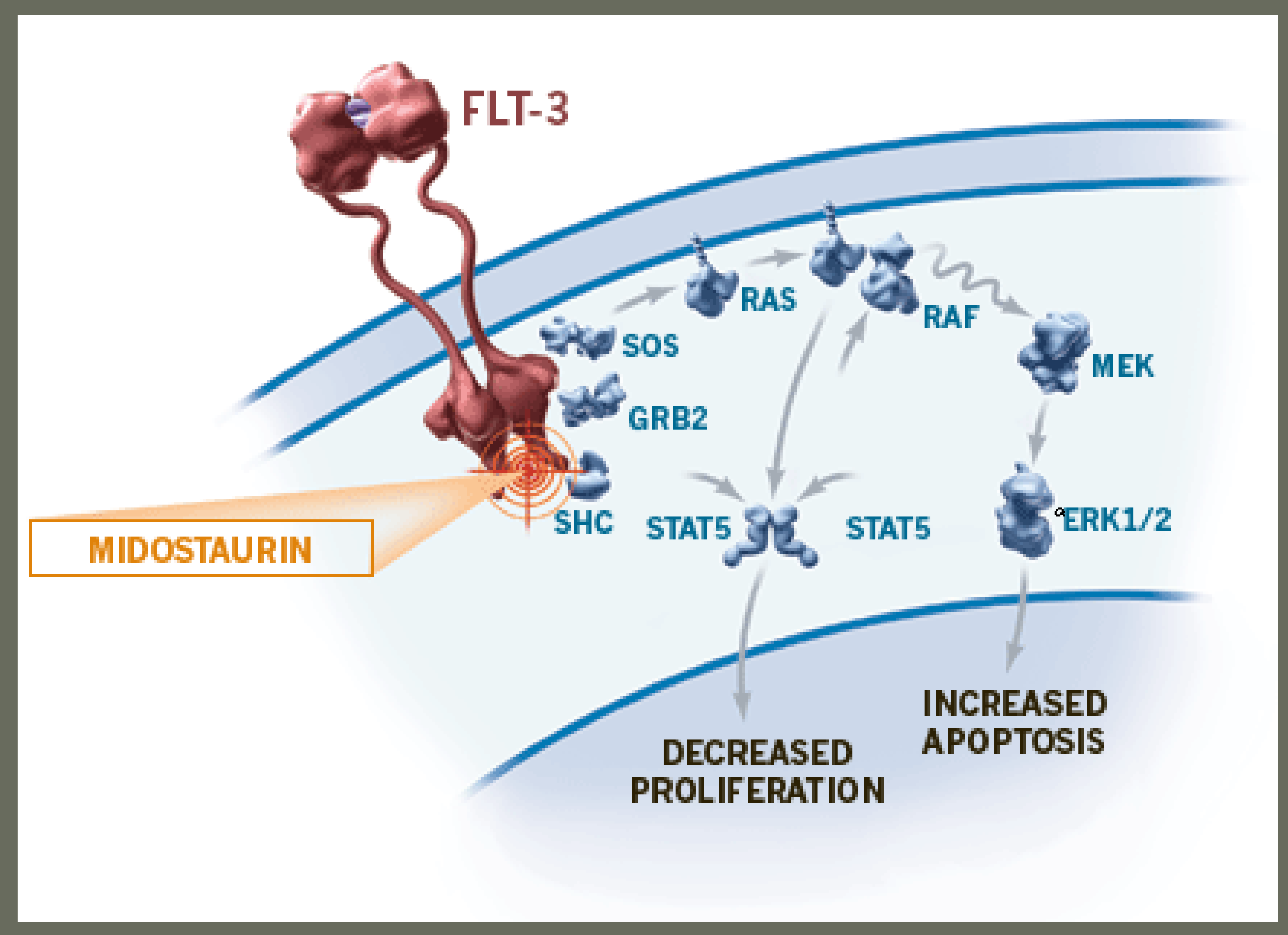
Detekce

Záchyt

- metodou PCR, vizualizace na 2 % agarózovém gelu







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

Nucleophosmin (NPM1)

•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 **translocatted** – formation of oncogenic fusion proteins

•**NPM gene contains 12 exons in maps to 5q35**

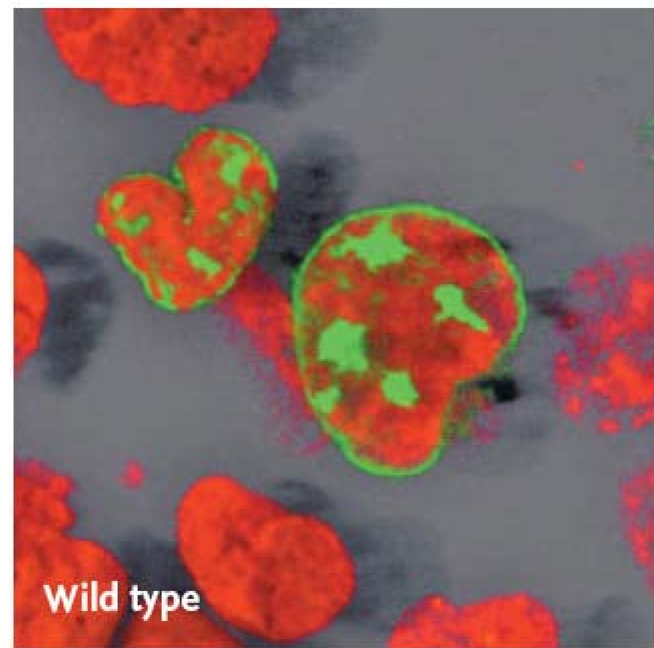
•C-terminus contains a short aromatic stretch with two tryptophans at positions 288 and 290, which are crucial for NPM binding to the nucleolus (nucleolar localization signal)

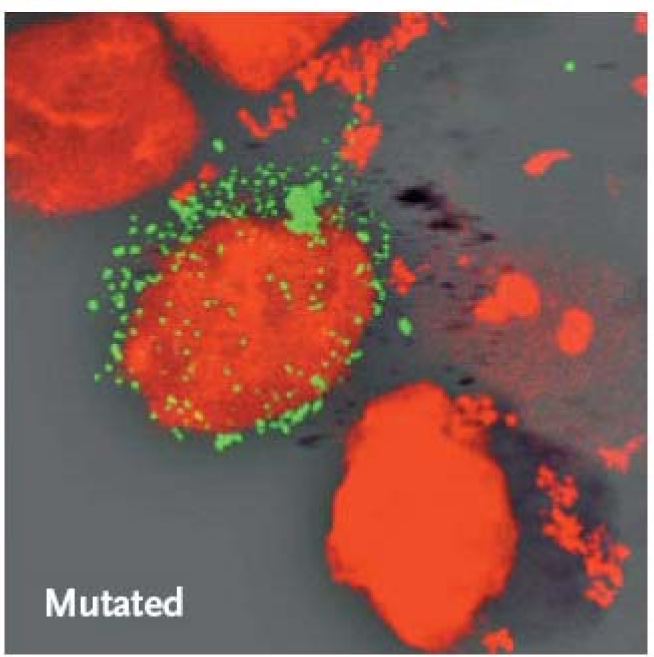
•Is more highly expressed in proliferating cells

•Is involved in the apoptotic response to stress and oncogenic stimuli and can modulate the activity and stability of the oncosupressor protein p53

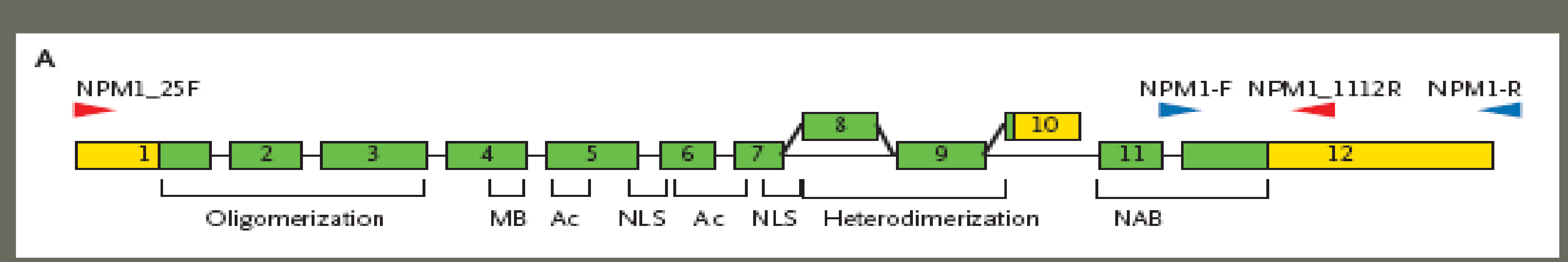
•**Mutations are consistently heterozygous, are restricted to exon 12, except for 2 cases: exon 11(2007) and exon 9(2006), about 50 molecular variants of mutations to date in AML with >95% at nucleotide position 960**

•**Mutation A duplicates a TCTG tetranucleotide at positions 956 to 959 75-80% of adult NPMc+ AML cases**





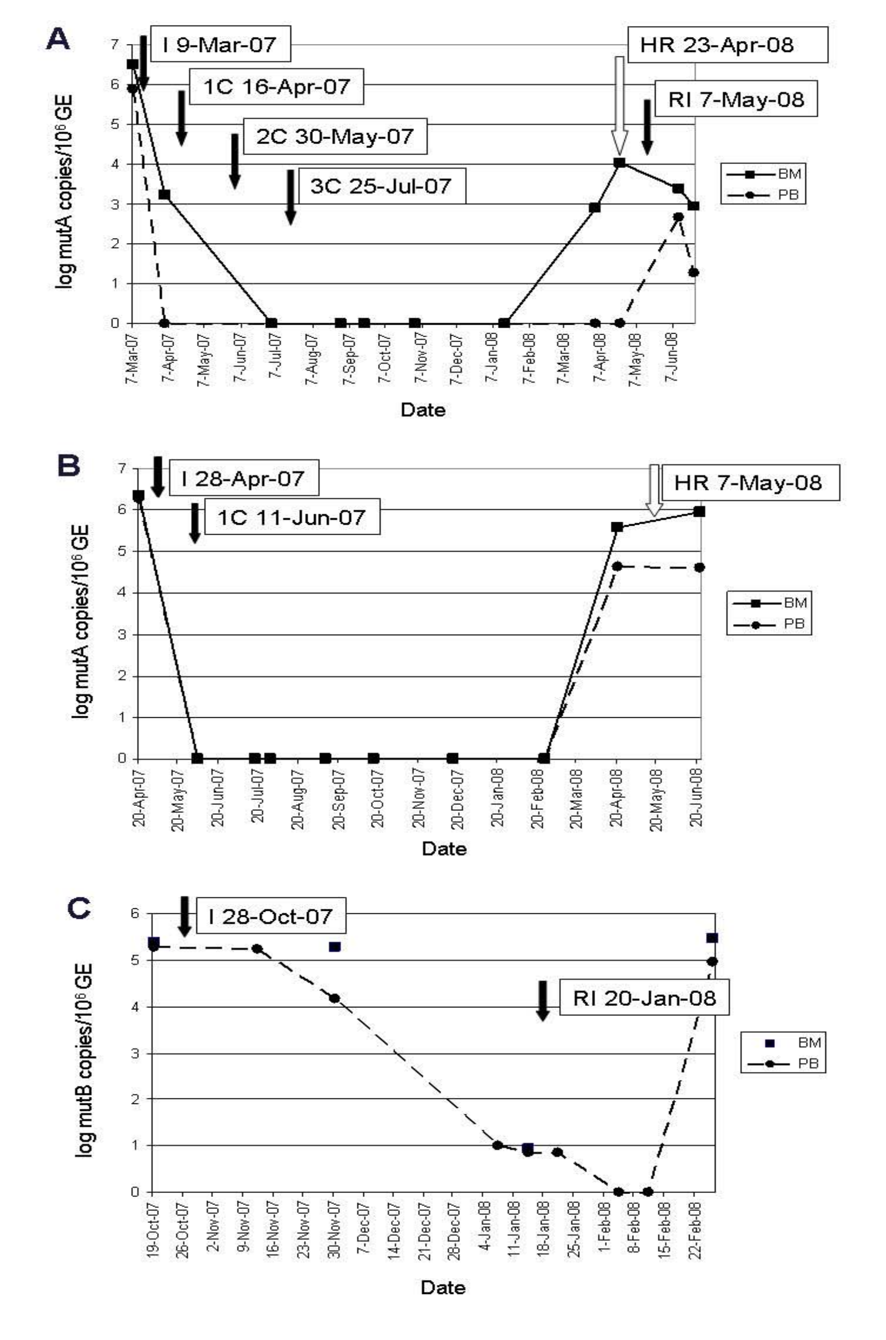
Falini, NEJM, 2005



B

Type of Mutation	GenBank Accession No.	Sequence	Predicted Protein
None (wild type)	NM_002520	GATCTCTG....GCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLWQWRKSL
Mutation A	AY740634	GATCTCTGTCTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCLAVEEVSLRK
Mutation B	AY740635	GATCTCTGCATGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCMAVEEVSLRK
Mutation C	AY740636	GATCTCTGCGTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCVAVEEVSLRK
Mutation D	AY740637	GATCTCTGCCTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCLAVEEVSLRK
Mutation E	AY740638	GATCTCTG....GCAGTCTCTTGCCCAAGTCTCTTTAAGAAAATAG	-DLWQSLAQVSLRK
Mutation F	AY740639	GATCTCTG....GCAGTCCCTGGAGAAAGTCTCTTTAAGAAAATAG	-DLWQSLEKVSLRK

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



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).

CEBPA

Gen

- mutace +/-10 % NK-AML
- lokalizace: 19q13.1
- velikost: 2,38 kb, 1 exon

Protein

- 358 amk (42 kDa)
- funkce - transkripční faktor
  - ovlivňuje diferenciaci granulocytů

Mutace

- mnohočetné mutace
- převážně heterozygotní
- lokalizované především na N konci
- mutace x polymorfismus

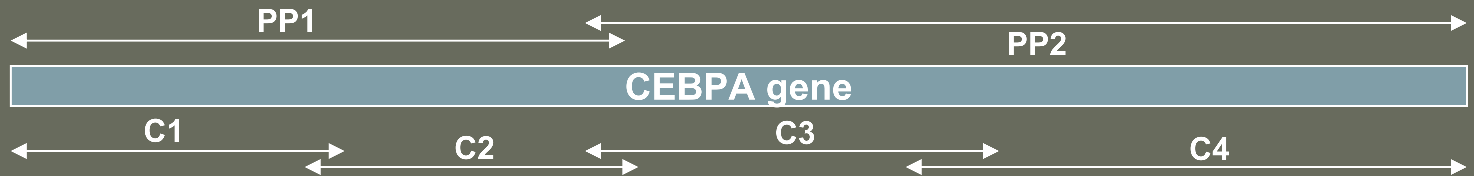
Prognóza: příznivá (?)

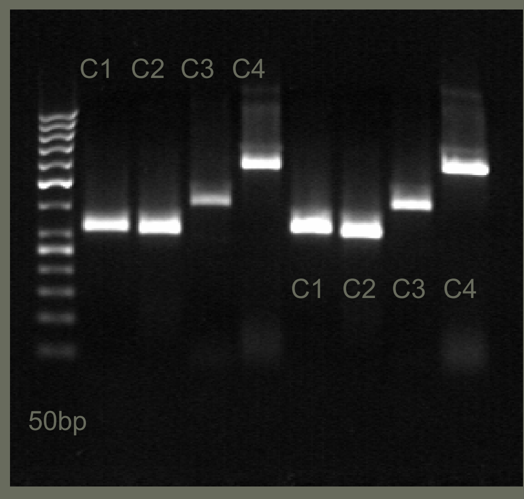
Chr 19
p13.3
p13.2
p13.13
p13.12
p13.11
p12
p11
q11
q12
q13.11
q13.12
q13.2
q13.31
q13.32
q13.33
q13.41
q13.42
q13.43

Detekce

Záchyt

- metodou High Resolution Melting Analysis



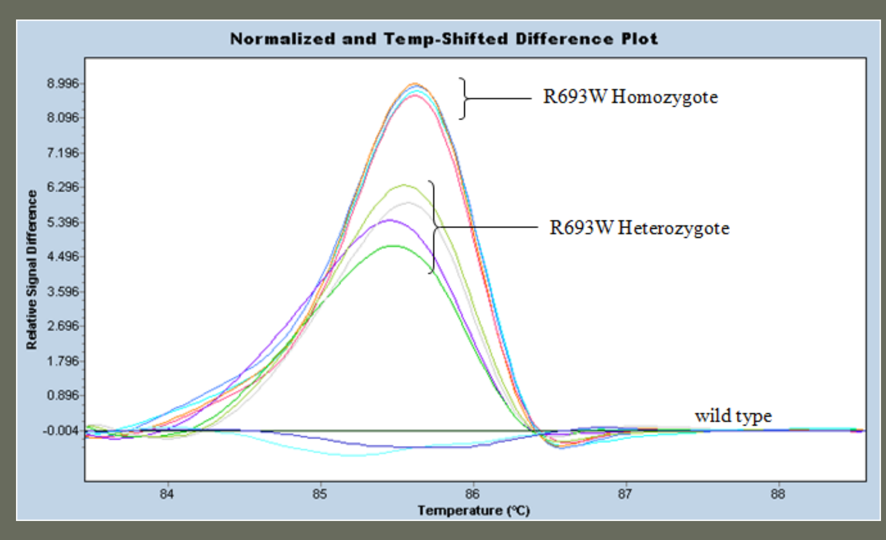


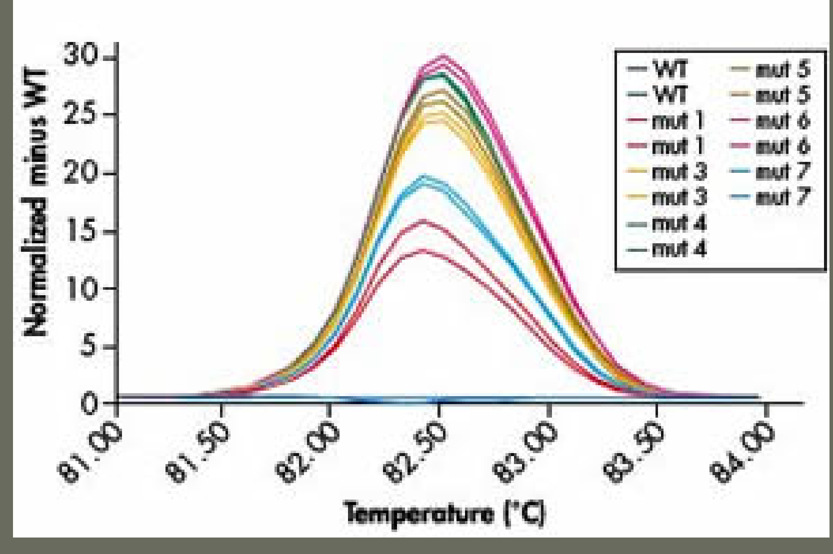
Reaction mixture C1-C4
- 5ul SensiMix HRM (Quantace, UK)
- 0.4ul EvaGreen Dye (Quantace, UK)
- 2x0.8ul specific primers (10uM)[1]
- 2ul distilled water
- 1ul DNA template

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

HRM (high-resolution melting)

- Měří rozdíl teplot tání mezi dsDNA se správně a chybně párovanými bazemi (polymorfismus nebo mutace)
- Princip – snížení vzájemné afinity obou řetězců DNA způsobené mutací (vazby mezi řetězci mají nižší energii) – snadno oddělitelné působením tepelné energie
- Screeningový test – typ variability stanovit sekvenováním





Další myeloproliferativní neoplázie

Chronic neutrophilic leukaemia

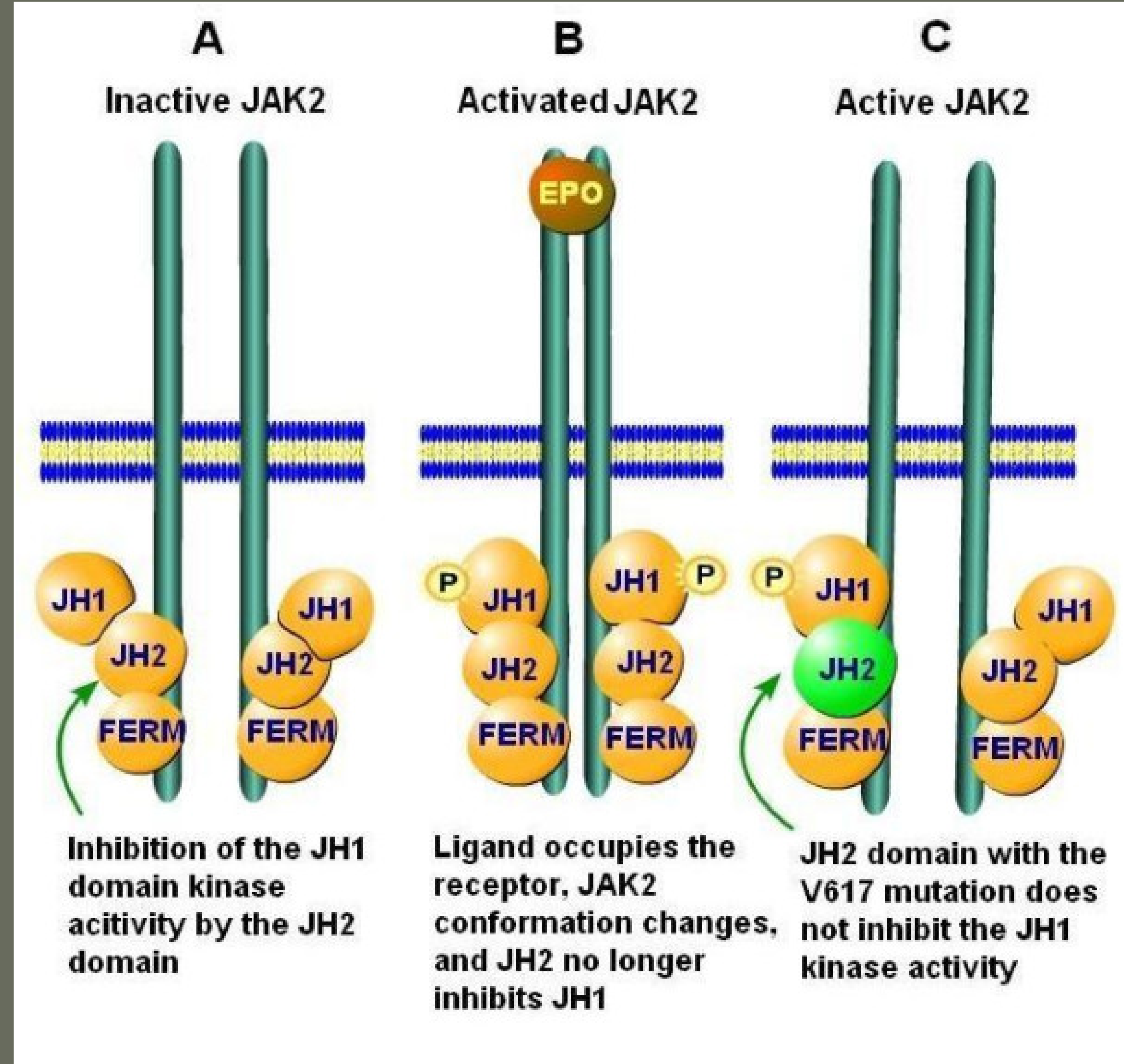
Polycythaemia vera (1.08/100 000/rok)

Primary myelofibrosis (0.37/100 000/rok)

Essential thrombocythaemia (1.65/100 000/rok)

Chronic eosinophilic leukaemia

Mastocytosis



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

gen *JAK2*

Rok 2005 období objevu mutací

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 .

Detekce mutace V617F *JAK-2*

Metoda alelické diskriminace

- 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)

Locked Nucleic Acid probes

- Jde o sondy pro real-time PCR i klasickou
- end-point PCR vyvinuté společností Proligo
- jež jsou vhodné všude tam, kde je potřeba přesná kvantifikace nebo mutační diskriminace.
- Ve srovnání s dosud používanými sondami
- mají zvýšenou teplotní stabilitu a hybridizační specifitu. Umožňují proto snazší navrhování oligonukleotidových sond pro aplikace s problematickými cílovými sekvencemi

Alelická diskriminace pro detekci *JAK2*^{V617F}

CTGAAAGTAGGAGAAAGTGCATCTTTATTATGGCAGAGAGAATTTTCTGAACTATTTATG
GACAACAGTCAAACAACAATTCTTTGTACTTTTTTTTTTCCTTAGTCTTTCTTTGAAGCA
GCAAGTATGATGAGCAAGCTTTCTCACAAGCATTTGGTTTTAAATTATGGAGTATGTTTC
TGTGGAGACGAGAGTAAGTAAAACTACAGGCTTTCTAATGCCTTTCTCAGAGCATCTGTT
TTTGTTTATATAGAAAATTCAGTTTCAGGATCACAGCTAGGTGTCAGTGTAAACTATAAT
TTAACAGGAGTTAAGTATTTTTGAAACTGAAAACACTGTAGGACTATTCAGTTATATCTT

primer F: 5´ - GAAGCAGCAAGTATGATGAGCAA – 3

primer R: 5´ - ACTGACACCTAGCTGTGATCC – 3´

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

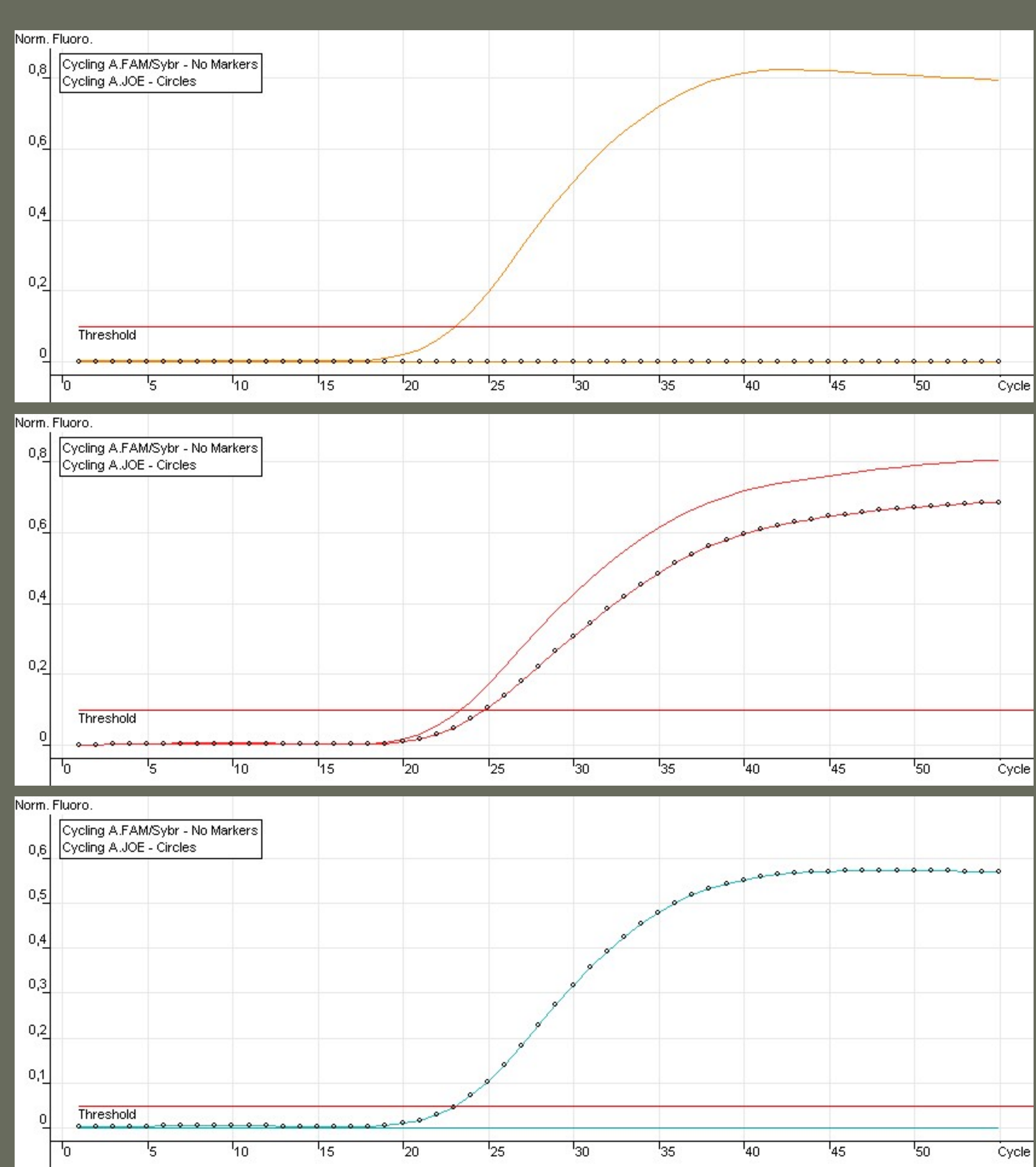
JAK2^{V617F} LNA sonda: HEX – ctcCacAgaAacAtaCtc – BHQ1

RQ – PCR analýza

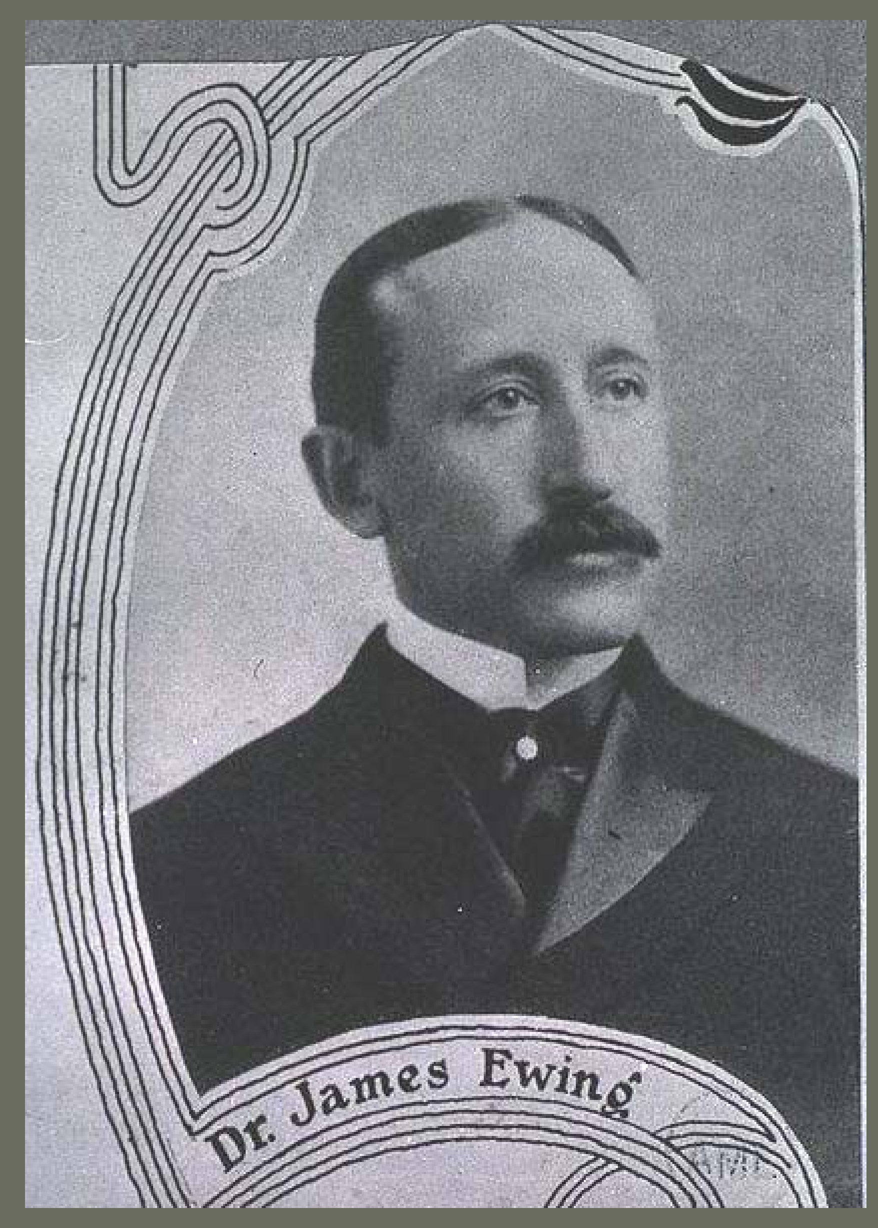
Pacient - wt

Pacient – heterozygot

Pacient – mut. homozygot



Problematika sarkomů a karcinomů



In 1921, James Ewing, MD, described a bone tumor that, unlike the common bone tumor, osteosarcoma, could be treated with radiation. This newly identified tumor became known as Ewing's tumor. At first, this tumor was only seen in bones. Soon, the same type of tumor was detected in the soft tissues and named extraosseous Ewing's (EOE).A tumor is described by size, where it originated, and whether it has spread. This is called staging. A tumor of the Ewing's family (TEF) is staged as either localized (involving only the site of origin and nearby tissues) or metastatic (involving spread to distant parts of the body).

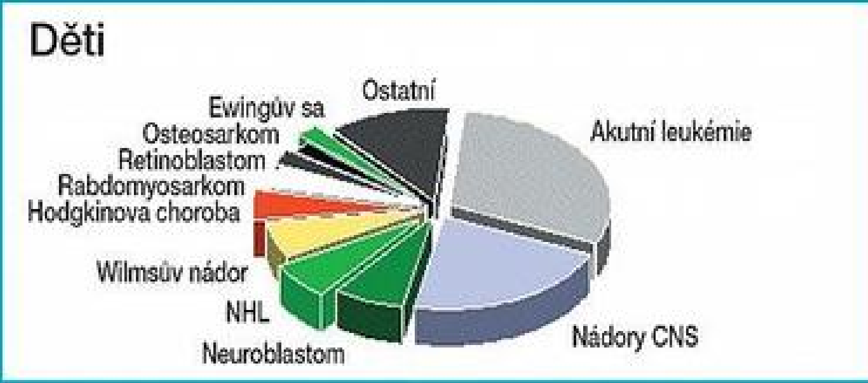
Ewingův sarkom

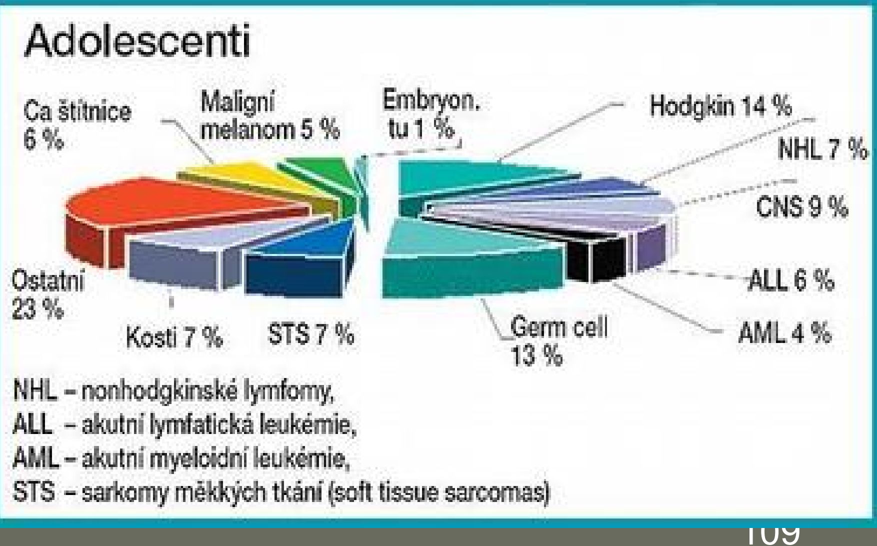
a příklad diferenciální diagnostiky metodou PCR

Ewingův sarkom/PNET
Alveolární rhabdomyosarkom
Desmoplastic Small Round Cell Tumor (DSRCT)

Druhý nejčastější primární maligní nádor kostí po osteosarkomu u starších dětí a mladých dospělých

Diferenciální diagnostika založena na detekci transkriptů fúzních genů ***EWS/FLI-1***, ***EWS/ERG***, ***PAX3/FKHR***, ***PAX7/FKHR***, ***EWS/WT-1***





Protinádorová terapie

- Chemoterapie (léčiva s cytotoxickým účinkem)
- Hormonální léčba (nádory odvozené od hormonálně dependentní tkáně)
- Diferenciační léčba retinoidy (stimulace diferenciace maligní buňky)
- Bioterapie (hemopoetické růstové faktory, interferony a další cytokiny)
- Radioterapie
- Inhibitory konstitutivně aktivovaných tyrozinkináz
- **Biologická léčba MCA**

Tyrosine kinases are important mediators of the signaling cascade, determining key roles in diverse biological processes like growth, differentiation, metabolism and apoptosis in response to external and internal stimuli.

Recent advances have implicated the role of tyrosine kinases in the pathophysiology of cancer. Though their activity is tightly regulated in normal cells, they may acquire transforming functions due to mutation(s), overexpression and autocrine paracrine stimulation, leading to malignancy.

Constitutive oncogenic activation in cancer cells can be blocked by selective tyrosine kinase inhibitors and thus considered as a promising approach for innovative genome based therapeutics.

The modes of oncogenic activation and the different approaches for tyrosine kinase inhibition, like small molecule inhibitors, monoclonal antibodies, heat shock proteins, immunoconjugates, antisense and peptide drugs are reviewed in light of the important molecules.

Receptor and nonreceptor tyrosine kinases (TKs) have emerged as clinically useful drug target molecules for treating certain types of cancer.

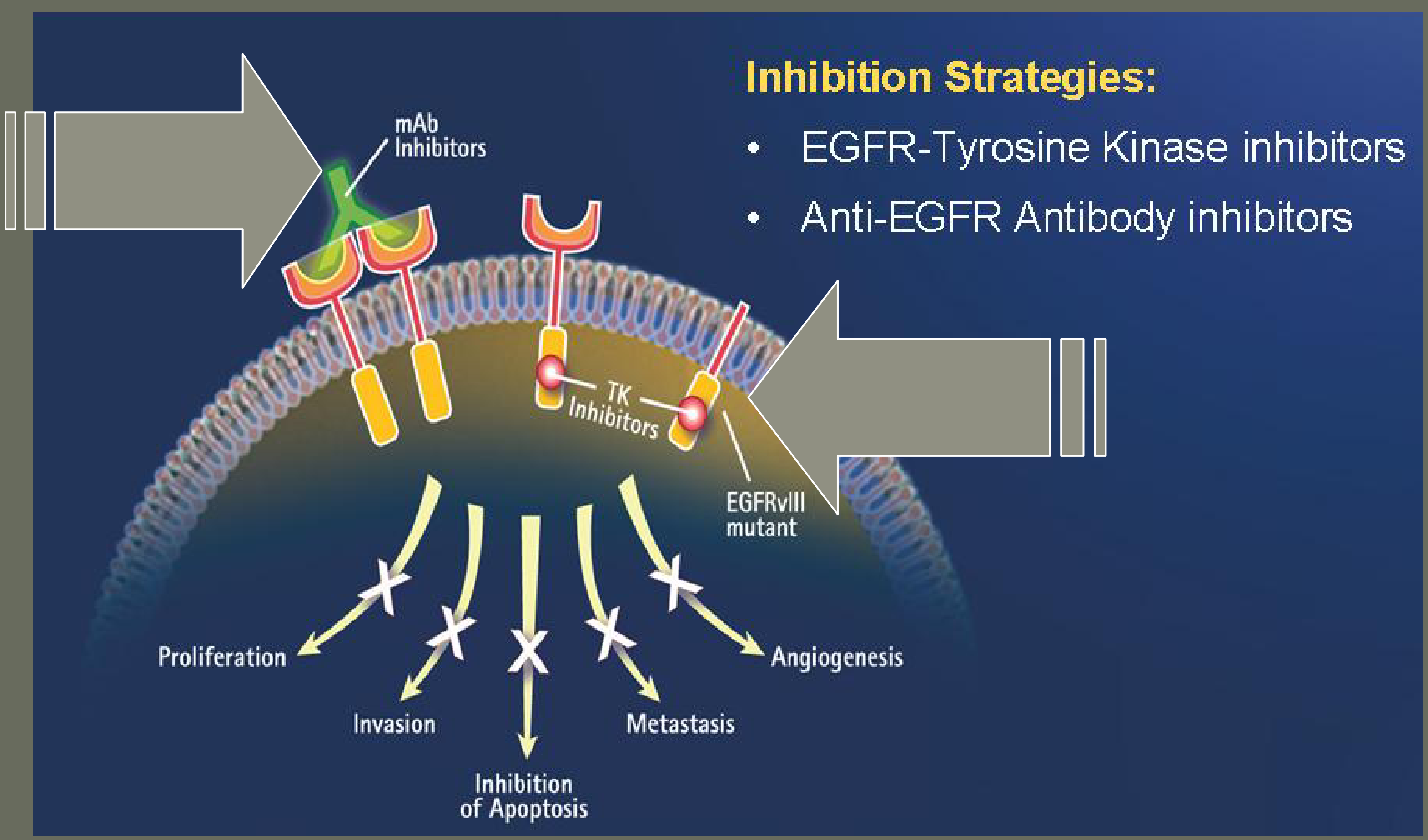
Epidermal growth factor receptor (EGFR)-TK is a transmembrane receptor TK that is overexpressed or aberrantly activated in the most common solid tumors, including non-small cell lung cancer and cancers of the breast, prostate, and colon.

Activation of the EGFR-TK enzyme results in autophosphorylation, which drives signal transduction pathways leading to tumor growth and malignant progression.

Randomized clinical trials of the EGFR-TK inhibitor gefitinib have demonstrated clinical benefits in patients with advanced non-small cell lung cancer whose disease had previously progressed on platinum- and docetaxel-based chemotherapy regimens.

v·d·e	Targeted therapy / extracellular chemotherapeutic agents/antineoplastic agents (L01) [hide]	
CI monoclonal antibodies ("-mab")	Receptor tyrosine kinase	ErbB: *HER1/EGFR* (Cetuximab, Panitumumab) • *HER2/neu* (Trastuzumab)
	Others for solid tumors	*EpCAM* (Catumaxomab, Edrecolomab) • *VEGF-A* (Bevacizumab)
	Leukemia/lymphoma	lymphoid: *CD20* (Ibritumomab, Ofatumumab, Rituximab, Tositumomab), *CD30* (Brentuximab), *CD52* (Alemtuzumab) myeloid: *CD33* (Gemtuzumab)
Tyrosine-kinase inhibitors ("-nib")	Receptor tyrosine kinase	ErbB: *HER1/EGFR* (Erlotinib, Gefitinib, Vandetanib) • *HER1/EGFR and HER2/neu* (Afatinib, Lapatinib, Neratinib) RTK class III: *C-kit and PDGFR* (Axitinib, Pazopanib, Sunitinib, Sorafenib, Toceranib) • *FLT3* (Lestaurtinib) *VEGFR* (Axitinib, Cediranib, Pazopanib, Regorafenib, Semaxanib, Sorafenib, Sunitinib, Toceranib, Vandetanib)
	Non-receptor	*bcr-abl* (Dasatinib, Imatinib, Nilotinib) *Src* (Bosutinib) *Janus kinase* (Lestaurtinib, Ruxolitinib) *EML4-ALK* (Crizotinib)
Other	*fusion protein against VEGF* (Aflibercept) • *proapoptotic peptide against ANXA2 and prohibitin* (Adipotide) • *exotoxin against IL-2* (Denileukin diftitox)	
M: NEO	tsoc, mrkr	tumr, epon, para — drug (L1i/1e/V03)

Inhibitory EGFR signalizace



onctalk.com/2007/03/03/iressa-tarceva-hx-part-i/

• Nemalobuněčný karcinom plic

- NSCLC je civilizační choroba s vysokou incidencí a úmrtností, v ČR je každoročně diagnostikováno více než 6000 nových onemocnění. Přes vývoj nových léků a léčebných kombinací zůstává prognóza velmi špatná.
- Kombinovaná chemoterapie, radioterapie a v posledních letech také biologicky cílenou léčbou. Náklady na konzervativní léčbu jsou značné, pravděpodobnost dosažení remise bývá menší než 50% a vedlejší účinky kombinované chemoterapie jsou často závažné.
- V současnosti se nabízejí některé genetické prediktory umožňující individuální předpověď vhodnosti terapie pro konkrétního nemocného a je tak možno indikovat biologicky cílenou léčbu a ušetřit pacienty nežádoucích účinků cytostatik či předem predikovat neúčinnost chemoterapie - somatické mutace (EGFR a KRAS) a genové amplifikace (EGFR).

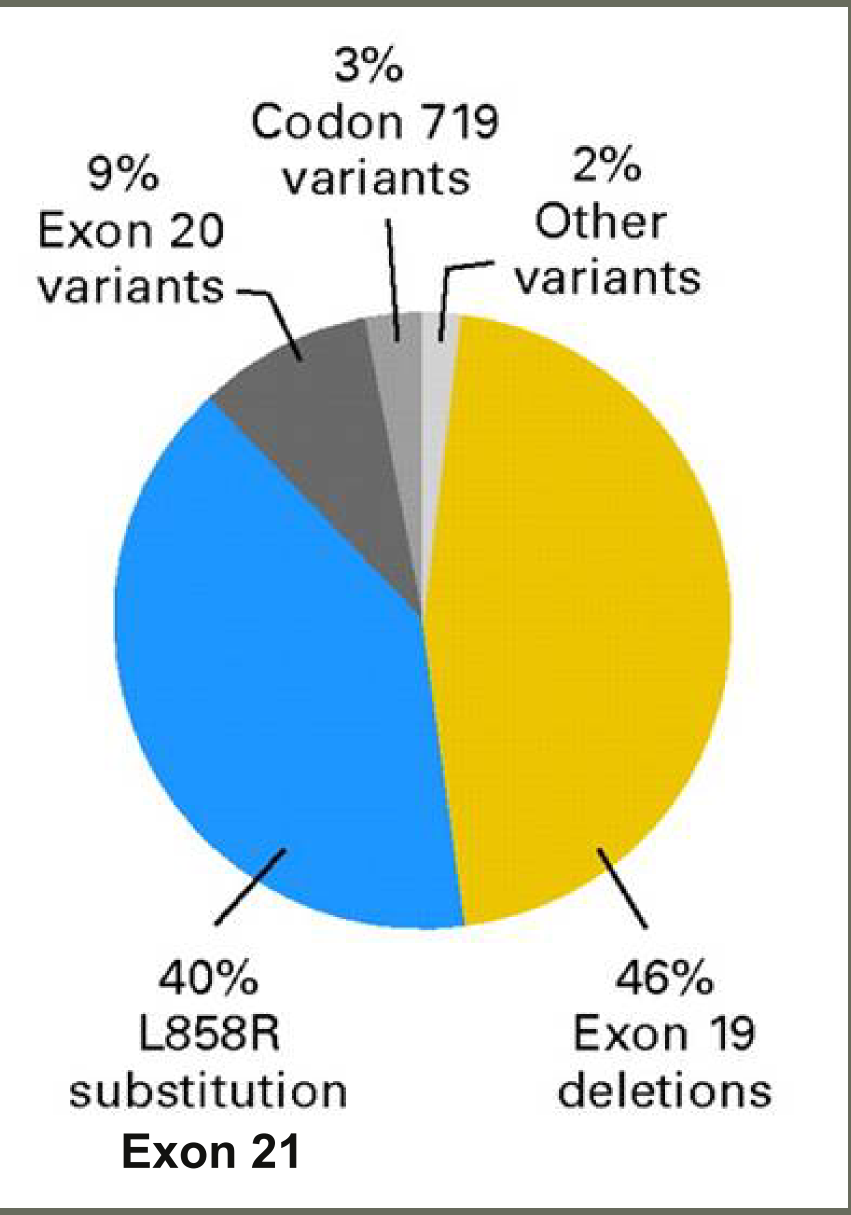
EGFR je povrchový receptor, jehož aktivací je spuštěna intracelulární signální kaskáda s vlivem na invazivitu, apoptózu a angiogenezi nádorového růstu.

Zvýšená exprese EGFR u 40-80% NSCLC.

Incidence aktivačních mutací EGFR u 10% (Evropa a Severní Amerika) až 40% (Východní Asie) NSCLC.

Přítomnost mutací spojena s nekuřáctvím, ženským pohlavím, asijským původem a histologicky prokázaným adenokarcinomem a s lepším přežitím.

KRAS mutace častější u kuřáků s NSCLC, u 20-30% NSCLC, rozporná data o účinnosti TK inhibice.



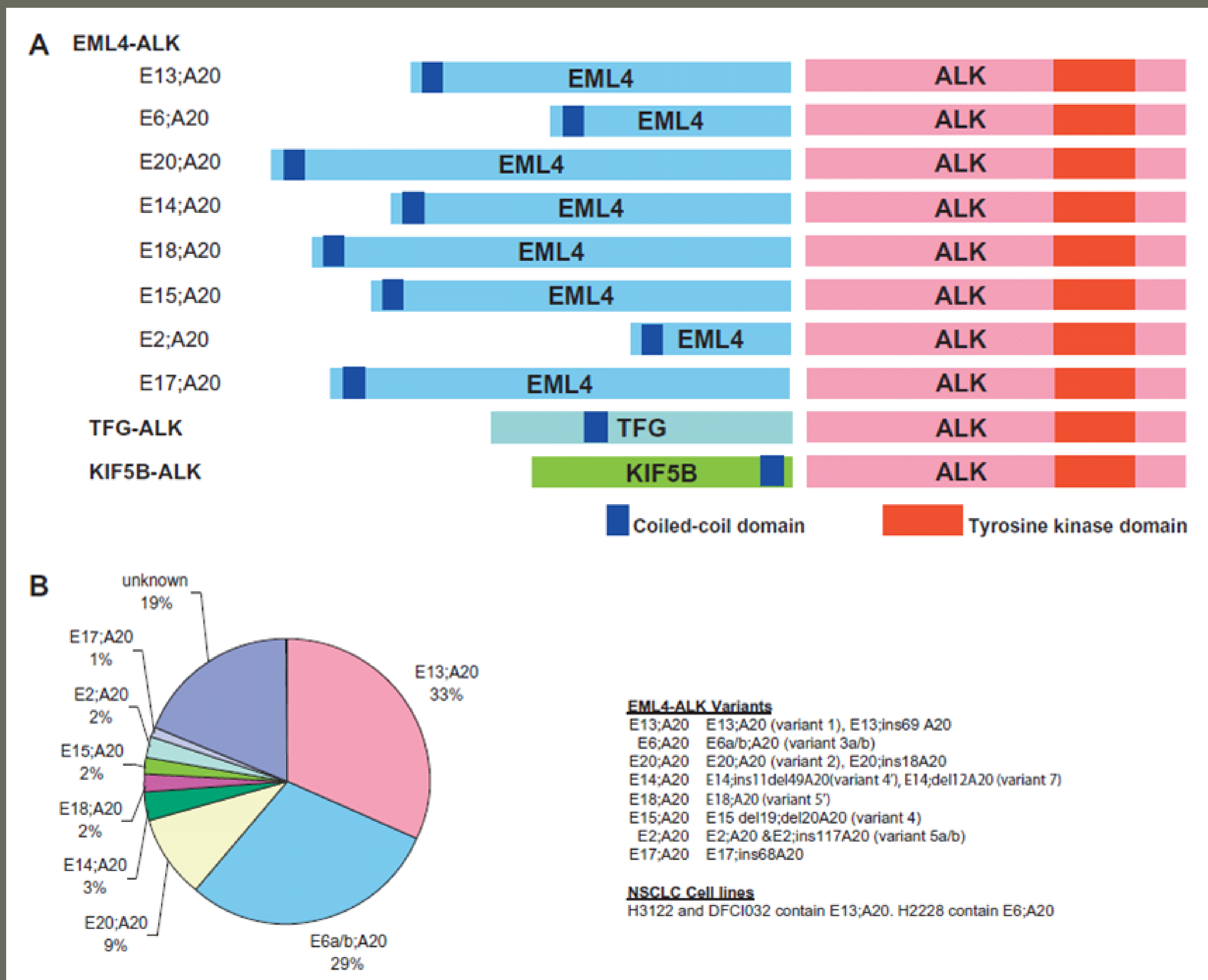
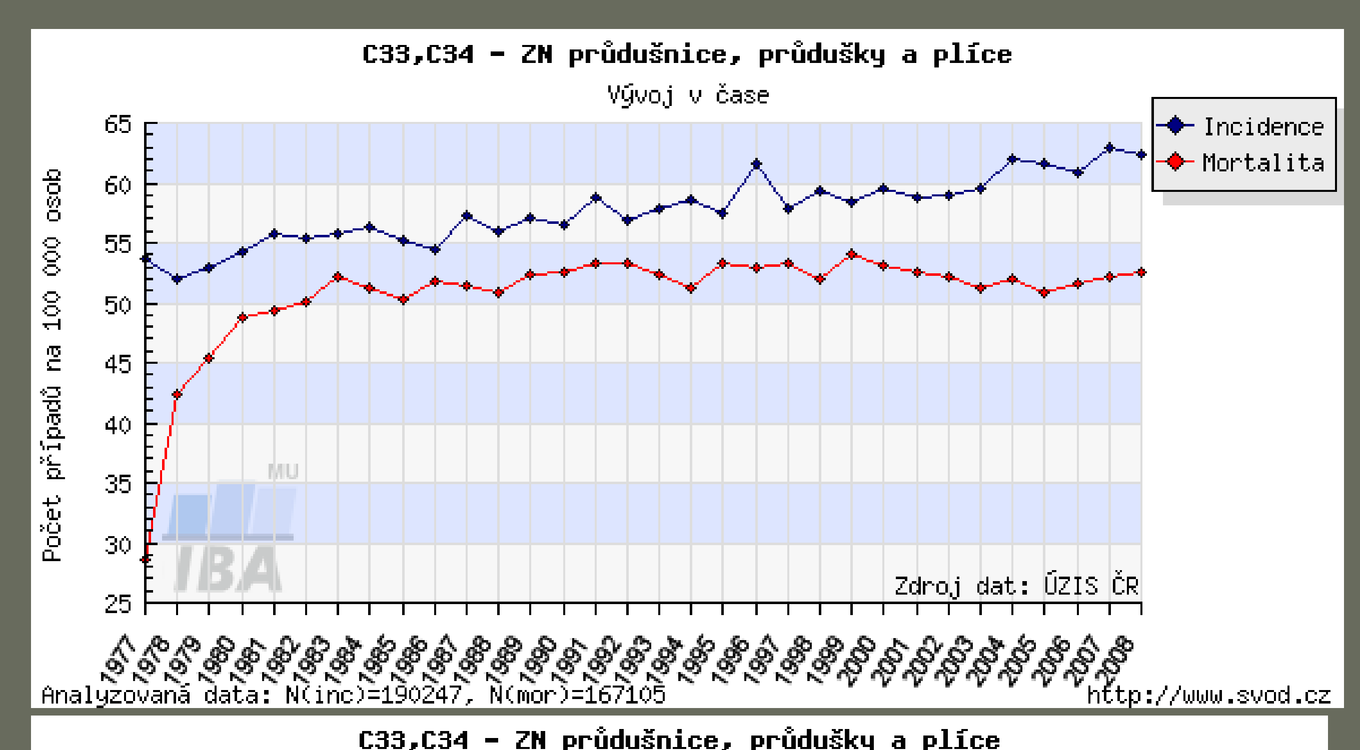
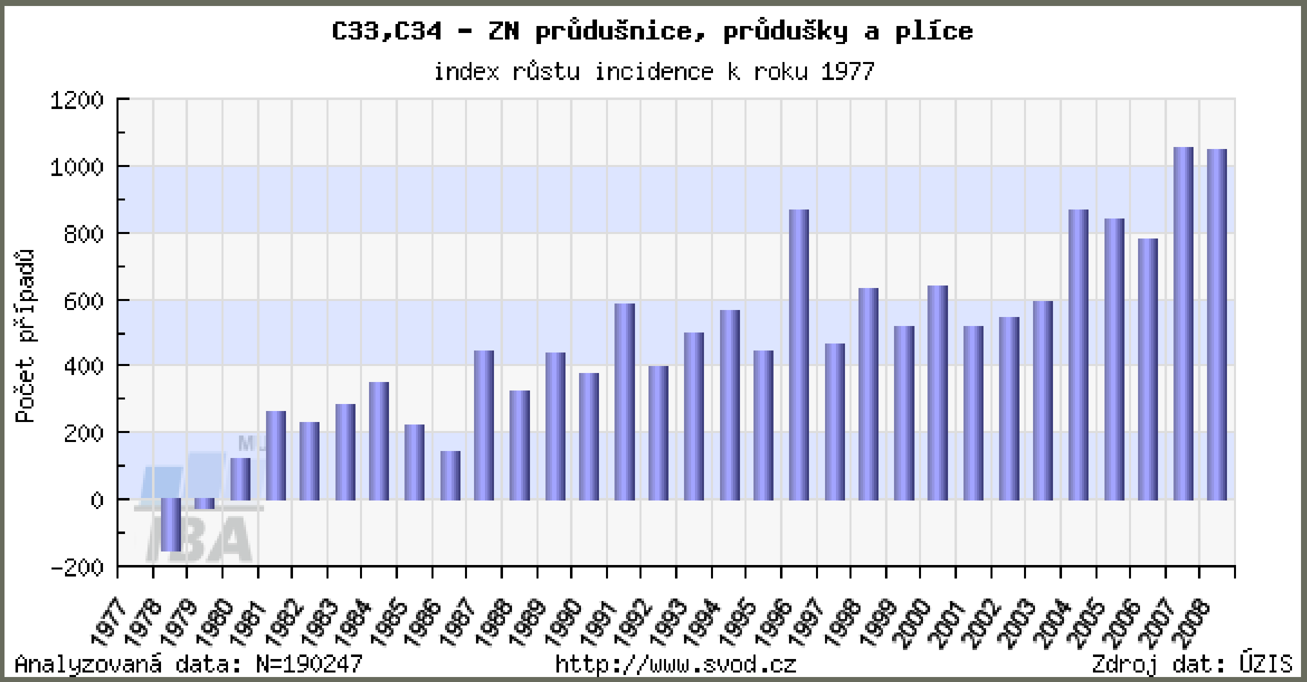


Fig. 3 – Different variants of *EML4-ALK* and non-*EML4* fusion partners. (A) Different variants of *EML4-ALK* are depicted. The nomenclature refers to the exon in *EML4* translocated to the exon in *ALK*. (B) Frequency of different *EML4-ALK* variants. The most common variants are E13;A20 (variant 1) and E6a/b; A20 (variant 3). Data obtained from.[4–11,30,32–36] Of note not all studies list the specific *EML4-ALK* variant.

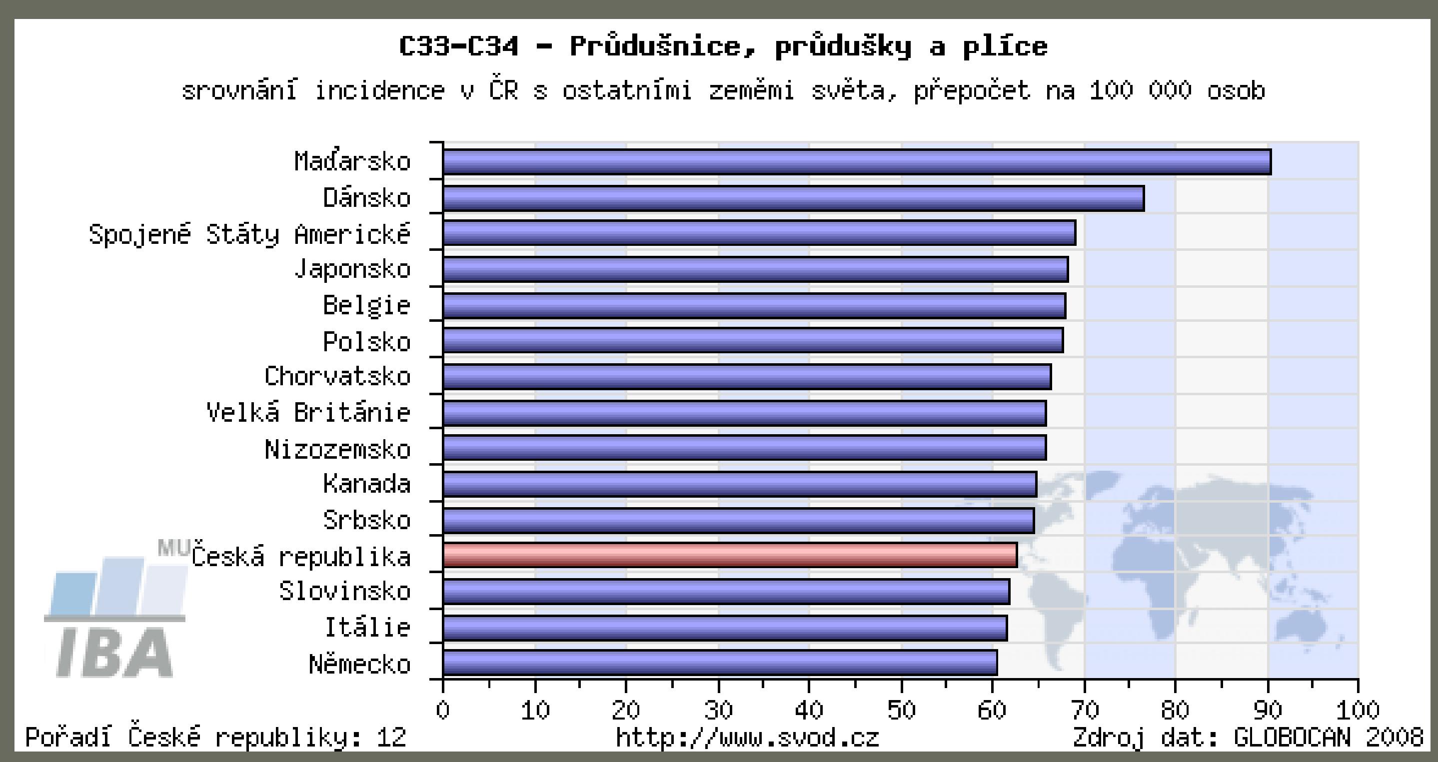


C33,C34 - ZN průdušnice, průdušky a plíce
Vývoj v čase
Incidence
Mortalita
Počet případů na 100 000 osob
Zdroj dat: ÚZIS ČR
Analyzovaná data: N(inc)=190247, N(mor)=167105
http://www.svod.cz



C33,C34 - ZN průdušnice, průdušky a plíce
index růstu incidence k roku 1977
Počet případů
Analyzovaná data: N=190247
http://www.svod.cz
Zdroj dat: ÚZIS

NÁRODNÍ ONKOLOGICKÝ REGISTR
ČESKÉ REPUBLIKY
Národní onkologický registr


C33-C34 - Průdušnice, průdušky a plíce
srovnání incidence v ČR s ostatními zeměmi světa, přepočet na 100 000 osob
Maďarsko
Dánsko
Spojené Státy Americké
Japonsko
Belgie
Polsko
Chorvatsko
Velká Británie
Nizozemsko
Kanada
Srbsko
Česká republika
Slovinsko
Itálie
Německo
0
10
20
30
40
50
60
70
80
90
100
MU
IBA
Pořadí České republiky: 12
http://www.svod.cz
Zdroj dat: GLOBOCAN 2008

Kolorektální karcinom (mCRC)

rezistence

na anti-EGFR terapii

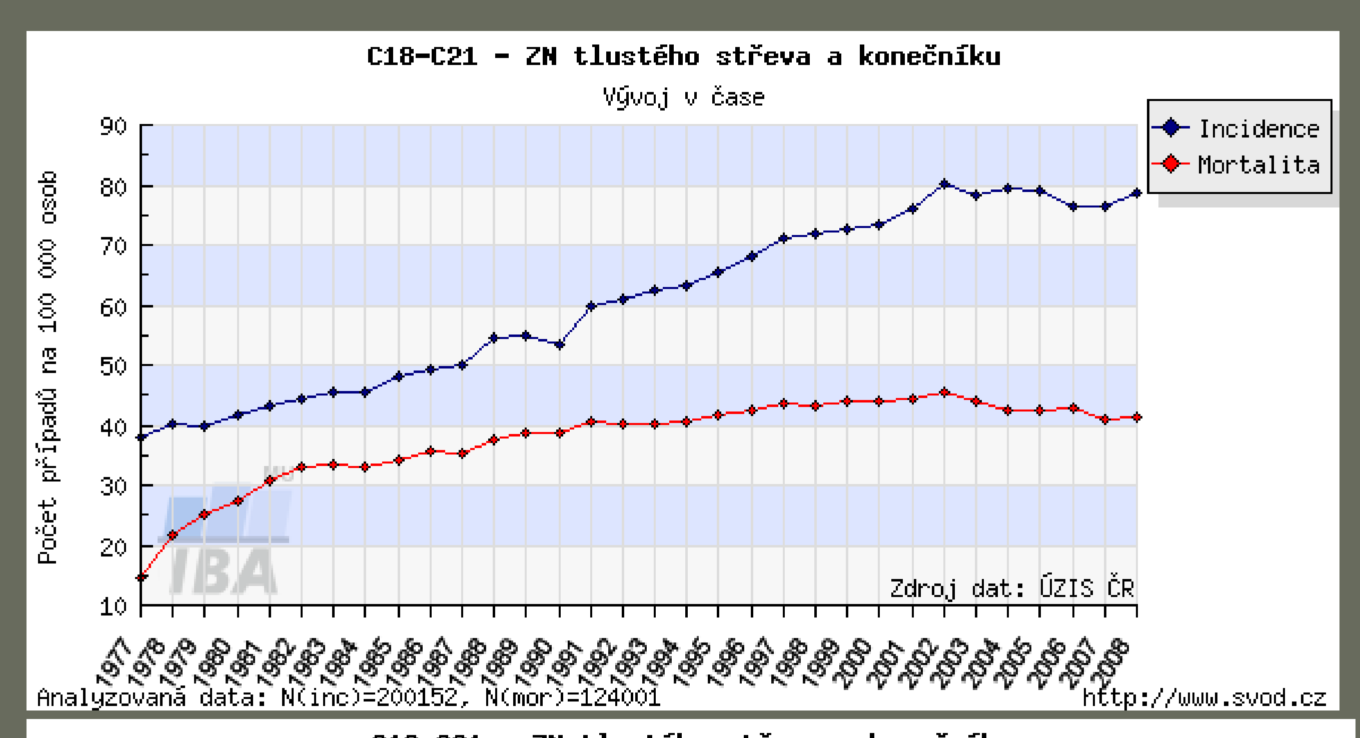
KRAS mutace

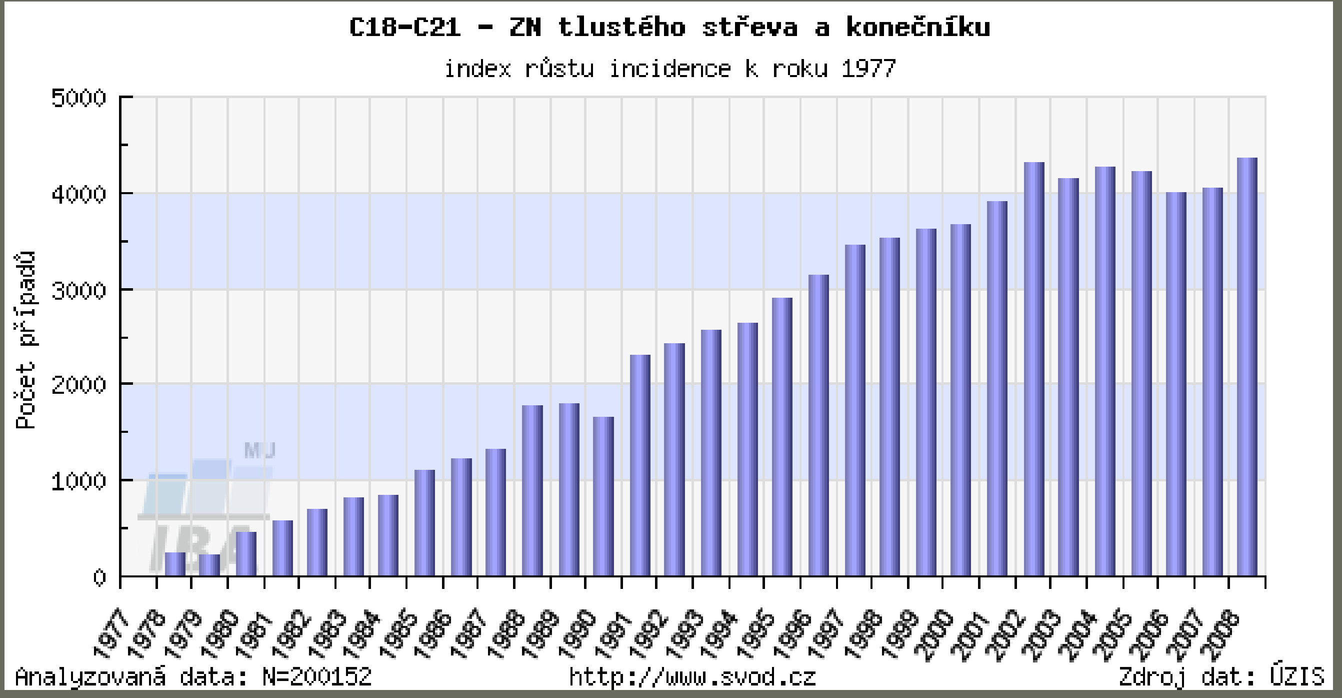
- Aktivační **mutace** u **30-40% pacientů s CRC způsobuje rezistence** na anti-EGFR terapii
  97% mutací v exonu 2: kodón 12 (GGT) a 13 (GGC)
- 30-50% pacientů bez odpovědi nemá mutaci *KRAS*; jiný mechanismus rezistence (geny BRAF, PIK3CA, KRAS – kodón 61, 146)

BRAF mutace

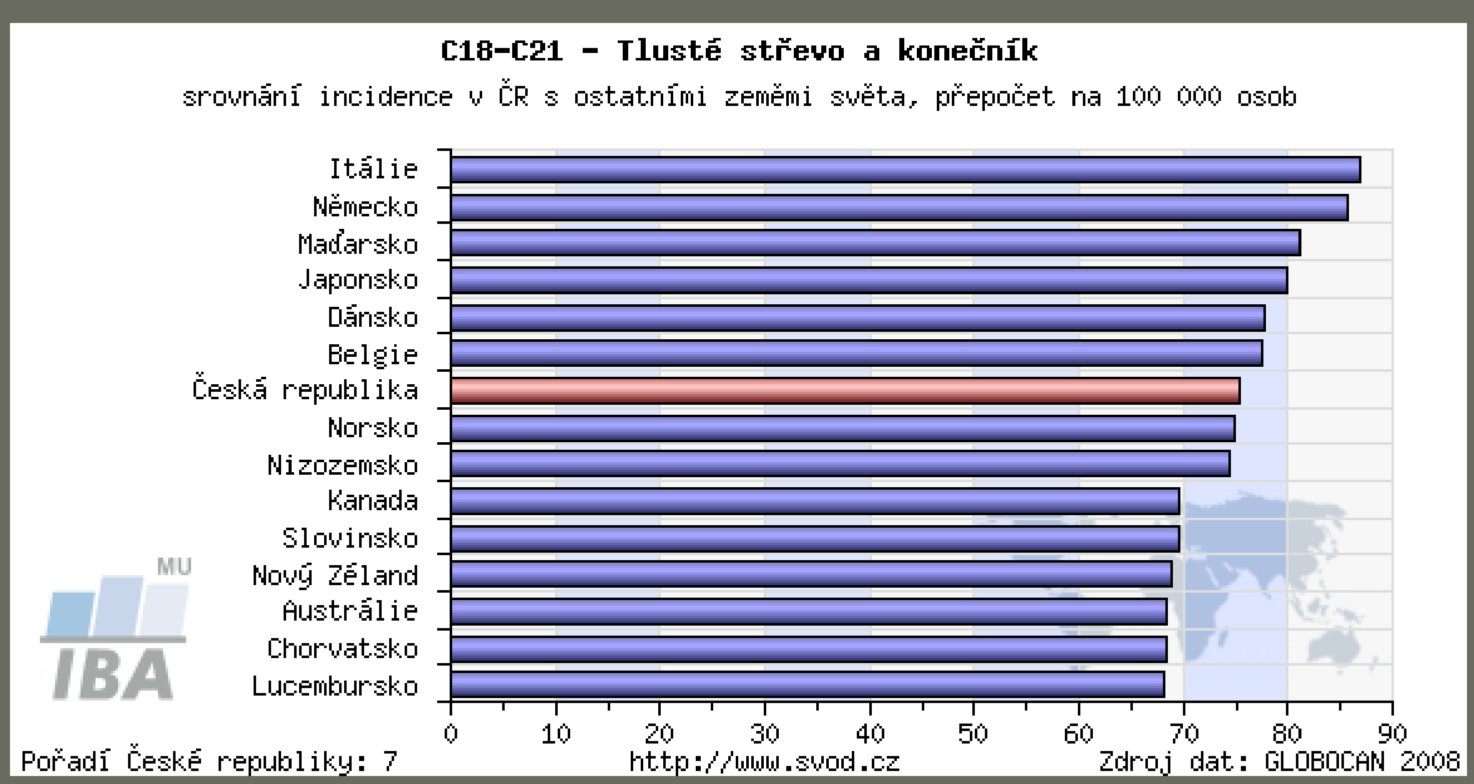
- Bodová mutace **V600E** (exon 15); 8-13 % u nemutovaných KRAS pacientů (*KRAS* a *BRAF* mutace se vyskytují současně výjimečně)

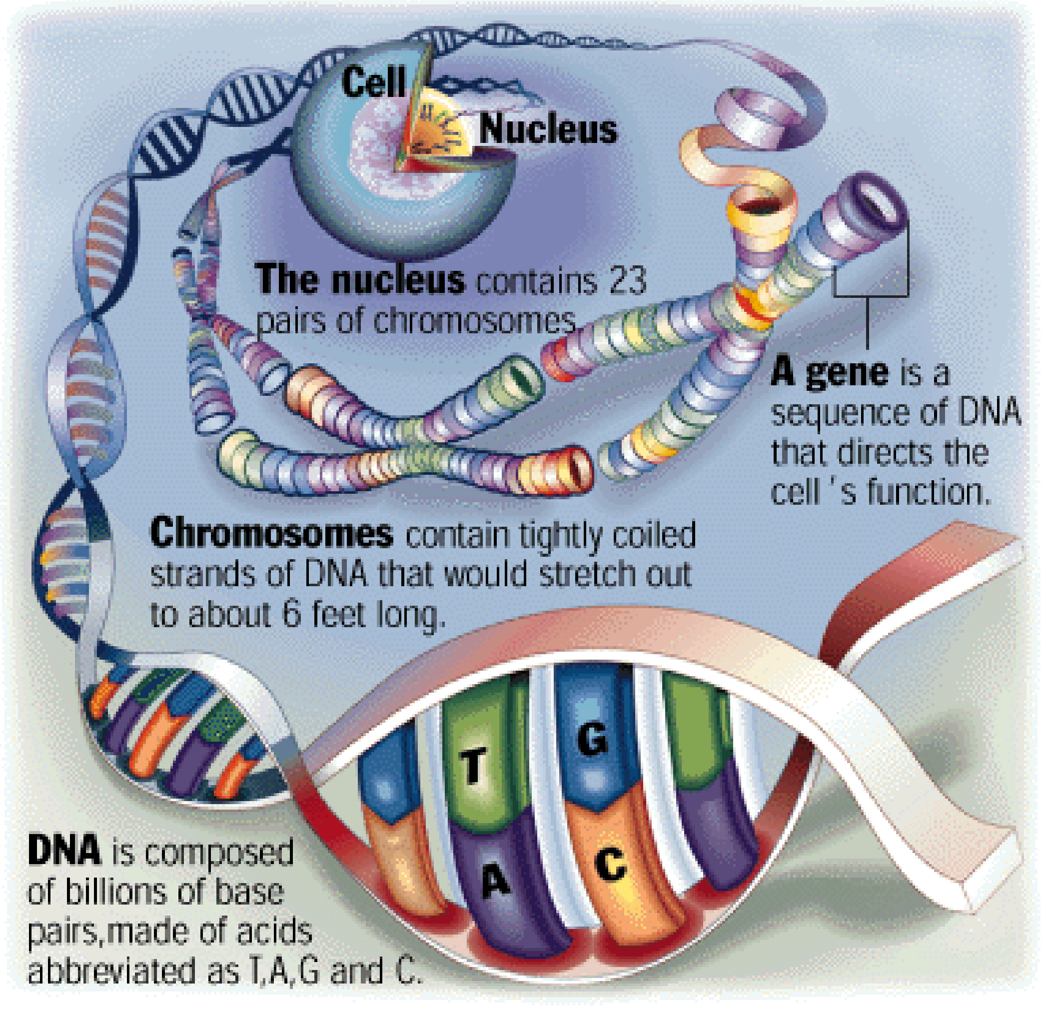
UK NATIONAL EXTERNAL QUALITY ASSESSMENT SCHEMES
www.ukneqas-molgen.org.uk
CPA
Accredited EQA Scheme Ref. No: 051


C18-C21 - ZN tlustého střeva a konečníku
Vývoj v čase
Incidence
Mortalita
Počet případů na 100 000 osob
90
80
70
60
50
40
30
20
10
Zdroj dat: ÚZIS ČR
1977 1978 1979 1980 1981 1982 1983 1984 1985 1986 1987 1988 1989 1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008
Analyzovaná data: N(inc)=200152, N(mor)=124001
http://www.svod.cz


C18-C21 - ZN tlustého střeva a konečníku
index růstu incidence k roku 1977
Počet případů
5000
4000
3000
2000
1000
0
1977 1978 1979 1980 1981 1982 1983 1984 1985 1986 1987 1988 1989 1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008
Analyzovaná data: N=200152
http://www.svod.cz
Zdroj dat: ÚZIS

NÁRODNÍ ONKOLOGICKÝ REGISTR ČESKÉ REPUBLIKY
Národní onkologický registr


C18–C21 – Tlusté střevo a konečník
srovnání incidence v ČR s ostatními zeměmi světa, přepočet na 100 000 osob
Itálie
Německo
Maďarsko
Japonsko
Dánsko
Belgie
Česká republika
Norsko
Nizozemsko
Kanada
Slovinsko
Nový Zéland
Austrálie
Chorvatsko
Lucembursko
0 10 20 30 40 50 60 70 80 90
Pořadí České republiky: 7
http://www.svod.cz
Zdroj dat: GLOBOCAN 2008
MU
IBA



Postgenomická éra neboli **období proteomiky**

znalost genomu umožní sestavit transkriptom a proteom

rozvoj bioinformatiky

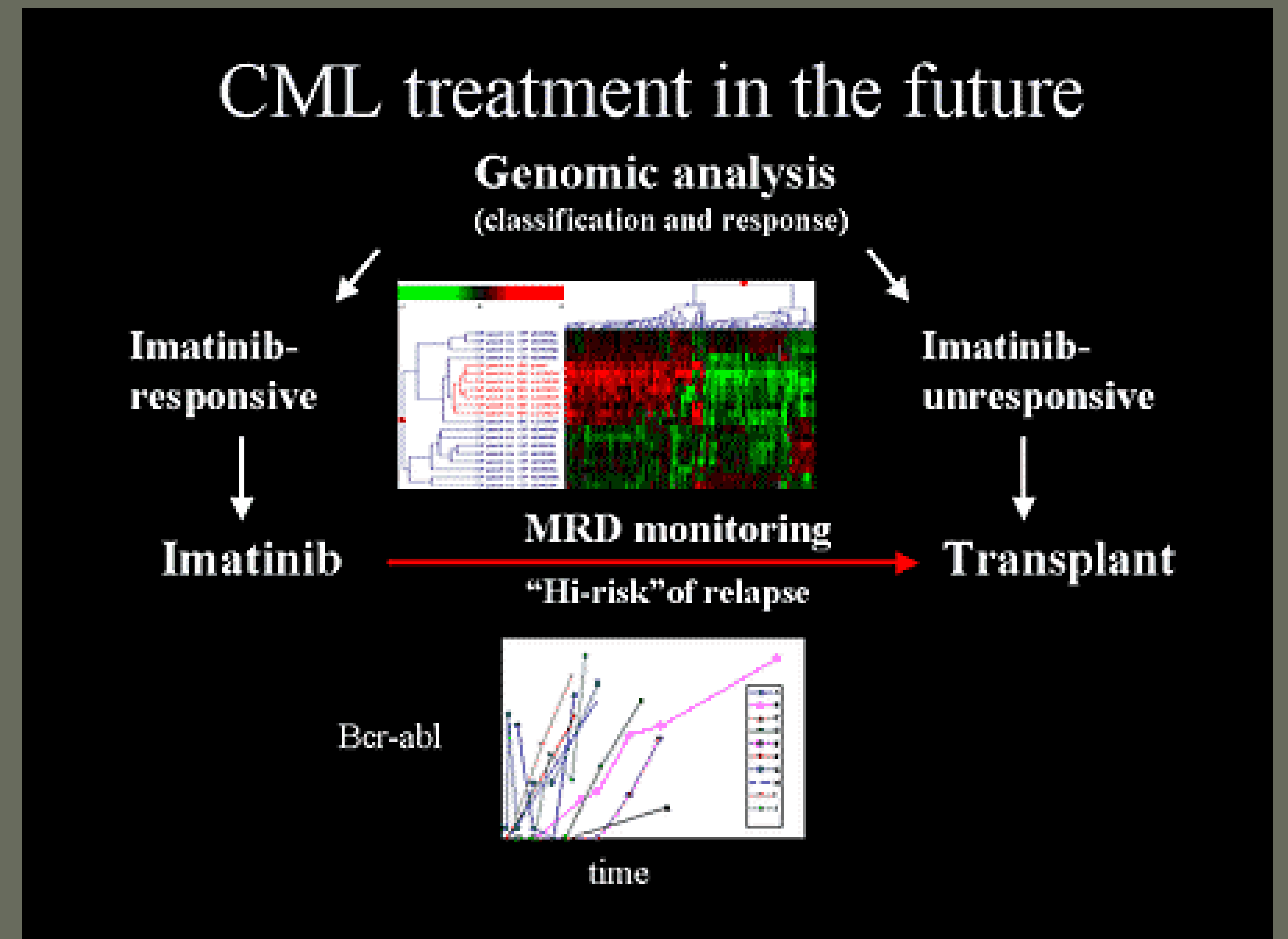
identifikace proteinů, jejich orgánová a tkáňová lokalizace, interakce mezi proteiny, funkce

možná pak pochopíme jak funguje buňka

molekulární medicína

individualizovaná medicína

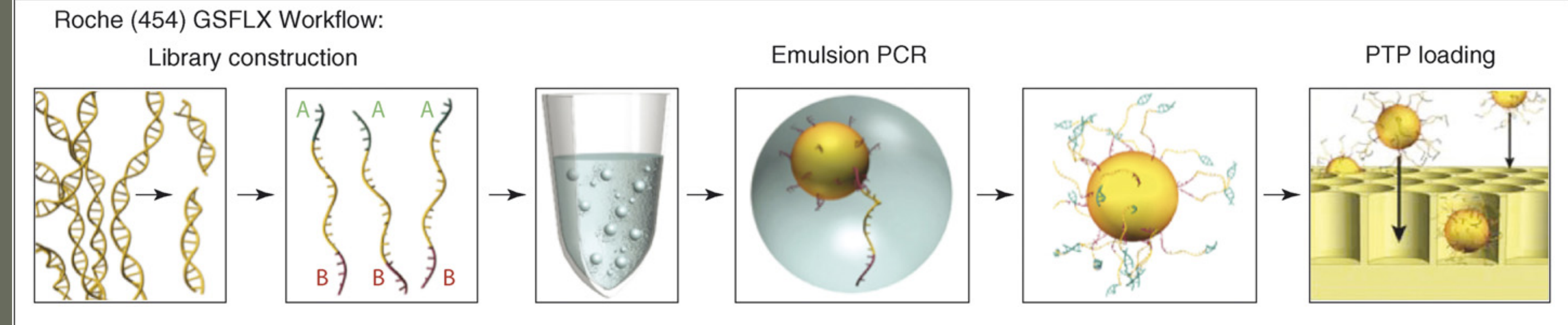
časová prodleva mezi možností diagnostikovat a terapeuticky zasáhnout

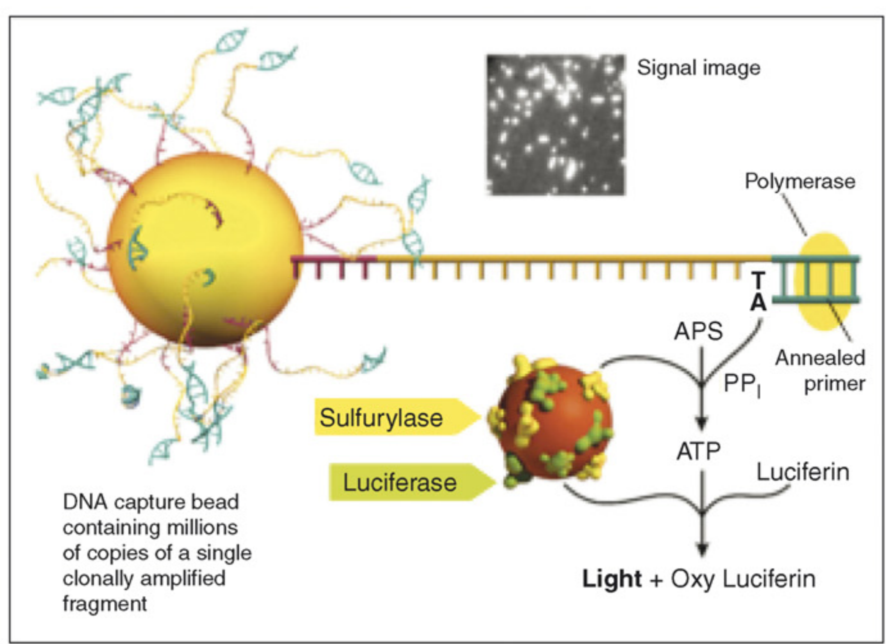


Goldman, J. et al.: Evolution of Management of CML, Tenth International Congress on Hematologic Malignancies

Next generation sequencing – High throughput sequencing

Masivní paralelní sekvenování miliónů bází za minimální cenu sekvenace





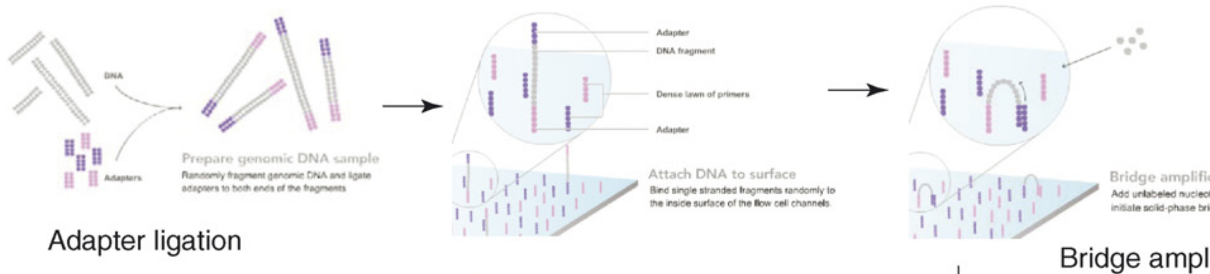
Pyrosequencing reaction

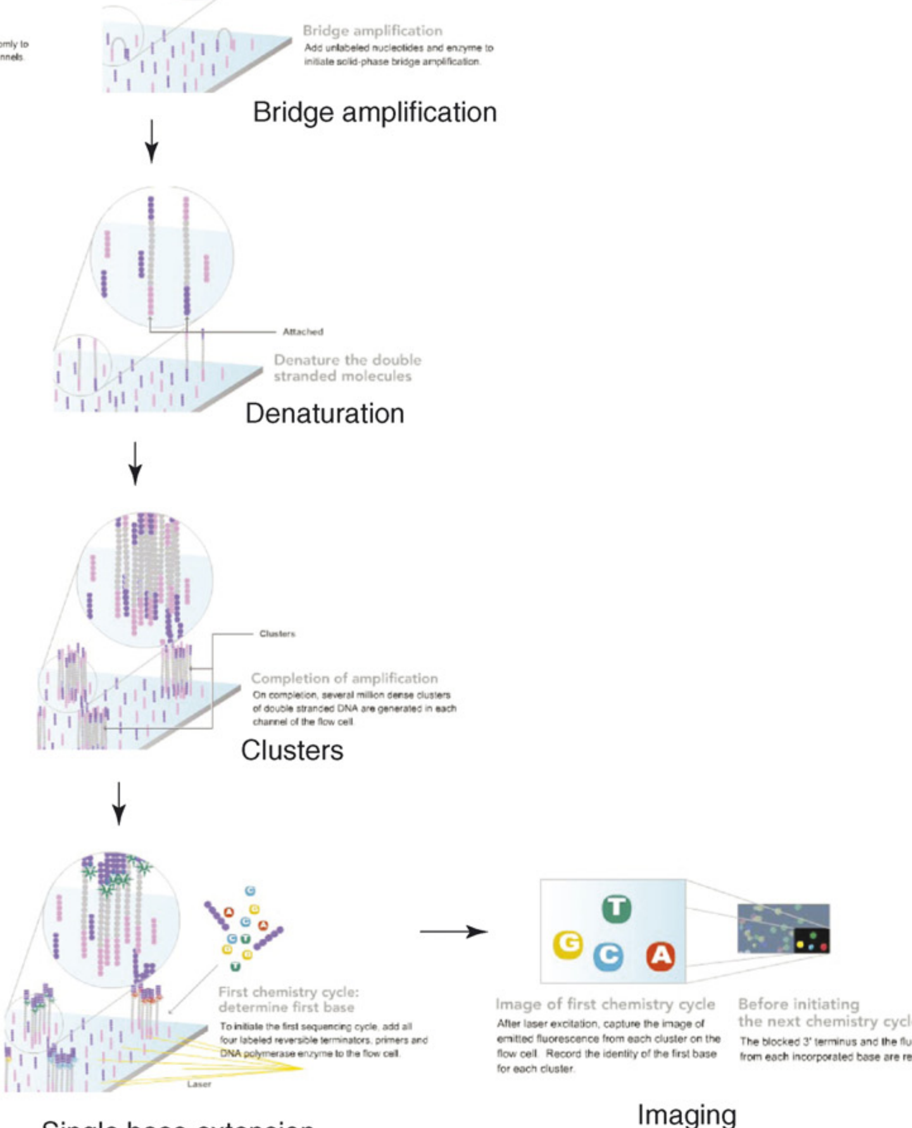
TRENDS in Genetics

Roche(454) 2004

Uveden na trh 2004. Uvolňování pyrofosfátových molekul z nukleotidů (emulzní PCR amplifikace DNA řetězců vázaných na stovky tisíc agarózových kuliček), produkce světla štěpením oxyluciferinu luciferázou.

Illumina Genome Analyzer Workflow





Illumina (Bridge amplification) 2006

Uveden na trh 2006.
Založen na konceptu „sequencing by synthesis“ (SBS) krátkých DNA fragmentů.

TRENDS in Genetics

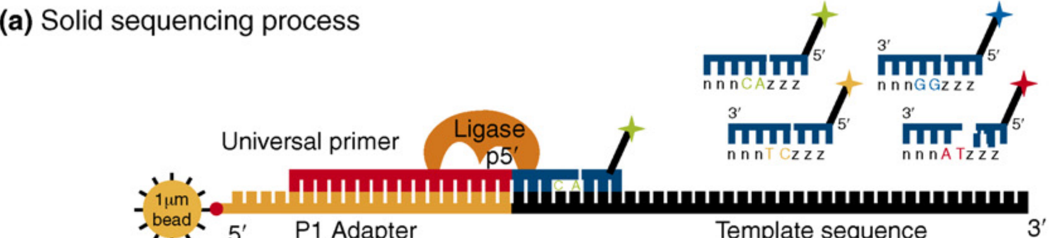
SOLid

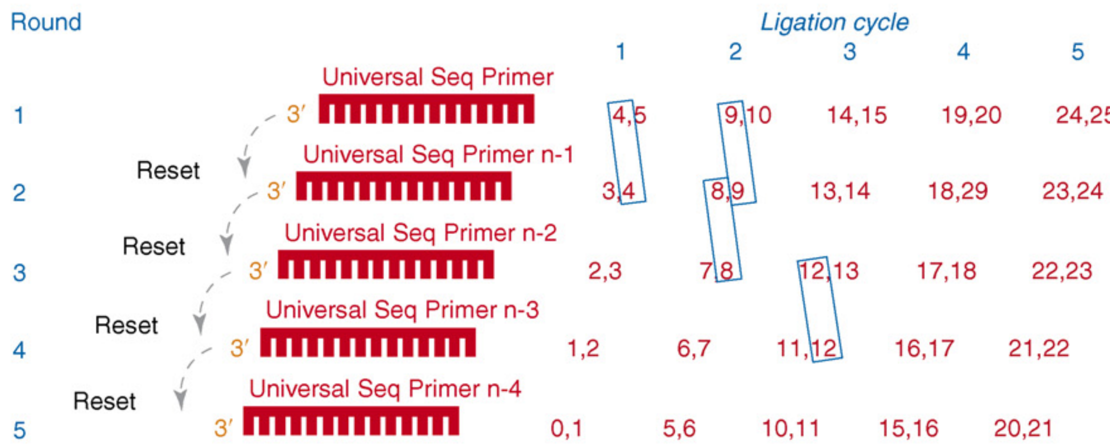
Applied Biosystems SOLid sequencer.

Uveden na trh 2007.

Sekvenace katalyzovaná DNA ligázou (Sequencing by Oligo Ligation and Detection)

2007





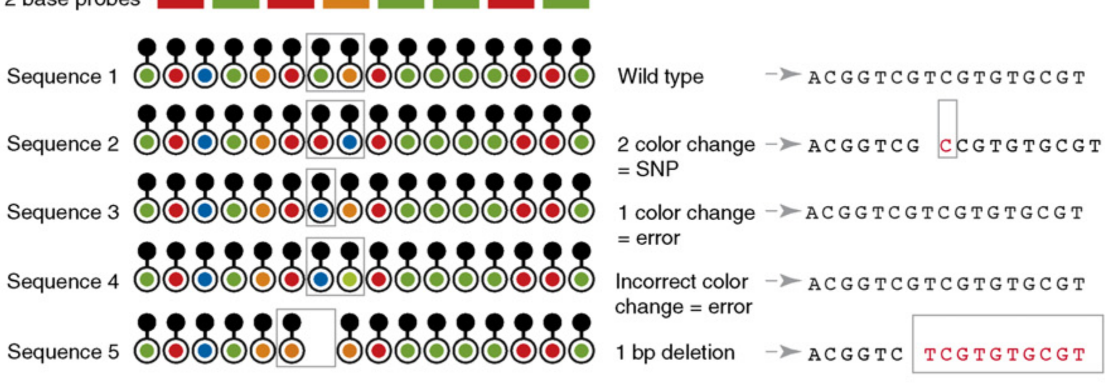


Table 1. Comparing metrics and performance of next-generation DNA sequencers

	Platform		
	Roche(454)	Illumina	SOLiD
Sequencing chemistry	Pyrosequencing	Polymerase-based sequencing-by-synthesis	Ligation-based sequencing
Amplification approach	Emulsion PCR	Bridge amplification	Emulsion PCR
Paired ends/separation	Yes/3 kb	yes/200 bp	Yes/3 kb
Mb/run	100 Mb	1300 Mb	3000 Mb
Time/run (paired ends)	7 h	4 days	5 days
Read length	250 bp	32–40 bp	35 bp
Cost per run (total direct[a])	$8439	$8950	$17 447
Cost per Mb	$84.39	$5.97	$5.81

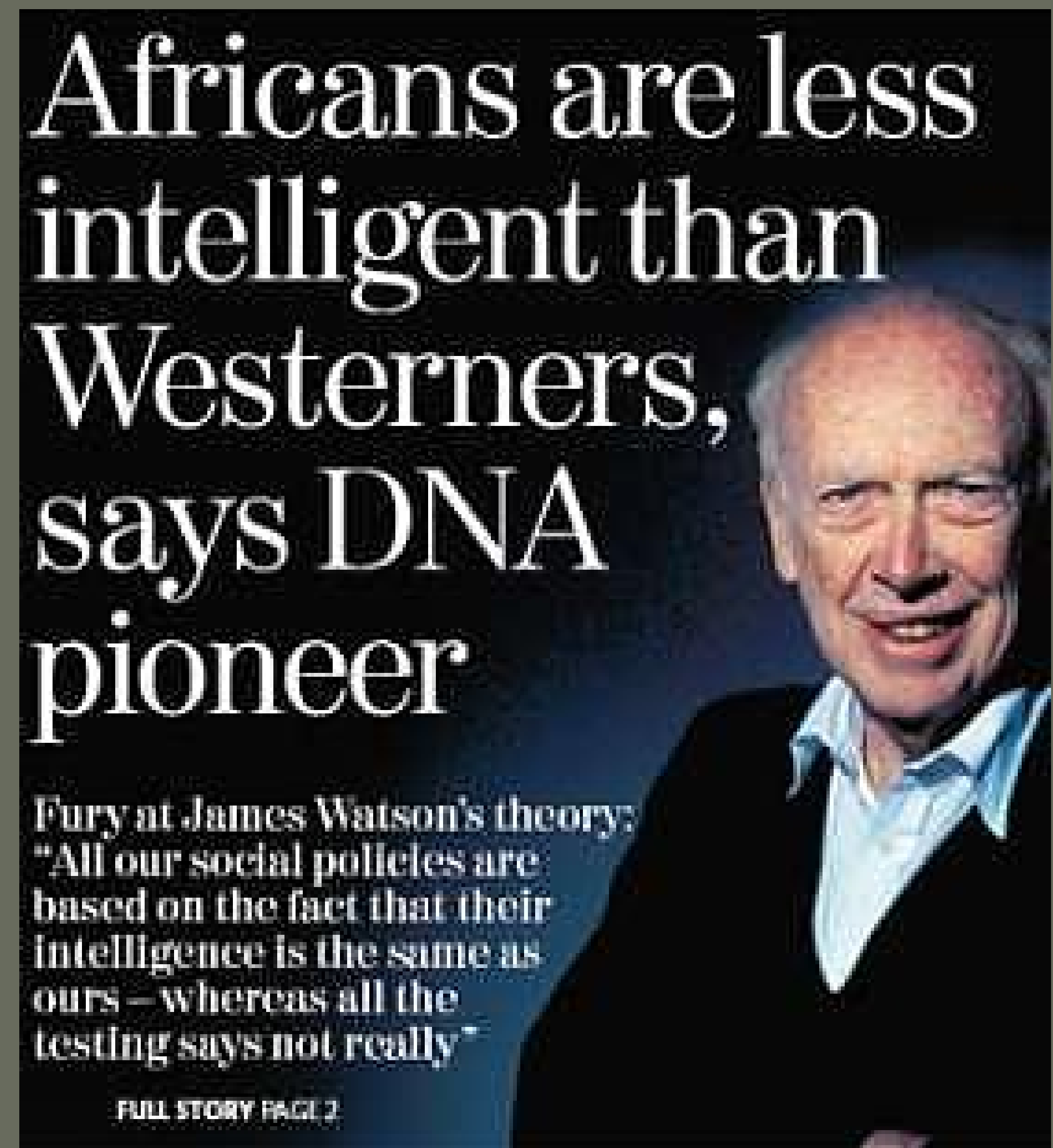
[a]Total direct costs include the reagents and consumables, the labor, instrument amortization cost and the disc storage space required for data storage/access.

Mardis The impact of next-generation sequencing technology on genetics, Trends Genet., 2008, 24:133-41

Wheeler, et al. The complete genome of an individual by massively parallel DNA sequencing. Nature, 452 (7189): 872-6, 2008

The association of genetic variation with disease and drug response have given great optimism for the impact „genomic medicine“. However, the formidable size of the diploid human genome, app. 6 gigabases has prevented the routine application.

Here we report DNA sequence of a diploid genome of a single individual, James D. Watson, using massively parallel sequencing in picolitre-size vessels. We identified 3.3 million SNP, of which 10.654 cause amino-acid substitution within the coding sequence.



October 2007

Watson si často nebere servítky a říká, co mu jazyk přinese. Jako učenci ze staré školy se mu hnusí politická korektnost, Watson vyjádřil pochyby, zda opravdu nutně musí být intelektuální kapacity všech lidí všude naprosto shodné, přičemž nepokrytě naznačil, že černí Homo sapiens v tomhle ohledu pokulhávají. Za to byl donucen odejít z vedení prestižní Cold Spring Harbor Laboratory.

Celá kauza má poněkud pikantní dohru. Watsonův genom byl nedávno kompletně přečten a zveřejněn na internetu. Islandská genetická firma deCODE Genetics provedla analýzy Watsonova genomu a její experti došli k závěru, že Watson má 16krát více „afrických“ variant genů, než je u průměrného bělocha zvykem. „Takové množství afrických variant genů můžete očekávat u někoho, kdo měl za prapradědečka Afričana,“ interpretoval výsledky analýz Kari Stefansson z deCODE Genetics.

Bleeker, et al. Mutational profiling of cancer candidate genes in glioblastoma, melanome and pancreatic carcinoma reveals a snapshot of their genomic landscapes. Hum Mutat., 30 (2): E451-9, 2009

Exomic sequencing: evaluation for tumor-specific mutations in all exons of protein coding genes (i.e. sequencing only the protein coding regions or exons)
Analysis of 18.191 well annotated coding sequences (RefSeq) **in breast and colorectal cancers** has led to the identification of somatic mutations in 1.718 genes. 280 of these have been denominated **candidate cancer genes** (CAN).

Torkamani and Schork, Prediction of Cancer Driver Mutations in Protein Kinases. Cancer Res, 68 (6): 1675-1682, 2008

A large number of somatic mutations accumulate during the tumorigenesis. A subset of these mutations contribute to tumor progression (know as „**driver**“ mutation) whereas the majority of these mutations are effectively neutral (know as „**passenger**“ mutations).
We provide a ranked list of candidate driver mutations.Researchers can begin to distinguish the driver mutations that push cells towards cancer from passenger mutations that are a by-product of cancer cell development.

Table 1.

Known cancer drivers and passenger

Kinase	Mutation	Driver?	Prediction	CanPredict
BRAF	R461I	Yes	Yes	Yes
BRAF	I462S	Yes	Yes	Yes
BRAF	G463E	Yes	Yes	Yes
BRAF	G465V	Yes	Yes	Yes
BRAF	L596R	Yes	Yes	Yes
BRAF	L596V	Yes	Yes	Yes
BRAF	V600E	Yes	Yes	Yes
BRAF	K600E	Yes	Yes	Yes
EGFR	G719C	Yes	Yes	Yes
EGFR	G719S	Yes	Yes	Yes
EGFR	**T790M**	Yes	Yes	No
EGFR	L858R	Yes	Yes	Yes
FGFR2	S267P	Yes	Yes	Yes
FGFR3	*R248C*	*Yes*	*Yes*	*ND*
FGFR3	*S249C*	*Yes*	*Yes*	*ND*
FGFR3	E322K	Yes	Yes	Yes
FGFR3	K650E	Yes	Yes	Yes
ErbB2	L755P	Yes	Yes	Yes
ErbB2	**G776S**	Yes	Yes	No
ErbB2	**N857S**	Yes	Yes	No
ErbB2	E914K	Yes	Yes	Yes
KIT	V559D	Yes	Yes	Yes
KIT	V560G	No	No	No
KIT	D816V	Yes	Yes	Yes
LKB1/STK11	Y49D	Yes	Yes	Yes
LKB1/STK11	G135R	Yes	Yes	Yes
PDGFRa	V561D	Yes	Yes	Yes
PDGFRa	D842V	Yes	Yes	Yes
RET	**M918T**	**Yes**	**Yes**	**No**

Roukos, Novel clinico-genome network modeling for revolutionizing genotype-phenotype-based personalized cancer care. Expert Rev Mol Diagn., 10 (1): 33-48, 2010

Cancer arise from changes in the DNA sequence in the genomes of cancer cells. All of these genetic alterations can now be discovered using next-generation DNA sequencing technology.

This can achieve two major goals:

first, to **complete the catalogue of driver mutations**, and second,

to **explore the functional role of cancer genes.**

Mutace v genech *IDH1* a *IDH2* u AML

ORIGINAL ARTICLE

Recurring Mutations Found by Sequencing an Acute Myeloid Leukemia Genome

Elaine R. Mardis, Ph.D., Li Ding, Ph.D., David J. Dooling, Ph.D., David E. Larson, Ph.D., Michael D. McLellan, B.S., Ken Chen, Ph.D., Daniel C. Koboldt, M.S., Robert S. Fulton, M.S., Kim D. Delehaunty, B.A., Sean D. McGrath, M.S., Lucinda A. Fulton, M.S., Devin P. Locke, Ph.D., Vincent J. Magrini, Ph.D., Rachel M. Abbott, B.S., Tammi L. Vickery, B.S., Jerry S. Reed, M.S., Jody S. Robinson, M.S., Todd Wylie, B.S., Scott M. Smith, Lynn Carmichael, B.S., James M. Eldred, Christopher C. Harris, B.S., Jason Walker, B.A., B.S., Joshua B. Peck, M.B.A., Feiyu Du, M.S., Adam F. Dukes, B.A., Gabriel E. Sanderson, B.S., Anthony M. Brummett, Eric Clark, Joshua F. McMichael, B.S., Rick J. Meyer, M.S., Jonathan K. Schindler, B.S., B.A., Craig S. Pohl, M.S., John W. Wallis, Ph.D., Xiaoqi Shi, M.S., Ling Lin, M.S., Heather Schmidt, B.S., Yuzhu Tang, M.D., Carrie Haipek, M.S., Madeline E. Wiechert, M.S., Jolynda V. Ivy, M.B.A., Joelle Kalicki, B.S., Glendoria Elliott, Rhonda E. Ries, M.A., Jacqueline E. Payton, M.D., Ph.D., Peter Westervelt, M.D., Ph.D., Michael H. Tomasson, M.D., Mark A. Watson, M.D., Ph.D., Jack Baty, B.A., Sharon Heath, William D. Shannon, Ph.D., Rakesh Nagarajan, M.D., Ph.D., Daniel C. Link, M.D., Matthew J. Walter, M.D., Timothy A. Graubert, M.D., John F. DiPersio, M.D., Ph.D., Richard K. Wilson, Ph.D., and Timothy J. Ley, M.D.

ABSTRACT

BACKGROUND

The full complement of DNA mutations that are responsible for the pathogenesis of acute myeloid leukemia (AML) is not yet known.

METHODS

We used massively parallel DNA sequencing to obtain a very high level of coverage (approximately 98%) of a primary, cytogenetically normal, de novo genome for AML with minimal maturation (AML-M1) and a matched normal skin genome.

RESULTS

We identified 12 acquired (somatic) mutations within the coding sequences of genes and 52 somatic point mutations in conserved or regulatory portions of the genome. All mutations appeared to be heterozygous and present in nearly all cells in the tumor sample. Four of the 64 mutations occurred in at least 1 additional AML sample in 188 samples that were tested. Mutations in *NRAS* and *NPM1* had been identified previously in patients with AML, but two other mutations had not been identified. One of these mutations, in the *IDH1* gene, was present in 15 of 187 additional AML genomes tested and was strongly associated with normal cytogenetic status; it was present in 13 of 80 cytogenetically normal samples (16%). The other was a nongenic mutation in a genomic region with regulatory potential and conservation in higher mammals; we detected it in one additional AML tumor. The AML genome that we sequenced contains approximately 750 point mutations, of which only a small fraction are likely to be relevant to pathogenesis.

CONCLUSIONS

From the Departments of Genetics (E.R.M., L.D., V.J.M., R.K.W., T.J.L.), Medicine (R.E.R., P.W., M.H.T., S.H., W.D.S., D.C.L., M.J.W., T.A.G., J.F.D., T.J.L.), and Pathology and Immunology (J.E.P., M.A.W., R.N.); the Genome Center (E.R.M., L.D., D.J.D., D.E.L., M.D.M., K.C., D.C.K., R.S.F., K.D.D., S.D.M., L.A.F., D.P.L., V.J.M., R.M.A., T.L.V., J.S. Reed, J.S. Robinson, T.W., S.M.S., L.C., J.M.E., C.C.H., J.W., J.B.P., F.D., A.F.D., G.E.S., A.M.B., E.C., J.F.M., R.J.M., J.K.S., C.S.P., J.W.W., X.S., L.L., H.S., Y.T., C.H., M.E.W., J.V.I., J.K., G.E., M.A.W., R.K.W., T.J.L.); Siteman Cancer Center (P.W., M.H.T., M.A.W., S.H., W.D.S., R.N., D.C.L., M.J.W., T.A.G., J.F.D., R.K.W., T.J.L.); and the Division of Biostatistics (J.B.) — all at Washington University, St. Louis. Address reprint requests to Dr. Ley at Washington University, 660 S. Euclid Ave., Campus Box 8007, St. Louis, MO 63110, or at timley@wustl.edu.

This article (10.1056/NEJMoa0903840) was published on August 5, 2009, at NEJM.org.

N Engl J Med 2009;361.

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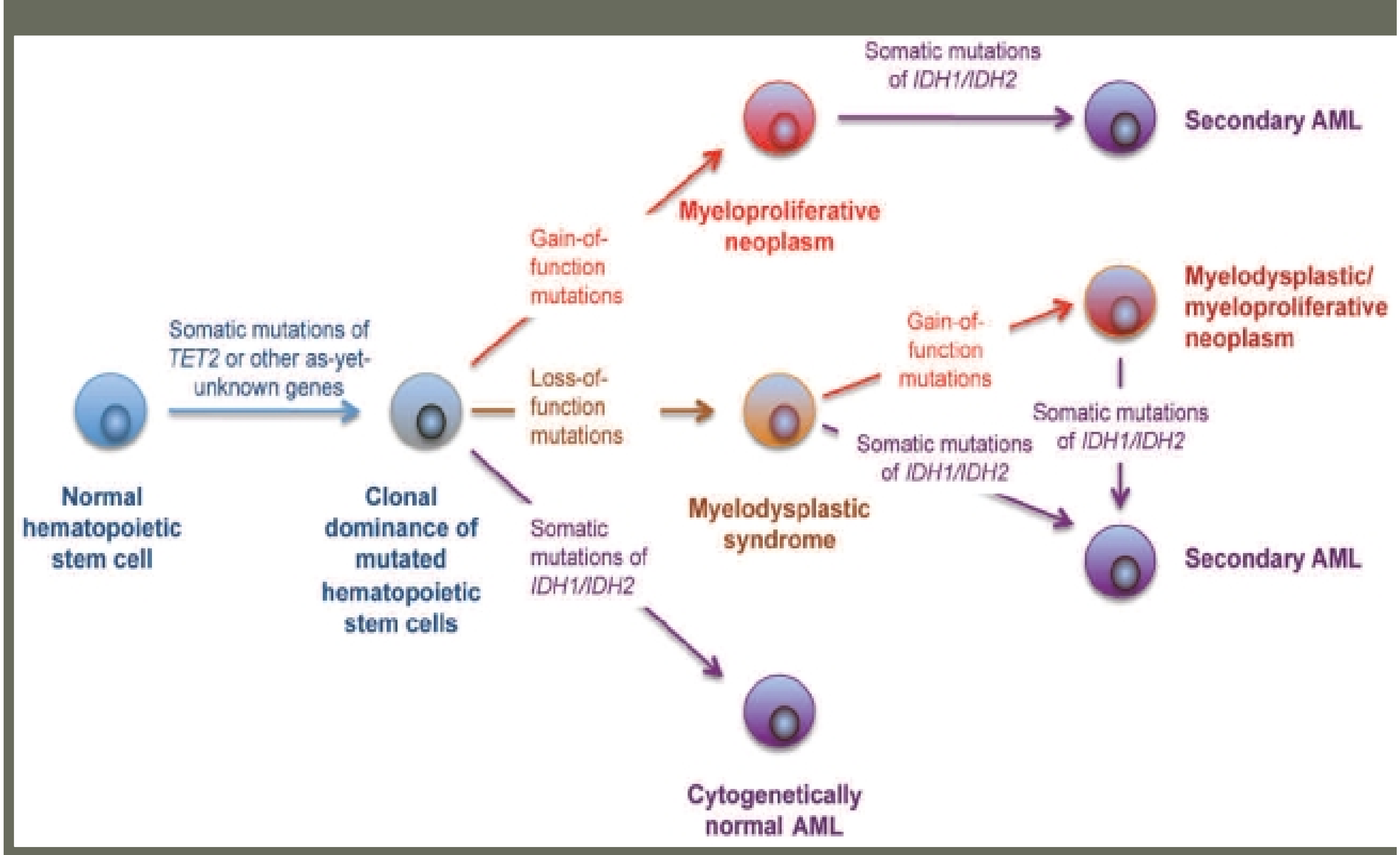
2009



Schematic representation of the molecular bases of myeloid neoplasms and hypothesized role of *IDH1/IDH2* mutations.
***Haematologica.* 2010 October; 95(10): 1623–1627.**

NATURE GENETICS | ARTICLE

Exome sequencing identifies somatic mutations of DNA methyltransferase gene *DNMT3A* in acute monocytic leukemia

Xiao-Jing Yan, Jie Xu, Zhao-Hui Gu, Chun-Ming Pan, Gang Lu, Yang Shen, Jing-Yi Shi, Yong-Mei Zhu, Lin Tang, Xiao-Wei Zhang, Wen-Xue Liang, Jian-Qing Mi, Huai-Dong Song, Ke-Qin Li, Zhu Chen & Sai-Juan Chen

Affiliations | Contributions | Corresponding authors

Nature Genetics 43, 309–315 (2011) | doi:10.1038/ng.788

2011

J Clin Oncol. 2011 Jul 20;29(21):2889-96. Epub 2011 Jun 13.

Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia.

Thol F, Damm F, Lüdeking A, Winschel C, Wagner K, Morgan M, Yun H, Göhring G, Schlegelberger B, Hoelzer D, Lübbert M, Kanz L, Fiedler W, Kirchner G, Krauter J, Ganser A, Heuser M.

Hannover Medical School, Hannover, Germany.

Leukemia. 2012 Jan 13. doi: 10.1038/leu.2011.382. [Epub ahead of print]

Prognostic significance of DNA methyltransferase 3A mutations in cytogenetically normal acute myeloid leukemia: a study by the Acute Leukemia French Association.

Renneville A, Boissel N, Nibourel O, Berthon C, Helevaut N, Gardin C, Cayuela JM, Hayette S, Reman O, Contentin N, Bordessoule D, Pautas C, Botton SD, Revel TD, Terre C, Fenaux P, Thomas X, Castaigne S, Dombret H, Preudhomme C.

1] Laboratory of Hematology, Biology and Pathology Center, CHRU of Lille, Lille, France [2] University of Lille Nord de France, Lille, France [3] Inserm, U837, Team 3, Cancer Research Institute of Lille, Lille, France.

Whole-exome sequencing identifies somatic mutations of *BCOR* in acute myeloid leukemia with normal karyotype

*Vera Grossmann,[1] *Enrico Tiacci,[2] Antony B. Holmes,[3] Alexander Kohlmann,[1] Maria Paola Martelli,[2] Wolfgang Kern,[1] Ariele Spanhol-Rosseto,[2] Hans-Ulrich Klein,[4] Martin Dugas,[4] Sonja Schindela,[1] Vladimir Trifonov,[3] Susanne Schnittger,[1] Claudia Haferlach,[1] Renato Bassan,[5] Victoria A. Wells,[6] Orietta Spinelli,[5] Joseph Chan,[3] Roberta Rossi,[2] Stefano Baldoni,[2] Luca De Carolis,[2] Katharina Goetze,[7] Hubert Serve,[8] Rudolf Peceny,[9] Karl-Anton Kreuzer,[10] Daniel Oruzio,[11] Giorgina Specchia,[12] Francesco Di Raimondo,[13] Francesco Fabbiano,[14] Marco Sborgia,[15] Arcangelo Liso,[16] Laurent Farinelli,[17] Alessandro Rambaldi,[5] Laura Pasqualucci,[2,6] Raul Rabadan,[3] Torsten Haferlach,[1] and Brunangelo Falini[2]

[1]MLL Munich Leukemia Laboratory, Munich, Germany; [2]Institute of Hematology, University of Perugia, Perugia, Italy; [3]Center for Computational Biology and Bioinformatics, Columbia University, New York, NY; [4]Department of Medical Informatics and Biomathematics, University of Münster, Münster, Germany; [5]Hematology Department, Ospedali Riuniti, Bergamo, Italy; [6]Institute for Cancer Genetics, Columbia University, New York, NY; [7]Klinikum rechts der Isar der TU München, III. Medizinische Klinik und Poliklinik, Munich, Germany; [8]Universitätsklinikum Frankfurt, Medizinische Klinik II, Frankfurt, Germany; [9]Klinikum Osnabrück GmbH, Hämatologie und Internistische Onkologie, Osnabrück, Germany; [10]Uniklinik Köln, Klinik I für Innere Medizin, Köln, Germany; [11]Klinikum Augsburg, II. Medizinische Klinik, Augsburg, Germany; [12]Institute of Hematology, University of Bari, Bari, Italy; [13]Institute of Hematology, University of Catania, Catania, Italy; [14]Institute of Hematology, Cervello Hospital, Palermo, Italy; [15]Institute of Hematology, Ospedale di Pescara, Pescara, Italy; [16]Institute of Hematology, University of Foggia, Foggia, Italy; and [17]Fasteris SA, Plan-les-Ouates/Geneva, Switzerland

Among acute myeloid leukemia (AML) patients with a normal karyotype (CN-AML), *NPM1* and *CEBPA* mutations define World Health Organization 2008 provisional entities accounting for approximately 60% of patients, but the remaining 40% are molecularly poorly characterized. Using whole-exome sequencing of one CN-AML patient lacking mutations in *NPM1*, *CEBPA*, *FLT3*-ITD, *IDH1*, and *MLL*-PTD, we newly identified a clonal somatic mutation in *BCOR* (*BCL6* corepressor), a gene located on chromosome Xp11.4. Further analyses of 553 AML patients showed that *BCOR* mutations occurred in 3.8% of unselected CN-AML patients and represented a substantial fraction (17.1%) of CN-AML patients showing the same genotype as the AML index patient subjected to whole-exome sequencing. *BCOR* somatic mutations were: (1) disruptive events similar to the germline *BCOR* mutations causing the oculo-facio-cardio-dental genetic syndrome; (2) associated with decreased *BCOR* mRNA levels, absence of full-length BCOR, and absent or low expression of a truncated BCOR protein; (3) virtually mutually exclusive with *NPM1* mutations; and (4) frequently associated with *DNMT3A* mutations, suggesting cooperativity among these genetic alterations. Finally, *BCOR* mutations tended to be associated with an inferior outcome in a cohort of 422 CN-AML patients (25.6% vs 56.7% overall survival at 2 years; *P* = .032). Our results for the first time implicate *BCOR* in CN-AML pathogenesis. (*Blood*. 2011;118(23):6153-6163)

The corepressors BCOR and BCORL1: two novel players in acute myeloid leukemia

Enrico Tiacci,[1*] Vera Grossmann,[2*] Maria Paola Martelli,[1] Alexander Kohlmann,[2] Torsten Haferlach,[2] and Brunangelo Falini[1]

[1]Institute of Hematology, University of Perugia, Italy; [2]MLL Munich Leukemia Laboratory, Munich, Germany

E-mail: faliniem@unipg.it doi:10.3324/haematol.2011.057901

* E. Tiacci and V. Grossmann contributed equally to BCOR gene studies

BCOR
(1755 aa)

3 2

BBD AF9BD ANK

BBD: BCL6 binding domain
AF9BD: AF9 binding domain
ANK: ankyrin repeats

FRAMESHIFT MUTATIONS
NONSENSE MUTATIONS
SPLICE-SITE MUTATIONS
MISSENSE MUTATIONS

BCORL1
(1711 aa)

2 2

CBS NLS LXXLL ANK LXXLL

CBS: CTBP1 binding site
NLS: nuclear localization signal
LXXLL: nuclear receptor recruitment motif
ANK: ankyrin repeats

Tiacci E et al. Haematologica 2012;97:3-5

2012

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JUN 23

Moving Next-Gen Sequencing into the Clinic

Filed Under Press Release

The FDA has begun to develop their program to evaluate sequencing based diagnostics. At a recent meeting, the Association for Molecular Pathology (AMP) advised FDA officials on many important considerations for evaluating the analytical validity of next-generation sequencing:

The analytical validation requirements for NGS will vary based on the clinical application at issue, such as a mutation panel for a Mendelian disease versus transcriptome analysis.

Performance of, and coverage needs for, a given platform are likely to differ depending on:

- the nucleic acid analyzed
- the characteristics of the DNA regions and the type of variations

AMP comments at FDA meeting on next-generation sequencing

June 23, 2011 (Bethesda, MD): Today, on behalf of the Association for Molecular Pathology (AMP), Dr. Elaine Lyon gave public comments at the US Food and Drug Administration's (FDA) meeting on "Ultra High Throughput Sequencing for Clinical Diagnostic Applications – Approaches to Assess Analytical Validity." As they begin developing their program to evaluate sequencing based diagnostics, AMP advised FDA officials on many important considerations for evaluating analytical validity.

The analytical validation requirements for NGS will vary based on the clinical application at issue, such as a mutation panel for a Mendelian disease versus transcriptome analysis. Also, performance of, and coverage needs for, a given platform are likely to differ depending on the nucleic acid analyzed, the characteristics of the DNA regions and the type of variations interrogated, the relative allele proportions of particular variants, and whether quantitative or qualitative results are desired. For these reasons, Dr. Lyon noted "this necessitates flexibility and individualization in the development of validation protocols, guidelines, and controls on an application-by-application basis."

While the analytical validity of a NGS instrument may be intrinsically very high, its data conversion and analysis software may have design flaws or performance limitations. As such, AMP told the FDA that for optimal FDA review of the test system, the analytical validity of the instrument and the performance of the bioinformatics software should be evaluated both independently and as a complete system.

AMP also pointed out the role of molecular pathology professionals in determining the most appropriate platform and technologies for answering the clinical question at issue and advised the FDA to be careful not to limit the practice of medicine. "Optimal patient care requires the ability of molecular pathology professionals to use their professional opinion of the most suitable technological approach," added Dr. Lyon, "and any FDA policy to review analytical validity should include a role for the molecular pathology professionals performing the test."

AMP calls on the FDA to partner with professional associations to benefit from their wealth of experience and expertise on next generation sequencing (NGS) and its clinical applications. "We have an important reservoir of experience and expertise within our organization," explained Dr. Lyon, "and we encourage the FDA to allow us to collaborate to ensure that this technology is safely, effectively, and appropriately used for the benefit of our patients." AMP is also currently working on professional practice guidelines to address the many ethical, social and legal implications for the clinical use of NGS, which will be beneficial for the entire medical community including the FDA.

Expert Rev Mol Diagn. 2011 Apr;11(3):333-43.

Next-generation sequencing and its applications in molecular diagnostics.

Su Z, Ning B, Fang H, Hong H, Perkins R, Tong W, Shi L.

Z-Tech, an ICF International Company at US FDA's National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079, USA.

Abstract

DNA sequencing is a powerful approach for decoding a number of human diseases, including cancers. The advent of next-generation sequencing (NGS) technologies has reduced sequencing cost by orders of magnitude and significantly increased the throughput, making whole-genome sequencing a possible way for obtaining global genomic information about patients on whom clinical actions may be taken. However, the benefits offered by NGS technologies come with a number of challenges that must be adequately addressed before they can be transformed from research tools to routine clinical practices. This article provides an overview of four commonly used NGS technologies from Roche Applied Science//454 Life Sciences, Illumina, Life Technologies and Helicos Biosciences. The challenges in the analysis of NGS data and their potential applications in clinical diagnosis are also discussed.

Clin Biochem Rev. 2011 Nov;32(4):177-95.

Next-generation sequencing for cancer diagnostics: a practical perspective.

Meldrum C, Doyle MA, Tothill RW.

Molecular Pathology, Hunter Area Pathology Service, Newcastle, NSW 2310;

Abstract

Next-generation sequencing (NGS) is arguably one of the most significant technological advances in the biological sciences of the last 30 years. The second generation sequencing platforms have advanced rapidly to the point that several genomes can now be sequenced simultaneously in a single instrument run in under two weeks. Targeted DNA enrichment methods allow even higher genome throughput at a reduced cost per sample. Medical research has embraced the technology and the cancer field is at the forefront of these efforts given the genetic aspects of the disease. World-wide efforts to catalogue mutations in multiple cancer types are underway and this is likely to lead to new discoveries that will be translated to new diagnostic, prognostic and therapeutic targets. NGS is now maturing to the point where it is being considered by many laboratories for routine diagnostic use. The sensitivity, speed and reduced cost per sample make it a highly attractive platform compared to other sequencing modalities. Moreover, as we identify more genetic determinants of cancer there is a greater need to adopt multi-gene assays that can quickly and reliably sequence complete genes from individual patient samples. Whilst widespread and routine use of whole genome sequencing is likely to be a few years away, there are immediate opportunities to implement NGS for clinical use. Here we review the technology, methods and applications that can be immediately considered and some of the challenges that lie ahead.

Clin Genet. 2012 Feb 29. doi: 10.1111/j.1399-0004.2012.01865.x. [Epub ahead of print]

Next Generation Sequencing: Ready for the clinics?

Desai AN, Jere A.
Persistent LABS, Persistent Systems Ltd., Pune.

Abstract

Next Generation Sequencing (NGS) has transformed genomic research by decreasing the cost of sequencing and increasing the throughput. Now, the focus is on using NGS technology for diagnostics and therapeutics. In this review, we discuss the possible clinical applications of NGS and the potential of some of the current systems to transition to the clinic. Clinical use of NGS technologies will enable identification of causative mutations for rare genetic disorders through whole genome or targeted genome resequencing, rapid pathogen screening and cancer diagnosis along with the identification of appropriate therapy. Routine clinical use of NGS technologies is appealing, but mandates high accuracy, simple assays, small inexpensive instruments, flexible throughput, short run times and most importantly, easy data analysis as well as interpretation. A number of NGS systems launched recently have least some of these characteristics namely; small instruments, flexible of throughput and short run time, but still face a few challenges. Moreover, simplified data analysis tools will need to be developed to minimize the requirement of sophisticated bioinformatics support in clinics. In summary, for successful transition of NGS to clinic, a sustained collaboration between research labs, clinical practitioners and vendors offering sequencing based genetic tests is required.

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The Challenge of Clinical Annotation

The day started off somber enough, with a morning session on "Clinical Translation of Genomics" (chaired by **Chad Nusbaum**) in which **everyone began to appreciate the truly difficult problem of accurate clinical annotation** of genetic variation. Sequencing and variant detection are accessible and straightforward to most labs. But interpretation of the results, even for rare genetic diseases, has proven challenging. **Lynn Jorde** gave a personal touch to his lab's work on the Miller syndrome family. **Heidi Rehm** spoke about the need to expand current targeted clinical tests.

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Next-gen Sequencing: Lots of Data, Not Many Findings

The evening concurrent sessions have been well-covered by Twitter feeds (search: #AGBT). For the most part, I stuck around the "Medical Sequencing and Genetic Variation" session, chaired by **Elaine Mardis** (WashU). The recurrent theme, ironically, seemed to be "**We're not sure what to make of this.**" It didn't matter if the phenotype was super-longevity (Devine), autism (Edwards), or an undiagnosed neurological condition in monozygotic twins (Margulies). The technologies were advanced, the analyses were [usually] thorough, but there just aren't any definitive novel findings. More work to be done. More samples needed. No one's blaming the sequencing technology.

And so, while the would-be-overlords of sequencing duke it out over throughput, accuracy, and cost, most of the delegates are really thinking about what's coming: the daunting task of systematic analysis and interpretation.

AGBT Advances in Genome Biology & Technology

Six Innovative Laboratories Win GenomeQuest Grants to Bring Next Generation Sequencing Diagnostics to the Clinic

GenomeQuest

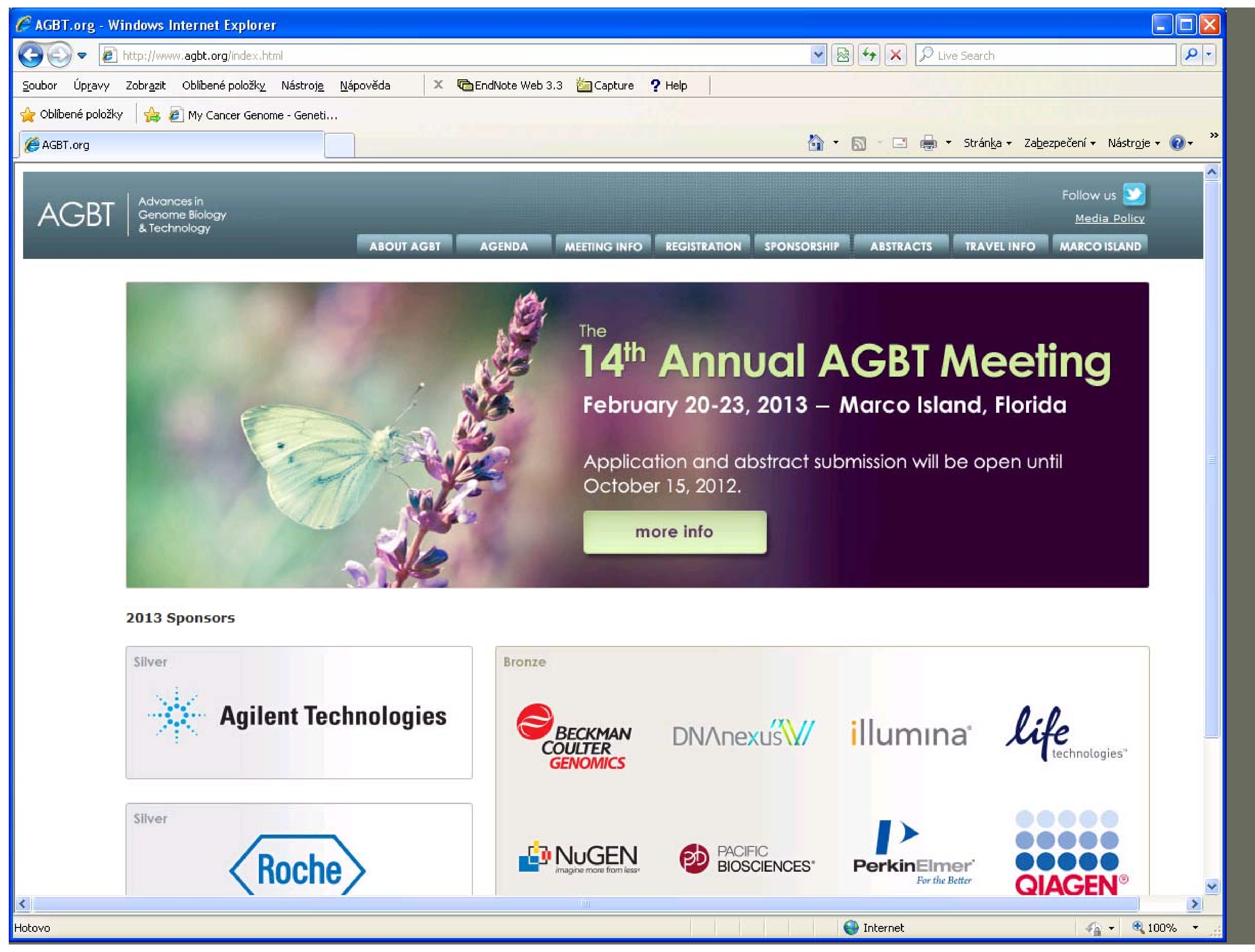
Program Provides GenomeQuest Software, Services, and Expertise to Convert and Consolidate Sanger Gene Test Panels

WESTBOROUGH, Mass., Jan. 10, 2012 — GenomeQuest Inc., a global provider of large-scale genomic software applications, announced today that it has awarded six grants totaling more than $120,000 in software and services to help six laboratories facilitate the transition from multiple Sanger-based gene tests to consolidated next generation sequencing (NGS) based tests.

"As a clinician, I am intrigued by the potential benefits of NGS for the long-term and the practical benefits in the near-term," said Dr. Roberta Pagon, Professor, Department of Pediatrics, University of Washington, Principle Investigator of GeneTests and GeneReviews. "At this point it appears that the entry path to NGS for a number of labs listed in the GeneTests Laboratory Directory will be through consolidation of existing single gene testing into multi-gene panels — such panels will benefit many patients whose physicians are seeking to identify the genetic basis of an inherited disorder. The GenomeQuest Lab Grant program offers a complete NGS bioinformatics solution that promises to help labs advance to NGS and next generation testing services."

GenomeQuest will provide the software, annotation data, and infrastructure required to process and store NGS data, produce diagnostic reports, and perform follow-up research on individual and aggregate results. Recipients will use the resources to more quickly and cost-effectively perform a variety of diagnostics for rare genetic disorders, autism, cystic fibrosis, congenital heart disease, muscular dystrophy, and harmful bacterial pathogens.

"We look forward to collaborating with the six grant recipients who were selected from a competitive field of applicants based on their clear, actionable vision for utilizing NGS-based diagnostics in the clinic," said GenomeQuest CEO Richard Resnick. "Lab directors are recognizing the enormous opportunity to improve patient care by screening larger and larger regions of the genome while simultaneously lowering costs and turn-around times with next generation sequencing. This program supports GenomeQuest's commitment to empowering laboratories with the knowledgebase and interpretation tools they need to bring new NGS-based diagnostics to the clinic."



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Br J Haematol. 2013 Jan 7. doi: 10.1111/bjh.12194. [Epub ahead of print]

Next-generation sequencing - feasibility and practicality in haematology.

Kohlmann A, Grossmann V, Nadarajah N, Haferlach T.

MLL Munich Leukaemia Laboratory, Munich, Germany.

Abstract

Next-generation sequencing platforms have evolved to provide an accurate and comprehensive means for the detection of molecular mutations in heterogeneous tumour specimens. Here, we review the feasibility and practicality of this novel laboratory technology. In particular, we focus on the utility of next-generation sequencing technology in characterizing haematological neoplasms and the landmark findings in key haematological malignancies. We also discuss deep-sequencing strategies to analyse the constantly increasing number of molecular markers applied for disease classification, patient stratification and individualized monitoring of minimal residual disease. Although many facets of this assay need to be taken into account, amplicon deep-sequencing has already demonstrated a promising technical performance and is being continuously developed towards routine application in diagnostic laboratories so that an impact on clinical practice can be achieved.

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Disease	Technique	Candidate gene (selected)	Discovery cohort cases	Further results	Clinical impact
AML	Whole-genome sequencing	*IDH1*	1	The AML genome (FAB M1) that was sequenced contained ~750 point mutations, of which only a small fraction are likely to be relevant to pathogenesis	*IDH1* mutation was present in 15 of 187 additional AML genomes tested; strongly associated with normal cytogenetic status (it was present in 16% (13/80) cytogenetically normal samples)
AML	Whole-genome sequencing	*DNMT3A*	1	62/281 (22·1%) screened cases had *DNMT3A* mutations predicting translational consequences; no *DNMT3A* mutations were found in the group of 79 patients with a favourable-risk cytogenetic profile	*DNMT3A* mutations were highly recurrent in patients with *de novo* AML with an intermediate-risk cytogenetic profile and were independently associated with a poor outcome
AML	Whole-exome sequencing	*BCOR*	1	*BCOR* disruptive mutations were relatively rare in unselected CN-AML patients (3·8%)	*BCOR* disruptive mutations accounted for a significant fraction (17·1%) of the least genetically characterized subgroup of CN-AML, i.e. those carrying no *NPM1*, *CEBPA*, *FLT3*-ITD, or *IDH1* mutations and no *MLL*-PTD and may confer an inferior prognosis
AML	Whole-genome sequencing	*NPM1*, *DNMT3A*, *IDH1*, *TET2*, *IDH2*, *RUNX1*, *ASXL1*, *SMC1A*, *SMC3*, *and STAG2*	24	Nine recurrently mutated genes were detected both in FAB M1 and M3 genomes. Recurring mutations were identified in genes that encode all four members of the cohesin complex (*STAG2*, *SMC3*, *RAD21*, and *SMC1A*)	This data suggested that most of the somatic events in AML genomes appear to be random, pre-existing, background mutations in the haematopoietic cell that acquired the key initiating mutation

Disease	Technique	gene (selected)	Discovery cohort cases	Further results	Clinical impact
CLL	Whole-exome sequencing	*NOTCH1*, *SF3B1*	105	*SF3B1*, encoding a subunit of the spliceosomal U2 small nuclear ribonucleoprotein (snRNP), is somatically mutated in 9·7% of affected individuals (27/279)	Individuals with *SF3B1* somatic mutations had significantly shorter time to disease progression and lower 10-year overall survival rates. Cox analyses suggested that the mutational status of *SF3B1* has a prognostic value independent of clinical stage, or ZAP70 or CD38 expression
CLL	Whole-genome and whole-exome sequencing	*SF3B1*	91	*SF3B1*, which functions at the catalytic core of the spliceosome, was the second most frequently mutated gene with mutations occurring in 15% of patients	Pre-mRNA splicing highlighted as a critical cellular process contributing to CLL
CLL	Whole-exome and Sanger sequencing	*NOTCH1*	5	Mutational activation of *NOTCH1* in 8·3% of CLL at diagnosis; detected at significantly higher frequency during disease progression toward Richter transformation (31·0%), as well as in chemorefractory CLL (20·8%)	Results confirmed previous findings on the low frequency and prognostic relevance of *NOTCH1* mutations at diagnosis, but identified *NOTCH1* mutations as a predictor of unfavourable outcome independent of *IGHV* mutation status and *TP53* disruption
Multiple myeloma	Whole-genome and whole-exome sequencing	*NRAS*, *KRAS*, *FAM46C*, *DIS3*, *TP53*	38	Discovery of frequent mutations in genes involved in RNA processing, protein translation and the unfolded protein response (16 out of 38 patients; 42%)	4% (7/161) of myeloma cases harboured common *BRAF* mutations (V600E, K601N) and may benefit from treatment with BRAF inhibitors
Waldenström Macroglobulinaemia	Whole-genome sequencing	*MYD88*	30	Sanger sequencing identified *MYD88* L265P in tumour samples from 49 of 54 patients with Waldenström macroglobulinaemia and in 3 of 3 patients with non–IgM-secreting LPL (91% of all	*MYD88* L265P is a commonly recurring mutation that can be useful in differentiating Waldenström macroglobulinaemia and non-IgM LPL from B-cell disorders that have some of the same features

Targeted Sequencing of Leukemia-Associated Genes Using 454 Sequencing Systems

GS GType TET2/CBL/KRAS and RUNX1 Primer Sets for the GS Junior and GS FLX Systems.

Introduction

Targeted sequencing is a focused and efficient method for investigating variation in a few selected exons or full genes of interest. 454 Sequencing Systems, including the GS FLX and GS Junior Systems, have proven to be powerful solutions for deep sequencing of PCR amplicons due to their long, highly accurate sequencing reads.

The GS GType RUNX1 Primer Set and GS GType TET2/CBL/KRAS Primer Set are research assays designed to provide researchers with a comprehensive picture of genetic variations in four key human genes - *RUNX1*, *TET2*, *CBL*, and *KRAS* - with potential impact in human health. These genes are associated with a range of leukemias and myeloid malignancies.

The complete solution, which includes primer sets, associated protocols, and dedicated software, is designed to aid in the investigation of genetic variation associated with developmental defects, disease progression, and residual disease. The assays are well suited for use with either the GS Junior or the GS FLX and GS FLX+ Systems using genomic DNA isolated from Peripheral Blood Mononuclear Cells (PBMCs).

The assays have been co-developed with and extensively tested at the MLL Munich Leukemia Laboratory (Munich, Germany, www.mll.com). Additionally, the TET2/CBL/KRAS assay is the result of the International RObustness of Next-Generation Sequencing (IRON) study (Leukemia. 2011; 25(12): 1840–8).

GENES, CHROMOSOMES & CANCER 51:689–695 (2012)

Next-Generation Sequencing for Minimal Residual Disease Monitoring in Acute Myeloid Leukemia Patients with *FLT3*-ITD or *NPM1* Mutations

Felicitas Thol,[1] Britta Kölking,[1] Frederik Damm,[2] Katarina Reinhardt,[3] Jan-Henning Klusmann,[3] Dirk Reinhardt,[3] Nils von Neuhoff,[4] Martijn H. Brugman,[5] Brigitte Schlegelberger,[4] Sebastian Suerbaum,[6] Jürgen Krauter,[1] Arnold Ganser,[1†] and Michael Heuser[1*]

[1]Department of Hematology, Hemostasis, Oncology, and Stem Cell Transplantation, Hannover Medical School, Hannover, Germany
[2]Institut Gustave Roussy, Villejuif, France
[3]Department of Pediatric Hematology and Oncology, Hannover Medical School, Hannover, Germany
[4]Institute of Cell and Molecular Pathology, Hannover Medical School, Hannover, Germany
[5]Department of Experimental Hematology, Hannover Medical School, Hannover, Germany
[6]Institute for Medical Microbiology and Hospital Epidemiology, Hannover Medical School, Hannover, Germany

Systematic assessment of minimal residual disease (MRD) in acute myeloid leukemia (AML) patients has been hampered by lack of a reliable, uniform MRD marker applicable to all patients. We evaluated next-generation sequencing (NGS) for MRD assessment in AML patients ($n = 80$ samples). The ability of NGS technologies to generate thousands of clonal sequences makes it possible to determine the allelic ratio of sequence variants. Using NGS, we were able to determine the allelic ratio of different *FLT3*-internal tandem duplication (ITD) clones within one patient sample, in addition to resolution of *FLT3*-ITD insertion site, length, and sequence in a single analysis. Furthermore, NGS allowed us to study emergence of clonal dominance. Parallel assessment of MRD by NGS and quantitative real-time polymerase chain reaction in *NPM1* mutated patients was concordant in 95% of analyzed samples ($n = 38$). The frequency of mutated alleles was linearly quantified by NGS. As NGS sensitivity is scalable depending on sequence coverage, it reflects a highly flexible and reliable tool to assess MRD in leukemia patients. © 2012 Wiley Periodicals, Inc.

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