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MOLECULAR PATHOPHYSIOLOGY OF MYELOPROLIFERATIVE DISORDERS

Ph.D. Thesis

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Prohlašuji, že jsem předloženou práci vykonala samostatně pod vedením školitele Doc. Vladimíra Divokého, Ph.D. s použitím citované literatury.

I plege my word, that this work has been my own under the supervision of Assoc. Prof. Vladimír Divoký, Ph.D., and that I have used all mentioned references.

In Olomouc……………..   ……………...…………………..

Jitka Veselovská

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1 SUMMARY

My work has focused on the molecular background of pathophysiology of chronic myeloproliferative disorders. I have dealt with two different areas. The first is the study of molecular mechanisms of resistance of chronic myeloid leukemia (CML) to targeted therapies i.e. to imatinib (IM) and dasatinib (Das). The second is the investigation of the role of *JAK2* V617F mutation in ethiopathogenesis of essential thrombocythemia (ET) and polycythemia vera (PV).

CML is the first leukemia known to be associated with typical genetic pathology: the Philadelphia chromosome. This chromosome results from reciprocal translocation leading to formation of an abnormal fusion gene *BCR-ABL*. The protein product of this gene is Bcr-Abl kinase, which is sufficient to induce leukemogenesis in animal models *in vivo*. Modern therapies have been developed that specifically target this abnormal kinase. Small-molecular-weight inhibitors of Bcr-Abl kinase i.e. IM and Das are already in use in clinical setting. Development of resistance to these targeted therapies represents new challenge for the clinicians.

In our laboratory we have developed a functional test of sensitivity of CML patients' leukocytes to these inhibitors *in vitro*. This method is based on incubation of isolated patients' leukocytes with or without the drug and evaluation of the drug activity by detection of inhibition of phosphorylation of target molecules (performed by western blotting). From 2004 to 2008, 116 different tests were performed (102 detections of phosphorylated Crkl, 14 detections of phosphorylated Src family of kinases i.e. P-Src) from clinical samples of overall 63 CML patients. The course of treatment of these patients can be readjusted according to individual patient's sensitivity.

In addition to the study of overt resistance, we have set out to investigate possible molecular background for *BCR-ABL* persistence on IM treatment. We have selected CML patients without additional chromosomal aberrations treated with IM for at least 6 months with persistant *BCR-ABL*-positive population of cells. We have selected *BCR-ABL*-positive cells, which were able to escape IM induced apoptosis by their cultivation with IM *in vitro*. DNA isolated from these cells was analyzed by CGH microarray. This method detects amplifications and deletions of genes, which could play a role in the disease persistence. We have found some interesting candidate genes, which will be subjected to further analysis in the future.

The second theme, which I was dealing with, is the role of *JAK2* V617F mutation in ethiopathogenesis of myeloproliferative disorders. This mutation is associated with almost all PV cases and about half of the adult ET patients, and is usually connected with worse clinical symptoms. We have focused on analysis of a very rare disease: childhood ET. Cohort of 15 patients diagnosed with this disease was studied in detail. *JAK2* V617F mutation was detected only in one patient on the level of peripheral blood leukocytes and in separated platelets and granulocytes. Monoclonal hematopoiesis was noted in only another one female patient. Erythroid progenitors of most of the patients displayed hypersensitivity to erythropoietin (Epo) *in vitro*; Epo-independent erythroid colonies (EECs) were detected in eight patients. Rare colonies heterozygous or homozygous for the *JAK2* V617F mutation were observed among EECs of four patients. Our data suggests that childhood ET patients could bear minor *JAK2* V617F-positive subclones. The presence of this mutation was not associated with any clinical complications. The patients will be followed in order to detect any prognostic or clinical value of this mutation in pediatric ET and for possible development into PV.

Final case, which I have studied in detail, was a patient diagnosed with both *JAK2* V617F-positive PV and B-cell chronic lymphocytic leukemia (B-CLL). The simultaneous occurrence of both chronic myeloid and lymphoid proliferations is a very rare event which offers possibility to study origins of these diseases. We have set out to determine if these two disorders arose from a single hematopoietic progenitor.

The granulocytes, monocytes, T and B lymphocytes were separated. The *JAK2* V617F mutation was detected neither in T nor B lymphocytes. In contrast, the monocytes and granulocytes were heterozygous for the *JAK2* V617F mutation. In addition, typical cytogenetic marker for B-CLL (*RB1* deletion, both homozygous and heterozygous) was detected only in lymphocytes. Clonal heterogeneity was also observed in the myeloid populations. Using *JAK2* genotyping of individual myeloid colonies, we detected heterozygous, homozygous and wt subclones with respect to *JAK2* V617F mutation. There were some EECs without the *JAK2* V617F mutation suggesting that *JAK2* mutation was a secondary event in this case. Our study proves that typical well-known markers of the two diseases (*RB1* deletion and *JAK2* V617F) develop as secondary events and only in lymphoid or myeloid lineage, respectively.

In summary, I have studied the molecular background of myeloproliferative disorders focusing on the resistance of CML patients to targeted therapies and the role of *JAK2* V617F mutation in ET and PV.

Key words: myeloproliferative disorder, CML, imatinib resistance, dasatinib, PV, ET, *JAK2* V617F, Epo hypersensitivity, EEC

2 SOUHRN

V této práci jsem se zabývala molekulární patofyziologií chronických myeloproliferací. Zaměřila jsem se na dvě různé oblasti. První je studium molekulárních mechanismů rezistence nemocných s chronickou myeloidní leukémií (CML) na cílenou léčbu imatinibem (IM). Druhou oblastí je otázka významu mutace V617F v genu kódující Jak2 kinázu v patogenezi esenciální trombocytémie (ET) a polycytémie vera (PV).

CML byla první leukémie, která byla asociována s genetickou abnormalitou tj. s Filadelfským chromosomem. Tento atypický chromosom vzniká reciprokou translokací chromosomu 9 a 22. Na molekulární úrovni zde vzniká abnormální fúzní gen *BCR-ABL*, který kóduje konstitutivně aktivní Bcr-Abl kinázu. Transformační potenciál Bcr-Abl kinázy byl prokázán *in vitro* i *in vivo* a je proto ideálním cílem terapie. Nízkomolekulární inhibitory Bcr-Abl kinázy (IM a DAS) jsou moderní léčiva již používaná v klinice. Základním problémem těchto moderních terapií je rozvoj rezistence.

V naší laboratoři jsme vyvinuly funkční test citlivosti buněk nemocných na jednotlivé inhibitory *in vitro*. Tato metoda je založena na inkubaci izolovaných leukocytů pacienta s léčivem a bez léčiva a na hodnocení účinnosti léčiva na základě inhibice fosforylace cílových molekul (pomocí metody western blot). Od roku 2004 do roku 2008 bylo provedeno 116 testů (102 detekcí fosforylace molekuly Crkl, 14 detekcí fosforylace Src kináz) na klinických vzorcích z celkem 63 pacientů. Tento *in vitro* test pomáhá v rozhodování o terapii pacienta s CML.

Dále jsem se zajímala nejen o plně rozvinutou rezistenci na léčbu IM, ale i o molekulární mechanismy umožňující přežívání *BCR-ABL*-pozitivních klonů v průběhu léčby IM. Vybrali jsme CML nemocné bez přídatných chromosomálních aberací, kteří byli léčeni IM nejméně 6 měsíců a u kterých přetrvával klon *BCR-ABL*-pozitivních buněk v periferii (do 30%). Vyselektovali jsme *BCR-ABL*-pozitivní buňky schopné přežívat v přítomnosti IM pomocí jejich kultivace s IM *in vitro*. Využili jsme metody CGH microarray k detekci amplifikací a delecí genů přítomných v těchto populacích. Tímto způsobem jsme našli zajímavé kandidátní geny, které budeme dále analyzovat v budoucnu.

Další oblastí výzkumu, na kterou jsem se zaměřila, je role mutace *JAK2* V617F v etiopatogenezi myeloproliferativních onemocnění. Tato mutace se nachází ve většině případů PV a v polovině případů dospělých nemocných s ET a bývá asociována s klinickými komplikacemi. V našem případě jsme studovali kohortu 15 pacientů s diagnózou velmi

vzácného onemocnění: dětskou ET. Mutace *JAK2* V617F byla detekována jen u jedné pacientky na úrovni leukocytů z periferní krve a na úrovni separovaných populací destiček a granulocytů. Monoklonální hematopoéza byla přítomna jen u další jedné pacientky. Erytroidní progenitory většiny pacientů vykazovaly hypersenzitivitu na erytropoetin (Epo) *in vitro*; tzv. endogenní erytroidní kolonie byly přítomny u osmi nemocných. U pěti pacientů jsme detekovali přítomnost několika vzácných kolonií heterozygotních a homozygotních na *JAK2* V617F mutaci. Naše výsledky ukazují, že dětští pacienti s ET mohou mít velmi vzácné subklony obsahující *JAK2* V617F mutaci. Přítomnost této mutace nebyla asociována s žádnými klinickými komplikacemi. Budeme tyto nemocné sledovat, abychom zjistili vliv této mutace na prognózu, klinický průběh, popřípadě možnou progresi onemocnění do klinického obrazu PV.

Výskyt chronické myeloidní a lymfatické proliferace u jediného pacienta je velmi vzácný úkaz, který umožňuje studium buněčného původu těchto onemocnění. Zabývali jsme se případem pacientky s diagnosou *JAK2* V617F pozitivní PV a zároveň i B-buněčné chronické lymfatické leukémie (B-CLL). Položili jsme si otázku, zda u této nemocné tato dvě onemocnění pocházejí ze společné progenitorové buňky, nebo zda se jedná o na sobě nezávislé genetické události ve dvou různých progenitorech, které vedly ke vzniku obou malignit.

Populace granulocytů, monocytů, T a B lymfocytů byly izolovány z periferní krve. Mutace *JAK2* V617F nebyla detekovaná v T ani B lymphocytární populaci, narozdíl od monocytů a granulocytů, které byly heterozygotní pro tuto mutaci. Polycytémie byla charakterizovaná růstem endogenních erytroidních kolonií, které obsahovaly nemutovaný i mutovaný gen *JAK2*. Typický cytogenetický marker B-CLL (delece *RB1* genu) byl detekován jen v populacích lymfocytů. Naše studie přímo neurčuje, zda u této nemocné tato dvě onemocnění pochází z různých kmenových buněk, ale ukazuje, že typické známé markery obou onemocnění (delece *RB1* a mutace *JAK2* V617F) se objevují jako sekundární události a to jen v lymfoidních nebo myeloidních buňkách.

Závěrem, studovala jsem molekulární mechanismy myeloproliferativních onemocnění se zaměřením na rezistenci CML pacientů na léčbu IM a na roli mutace *JAK2* V617F v patofyziologii ET a PV.

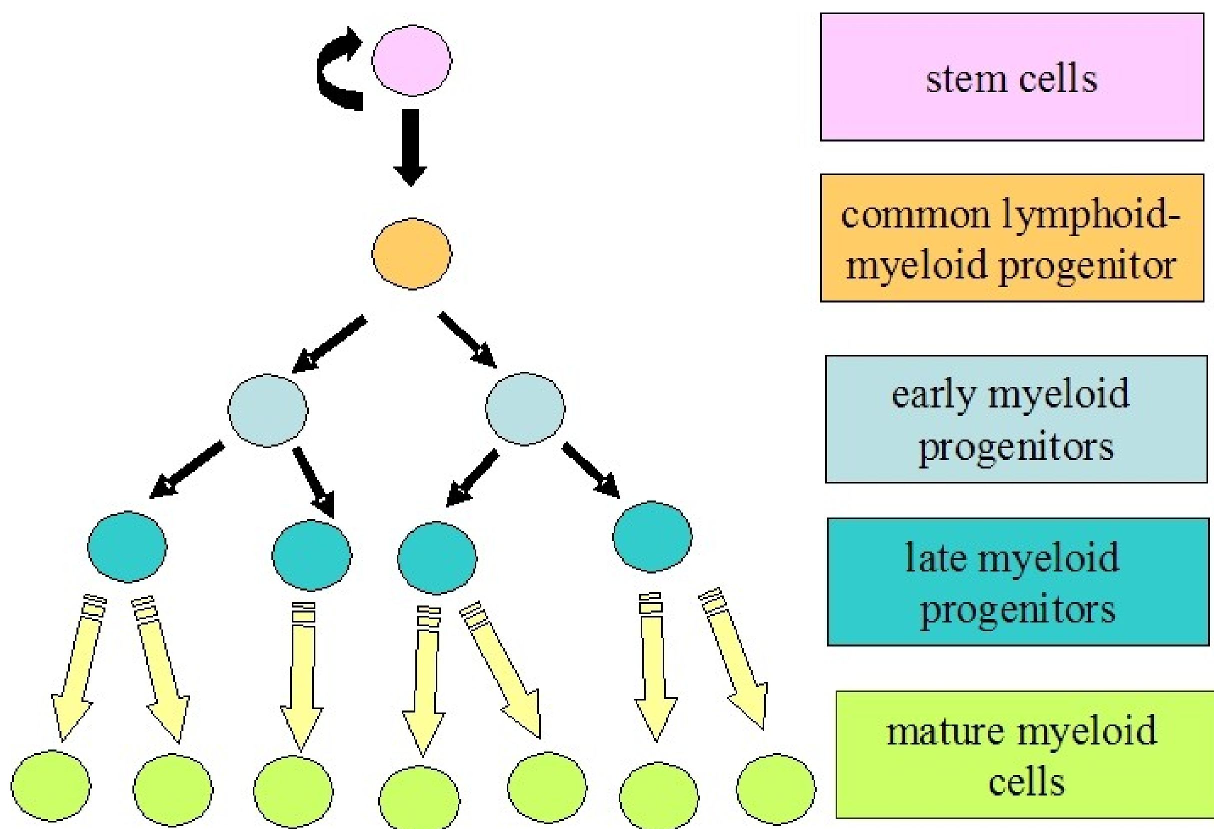
Klíčová slova: myeloproliferativní onemocnění, CML, rezistence na imatinib, dasatinib, PV, ET, *JAK2* V617F, hypersenzitivita na Epo, EEC

3 INTRODUCTION

7.1.1 Hematopoiesis

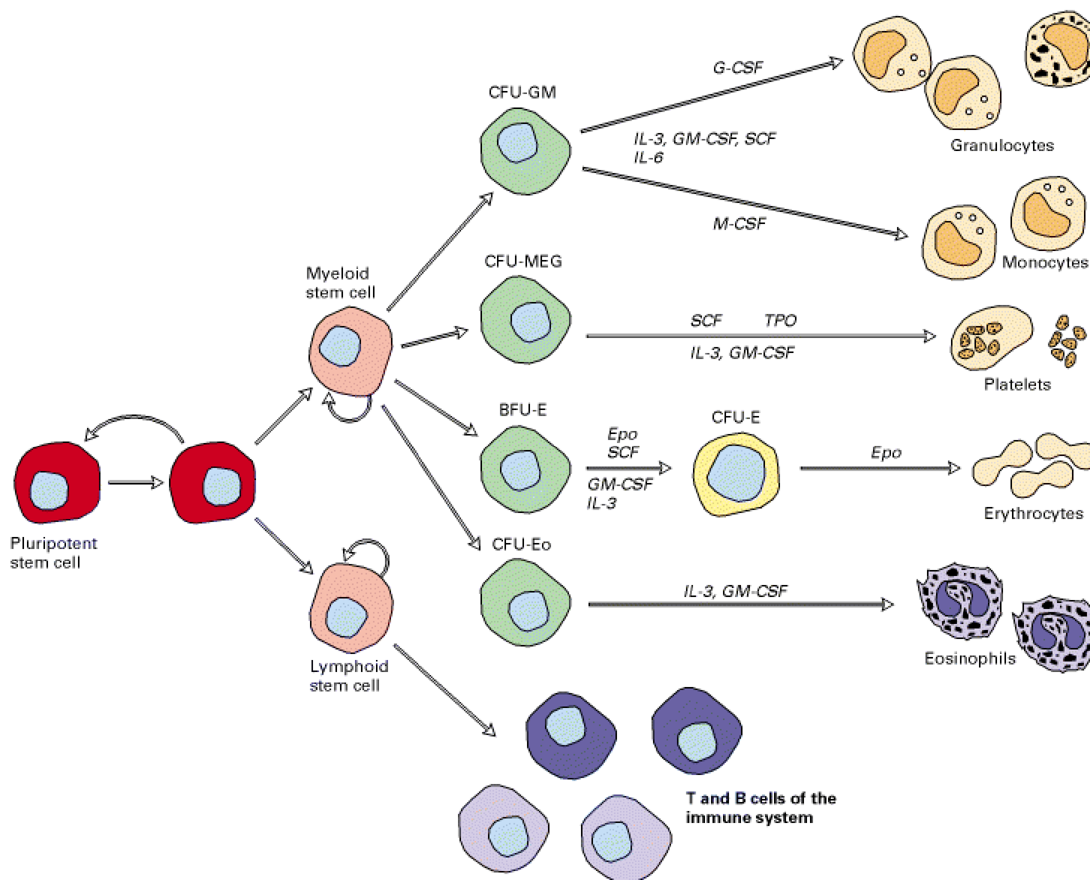
Blood formation (hematopoiesis) is a dynamic process of continuous renewal of all blood elements which is tightly regulated by extracellular (growth factors, hormones etc.) and intracellular (transcription factors, kinases etc.) signals. The basic stones of hematopoiesis are the self-renewal ability of hematopoietic stem cells and the coordination of proliferation and differentiation.

Figure 1: Schematic representation of differentiation of hematopoietic stem cells into myeloid lineage.



Adult hematopoietic stem cells are located in adult bone marrow. Their unique ability to undergo asymmetric division resulting in one daughter stem cell and one partially differentiated progenitor is firmly regulated by extracellular signals such as growth hormones, and adhesion and junction complexes of cells in the hematopoietic stem cell niche [reviewed by Ho AD et al., 2007]. Another unique stem cell property is multipotency: meaning that hematopoietic stem cells can give rise to all different blood elements.

Figure 2: Hematopoesis [Lodish H et al., 2000]. CFU-GM: colony-forming unit granulocyte-macrophage; CFU-MEG: colony-forming unit megakaryocyte; BFU-E: burst-forming unit erythrocyte; CFU-E: colony-forming unit erythrocyte; CFU-Eo: colony-forming unit eosinophil; growth factors: EPO: erythropoietin, G-CSF: granulocyte-colony stimulating factor, GM-CSF: granulocyte macrophage colony-stimulating factor, interleukins: IL-3, IL-6, SCF: stem cell factor.



During differentiation hematopoietic cell undergoes series of decisions to commit to one of the lineages, always narrowing the differentiation potential. This is a continuous process of many different progenitor cells beginning with dedifferentiated and multipotent stem cell, giving rise to intermediate progenitors (common lymphoid-myeloid progenitor, Fig. 1), which differentiate into more committed progenitors (myeloid, lymphoid progenitor) and ending with terminally differentiated cells. The process of differentiation is very closely regulated by specific growth factors, hormones, cytokines (Fig. 2) and by expression of specific transcription factors [reviewed by Buza-Vidas N et al., 2007; Loose M et al., 2007].

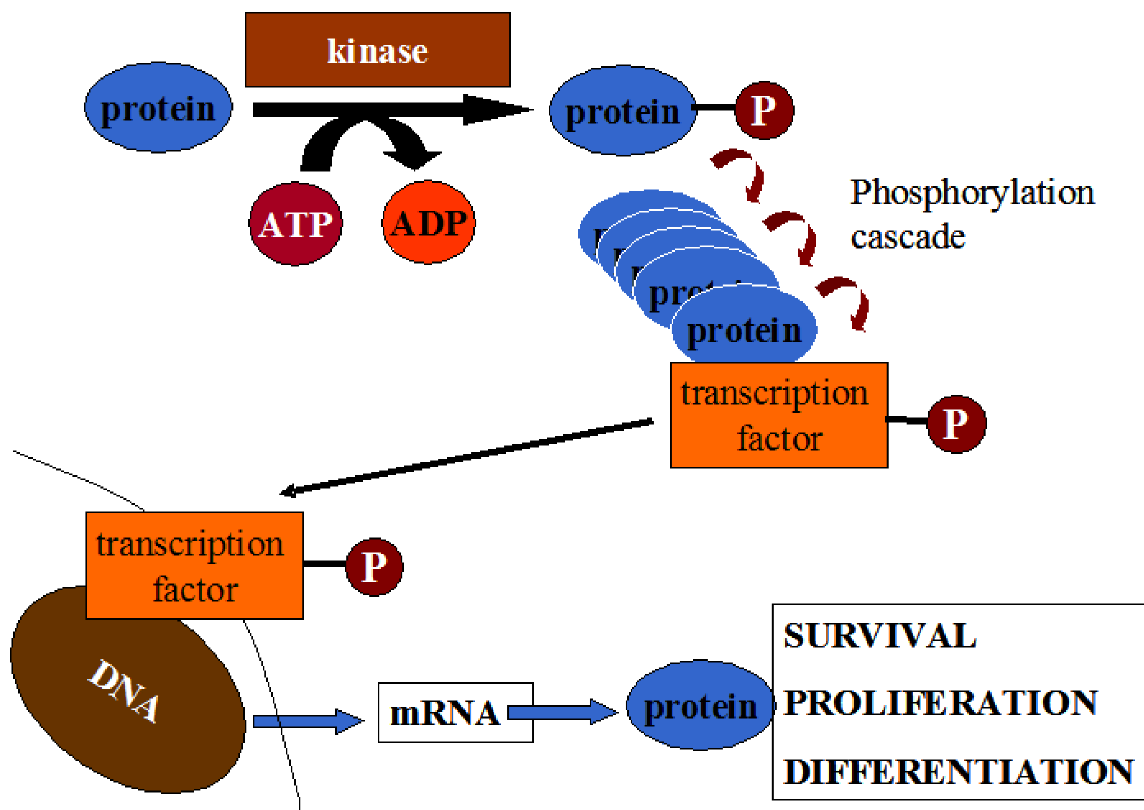
Deregulation of proliferation (cell cycle), block of differentiation, and escape from apoptosis of a hematopoietic progenitor can all participate in the development of a hematological malignancy.

7.1.1 Cell Signaling and Cancerogenesis

The decisions of a cell to live (enter the cell cycle) or die, to proliferate or arrest the cell cycle, are based on an intricate concert of environmental signals and intrinsic signaling pathways. Once the sum of the signals crosses a specific threshold, the program has to be inevitably completed.

A general signaling pathway is triggered by a factor such as cytokine, growth hormone etc., which is recognized by specific cell receptors. These are associated with enzymes such as kinases which pass the signal onto signaling pathway (Fig. 3). One of the basic mechanisms of the signal transduction is the gain/loss of a phosphate group. Kinases catalyze the phosphorylation of a substrate i.e. formation of macroergic phospho-ester bond. The signaling cascade results in phosphorylation/activation of transcription factors, which translocate into the nucleus and trigger the expression of specific genes. The protein products of these genes participate in the regulation of proliferation, survival and differentiation of a cell.

Figure 3: The principle of signaling pathways and the function of kinases.



All parts of the signaling pathway have been indicated to be encoded by various proto-oncogenes. When activated, these genes can trigger the complex multi-step process of cellular transformation. The cell possesses protective machinery called tumor suppressor barrier, which guards the integrity of the genome and counteracts aberrant cellular proliferation. If necessary the tumor suppressor barrier causes the cell to arrest the cell cycle either transiently or permanently (cell senescence [Smith JR and Pereira-Smith OM, 1996]) or induces cell death (apoptosis).

Many, perhaps all, signaling pathways that drive cell proliferation posses "safety valves" i.e. intrinsic growth-suppressive properties. Therefore genetic or epigenetic change activating expression of a single oncogene may not posses selective advantage over normal cells, because oncogene signaling may activate tumor suppressor barrier. Typical example is oncogene-induced senescence. For example activated oncogene H-*RAS* was found to induce premature senescence in primary rodent and human cells [Serrano M et al., 1997].

In addition, programs such as cell proliferation and cell death are linked and interdependent [Evan GI et al., 1992, 2002]. These two pathways may not be overlapping but rather share some signaling molecules and the final decision of the cell is dependent on the total sum of extrinsic and intrinsic signals. The complexity of these processes is shown by the examples of oncogenes *E2F* and *MYC*. Both can induce either pro-apoptotic or pro-senescent signals depending on the cell type, the levels to which they are expressed, and the extent of other pro-apoptotic and growth signals received by the cell [Dimri GP et al., 2000, Wu X et al., 1994]. It seems that apoptosis, senescence and DNA repair mechanisms co-operate in early neoplasmic lesions under 'oncogene stress' to stop the transformation process.

Several studies have shown that for a full transformation of human somatic cell the tumor suppressor barrier has to be considerably weakened (transformation by SV40 large T, small t), at the same time the senescence has to be abrogated (*hTERT*) and simultaneously activated oncogene expressed (*H-RAS*) [Hahn WC et al., 1999; reviewed in Boehm JS and Hahn WC, 2005].

7.1.1 Molecular Background of Selected Hematological Malignancies

The development of cytogenetic and molecular genetic methods has shed a lot of new insights into the molecular genetic background of hematological malignancies. The cloning and sequencing of several hundreds of chromosomal aberrations and molecular lesions

described in patients suffering from hematological malignancies led to characterization of many new putative oncogenes. Based on the *in vitro* and *in vivo* functional analysis these mutations were divided into at least two distinct groups [reviewed in Chalandon Y and Schwaller J, 2005].

Genetic alterations which provide a proliferation and/or survival advantage belong to the first group (Table I). In general, mutations targeting protein tyrosine kinases (PTK) are gain-of-function mutations leading to constitutive activation of the protein. Overexpression of activated PTK has been shown to be sufficient to transform cells *in vitro* and to induce lethal myelo- and/or lymphoproliferative disorders in animals. Currently, there are more than 10 different known leukemogenic PTK described in class I mutations. PTK constitutive activation is caused either by fusion to a different N-terminal partner protein, which provides oligomerization domain for constitutive auto-phosphorylation, or by activating point mutations such as point mutations in their kinase domain or internal tandem repeats (length mutations) in the juxtamembrane domain (*FLT3*, *KIT*). Most of these muations are associated with chronic myeloproliferative diseases, except activating mutations of *FLT3* and *KIT*, which are in hematological malignancies described exclusively in acute leukemias.

Mutations which belong to the second group, in contrast to the first group do not provide survival or proliferative advantage rather they regulate self-renewal and impair cellular differentiation. These usually loss-of-function mutations alter genes encoding transcription regulators, which play critical role in the differentiation and development of hematopoietic cells. In contrast to the first group the *in vivo* models of these aberrations do not cause the leukemic phenotype but rather they may develop after a long latent period into myelodysplasia with differing risk to develop into the picture resembling acute leukemia. These animal models suggest that the development of acute leukemia on the basis of these genetic alterations requires other genetic changes. There is accumulating evidence that class I and class II mutations co-operate in development of leukemic phenotype [Mitani K et al, 1994, Neering SJ et al, 2007].

7.1.1 Myeloproliferative Disorders (MPDs)

The term “myeloproliferative disorder (MPD)” was first used by W. Dameshek in 1951, who stressed the clinico-pathological similarities among polycythemia vera (PV), essential thromobocythemia (ET), idiopathic myelofibrosis (IMF), and chronic myeloid leukemia

(CML). Later on, a common biological base for these diseases was identified as clonal expansion of one or more myeloid cell types, while the differentiation of these cells was not significantly affected. The genetic background is underlined by acquired mutation in a hematopoietic progenitor. The abnormal proliferation and survival of one or more myeloid lineages in the bone marrow leads to overproduction of granulocytes, erythrocytes and/or platelets in the peripheral blood, and is frequently associated with splenomegaly and hepatomegaly. Recent advances in our understanding of molecular-genetic basis of MPDs have shed a lot of light into disease pathophysiology and provided new targets for novel specifically targeted therapy.

7.1.1 Chronic Myeloid Leukemia (CML)

Chronic myeloid leukemia was the first leukemia to be described [Virchow R, 1845] and the first hematological malignancy, which was shown to be associated with defined genetic abnormality – shortened chromosome 22 called the Philadelphia (Ph) chromosome [Nowel and Hungerford, 1960]. CML is characterized by increased proliferation of myeloid cells in the bone marrow and their increased blood counts. The typical clinical course of the disease comprises of three different stages depending on the laboratory and clinical findings. The chronic disease (CP) is defined as less than 5% of blast cells (immature myeloid cells) in peripheral blood. If untreated, the illness will progress after 4-6 years into an accelerated stage (AP) with worsening clinical picture with less than 30% blasts. In less than a year the illness will end up in fatal blast crisis (BC) with more than 30% blasts and a clinical picture similar to acute leukemia and with resistance to chemotherapy (Fig. 4).

Figure 4: Clinical course of CML [adapted from Capdeville R. et al., 2002].

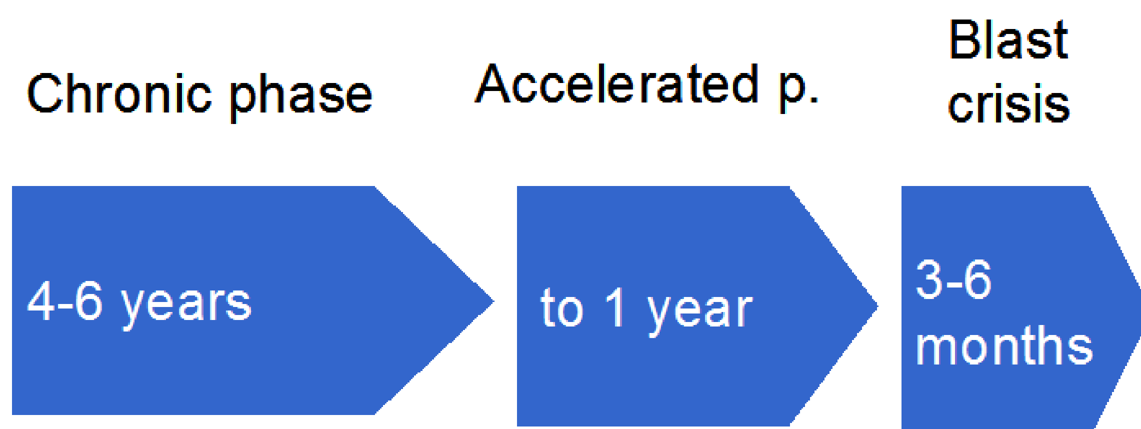


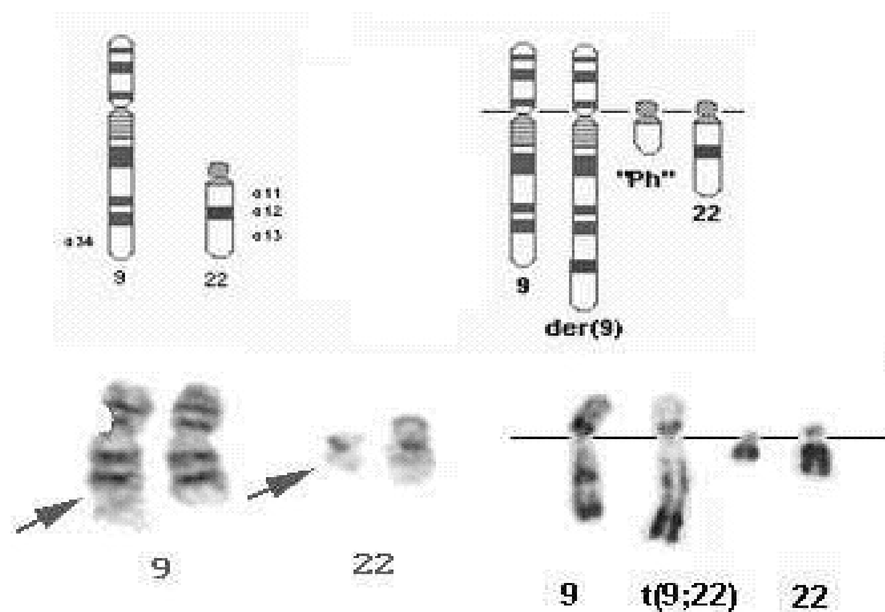
Table I. Genetic alterations in hematologic malignancies			
Class I mutations: providing cellular proliferation and/or survival advantage			
A. Tyrosine kinase fusion genes			
PTK invoved	**Fusion gene**	**Disease phenotype**	**Animal model**
ABL	*BCR/ABL*	CML, ALL	Daley GQ et al., 1990; Heisterkamp N et al., 1990
	TEL/ABL	atypical CML	Million RP et al., 2002
PDGFβR	*TEL/PDGFβR*	CMML/atypical CML	Ritchie KA et al., 1991; Tomasson MH et al., 1991
	H4/PDGFβR	CMML/atypical CML	Schwaller J et al., 2001
PDGFαR	*FIP1L1/PDGFαR*	HES	Cools J et al., 2003
JAK2	*TEL/JAK2*	atypical CML, ALL, AML	Schwaller J et al., 1998
TRKC	*TEL/TRKC*	AML	Liu Q et al., 2000
B. Gain of function mutations			
FLT3	ITD (80%), activation loop kinase domain (15%)	AML	Lee BH et al., 2005
KIT	JM region, activation loop kinase domain	AML, mast cell leukemia	Demehri S et al., 2006
JAK2	*JAK2* V617F mutation	PV, ET, IMF	Lacout C et al., 2006
Class II mutations: impairing cellular differentiation and subsequent apoptosis			
A. Transcription factor fusion genes			
CBF	*AML1/CBFβ*	AML	reviewed by Downing JR, 2003
RAR	PML/RAR alpha, PLZF/RAR alpha, NPM/RAR alpha	APL	Rego EM et al., 2006
MLL	MLL/CBP	AML	reviewed by Liedman D, Zeleznik-Le N, 2001
HOX	NUP98/HOXA9	MPD, AML	Kroon E et al., 2001
B. Loss of function mutations			
AML1	*AML1/ETO*	AML	Higuchi M et al., 2002; Schessl C et al., 2005
PU.1	hypomorphic alleles – reduced expression	AML	Rosenbauer F et al., 2004
GATA 1	mutation in translation initiation codon	thrombocytopenia, AMegL	Stachura DL et al., 2006; Majewski IJ et al., 2006

Adapted from Chalandon Y et al., 2005. ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; AMegL: acute megakaryoblastic leukemia; APL: acute promyelocytic leukemia; CML: chronic myeloid leukemia; CMML: chronic myelomonocytic leukemia; ET: essential thrombocythemia; HES: hypereosinophilic syndrome; IMF: idiopathic myelofibrosis; MPD: myeloproliferative disorders; PTK: protein tyrosine kinase PV: polycythemia vera;.

The Ph chromosome is found in 95% of patients with CML and 25% of adult patients with acute lymphocytic leukemia (ALL). Later on this mutations was cytogenetically characterized as reciprocal translocation of chromosomes 22 and 9 [t(9;22)(q34;q11)] [Rowley JD, 1973; de Klein A et al., 1982; Groffen J et al., 1982]. The translocation fuses the *ABL* (Abelson) tyrosine kinase proto-oncogene on chromosome 9 with the breakpoint cluster region (*BCR*) gene on chromosome 22 (Fig. 5), generating an oncogene that encodes the constitutively active Bcr-Abl tyrosine kinase [Shtivelman E et al. 1985, Ben-Neriah Y et al. 1986].

The Ph chromosome is an acquired somatic mutations arising in hematopoietic stem cell [Fialkow PJ et al. 1967]. It has been shown that *BCR-ABL* as a sole genetic lesion is sufficient for oncogenic transformation in cell lines, primary cells, and mouse transplant or transgenic models [Daley GQ et al., 1990; Heisterkamp N et al., 1990; Kelliher MA et al., 1990; Lugo TG et al., 1990; Gishizky ML et al., 1993; Pear WS et al., 1998; Li S et al., 1999], and that the tyrosine-kinase activity of Bcr-Abl is crucial for its transforming activity [Lugo TG et al., 1990]. The Bcr-Abl tyrosine kinase activity is thus a perfect target for rational drug development. Most of our understanding of the mode of action of Bcr-Abl has been based on two strategies: correlation of the common structural motifs between related genes and analysis of naturally occurring and engineered mutations.

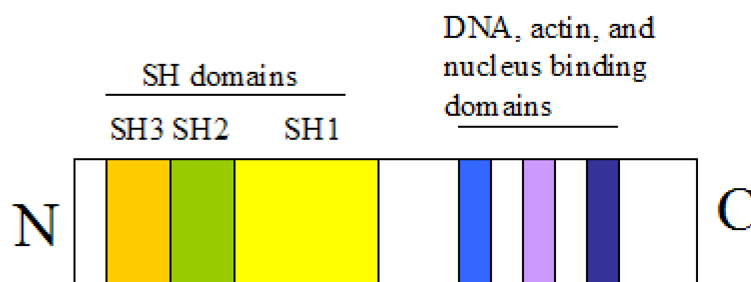
Figure 5: Philadelphia chromosome translocation resulting in *BCR-ABL* fusion gene. The figure is taken from www.cmlsupport.com.



3.1.1 *c-ABL* Gene

The *c-ABL* gene on chromosome 9 is the human homologue of a gene originally identified in a murine oncogneic virus, the Abelson Murine Leukemia Virus. This retrovirus was isolated from a prednisolone treated mouse which developed lymphoma following inoculation with Moloney Murine Leukemia Virus. Comparison between *v-ABL* and *c-ABL* sequences revealed that the first two exons of a *c-ABL* were deleted in the viral *ABL* oncogene [Wang JY et al. 1984].

Figure 6: The structure of *c-ABL* gene.



The 145 kDa c-Abl protein belongs to the non-receptor family of tyrosine kinases and contains src homology domains SH1, SH2 and SH3 [Pawson T, 1995]. SH1 encodes the kinase domain. SH2 and SH3 domains play a key role in assembling the signaling protein complexes. SH2 binds to phophotyrosine residues and SH3 binds to proline-rich sequences. In addition, SH3 domain probably plays role in negative regulation of c-Abl kinase activity. The binding specificity of these domains appears to be determined by amino acids immediately adjacent to either phosphotyrosine or prolines. The carboxy-terminus of *c-ABL* contains nuclear localization signals, proline-rich sequences, a DNA binding domain, a p53 binding site, and an actin binding domain (Fig. 6) [reviewed in Raitano et al, 1997]. c-Abl is expressed in all tissues examined [Westin et al. 1982]. c-Abl is predominantly nuclear protein. When the N-terminal domain of the Abl protein is encoded by exon 1a, the protein is localized to the nucleus, when encoded by exon 1b, the protein is myristoylated and directed to the plasma membrane [Van-Etten RA et al., 1989; Jackson P and Baltimore D, 1989].

Targeted disruption of *c-ABL* in mice shows that c-Abl is required for normal growth and development. Mice homozygous for null mutation possess high neonatal mortality rates

and increased susceptibility to infections, presumably from lymphopenia, suggesting a role of c-Abl in B-lymphocyte development [Tybulewicz et al. 1991, Swartzberg PL et al. 1991].

c-Abl kinase is predominantly localized to the nucleus, binds DNA and its activation is in normal cells tightly regulated. It plays a role in the signaling pathways activated by DNA damage, cell cycle progression to S phase, and integrin-mediated adhesion. c-Abl is activated after radiation or chemotherapy induced DNA damage downstream ataxia teleangiectasia protein (ATM). ATM directly phosphorylates c-Abl, then c-Abl binds p53 and enhances its transcriptional activity for target genes such as p21 [Baskaran et al., 1997; Jing Y et al., 2007]. In addition, in response to genotoxic stress c-Abl is bound to and phosphorylates DNA dependent protein kinase DNA-PK, which is important in double stranded DNA break repair and DNA recombination [Kharbanda S et al. 1997]. The c-Abl activation is also necessary for triggering stress kinase pathways such as the SAPK/JNK pathway [Kharbanda S et al., 1995]. When wild-type c-Abl is overexpressed in fibroblasts, it remains primarily in the nucleus and induces G1 growth arrest, by interaction with p53 and pRB proteins [Sawyers et al., 1994; Welch et al., 1993]. c-Abl also plays role in regulation of apoptosis [Kawai H. et al., 2002] via direct phosphorylation of IkappaBalpha leading to induction of IkappaBalpha accumulation in the nucleus. As a consequence, NF-kappaB transcription activity is abolished, leading to an increased cellular sensitivity to the induction of apoptosis. In response to genotoxic stress c-Abl binds to the p53 homolog, p73, phosphorylates it and thus promotes apoptosis [Agami R et al., 1999]. Furthermore, c-Abl is implicated in cellular adhesion. c-Abl transiently moves from the nucleus to the cytoplasm and its kinase activity increases in response to integrin-mediated adhesion. c-Abl binds and phosphorylates proteins which localize to focal adhesion plaques [Lewis JM et al., 1996]. Furthermore, c-Abl probably contributes to the integrin activation of MAP and Ras kinases [Renshaw MW et al., 2000].

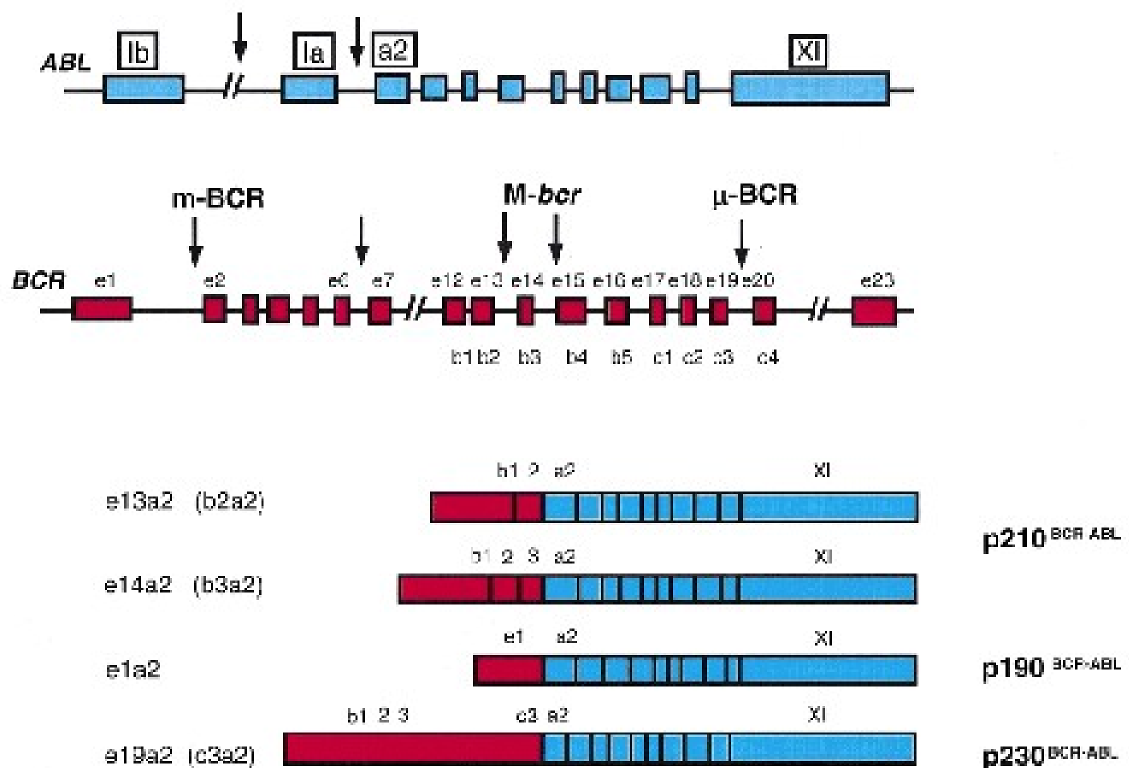
3.1.2 *BCR-ABL*

In contrast to wild type c-Abl which is primarily localized in the nucleus and is expressed ubiquitously, Bcr-Abl is found in the cytoplasm of Ph-positive somatic cells [Ren R, 2005]. The fusion to Bcr provides Abl kinase with a coiled-coil oligomerization domain in the C-terminus which allows dimerization, auto-phosphorylation, and thus constitutive activation of Bcr-Abl kinase [McWhirter JR and Wang JY, 1997]. In addition, Bcr C-terminus

of the fusion protein promotes binding to actin and may interfere with the negative regulatory function of the SH3 domain of Abl by intramolecular folding [Pendergast AM et al., 1991].

In Ph translocation different chromosomal breakpoints lead to different forms of *BCR-ABL* (Fig. 7). The translocation fuses exon 2-11 of *c-ABL* to N-terminal coding exons of the *c-BCR* gene. The chromosome 9 breakpoints are localized in a large 200 kb region within the *ABL* alternative first exons (1a and 1b) [Bernards A, 1987]. In contrast, the break points on chromosome 22 are clustered within three regions of the *BCR* gene [reviewed in Melo JV, 1997]. In CML patients a 210 kDa Bcr-Abl protein (p210) is produced by fusion of *c-ABL* to *BCR* gene with a major *BCR* break point (M-*BCR*). M-*BCR* is a 5.8 kb region spanning exons 12-16. There are two junction variants of M-*BCR*: b2a2 and b3a2, without any documented clinical relevance [Tefferi A, 1990]. A smaller 185-190 kDa protein (p185 or p190) is present in about 50% of Ph-positive ALL patients and results from the minor *BCR* break point (m-*BCR*) found in the region between exons e1 and e2 of the *BCR* gene. Alternative break points are quiet rare, the most well described is p230, which is associated with a specific subtype of CML with a micro *BCR* break point (µ-*BCR*) between exons e19 and e20 [reviewed in Allen P et al. 1992].

Figure 7: The various variants of *BCR-ABL* fusion genes [Mughal TJ. et al. 2001].



3.1.3 Bcr-Abl Signaling

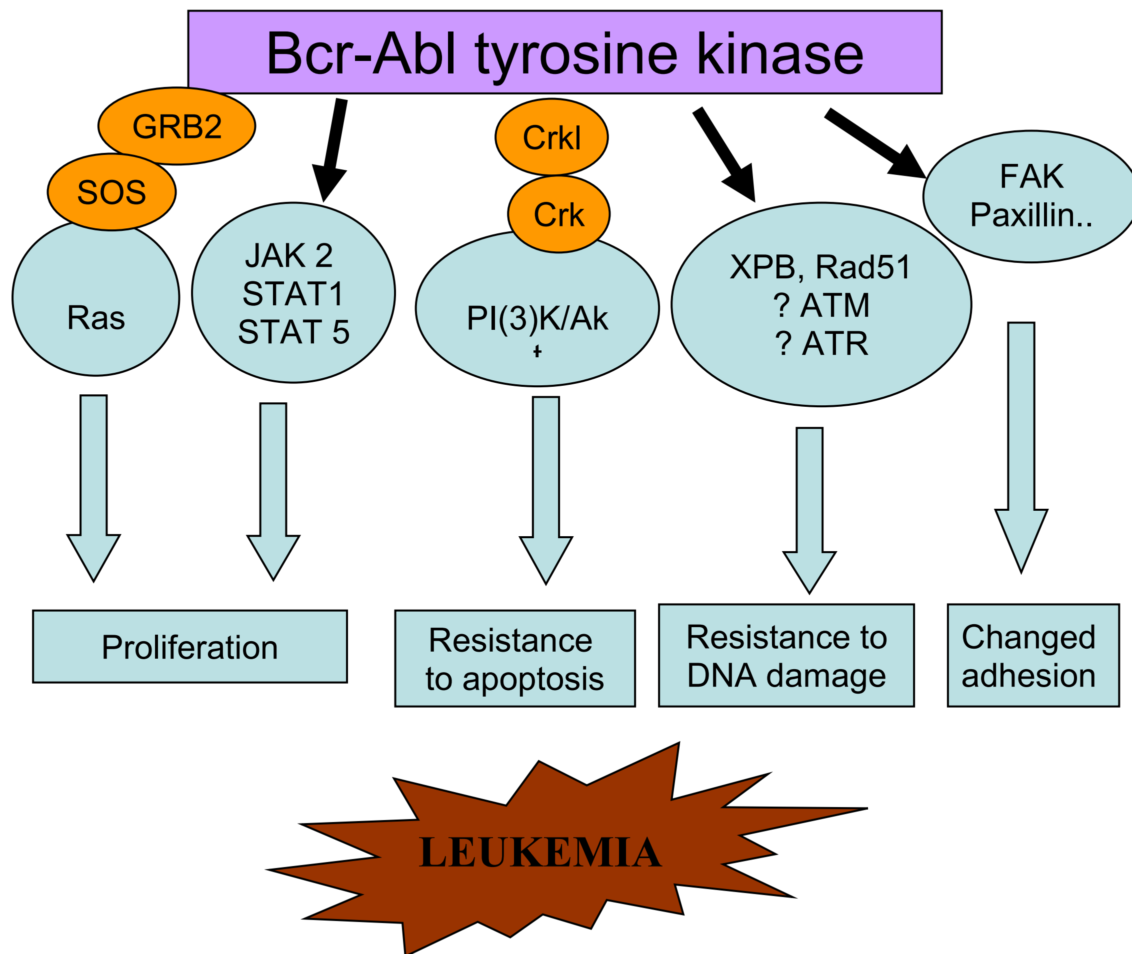
Bcr-Abl localizes to the cytoskeleton and displays an up-regulated tyrosine kinase activity that leads to the recruitment of downstream effectors of cell proliferation, cell survival, resistance to DNA damage and consequently to leukemogenesis. The following physiological properties have been demonstrated for both p210 and p190 forms of Bcr-Abl:

- The induction of neoplastic transformation and cell proliferation.
- The induction of growth factor independence and inhibition of apoptosis in growth factor dependent hematopoietic cell lines.
- The inhibition of adhesion of chronic myeloid progenitor cells to marrow stroma.

Bcr-Abl singal transduction involves multiple and redundant intracellular pathways (Fig. 8) including adaptor proteins, such as growth receptor binding protein-2 (GRB-2), the crk like protein (CRKL) and SHC. Each adaptor molecule forms complexes with Bcr-Abl and has the potential to link Bcr-Abl to downstream signaling molecules e.g. to Ras through recruitment of guanine nucleaotide exchange factors. Based on *BCR-ABL* mouse leukemia model studies, the Ras pathway is critically required for the onset of leukemia in these mice. The importance of Ras signaling in the cancerogenesis is underlined by the fact that mutations that result in constitutive activation of Ras are associated with approximately 30% of all human cancers, including 20-30% of cases of AML, MPDs and myelodysplastic syndrome (MDS).

Crkl is an important Bcr-Abl substrate because it is the most prominent phosphoprotein in clinical CML samples [Nichols GL et al., 1994]. Crkl is specifically phosphorylated by Bcr-Abl kinase in neutrophils [ten Hoeve J et al., 1994; Oda T et al., 1994]. Crkl (v-CRK Avian Sarcoma Virus CT10 Oncogene Homolog-like) contains one SH2 domain and two SH3 domains. The amino terminal Crkl SH3 domain binds directly to a proline-rich region in the C-terminus of *BCR-ABL*. Phosphorylated Crkl activates Ras and JUN-dependent signaling pathways. Overexpression of Crkl, similar to constitutive activation of Crkl by Bcr-Abl, leads to the transformation of fibroblasts in a Ras-dependent manner [Senechal K. et al., 1996]. In addition, Crkl mediates the contact of Bcr-Abl to paxillin within the focal adhesion complexes [Salgia R et al., 1995].

Figure 8: Bcr-Abl signaling.



In addition to Ras signaling, the adaptor molecules activate a range of other signalling pathways that activate proteins such as PI3K, Akt, JNK, Src family kinases, protein and lipid phosphatases, and their respective downstream targets, as well as transcription factors such as the STATs, NF-kappaB and myc [reviewed in Melo JV and Deininger MW, 2004].

Bcr-Abl can also trigger direct translational control of key molecules. For example Bcr-Abl possesses unique translational control mechanism that increases the synthesis of Mdm2. Mdm2 is a negative regulator of p53, a well-known tumor suppressor, a key inducer of cell cycle arrest or apoptosis. The Mdm2 down-regulation was observed in Bcr-Abl-expressing cells, and in accelerated phase and blast crisis CML samples [Trotta R et al., 2003].

Bcr-Abl activation also impacts the differentiation potential of myeloid cells. Bcr-Abl blocks expression of the transcription factor C/EBPα in a MAPK-hnRNP-E2 dependent manner [Chang JS et al., 2007]. The transcription factor C/EBPalpha controls differentiation and

proliferation in normal granulopoiesis in a stage-specific manner. Loss of C/EBPalpha function in myeloid cells *in vitro* and *in vivo* leads to a block of myeloid differentiation similar to that which is observed in malignant cells from patients with acute myeloid leukemia [reviewed in Mueller BU and Pabst T, 2006].

Bcr-Abl expression is associated with genomic instability and accumulation of mutations which inevitably lead to chemoresistant blast crisis in CML patients. The exact mechanism of Bcr-Abl in DNA repair and DNA damage resistance is not yet fully clear. *BCR-ABL* positive cells accumulate more DNA double strand breaks (DSBs) after genotoxic treatment but possess more efficient repair mechanisms than normal cells and survive. This phenomenon is associated with prolonged cell cycle at the G2/M phase checkpoint, and the production of proapoptotic factors of the Bcl-2 family members. In addition, Bcr-Abl upregulates STAT5 signaling, leading to increased expression of Rad51 and thus increased rate of homologous recombination repair [Slupianek A et al., 2002].

Moreover, it has been shown (through yeast two-hybrid screening) that both Bcr and Bcr-Abl can interact with the Xeroderma Pigmetosum B protein (XPB) [Takeda N et al., 1999]. XPB is known to be critical in the nucleotide excision repair (NER). It has been suggested that Bcr-Abl effect on NER is cell-type dependent [Canitrot Y et al., 2003] reducing NER activity in lymphoid cells, leading to hypersensitivity to UV and mutagenesis. In contrast, Bcr-Abl expression in myeloid cells facilitated NER and induced resistance to UV.

Bcr-Abl, like c-Abl associates with ATM bud does not disrupt the function of ATM in DNA repair. There are still some controversies concerning Bcr-Abl modulation of function of ATM homolog, ATR. Some researches show higher ATR-Chk1 activation (important DNA repair pathway) in *BCR-ABL*-positive cells after genotoxic treatment [Nieborowska-Skorska M et al., 2006] leading to enhanced double strand break repair. Others suggest that Bcr-Abl translocates to the nucleus and disrupts an ATR-dependent intra-S phase checkpoint leading to increased DNA double-strand breaks and a radioresistant DNA synthesis phenotype [Dierov J et al., 2004].

Bcr-Abl possesses an intrinsic kinase activity and moreover, it can also interact with other cytoplasmic tyrosine kinases such as JAK2 [Xie S et al., 2001], Fes [Lionberger JM et al., 2000], and Src family.

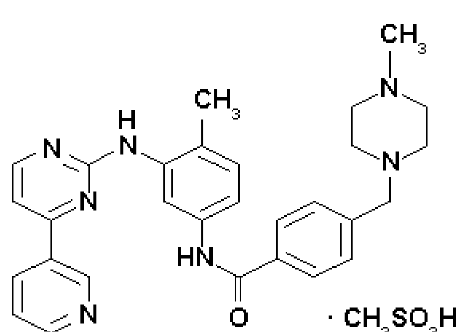
3.1.4 CML Treatment

Historically the therapy of CML was empirically based. In 1800s the main CML treatment was Fowler's solution, which contained active substance: arsenic trioxide. Today there is a revival of interest in the use of this substance (Trisenox) for CML [Konig H et al., 2007]. During the 1900s, radiation, busulfan, hydroxyurea, interferon-alfa (INF-α), and stem-cell transplantation were developed and also used for CML therapy. With increasing knowledge of genetic and molecular genetic background of cancer development a new era for cancer treatment has arisen. CML is a perfect candidate for targeted therapy because in contrast to other cancers where tumorigenesis involves disruption of multiple genes and signaling pathways, it has been shown that *BCR-ABL* as a sole genetic defect can induce a CML-like phenotype in mice. Moreover, it has been proved that the kinase activity of Bcr-Abl is essential for the oncogenic effect [Lugo TG et al., 1990]. The first drug specifically targeted against leukemic cells used in clinical setting is imatinib mesylate (IM, STI571, CGP57146B, Glivec, Gleevec; Novartis Pharma AG), a small inhibitor of Bcr-Abl kinase.

3.1.5 Imatinib Mesylate as Paradigm of Targeted Therapy

IM is a chemical derivative of 2-phenylaminopyrimidine (Fig. 9). The original chemical formula was found in the screen for inhibitors of protein kinase C (PKC) [Buchdunger E et al., 1996]. By chemical modification a high selectivity against tyrosine kinases and higher bio-availability was reached. IM emerged as the most promising compound for clinical development.

Figure 9: Chemical structure of imatinib mesylate:



4-[(4 Methyl-1-piperazinyl) methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamid

(The figure taken from de.wikipedia.org.)

Studies using purified enzymes showed that IM potently inhibits all of the Abl tyrosine kinases including c-Abl, v-Abl and p 210 Bcr-Abl [Buchdunger E et al., 1996]. Later on, similar effect was proved in cell lines *in vitro* against other Abl fusion proteins, such as p185 Bcr-Abl and Tel-Abl [Carroll M et al., 1991]. In addition, IM also inhibits signal transduction mediated by receptor kinases c-Kit and platelet-derived growth factor receptors (PDGFR) [Buchdunger E et al., 2000].

It was shown that the inhibition of autophosphorylation of Bcr-Abl is closely related to the antiproliferative activity of IM. Incubation of submicromolar concentration of IM possesses antiproliferative activity and selectively induces apoptosis in *BCR-ABL*-positive cell lines and induces cell killing in primary leukemia cells from Ph-positive CML patients [Druker BJ et al., 1996]. In addition, the efficacy and selectivity of IM against *BCR-ABL*-positive tumor growth was proved in animal models [Druker BJ et al., 1996; le Coutre et al., 1999]. In these mouse models of Bcr-Abl leukemogenesis IM prolonged the tumor-free and over-all survival significantly, together with a marked improvement in peripheral-white-blood-cell counts and splenomegaly [Wolff NC and Ilaria RL Jr., 2001].

At first the mode of IM action was thought to compete with ATP at the ATP-binidng site of Abl kinase. Later more precise crystallographic studies revealed that IM binds only to and stabilizes the inactive conformation of Bcr-Abl kinase, shifting the equilibrium from the active conformation and inhibiting thus the kinase activity [Schindler T et al., 2000; Nagar B et al., 2002].

The clinical trials with IM began very early on in 1998 and enrolled patients in chronic phase of CML who failed therapy with INF-α. The results were very promising where patients receiving dose of 300 mg or higher reached cytogenetic reponses (31% of patients) including complete responses in 13%. The standard dose of 400 mg a day was proposed which corresponds to peak levels at steady state approximately 4.6 µM IM and trough levels approximately 2.13 µM. (The effective concentration from *in vitro* studies was 1 µM IM). This dosage provided continuous inhibition of Bcr-Abl protein with only mild side effects and no maximum tolerated dose was identified [Druker BJ et al., 2001; Peng B et al., 2004]. Phase II large multinational studies used IM as single agent for all stages of CML: chronic phase after failure of INF-α treatment (400 mg /day) [Kantarijan H et al., 2002], accelerated phase [Talpaz M et al., 2002], blast crisis of CML [Sawyers C et al., 2002] and Ph-positive acute lymphoblastic leukemia (ALL) (600 mg/day) [Ottmann OG et al., 2002]. The data

from these studies showed that the number of hematological and cytogenetic responses were higher if the treatment was started earlier in the onset of the disease. Moreover, overall and event-free survival was associated with achievement of complete cytogenetic response (CCR). IM therapy resulted in a clinically relevant hematologic response rate even in Ph-positive ALL patients, but development of resistance and subsequent disease progression were rapid within weeks to months. The observed side effects were usually mild (nausea, vomiting, oedema etc.) but rare serious events were also reported (liver toxicity, fluid-retention syndromes). Neutropenias and thrombopenias were more common in advanced diseases with already compromised bone-marrow reserve. The results of the phase I and II trials led to the approval of the Food and Drug Administration of IM for the treatment of CML in advanced phase and after failure of IFN. Phase III clinical trial (called IRIS trial: The International Randomized Study of Interferon versus STI-571) compared the efficacy of IM treatment as first line in comparison with combination of INF-α and cytarabine. In the course of the trial many patients crossed from the arm of INF-α plus cytarabine to the arm of IM due to lack of INF-α treatment efficiency or INF-α intolerance. IM showed superior effects as observed by the rate of complete hematological response (CHR), major cytogenetic response (MCR) and CCR (81% at 30 months) as well as progression-free survival. Based on these results the drug was approved by health-care authorities for first-line treatment of CML in Europe and US.

In addition to the efficacy of IM treatment of Ph-positive CML and ALL, IM has shown some clinical promise in other diseases (Table II). First of all IM is already in use for treatment of gastroinstestinal stromal tumors (GIST). GISTs are mesenchymal neoplasms that can arise from any organ in the gastrointestinal tract or from the mesentery or omentum. The molecular background is associated with activation of c-Kit, a transmembrane receptor tyrosine kinase, which is activated by proliferation signal called stem cell factor (SCF). Majority of GISTs express c-Kit and 90% of the cases c-Kit is activated by point mutation in exons 9 or 11 [Hirota S et al., 1998]. These mutations render c-Kit function independent of activation by SCF, leading to a high cell division rate and possibly genomic instability. It is likely that additional mutations are "required" for a cell with a c-Kit mutation to develop into a GIST, but the c-Kit mutation is probably the first step of this process. GISTs respond to single- or multiagent chemotherapy in less than 5%. In contrast, clinical trials have shown that IM therapy as a single agent reached objective response in 53%-65% of GIST patients

[van Oosterom AT et al., 2002; Demetri GD et al., 2002] including GIST patients with c-Kit mutations.

From the *in vitro* studies it was obvious that IM inhibits also other tyrosine kinases such as PDGFR family. There are several other diseases where IM treatment is efficient which are associated with activation of these kinases. These include chronic myelomonocytic leukemia (CMML) with *ETV6-PDGFRB* fusion gene and hypereosinophilic syndrome (HES) with fusion gene *FIP1L1-PDGFRA*. IM is also used in the treatment of dermatofibrosarcoma protruberans (DFSP). The molecular mechanisms is also associated with abnormal activation of PDGFB but this time due to over production of its ligand PDGF-BB as a fusion variant resulting from translocation t(17;22) producing *col1A1-PDGFB* fusion gene.

Table II: Diseases in which IM treatment has proven efficient.

Kinase	Disease	Mechanism of activation of the kinase
Abl	CML	chromosomal translocation: t(9;22) - *BCR-ABL*
	ALL	chromosomal translocation: t(9;22) - *BCR-ABL*
c-Kit	GIST	point mutation in kinase domain
PDGFRA	GIST	point mutation
	HES	intrachromosomal deletion leading to fusion gene: *FIP1L1-PDGFRA*
PDGFRB	CMML	chromosomal translocation: t(5;12) – *ETV6-PDGFRB*
	DFSP	chromosomal translocation: t(17;22) – *col1A1-PDGFB*

Abbreviations: ALL - acute lymphoblastic leukemia, CML - chronic myeloid leukemia, CMML - chronic myelomonocytic leukemia, DFSP - dermatofibrosarcoma protruberans, GIST - gastrointestinal stromal tumor, HES - hypereosinophilic syndrom. Adapted from Druker BJ, 2003.

In summary, IM has revolutionized CML therapy, because 97% patients receiving frontline IM reach complete hematological response with 82% complete cytogenetic response (CCR). In contrast, INF-α induces CCR at rates of 5% to 20% in early chronic-phase CML and is associated with serous toxicities. Although allogenic stem-cell transplatantion is potentially curative, it remains limited by suitable donor availability and by transplant-associated mortality and morbidities.

3.1.6 Resistance to IM Treatment in CML

Although the era of IM treatment brought new hope for CML patients, this therapy has its limitations. Response rates and durability of the response to IM are highly dependent on the stage of disease at which treatment is initiated [Sawyers CL et al., 2002; Kantarjian H et al., 2002, Talpaz M et al., 2002]. Emergence of resistance is one of the problems clinicians are encountering. The rate of response to treatment is closely monitored on the level of cytogenetics (percentage of *BCR-ABL*-positive cells measured by classical cytogentics or fluorescent-in-situ-hybridization i.e. FISH), on the level of molecular response (real-time quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR), which measures the amount of *BCR-ABL* transcripts, or more sensitive detection of *BCR-ABL* mRNA: nested RT-PCR). The response rates are given as hematological response depending on the blood counts and spleen size, cytogenetic response (major cytogenetic response MCR: <34% Ph-positive metaphases; complete cytogenetic response CCR: 0% Ph-positive metaphases), or molecular response (major molecular response MMR: > 3 log reduction of *BCR-ABL* transcript or a *BCR-ABL/ABL* ratio of < 1%; complete molecular response CMR: no *BCR-ABL* transcript detected).

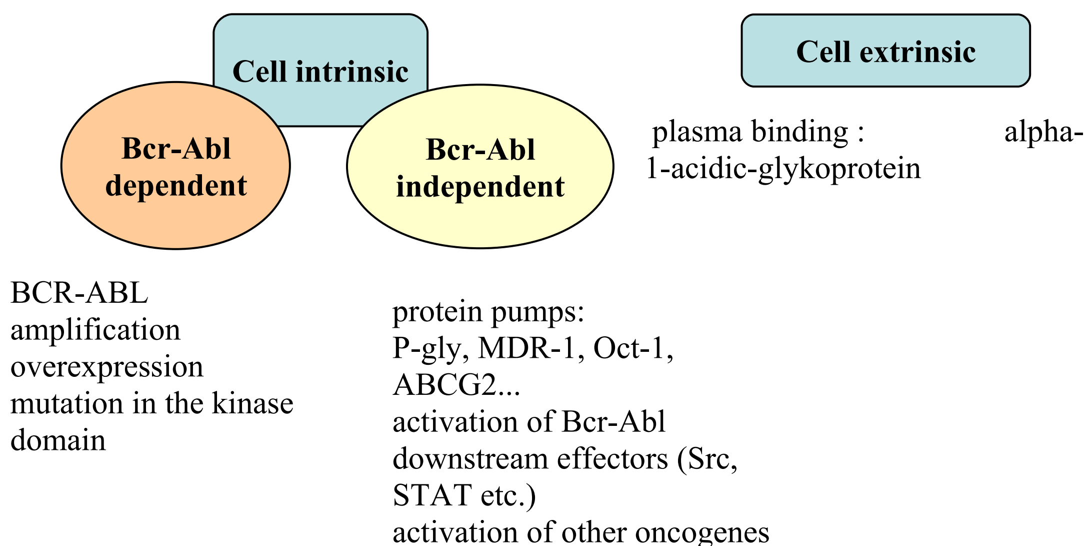
Primary resistance or refractory disease is characterized by complete lack of response. According to the IRIS trial [Druker BJ et al., 2006] 2% of early phase CML patients treated with IM from diagnosis failed to achieve hematologic response and 8-13% of them failed to achieve MCR or CCR.

Secondary resistance develops after initial response i.e. loss of hematologic or cytogenetic response and/or progression from chronic to advaced-stage disease. In the IRIS trial 5-year update [Druker BJ et al., 2006] 18% of CML patients with frontline IM treatment from diagnosis had progression events including loss of CCR and CHR and progression from chronic to advanced disease. For patients with advanced disease treated with IM as salvage therapy, rates of resistance and relapsing disease is dramatically higher, occurring in 75% or more of AP patients and 95% of myeloid BC patients [Silver RT et al., 2004]. The risk factors for developing IM resistance according to Branford et al. [2003] are prolonged time from CML diagnosis to IM therapy, clonal evolution (additional chromosomal aberrations in either Ph-positive or –negative clone), lack of MMR, rising levels of real-time RT-PCR (> 2 fold rise in the number of *BCR-ABL* transcripts).

Another important issue is the persistence of the *BCR-ABL* clone even in patients who are in long-term remission. They remain positive in nested RT-PCR suggesting that Ph-positive leukemic cells persist in 10^{-2} to 10^{-5} cells.

The molecular mechanisms underlying resistance can be very heterogeneous and complex. They can be divided into mechanisms intrinsic to the cell and mechanisms which function outside the cell (Fig. 10). The intrinsic mechanisms can be either dependent or independent of the activity of Bcr-Abl kinase. The former group includes amplification of *BCR-ABL* gene, overexpression of Bcr-Abl protein, mutations within the Bcr-Abl kinase domain. The latter group consists of expression of multi-drug-resistant protein (*MDR-1*, P-glykoprotein) or other chemical transporters which pumps the drug in/out of the cell (Oct-1), activation mutation of downstream targets of Bcr-Abl or any other oncogene (e.g. additional chromosomal aberration). One of the most discussed compounds which may play role in the extrinsic mechanisms of IM resistance is alpha-1-acidic-glykoprotein, which binds IM in the plasma and may lower its cellular bio-availability.

Figure 10: Mechanisms of resistance to IM in CML therapy. P-gly: P-glykoprotein, MDR-1: multi-drug-resistance.



3.5.6.1 *BCR-ABL* Amplification and Overexpression

The amplification of *BCR-ABL* gene has been documented *in vitro* in several cellular model of IM resistance [le Coutre et al., 2000; Weisberg E and Griffin JD, 2000; Mahon FX et al., 2000; Scappini B et al, 2004]. The first one was the cultivation of *BCR-ABL*-positive LAMA84 cell line in increasing concentrations of IM [le Coutre et al., 2000]. The resistant cell line LAMA84R can be grown in 0.7 μM IM and is characterized by 5 times higher expression of Bcr-Abl protein than in parental LAMA84 and this kinase remained sensitive to IM inhibition. The cause of Bcr-Abl overexpression in LAMA84R is amplification of the *BCR-ABL* gene (13-15 copies of the fusion gene in the resistant cells). The overexpression of *BCR-ABL* gene does not have to be always connected with amplification of the gene. For instance, K562 cell lines resistant to IM [Weisberg E and Griffin JD, 2000] displayed a 2-fold to 3-fold increase in Bcr-Abl protein but did not show any detectable gene amplification. This can be caused by change in regulation of transcription, translation or post-translational processing. On the other hand, high concentrations of Bcr-Abl protein are toxic for the cell if IM is withdrawn from the culture. Later on, the amplification of the *BCR-ABL* gene and overexpression of the Bcr-Abl protein was described in patient samples, with overall frequency of about 18% of CML patients clinically resistant to IM [reviewed in Gambacorti-Passerini CB et al., 2003].

3.5.6.2 *BCR-ABL* Point Mutations

One of the major causes which render the Bcr-Abl kinase insensitive to IM treatment is thought to be the acquisition of point mutations within the kinase domain. In the past it was suggested that mutations are present in 90% of CML patients with secondary resistance, more recent studies show that it is in 40% of cases [reviewed in Deininger M et al., 2005]. Up today over 50 different point mutations of Abl kinase have been described in cell lines as well as in clinical samples (Fig. 11) [reviewed in Hochhause A and la Rosee P, 2004].

The mechanisms of IM resistance driven by point mutations can be divided into two groups. If the mutation occurs in any of the approximately 21 amino acids which are involved in IM binding, the bond between the kinase and the drug is either weakened or completely abrogated e.g. by steric hindrance. A typical example and one of the most frequent mutations from this group is the substitution of threonin to isoleucine in the 315th amino acid of c-Abl (T315I) [Gorre ME et al., 2001]. This substitution not only breaks hydrogen bonding between IM

and threonin but the substituted isoleucine also contains extra hydrocarbon group in the side chain which sterically hinders IM from entering the ATP-binding pocket, while ATP binding to the kinase is not affected (Fig. 12). This mutation also occurred during cultivation of *BCR-ABL*-positive cell line in increasing concentrations of IM over a long period of time [Scappini B et al, 2004]. Other typical examples of mutations (amino acid exchanges) which directly interact with IM via Van der Waals bonds are F317L, Y253H/C/F and V289A. The clinical significance of these mutations varies. For example T315I mutation is completely resistant to IM inhibition, while F317L confers only moderate IM resistance.

Figure 11: The approximate frequencies of *BCR-ABL* mutations (given as amino acid changes) in CML resistant clinical samples as compiled from data reviewed by Hochhaus A and la Rosee P, 2004. P-loop: ATP-binding loop, A-loop: activation loop.

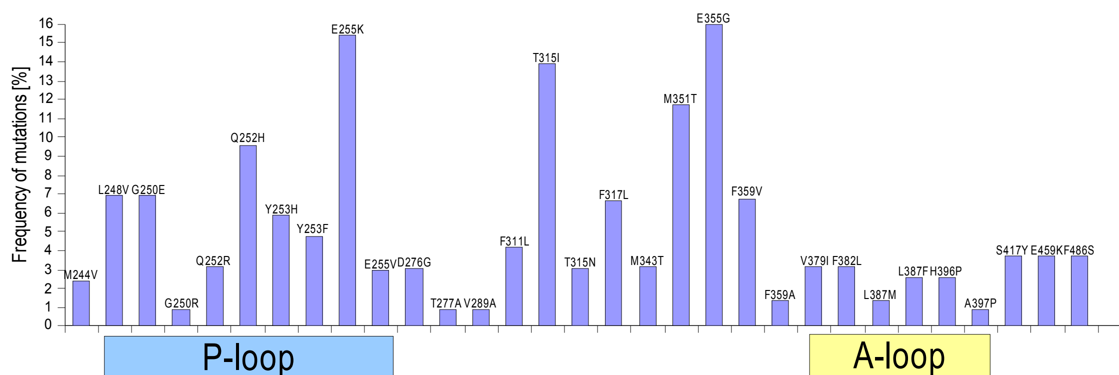
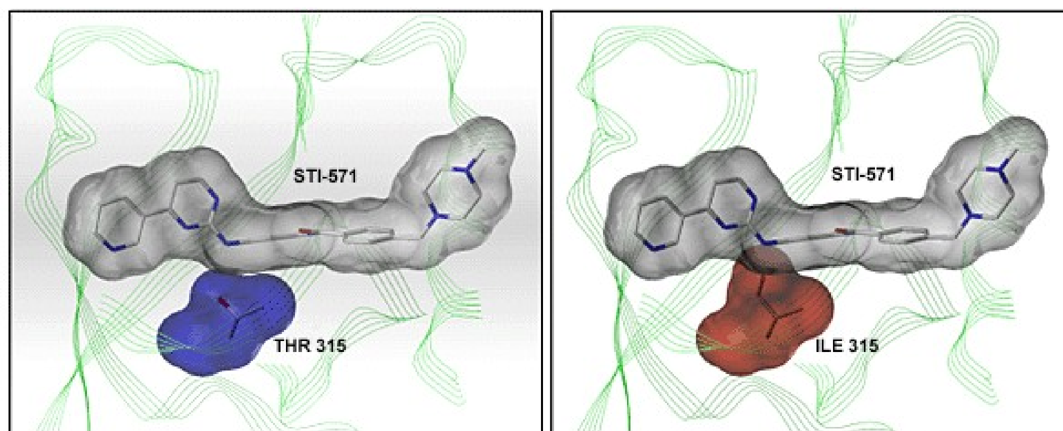


Figure 12: Model of IM binding site in wild type c-Abl (left) and c-Abl with T315I mutation in complex with IM (right). In the molecular structures representing IM and c-Abl residue 315, nitrogen atoms are shown in blue and oxygen atoms are shown in red. Van der Waals interactions are depicted in gray for IM (both panels), in blue for wild-type Abl residue Thr 315 (left), in the red for mutant Abl residue Ile 315 (right). The polypeptide backbone of the Abl kinase domain is represented in green [Figure taken from Gorre ME et al., 2001].



In the second group, there are mutations located in the kinase domain, which do not directly interfere with IM binding but rather change the conformation of the c-Abl kinase. The inactive conformation of c-Abl is stabilized by two flexible loop structures: ATP-binding phosphate loop (P-loop: 244th-255th amino acid) and the activation loop (A-loop: 381st-402nd amino acid) [Schindler T et al., 2000]. Mutations within these domains may destabilize their arrangements and thus the kinase domain cannot assume the inactive conformation required for IM binding.

The detection of mutations within the clinical samples is focused on the kinase domain of Bcr-Abl kinase. *In vitro* screen of randomly mutagenized *BCR-ABL* has shown that mutations outside this domain may also confer resistance [Azam M et al., 2003]. The resistance mechanisms driven by these mutations also include change in Bcr-Abl molecular conformation that impairs IM binding.

Characterization of the mutants by *in vitro* kinase assays, quantification of intracellular Bcr-Abl pohosphorylation and proliferation assays revealed different degrees of resistance to IM inhibitions depending on the type of the mutation [reviewed in Deininger M et al., 2005]. These results have not yet been fully correlated to clinical outcome. Mutant clones have also been detected in patients prior to IM therapy [Roche-Lestienne C et al., 2002] and in patients in CCR. In some patients the mutated clone overgrew the wild type while on IM therapy showing a proliferative advantage and lead to subsequent relapse. While in others the mutant clone detected prior to therapy disappeared on IM treatment [Willis SG et al., 2005]. The fact that the mutations present prior to therapy were not selected on the treatment suggested that at least in some cases other additional mechanisms were necessary for fully resistant phenotype [Lange T et al., 2005]. In some patients more than one mutation was detected. Some investigators showed that patients with P-loop mutations appeared to have a particularly bad prognosis compared to patients with other types of mutations [Branford S, Rudzki Z et al., 2003]. All these clinical parts of the puzzle seem to be put together by *in vitro* and *in vivo* study that showed that different mutations possessed different transformation potential, kinase activity, and substrate utilization, irrespective of the level of IM resistance [Griswold IJ et al., 2006]. The mutations that confer proliferative advantage over the wild-type *BCR-ABL* clone such as P-loop mutations (E255K and Y253F) possess greater transformation potential and thus are directly involved in progression of the disease. On the other hand, mutated *BCR-ABL* with no proliferative advantage over the wild type

kinase under normal conditions is selected only on IM treatment [Griswold IJ et al., 2006]. Finally, some mutant clones do not necessarily have a proliferative advantage even in the media with IM and their presence does not always account for resistance to IM at least in some patients [Khorashad JS et al., 2006].

Similar principle of resistance to IM treatment has been observed also in patients with GISTs and hypereosinophilic syndrome, who bore IM-resistant point mutations of c-Kit [reviewed in Fletcher JA and Rubin BP, 2007] and PDGFRalpha (T674I) [Cools J, DeAngelo DJ et al., 2003], respectively. If the occurrence of mutations as a principle of resistance to small kinase inhibitors is a general rule remains to be elucidated.

3.5.6.3 Protein Pumps Implicated in IM Resistance

Resistance to IM might also be connected with pharmacokinetic parameters. The influence of protein pumps belonging to the family of ABC transporters on the plasma and intracellular concentrations of IM is under investigation. These are membrane proteins that transport hydrophobic, lipid substances and xenobiotics across the cellular membrane. IM is a substrate of ABCG2 and ABCB1 (*MDR-1*; P-glycoprotein) drug pumps belonging to this family [Burger H, Nooter K., 2004] and these pumps are usually responsible for the drug efflux and thus for the decreased IM-cellular concentrations. IM treatment induces the expression of these transporters in intestinal cell line *in vitro*, suggesting that upregulation of these intestinal pumps could reduce the IM oral bioavailability [Burger H et al., 2005]. In addition, both ABCB1 and ABCG2 are highly expressed on primitive hematopoietic stem cells (HSCs). Recently, Brendel C et al. [2007] has demonstrated a dose-dependent, reversible inhibition of ABCG2-mediated Hoechst 33342 dye efflux in primary human and murine HSCs by IM, suggesting the role of this transporter in decreased cellular IM-concentrations in HSCs and thus possibly in IM resistance. In the case of P-glykoprotein (Pgp) the scientific evidence is diverging. There are *in vitro* studies on *BCR-ABL*-positive cell lines suggesting that expression of Pgp decreases the IM cellular concentrations and thus can lead to IM resistance [Mahon FX et al., 2000; Mahon FX et al., 2003; Illmer T et al., 2004]. In addition, biological effect of Pgp modulation (inhibition by cyclosporine A) during IM treatment was proved *in vivo* in a refractory *BCR-ABL*-positive ALL patient [Illmer T et al., 2004]. In contrast, other groups did not observe any effect during IM treatment either of Pgp overexpression in cell lines *in vitro* [Ferrao PT et al., 2003] or of loss of Pgp expression in hematopoietic stem cells in a murine transplantation CML model *in vivo* [Zong Y et al.,

2005]. There are more studies needed to explain the discrepancies among these scientific results.

The pharmacokinetics of IM is also influenced by protein binding in plasma. Approximately 95% of IM in clinically relevant concentration is bound to albumin and to alpha-1-acidic-glycoprotein (AAG). Animal models as well as studies of clinical samples *in vitro* and *in vivo* have showed that IM binds to AAG with high affinity and the plasma concentration of IM depends on the amount of AAG. AAG decreases the IM intracellular concentration and inhibits its activity [Gambacorti-Passerini C et al., 2000; Gambacorti-Passerini C, Zucchetti M. et al., 2003]. In contrast, Jorgensen HG et al. [2002] showed that purified AAG from CML patients did not inhibit IM effect on *BCR-ABL*-positive cell line *in vitro*.

The exact factors influencing the pharmacokinetics of IM are not yet clear, but it is already clear that these players have to be considered if evaluating clinical IM-resistance.

3.5.6.4 Clonal Evolution

Additional chromosomal abnormalities are commonly observed in CML patients in AP or BC and are associated with high risk of disease progression in patients in CHR [Marktel S et al., 2003]. Karyotypic abnormalities in addition to Ph chromosome have also been detected in patients resistant to IM treatment [Hochhaus A et al, 2002, Schoch C et al., 2003]. Recently, Duesberg P et al. [2007] have proposed that IM resistance in CML is rather of chromosomal origin than due to kinase mutations. Further, Fabarius A et al. [2005, 2007] has shown that IM can induce centrosome and karyotype aberrations (genetic instability) in various cell lines *in vitro*, which may explain the emergence of clonal chromosomal abnormalities in Ph-negative progenitor cells under IM therapy. The role of chromosomal abnormalities in CML patients is very difficult to generalize because it can be very individual. In addition, it is problematic to decide if the new aberration is responsible for IM-resistance or the disease progression. The appearance of *BCR-ABL* oncogene is usually the trigger point of the cancer development but additional aberration may include activation of oncogene, which maintains the cancer clone independently of Bcr-Abl inhibition.

3.5.6.5 Src Family of Kinases: Role in CML Disease and IM Therapy Resistance

Src kinases, Lyn and Hck, are activated in leukemic *BCR-ABL*-positive cells [Danhauser-Riedl S et al., 1996]. Src kinases are substrates of Bcr-Abl and their interaction is essential for

transformation signaling by Bcr-Abl [Lionberger JM et al., 2000] for example via Bcr-Abl/Hck/STAT5 signaling pathway [Klejman A, Schreiner SJ et al., 2002]. Activated STAT5 proteins translocate to nucleus and trigger expression of genes encoding proteins which regulate the growth factor independence, differentiation, adhesion/invasion and DNA repair/drug resistance. Hck and Lyn are constitutively activated (phosphorylated) in blast crisis CML patients and their increased expression correlates with the progression of the disease [Roginskaya V et al., 1999; Donato NJ et al., 2003]. Src kinases can be activated independently of Bcr-Abl and directly activate (phosphorylate) Bcr-Abl [Meyn MA 3rd et al., 2006]. Overexpression or activation of Src kinases mediates Bcr-Abl independence and IM resistance in some CML cell lines [Donato NJ et al., 2003; Dai Y et al., 2004] as well as in clinical specimens [Donato NJ et al., 2004]. In these patients, inhibition of Bcr-Abl and Src kinases is required for apoptosis. The important role of Src kinases in CML disease progression and in Bcr-Abl-dependent as well as Bcr-Abl-independent IM-resistance makes them a good target for therapy.

3.1.7 *BCR-ABL* Persistence

Even though most patients respond well to IM treatment, less than 5% reach a complete RT-PCR negativity for *BCR-ABL* [IRIS study, O'Brien SG et al., 2003]. This *BCR-ABL* persistence has to bee closely monitored as minimal residual disease.

It is not yet clear which mechanisms underline this leukemia persistence or if they are similar to mechanisms inducing IM resistance. A persistent subpopulation of *BCR-ABL*-positive primitive quiescent cells was identified in CML patients on IM therapy [Jiang X et al., 2007]. This subpopulation is sensitive to IM inhibition of proliferation but escapes the pro-apoptotic effect of IM. Thus, IM may be able to eliminate *BCR-ABL*-positive stem cell proliferation but is not sufficient to eradicate the fraction of quiescent cells [reviewed by Barnes DJ et al., 2006].

It has been hypothesized that this persistent subpopulation is represented by “leukemia stem cells (LSCs).” The LSC share many properties with normal HSC, including distinct immunophenotype, relatively quiescent cell cycle status, the ability of self-renewal, multipotentiality, and a strong proliferative capacity [reviewed in Reya T et al., 2001; Jordan CT, 2007]. Several murine models of CML have shown that different chemotherapies including IM treatment and radiotherapy do not target selectively LSCs but rather both HSCs

as well as LSCs. In addition, LSCs may have enhanced mechanisms of DNA repair and/or the ability to evade senescence induction [Neering SJ et al., 2007]. These murine models may be very useful in development of more selective regiments for eradication of malignant stem cells.

Considering the above mentioned facts, it is not clear if discontinuation of IM therapy after the CML patient has reached complete molecular response is safe. There are patients, who immediately relapse [Usuki K et al., 2005], at the same time there are patients, who do not show any disease for more than one year after withdrawal of IM treatment [Mauro MJ et al., 2004; Hess G et al., 2005; Rousselot P et al., 2007]. Recently, there is an ongoing clinical study called STIM (stop imatinib) in France, which should shed some more light into this problem [Rousselot P et al.]. The patients have been treated with IM for 6 years. The question is if the continuation of this treatment in patients with CMR for 5 more years will be sufficient for eradication of LSCs. In general, patients not in molecular remission should probably continue the drug indefinitely.

3.1.8 Treatment Options for CML Patients Resistant to IM

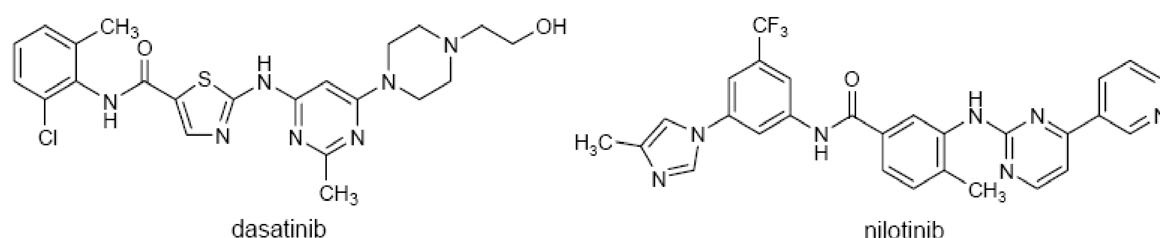
Some of the resistant patients to IM treatment may respond favorably to higher dosage [Kantarjian HM et al., 2003]. These include patients with *BCR-ABL* overexpression, amplification or mutations that are partially sensitive to IM. In case of T315I mutation complete cessation of IM treatment is proposed as this mutation confers a complete resistance. Sometimes the withdrawal of IM treatment may also help in the case of mutations with proliferative advantage in IM media over the wild-type Bcr-Abl but no advantage in media without IM. Discontinuation of IM therapy led to reversion from BC to CP in a case of CML [Liu NS, O'Brien S, 2002]. There are ongoing new clinical trials of second generation of Bcr-Abl inhibitors, which are effective against most of the known mutations and also trials of various combinational therapies. Autologous stem cell transplantation remains an important salvage option for patients who develop resistance to IM through Bcr-Abl mutations [Jabbour E et al., 2005]. In addition, the return to conventional anti-CML drug e.g. INF-alfa, hydroxyurea or busulfan may be tried. Finally, there are new experimental agents developing e.g dual Abl/Src inhibitors, bosutinib, farnesyl transferase inhibitors etc. (see below).

3.1.9 Second Generation of Small Inhibitors of Abl Kinase

Several second-generation kinase inhibitors have been developed and some of them are now in preclinical or in early stages of clinical trials. Hopefully, these novel drugs will help in eradicating residual disease and reducing the incidence of resistance. The two leading compounds with most advanced clinical evaluations are nilotinib (AMN107) and dasatinib (BMS354825 - already registered for CML therapy of IM-resistant patients in the Czech Republic). Both inhibitors have displayed promising activity in patients with IM–resistant chronic- and advanced-phase, blast crisis CML and IM–resistant Ph-positive ALL [Hochhaus A et al., 2007; Guilhot F et al., 2007; Cortes J, Rousselot P et al., 2007; Ottmann O et al., 2007; Kantarjian HM et al., 2007].

Nilotinib has been developed as a derivative of the molecular structure of IM (Fig. 13). It is a highly selective 26-fold more potent inhibitor of Bcr-Abl than IM and binds to the inactive conformation of Abl kinase [Weisberg E et al., 2005; Kantarjian HM et al., 2007]. Dasatinib (Das) possesses completely different molecular structure (Fig. 13), and is 325-fold more potent inhibitor of Bcr-Abl than IM. In addition, Das targets broader range of kinases including the Src family of kinases [Shah NP et al., 2004]. Das binds both to the active as well as to the inactive conformation of Abl kinase. The fact that Das inhibits Src kinases is an advantage because of the role of Src kinases in IM-resistance and disease progression as described above. On the other hand, less selective effect brings along higher degree of toxicity and increased rate of side effects.

Figure 13: Chemical structure of nilotinib and dasatinib [picture taken from http://www.chem.uoa.gr/chemicals/chem_imatinib.htm#15].



Both these inhibitors are effective against the most common causes of IM-resistance i.e. *BCR-ABL* amplification/overexpression and against most of the IM-resistant mutant forms of Bcr-Abl with the exception of mutation T315I. On the other hand, it is to be expected that

the selective pressure of treatment with these inhibitors will produce other typical mechanisms of resistance. Indeed, *in vitro* screen of resistant cell lines revealed some resistant Bcr-Abl mutations with the most dominant T315I [von Bubnoff N et al., 2006; Bradeen HA et al., 2007]. Some novel Das-resistant mutations were sensitive to IM inhibition. Combination therapy of IM plus other inhibitors is potentially a more potent strategy of treatment [O'Hare T et al., 2005] but due to lower toxicity the sequential administration of different inhibitors is preferred. Clinical study of 112 CML patients who received second-generation inhibitors after IM-failure showed that types of mutations that occur after treatment with these inhibitors were diverse [Cortes J et al., 2007], including those seen in patients resistant to IM therapy and others that are novel and only seen after introduction of a new inhibitor (Table III). Acquisitions of mutations are usually associated with therapy resistance, but treatment failure can occur in many instances in the absence of any mutations suggesting other mechanisms of resistance analogous to IM-resistance.

Table III: Mutations of Bcr-Abl kinase developing in IM-resistant CML patients treated with dasatinib or nilotinib

dasatinib	G250E, V299L, T315A, **T315I**, F317I, F317L, L364I, T495R
nilotinib	M244V, Q252H, Y253H, E255K, D276G, F311I, **T315I,** F359C, F359V, E453K, H396P, H396R

Compiled from Cortes J et al., 2007; Soverini S et al., 2007; Shah NP et al., 2007.

The enormous success of IM treatment has triggered development of many novel compounds which are in various phases of investigation see Table IV. These include bosutinib (SKI-606) [Boschelli DH et al., 2006], MK-0457 (VX-680), BIRB-796, ON012380, adaphostin [reviewed in Weisberg E et al., 2007]. For example ON012380 is a compound that does not bind to ATP-binding site of Abl kinase as IM and nilotinib do, but targets the substrate-binding site instead. This compound is effective in cellular and animal models against most of the IM-resistant Bcr-Abl mutants including the notorious T315I [Gumireddy K et al., 2005]. In addition, MK-0457 is another inhibitor, which is effective against T315I mutation *in vitro* and has already shown promising results in CML patients [Giles FJ et al., 2007].

Table IV: The range of inhibiton of kinases by IM and second-generation inibitors.	
Imatinib	Bcr-Abl, c-Kit, PDGFR
Dasatinib	Bcr-Abl, Src family of kinases, c-Kit, ephrin receptor kinases, PDGFR
Nilotinib	Bcr-Abl, c-Kit, PDGFR
Bosutinib	Bcr-Abl and Src family of kinases
MK-0457	Bcr-Abl, aurora kinases, Flt3 kinase
BIRB-796	Bcr-Abl, p38 MAP kinase
ON012380	Bcr-Ab, Lyn kinase
Adaphostin	Bcr-Abl and other tyrosine kinases
Adapted from Kantarjian HM et al., 2006	

3.1.10 Targeting the Upregulated Activity of Signaling Molecules Downstream Bcr-Abl

Deregulation of signaling molecules downstream Bcr-Abl such as Ras, PI3K, surivin etc. can play role both in disease progression, as well as, in resistance to treatment. The constitutive activity of these molecules is an attractive target for development of novel drug discoveries.

Bisphosphonates, such as zoledronate, have been shown to inhibit the oncogenicity of Ras, an important downstream effector of Bcr-Abl. Chuah C et al. [2005] observed that zoledronate is equally effective in inhibiting the proliferation of both IM-sensitive and resistant CML cells, regardless of their mechanism of resistance. This is achieved by the induction of S-phase cell cycle arrest and apoptosis, through the inhibition of prenylation of Ras and Ras-related proteins by zoledronate. Farnesyl transferase inhibitors (FTI) possess similar mode of action. An example is Lonafarnib, which inhibits farnesylation of intracellular proteins including Ras. Lonafarnib has shown activity against *BCR-ABL*-positive IM-resistant as well as IM-sensitive cells and is now in clinical trials [Borthakur G et al., 2006].

The fact, that PI3K pathway is essential for the growth of CML cells, but not for normal hematopoietic cells, makes it an ideal target for therapy. PI3K inhibitors (wortmannin, LY294002) synergize with IM by greatly increasing apoptosis of CP and BC CML patient cells [Klejman A et al., 2002]. The PI3K inhibitors may be too toxic for clinical use, so different more specific downstream targets are looked at such as mammalian target of rapamycin (mTOR). The combination of IM and rapamycin was found to act synergistically against committed CML progenitors from CP and BC patients. This drug combination was also effective *in vitro* against IM-resistant cell line bearing mutations (M351T, E255K) in Bcr-Abl kinase [Halbur L et al., 2003].

Survivin, a member of the inhibitor of apoptosis family of proteins, plays important roles in both cell proliferation and cell death and was found to be activated by Bcr-Abl kinase through MAPK cascade signaling [Carter BZ et al., 2006]. At the same time activation of surivivin was also found independently of Bcr-Abl kinase activity in progressive CML disease, as well as in IM-resistant cell lines. Carter BZ et al. [2006] showed that targeting survivin both overcomes IM resistance in IM-resistant CML cells and increases IM efficacy in IM-responsive CML cells *in vitro*.

3.1.11 Summary for CML

The discovery of molecular pathogenesis of CML has lead to development of the first specific therapy targeted against defined oncogene. The wonderful clinical success of this approach triggered an incredible bloom of investigations of molecular targets in other cancers including other myeloproliferative disorders and started intensive search for their specific inhibitors. IM heralds new era of patient-specific therapy based on precise knowledge of molecular pathology underlying the disease.

7.1.1 *BCR-ABL*-Negative Myeloproliferative Disorders

Myeloproliferative disorders which are not associated with Ph-chromosome are classical MPDs, and all the other *BCR-ABL*-negative diseases are grouped under the heterogeneous category of atypical MPDs (see Table V). Classical *BCR-ABL*-negative myeloproliferative disorders (MPDs) are polycythemia vera (PV, also called polycythemia rubra vera, or erythremia), essential thromobocythemia (ET), and idiopathic myelofibrosis (IMF, also known as myelofibrosis with myeloid metaplasia, agnogenic myeloid metaplasia, and primary myelofibrosis). These disorders are characterized by increased number of erythrocytes (polycythemia), thrombocytes (thrombocythemia) or of all myeloid lineages (PV). IMF is associated with decreased numbers of blood elements (progressive anemia) due to replacement of the bone marrow with fibrous connective tissue. PV and ET can in the course of the disease evolve into IMF or acute myeloid leukemia (AML). These specific disease entities are interrelated and may be very difficult to clinically distinguish. Moreover, recent studies revealed that some of the diseases overlapped also on the molecular level. These studies have clearly shown that these diseases are typical examples of defects in signaling pathways. These primary disorders caused by intrinsic defects in signaling in the hematopoietic stem cell are clinically very difficult to distinguish from secondary

or reactive expansion of myeloid cells caused by factors outside the hematopoietic stem cell such as polycythemia due to hypoxia, thrombocythemia caused by inflammations etc. The knowledge of precise genetic lesions underlying these disorders may help not only in diagnosis but also in developing specifically targeted therapy. In 2005 five different studies described a genetic lesion common for most of the patients with the diagnosis of classical MPDs. This is a mutation in inhibitory domain of JAK2 kinase, which renders the kinase constitutively active. In addition, precise genetic lesions in some of the atypical MPDs have also been described (Table V).

3.6.1 Colony-forming Assay of Hematopoietic Progenitors, Hypersensitivity to Erythropoietin (EPO)

MPDs as mentioned above are caused by intrinsic defects in signaling pathways of hematopoietic progenitors, which lead to increased proliferation and differentiation even in environment with no or low concentration of certain cytokines (hypersensitivity to cytokines). In contrast, normal progenitors will proliferate and differentiate only after stimulation with growth factors. This property can be tested in a functional assay, which can help in differentiating between primary and secondary (reactive) increase in blood cell numbers. In this test isolated hematopoietic progenitors are cultivated in semi-solid media with cocktail of cytokines [Migliaccio et al., 1988]. Each hematopoietic progenitor will be stimulated to divided and differentiate. The clonal daughter cells cannot migrate freely due to the nature of semi-solid medium and will form a lineage-specific recognizable colony. For example erythroid progenitor BFU-E (burst-forming unit-erythrocyte) forms a colony consisted of about 2000 to 4000 cells, which are discernable by microscope. The type, morphology, and number of these colonies can be evaluated. In our laboratory we test hypersensitivity to EPO in patients with suspected PV or ET. The progenitors are cultured in decreasing concentrations of exogenously added EPO.

In contrast to reactive polycythemia/thrombocythemia, PV and ET patients are characterized by erythroid progenitor hypersensitivity to EPO. PV can be differentiated from Primary Familial and Congenital Polycythemia (PFCP, also called benign erythrocytosis or familial erythrocytosis) and secondary polycythemia by growth of hemoglobinized erythroid colonies in media without addition of EPO (EECs – endogenous erythroid colonies) [Prchal JF and Axelrad AA, 1974; reviewed in Weinberg RS, 1997; Kralovics R and Prchal JT, 1998]. The formation of EECs is an accepted auxiliary diagnostic criterion in the 2006 WHO (World

Health Organization) decree for the MPD diagnosis [Michiels JJ et al., 2006]. EECs are not specific for PV because EECs can be found in some ET as well in some IMF patients. This phenotypic overlay either suggests a common molecular cause underlying all three types of MPD or might be a phenotypic feature that results from multiple molecular causes. It has been suggested that some ET patients with EECs may in fact represent masked PV and may develop into more typical clinical PV picture later on [Shih LY, Lee CT, 1994]. Some patients diagnosed with ET may have an early stage of IMF, or, alternatively, the diagnosis of IMF may be incorrectly assigned in cases with a previously undiagnosed spent phase of PV

Table V: *BCR-ABL*-negative myeloproliferative disorders and their semimolecular classification

Classical	frequently associated with *JAK2* V617F mutation
Polycythemia vera Essential thrombocythemia Idiopathic myelofibrosis	
Atypical	
I. Molecularly defined	
PDGFRA-rearranged eosinophilic/mast cell disorders	e.g. *FIP1L1-PDGFRA*
PDGFRB-rearranged eosinophilic disorders	e.g. *TEL/ETV6-PDGFRB*
Systematic mastocytosis with c-Kit mutation	e.g. *c-KIT* D816V
FGFR1-rearranged EMS/SCLL (or sometimes called 8p11 myeloproliferative syndrome)	e.g. *ZNF198/RFIM/RAMP-FGFR1*
II. Clinicopathologically assigned	infrequently associated with *JAK2* V617F mutation
Chronic neutrophilic leukemia (CNL) Chronic eosinophilic leukemia Hypereosinophilic syndrome (HES) Chronic basophilic leukemia Chronic myelomonocytic leukemia (CMML)	molecularly not defined
Juvenile myelomonocytic leukemia	associated with recurrent mutations of Ras signaling pathway molecules including PTPN1 and NF1
Systematic mastocytosis (SM) Unclassified myeloproliferative disorder	molecularly not defined

Adapted from Tefferi A and Barbui T, 2005.

PDGFRA/B: platelet-derived growth factor receptor alpha/beta; EMS/SCLL: 8p11 myeloproliferative syndrome/stem cell leukemia/lymphoma; PTPN1: protein tyrosine phosphatase, non-receptor type 1; NF1: neurofibromatosis type I

3.6.2 Clonality

MPDs are recognized as clonal disorders [Adamson JW et al., 1976]. A specific mutation of a hematopoietic progenitor cell triggers the development of the disease. This mutation provides the cell with proliferative advantage, giving rise to a clone with higher proliferative capacity. Since all myeloid and often also lymphoid lineages (B-lymphocytes, not usually T-lymphocytes or NK cells) display clonality, MPD is considered a hematopoietic stem cell disorder [Fialkow PJ et al, 1981; Raskind WH et al., 1985; Anger B et al., 19990; el-Kassar et al., 1997]. Additional mutations are probably acquired later in the course of the disease and are associated with progression of the disease from MPD into acute leukemia with either myeloid or lymphoid character. Patients diagnosed with PV or IMF are always clonal. In contrast, ET patients are usually polyclonal but may be clonal [Liu E et al., 2003; Harrison CN et al., 1999]. The differences between clonal and polyclonal ET in respect to disease prognosis, progression and therapy still have to be elucidated.

Detection of clonal genetic abnormality other than Ph-chromosome is one of the major criteria in MPD diagnosis as proposed by WHO in 2001 [Michiels JJ et al., 2006]. Clonality can be detected either indirectly by using markers that allow detection of X-chromosome inactivation in female patients, or directly by detecting chromosomal alterations or mutations on the DNA or RNA level.

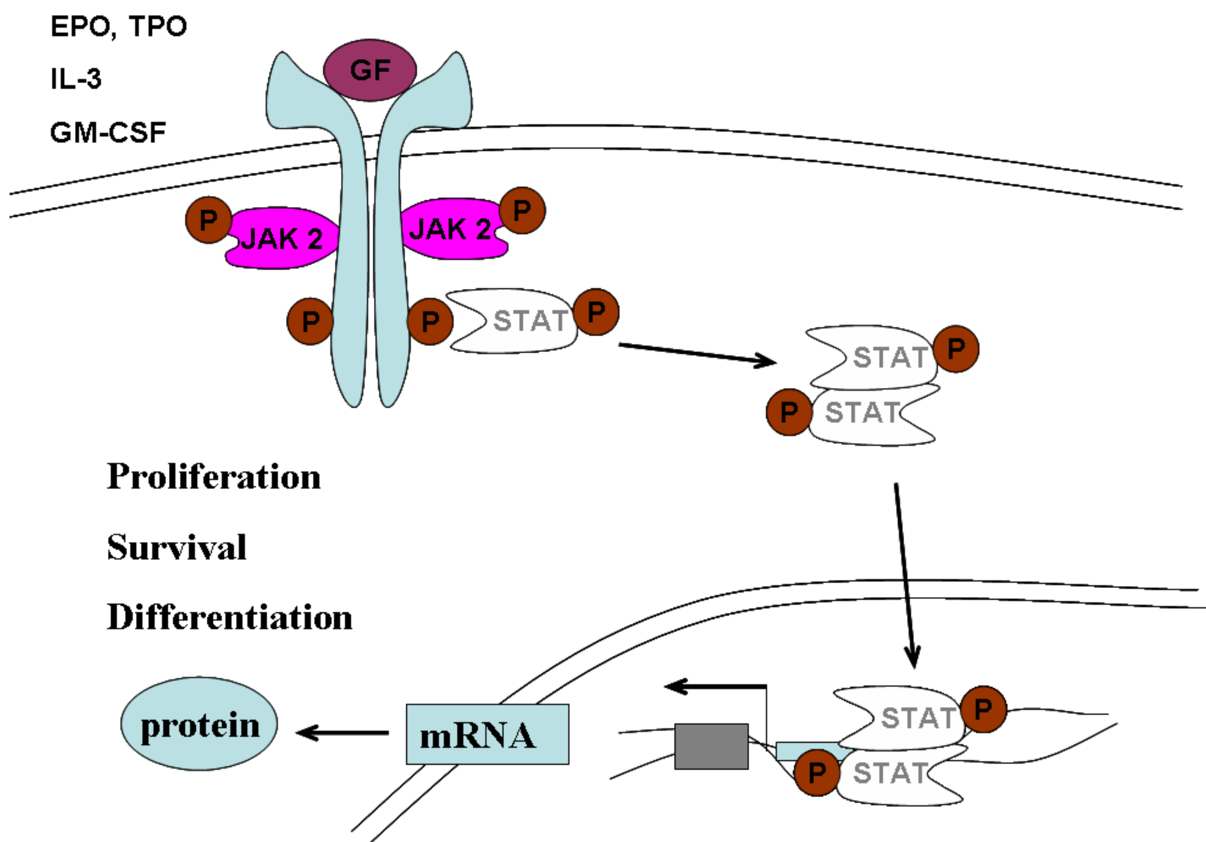
3.6.3 JAK2

In the year 1989 Wilkes cloned two novel cytoplasmic protein tyrosine kinases, and called them playfully **J**ust **A**nother **K**inase (Jak). Currently i.e. in 2007, there are more than 2800 publications about this family of kinases and thus the kinases were renamed in a more dignified manner by the Greek god Janus as **Ja**nus **K**inases. The Janus kinases are located in cytoplasm (Jak1, Jak2, Jak3 and tyrosine-kinase-2: Tyk2), and upon activation they associate with receptors of important growth factors such as type I receptors (for IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL- 13, GM-CSF, growth hormone, prolactin, EPO and thrombopoetin: TPO), and type II cytokine receptors (for IFN-α,-β, -γ). Binding of cytokines to these cell-surface receptors results in receptor dimerization and activation of JAK kinases. Activated JAKs phosphorylate the cytoplasmic domain of the receptor on tyrosine residues thus creating docking sites for other signaling/adaptor molecules such as STAT proteins. STATs are latent cytoplasmic transcription factors, which bind to activated receptors, and are subsequently

phosphorylated/ activated by Jaks. When activated they dimerize, translocate to nucleus and trigger expression of various key genes such as anti-apoptosis mediator *Bcl-XL*. Protein products of these genes regulate cellular proliferation, differentiation and/or survival (Fig. 14).

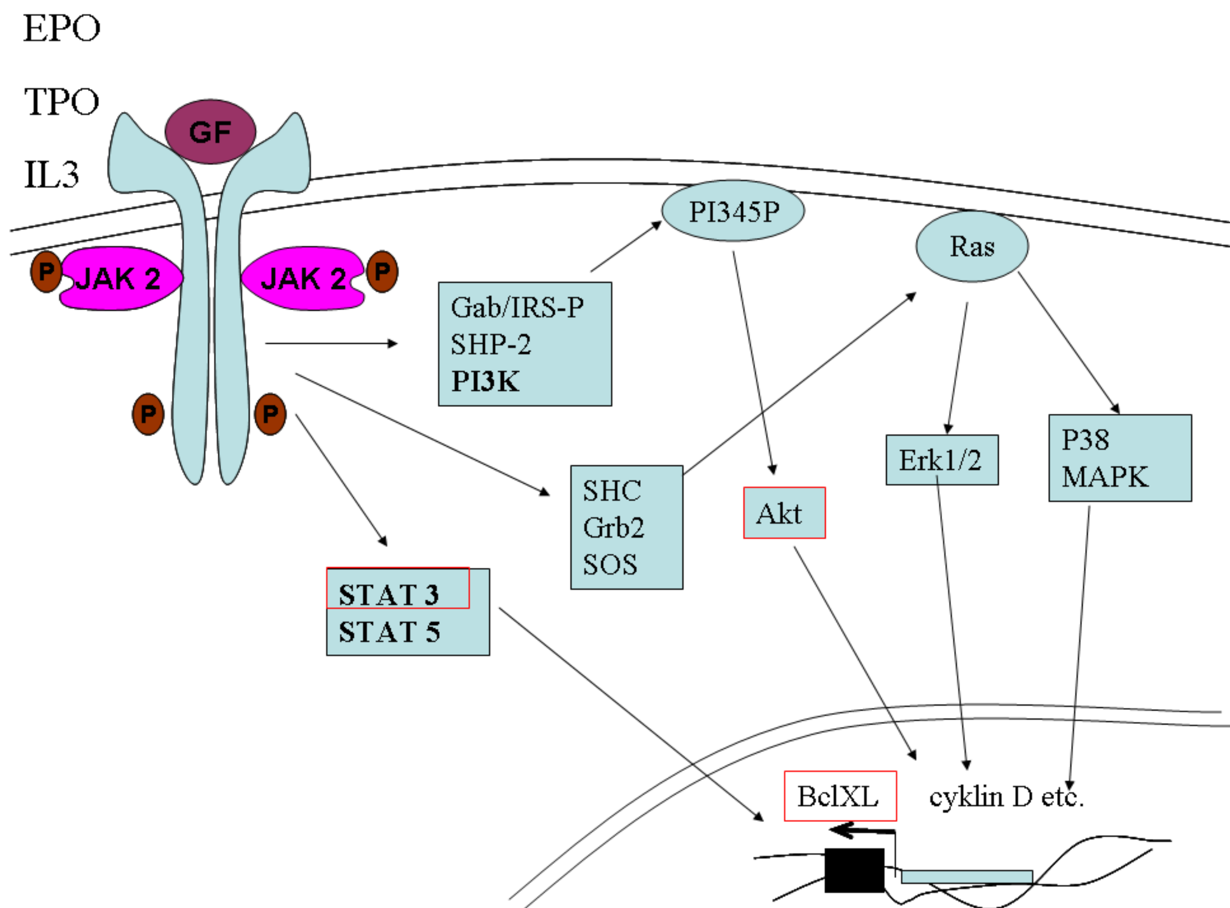
The docking sites created by phosphorylation of a growth factor receptor by Jak kinases are also available for other signaling molecules including scaffolding molecules (Fig. 15) such as GAP/IRS proteins. These molecules associate with SHP-2 phosphatase and phopho-inositol-3-kinase (PI3K), which activate signaling pathway involving phosphoinositol-3,4,5-triphosphate (PI345P)/Akt. Akt is a serine-threonine kinase which phosphorylates number of substrates including transcription factors, which trigger expression of proteins playing a key role in regulation of cellular proliferation. Other important scaffolding molecules are SHC/Grb2/SOS which activate famous Ras-signaling pathway (see above).

Figure 14: JAK/STAT signaling pathway. GF: growth factor, EPO: erythropoietin, TPO: thrombopoietin, IL-3: interleukin-3, GM-CSF: granulocyte-macrophage colony-stimulating factor, JAK: Janus kinase, STAT: signal transducer and activator of transcription, P: phosphate group.



Generation of strains of mice lacking the individual *JAK* family members showed that they possess essential, non-redundant roles in cytokine signaling [Suzuki K et al., 2000; Rodig SJ et al., 1998]. Targeted disruption of *JAK2* gene in mice revealed the essential role of this kinase in definite erythropoiesis [Parganas et al., 1998; Neubauer et al., 1998]. The mutation was embryonically lethal because of the lack of definite erythrocytes. Interestingly the lymphoid lineage differentiation and proliferation was not influenced. In comparison with the erythropoietin receptor-deficient mice, the *JAK2* deficiency possessed more severe phenotype. Frenzel K et al. [2006] created a mouse model based on dominant negative Jak2 protein, which also showed that *JAK2* is essential for normal mammalian development.

Figure 15: Downstream signaling triggered by hematopoietic growth factors. The red boxes mark signaling molecules, which are abnormally activated in bone marrow or peripheral blood cells in PV or ET patients. GF: growth factor, SHP-2: phosphatase, PI3K: phosphoinositol-3-kinase, PI345P: phophoinositol-3,4,5-triphosphate, SOS: son of seven-less, MAPK: mitogen activated protein kinase, GAP: GTPase-activating proteins. Adapted from Kaushansky K, 2005.



3.6.3.1 Jak2 Structure

Each family member of Jak kinases is composed of seven Jak homology (JH) domains. The tyrosine kinase catalytic activity is contained within the JH1 domain. The JH2, or pseudocatalytic domain, is nearly identical in amino acid sequence to JH1, but lacks the catalytic activity. The N-terminal of the kinase is responsible for association with cytokine receptors through JH5-7 domains, which contain the FERM domain (band four-point-one, ezrin, radixin, moesin).

Similarly to other kinases, the Jak kinases contain several autoinhibitory mechanisms. The crystal structures of the kinase domains have been solved for Jak3 [Boggon TJ et al., 2005] and for Jak2 [Lucet IS et al., 2006]. In order to be active, Jak kinase requires phosphorylation of activation loop within the kinase domain. This phosphorylation event indirectly promotes the activation conformation by removing steric constraints imposed by the unpohosphorylated loop. The pseudokinase domain JH2 plays an important role in negative regulation of the kinase activity possibly through interaction with the activation loop of JH1 domain [Lindauer K et al., 2001; Saharinen P et al., 2005]. The importance of JH2 negative regulation is emphasized by the biological consequences of its mutation as described below. The third level of negative regulation involves the FERM domain. Funakoshi-Tago M et al. [2006] observed that FERM domain could be phosphorylated upon association with receptors (erythropoietin, thrombopoietin, growth hormone receptors). This phosphorylation leads to dissociation of Jak2 kinase from the receptor and to degradation of the kinase. All these autoinhibitory mechanisms have to be overcome in order to activate the kinase, thus ensuring that the activation occurs only when the receptor is engaged with a ligand.

3.6.3.1 *JAK2* Translocations

Jak kinases play an essential role in mediating the growth-promoting signaling of a variety of cytokines. It is not surprising, that mutations leading to constitutive activation of Jaks could have pathological consequences. Certain hematopoietic malignancies are associated with chromosomal translocations of *JAK2* gene. The fusion partners are diverse, but all of them provide the *JAK2* gene with either dimerization or oligomerization domain. The resulting Jak2 kinase is thus constitutively activated even in the absence of cytokine signaling. Typical examples of these translocations are *JAK2* gene fusion to Ets family

member *Tel* (also called *ETV6*) [t(9;12)(p24;p13)], *Bcr* [t(9;22)(p24;q11.2)], and *PCM1* (pericentriolar material gene 1) [t(8;9)(p22;p240)], which have been detected in a variety of hematological malignancies including CML, AML, HES, MDS, ALL and other MPDs. The exact mechanisms of pathology of these disorders and the role of the *JAK2* fusion genes are still to be elucidated. For example the same Tel-Jak2 fusion protein is able to induce either CML-like disease [Schwaller J et al., 1998] or T-cell ALL-like disorder [Carron C et al., 2000] or B-cell lymphoma/leukemia in mice [dos Santos NR et al., 2006]. In addition, when expressed in primary human hematopoietic cells Tel-Jak2 drives erythropoietin-independent erythropoiesis and induces myelofibrosis *in vivo* [Kennedy JA et al., 2006]. The different consequences of the same fusion gene show the importance of possible variations in cell environmental factors or of secondary transforming events in the pathogenesis of these diseases.

3.6.3.1 *JAK2* V617F Mutation

Increased hematopoiesis in myeloproliferative disorders with no apparent chromosomal aberration was associated with constitutive or increased activation of STAT3 [Roder S et al., 2001], Akt [Dai C et al., 2005], Bcl-XL [Silva M et al., 1998] in bone marrow or peripheral blood cells (see Fig. 15 red boxes). These observations plus hypersensitivity of MPD progenitors to cytokines hinted that the underlying mechanism is connected to deregulated activity of a common kinase.

Indeed, in 2005 five different groups simultaneously described a common acquired somatic mutation in pseudokinase domain of the Jak2 kinase in patients with classical Ph-negative myeloproliferative disorders by using different approaches [Baxter EJ et al.; James C et al.; Kralovics R et al.; Levine RL et al.; Zhao R et al.]. Incredibly the mutation in all patients was the same leading to substitution of valine to phenylalanine at the position of the 615th amino acid (V615F) of the JH2 domain. In these original reports the *JAK2* V617F mutation was detected in majority of PV patients (65-97%), and in about a half of ET (23-57%) and IMF (43-57%) patients. Later on more sensitive detection techniques such as pyrosequencing [Jelinek J et al., 2005], ARMS-PCR [Vannucchi AM et al., 2006] etc. showed that this mutation is present in almost all cases of PV. In addition, *JAK2* mutation has now also been detected in cases with other MPDs including atypical MPDs and MDS and in a low percentage of AML patients [Jones AV et al., 2005; Steensma DP et al., 2005; Levine RL, Loriaux M et al., 2005]. Kralovics R et al. [2005] observed that the occurrence of *JAK2*

V617F mutation was linked to longer duration of the disease and to more clinical complications such as secondary fibrosis, bleeding, and thrombosis.

JAK2 V617F is a dominant gain-of-function mutation, which probably disrupts the negative regulatory role of the pseudokinase domain [Saharinen et al., 2003]. The mutated pseudokinase domain looses its ability to stabilize the inactive conformation of the kinase by direct interference with activation loop [Lindauer K et al., 2001]. In agreement with this, *JAK2* V617F behaves as a constitutively activated tyrosine kinase when expressed in cell lines [James C et al., 2005; Levine RL et al., 2005]. On the cellular level this mutation causes cytokine-independent and erythropoietin- (EPO-) or interleukin-3- (IL-3-) hypersensitive growth of cell lines and cultured bone marrow cells accompanied by constitutive activation of STAT5, PI3K, ERK, and Akt pathways [Kralovics R et al., James C et al., Zhao R et al., 2005; Wernig G et al., 2006]. Primary blood and spleen cells derived from *JAK2* V617F-positive mouse models (see below) displayed constitutive STAT5 activation and endogenous and EPO-hypersensitive erythroid colony formation [Wernig G et al., 2006; Lacout C et al., 2006].

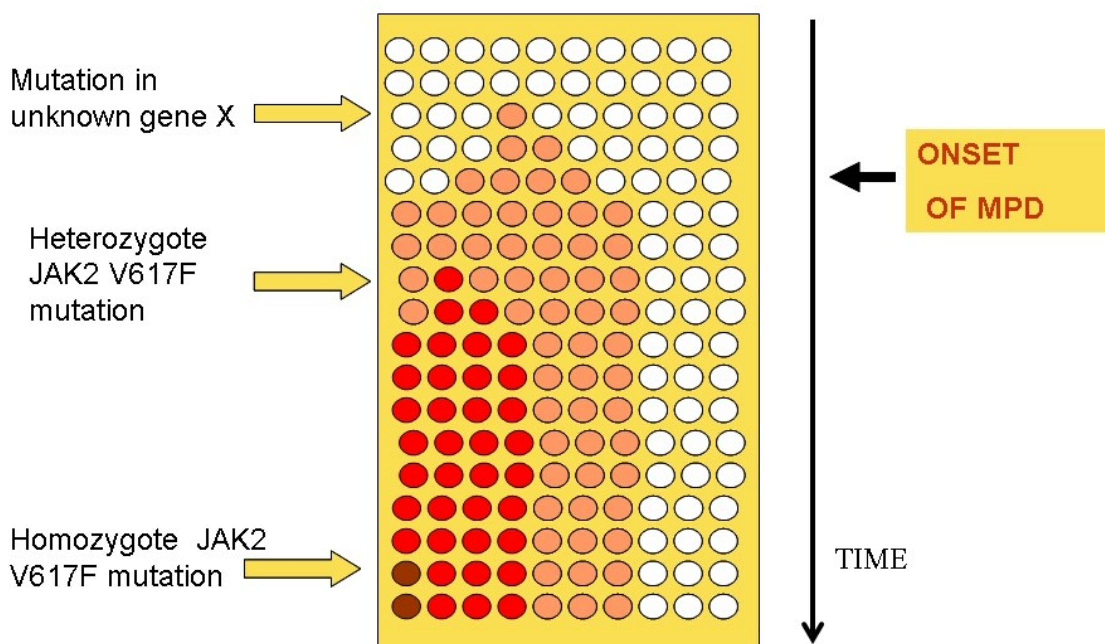
Significant fraction of PV patients is homozygous for the mutant allele in the peripheral blood [Scott LM et al., 2006]. The homozygosity is not caused by deletion of the wild type allele, but by mitotic recombination leading to the exchange of the 9p24 chromosomal region carrying the wild type *JAK2* allele with the 9p24 region carrying the mutated allele (uniparental disomy) [Kralovics R et al., 2005; Levine RL et al., 2005]. As a consequence, homozygous patients carry two copies of the mutated *JAK2* allele in the absence of the wild-type *JAK2* (9p24 loss of heterozygosity). Mitotic recombination is a very rare event occurring in 1 out of 1000 mitoses. It is therefore assumed, that the homozygous *JAK2* V617F clone possesses an additional proliferation and/or survival advantage over the heterozygous one [James C et al., 2005]. The proliferative advantage of the *JAK2* V617F-positive erythroid cells in the presence of optimal concentration of EPO was proved *in vitro* [Dupont S et al., 2007].

Murine models that retrovirally overexpressed *JAK2* V617F developed phenotype similar to PV with erythrocytosis, splenomegaly, extramedullary hematopoiesis, granlucytosis and delayed-onset bone marrow myelofibrosis and anemia, if high levels of *JAK2* V617F were expressed [James C et al., 2005; Lacout C et al., 2006; Wernig G et al., 2006]. In contrast, transient thrombocytosis was observed in mice with low expression of *JAK2*

V617F [Lacout C et al., 2006]. In addition, the level of *JAK2* V617F correlates with hemoglobin values and/or white blood cell counts in PV patients [Lippert E et al., 2006]. *In vitro* colony-forming assays have proved that *JAK2* V617F mutation triggers EPO hypersensitivity and that homozygosity increases EPO independence [Dupont S et al., 2007]. These data suggest that disease phenotype is closely liked to the allele burden of *JAK2* V617F.

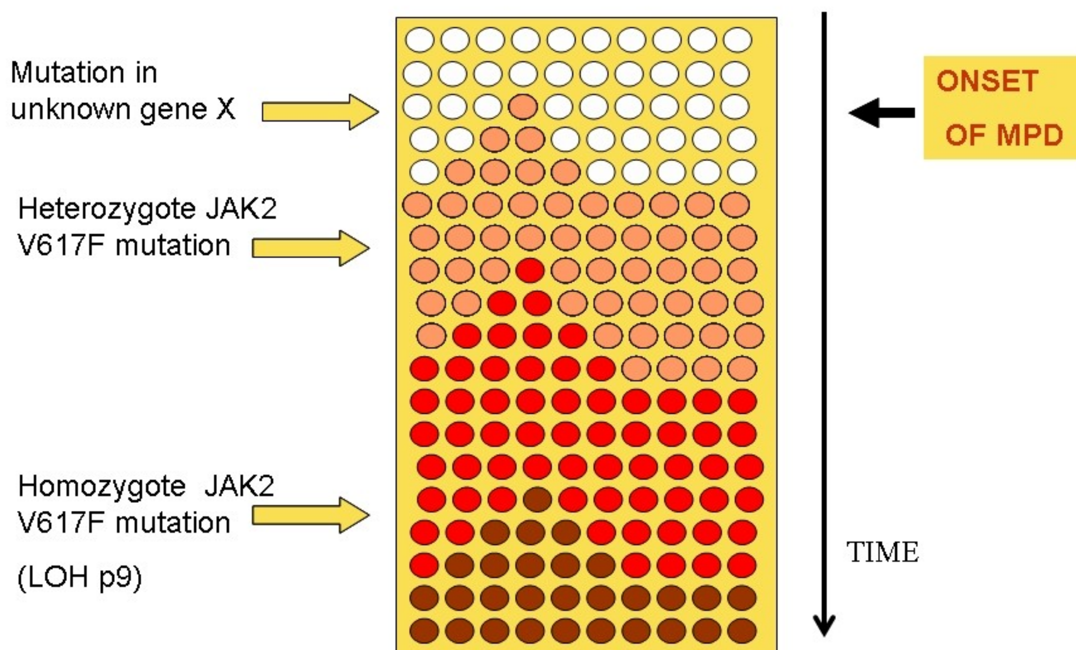
On the other hand, it is also clear that *JAK2* V617F mutation is not the initiating event of these diseases. Clonality studies based on X-chromosome inactivation pattern assays have shown that *JAK2* V617F clone is only a part of the clonal populations of blood cells [Kralovics R et al., 2006]. In addition, EECs from these patients are not only *JAK2* V617F-positive but also wild-type, suggesting that there is an additional cause of hypersensitivity. Finally in familial forms of *JAK2* V617F-positive MPDs no germline transmission of the mutation has been observed [Bellanne-Chantelot C et al., 2006]. The appearance of *JAK2* V617F mutation is therefore probably preceded by mutation in an as-yet-unknown gene. A model for the clonal development of PV and ET has been proposed by Skoda C [2005] see (Fig. 16, 17).

Figure 16: Model of clonal evolution in peripheral blood compartment of an ET patient. Adapted from Skoda RC, 2005.



One of the questions, which remains to be answered, is: In which progenitor does the *JAK2* V617F mutation occur? *JAK2* V617F mutation is specific to the cells belonging to the various myeloid lineages in the majority of patients with PV [Ishii T et al., 2006] but is not present in T or B lymphocytes. This evidence corresponds to the fact that T and B lymphocytes are usually polyclonal in PV in contrast to myeloid cells. On the other hand, *JAK2* V617F mutation was detected in B and natural killer (NK) cells in approximately half the patients with IMF studied and a minority of those with PV. Moreover, in a few cases patients with IMF had mutated peripheral T cells, as well [Delhommeau F et al., 2007]. There is evidence that the *JAK2* V617F mutation in PV and IMF is present in a lymphomyeloid stem/ progenitor cell and that the phenotype of the disease is probably related to the proliferative advantage given essentially to the myeloid lineage leading thus to a pure myeloproliferative and not to a lymphomyeloid proliferative disorder [Delhommeau F et al., 2007].

Figure: 17: Model of clonal evolution in peripheral blood compartment of a PV patient.



In accordance with these results the *JAK2* V617F mutation was described in other rare myeloproliferative diseases (unclassified MPDs, CMML, MDS, SM, CML, HES, acute megakaryocytic leukemia) but not in lymphoid once [Jones AV et al., 2005; Jelinek J et al., 2005; Steensma DP et al., 2005; Levine RL et al., 2005].

Although recent studies have shed some light on the role of *JAK2* V617F mutation in pathogenesis of MPDs, it is still not clear weather this mutation is only an additional mutation arising during the progression of the disease or why is it selected for in such an extend in these disorders.

3.6.3.1 Other *JAK2* Exon 12 Mutations

Recently, other mutations of *JAK2* gene exon 12 have been described in patients with *JAK2* V617F-negative PV or idiopathic erythrocytosis leading to substitutions K539L, H538QK539L [Scott LM et al., 2007], C616Y [Zhang SJ et al., 2007], D6120E [Grunebach F et al., 2007]; and deletions F537-K539delinsL, N542-E543del [Scott LM et al., 2007], I540-E543delinsMK, R541-E543delinsK [Butcher CM et al., 2007]. Some of these mutations have already been characterized on the cellular level and even in mouse models. These mutations cause growth-factor hypersensitivity *in vitro* and activate biochemical pathways associated with erythropoietin signaling. In comparison with *JAK2* V617F these exon 12 mutations result in stronger ligand-independent signaling through Jak2 i.e. higher levels of Jak2 and higher phosphorylation of downstream effectors ERK1 and ERK2 than does the V617F mutation [Scott LM et al., 2007]. In addition, these mutations have not yet been found in homozygous state or in ET patients. This corresponds to the notion that low levels of Jak2 signaling favor thrombocytosis, whereas more active signaling favors erythrocytosis.

Different exon 12 *JAK2* mutations have also been described in other diseases such as substitution D620E in *BCR-ABL*-negative *JAK2* V617F-negative atypical MPDs [Schnittger S et al., 2006]; 5-amino acid deletion *JAK2*DeltaIREED in a patient with Down syndrome with B-cell precursor acute lymphoblastic leukemia [Malinge S et al., 2007]; and K607N mutation in an AML patient [Lee JW et al., 2006]. The precise significance of these mutations in the pathology of these disorders is yet to be defined.

3.6.4 Pediatric MPDs

MPD may occur also in children but it is a very rare event in comparison with the adult MPDs [Teofili L et al., 2007; Hasle H et al., 2000]. It has been suggested that the biological mechanism underlying these disorders in children may differ from the adult diseases. In addition, many children with primary thrombocytosis and erythrocytosis can have a familial form of thrombocythemia and polycythemia [reviewed by Skoda R, Prchal JT, 2007]. Minority of familial ETs and PVs has already been characterized on the molecular

level. They are associated with specific mutations in thrombopoietin (TPO) gene, TPO receptor gene, or EPO receptor gene. These mutations lead to constitutive activation of TPO/EPO signaling. The genetic background of the sporadic forms of pediatric PV and ET is still under investigation.

3.6.4.1 Pediatric ET

Hematopoetic progenitors from pediatric ET patients are hypersensitive to cytokine signaling (e.g. to EPO). Most of the pediatric ET patients have been reported to possess polyclonal hematopoiesis with only a few clonal cases [Randi ML et al., 2006]. The *JAK2* V617F mutation was detected only in just a few children with ET by standard procedures and these patients were never homozygous. The molecular basis of pediatric ET remains to be elucidated. It is not clear if the diagnosis of pediatric ET includes several different disorders with similar clinical manifestations or if it is a distinct disease from sporadic adult ET. Another question which remains to be answered is the role of *JAK2* V617F mutation in this disorder.

2 AIMS OF THE STUDY

The aims of this thesis were to study molecular pathogenesis of myeloproliferative disorders.

Aim 1:

Molecular characterization of CML patients resistant to imatinib or dasatinib treatment.

Aim 2:

Cellular and molecular characterization of polycythemias and thrombocythemias

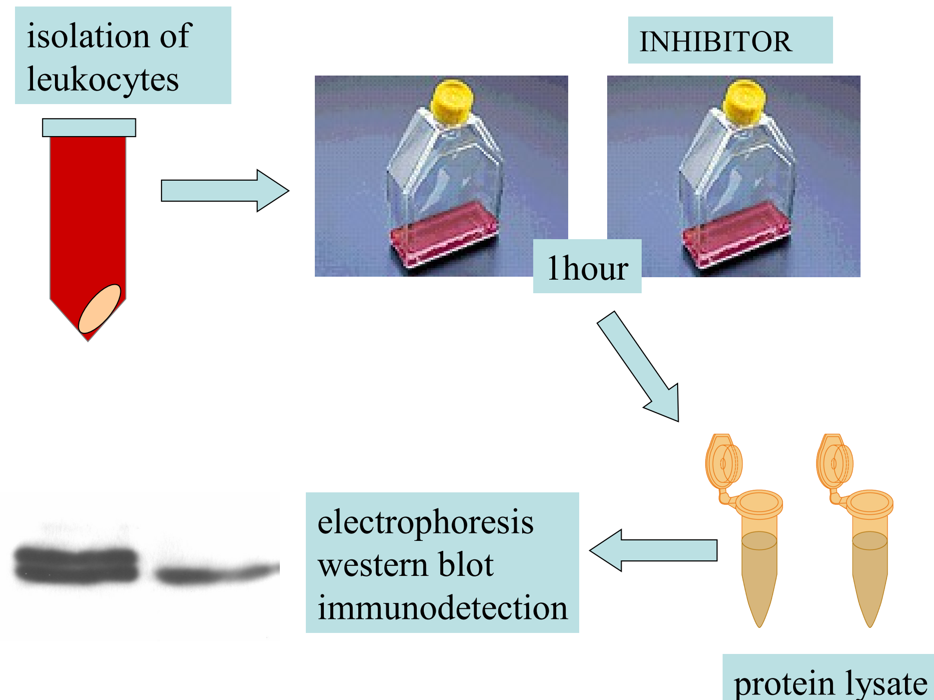
3 EXPERIMENTAL PROCEDURES

The Ethics Committee of the Palacky University reviewed and approved the study. All of the patients provided signed informed consent for giving blood for clearly defined research purposes.

5.1. Western Blot Analysis: Detection of Phosphorylation of Crkl and Src Family of Kinases

Sixteen ml of heparinized blood samples were obtained for each experiment (Fig. 18). Erythrocytes were lysed in lysis buffer (150 mM NH_4Cl, 10 mM NH_4HCO_3, 4 mM EDTA) for 10 to 15 min on ice. The leukocytes were centrifuged at 1800 rpm for 10 min at 4°C. The pellet was washed with the same lysis buffer. The isolated leukocytes were washed with serum-free medium Optimem I (Gibco, Invitrogen, Carlsbad, CA). Leukocytes (6 x 10^6) were incubated in 50 ml RPMI (Sigma-Aldrich, Germany), 10% fetal bovine serum (Gibco) with or without 10 μM IM at 37°C and 10% CO_2 for 1 hour. Cells were always kept on ice in pre-chilled falconi tubes and washed two times with ice-cold PBS (phosphate buffered saline) (centrifuged at 2000 rpm for 5 min at 4°C) and lysed in 100 μl IP buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% v/v glycerol, 0.1% v/v Tween 20) with phosphatase and protease inhibitors. Gel electrophoresis and immunoblotting with 500 x anti-Crkl 32H4 monoclonal antibody (Cell Signaling Technology, Beverly, MA) or 250 x Phospho-Src Family (Tyr416) Antibody (Cell Signaling Technology Inc., Danvers, MA), and secondary antibody (PIERCE Stabilized Goat Anti-Mouse/Rabbit HRP-conjugated, Rockford, IL) were performed. Detection was done with Super Signal West Dura Chemiluminiscence Substrate (PIERCE).

Figure 18: *In vitro* test of sensitivity of leukemia patients' cells to inhibitors (10 µM IM, or 250 nM Das). The leukocytes were isolated from peripheral blood by erythrocyte lysis and centrifugation. The patient cells were incubated with and without the inhibitor. Protein lysates were prepared and western blot with immunodetection was performed.



5.2. Mutation Analysis of ATP Binding Pocket of Bcr-Abl Kinase

5.2.1. Preparation of Total RNA from Leukocytes and Reverse Transcriptase (RT-) PCR

RNA was isolated from leukocytes obtained from 8 – 10 ml of peripheral blood with EDTA (ethylenediaminetetraacetic acid). Erythrocytes were lysed in lysis buffer (as described above) for 30 min on ice with occasional gentle mixing. The leukocytes were centrifuged at 3000 rpm for 10 min at 4°C. The pellet was washed with the same lysis buffer and twice with 1x phosphate-buffered saline (PBS). Total RNA was isolated from leukocytes lysed in Trizol reagents (Invitrogen) [Chomczynski P et al., 1987]. The total RNA was mixed with random primers (Promega, Madison, WI), in total volume of 16 µl. The mixture was incubated at 75°C for 10 min, and frozen on dry ice for 5 min, and left to melt on ice (4°C). The reverse transcription reaction consisted of 5 µl of the RNA with annealed random primers, 1 µl 10mM dNTPs (diluted in DEPC water, deionized autoclaved diethylpyrocarbonate-treated

water), 16 U RNasin (Promega), 20 U of AMV Reverse Transcriptase (Finzyme, Espoo, Finland) and took place for 40 min at 42°C. The resulting hybrid cDNA was used fort two-round PCR reaction for amplification of *BCR-ABL* kinase domain.

5.2.2. Two-round PCR: Amplification of *BCR-ABL* Kinase Domain

Two-round PCR was performed with mixture in ratio 1:1.4 of Taq DNA recombinant polymerase (Invitrogen) and Pfu Turbo Hotstart DNA Polymerase (Strategene, La Jolla,CA) see Table VI. First round primers for the fusion domain of the *BCR-ABL* transcript were:

forward primer B2A: 5´-TTCAGAAGCTTCTCCCTGACAT-3´),
reverse primer Bcr Abl R: 5´-CTTCTCTAGCAGCTCATACACC-3.´
Cycle: 95°C 3 min, 35x (95°C 45 sec, 56°C 40 sec, 72°C 45 sec), 72°C 7 min. The second round amplified the ATP binding pocket with two different sets of primers:

forward primer NTPE3+: 5´-AAGCGCAACAAGCCCACTGTCTAT-3,'
and **reverse primer Bcr Abl R** with annealing temperature 58°C;
forward primer Abl ATP F: 5´-TCACCATGAAGCACAAGCTG-3,'
and **reverse primer Abl ATP R**: 5´-TCAGGTAGTCCAGGAGGTTC-3´
with annealing temperature 54°C.

The PCR product was purified by electrophoresis in a 1% agarose sterile gel run in 1x TAE solution (40 mM TRIS-Acetate (trishydroxymethylaminomethane - acetate), 1 mM EDTA, pH 8.3). The specific band corresponding to the desired PCR product was cut and extracted from agarose with QIAGEN Gel Extraction Kit (QIAGEN, Valencia, CA). Direct sequencing of the PCR product from both sides was performed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Table VI: Two-round PCR reaction for amplification of *BCR-ABL* kinase domain.

	1st round	Final	2nd round		2nd round/ ATP binding pocket	
Pfu Poly [2.5 U/µl]	0.28	0.7 U		0.28		0.28
Taq Poly [5 U/µl]	0.2	1 U		0.2		0.2
cDNA	2		**10x diluted cDNA**	2	**10x diluted cDNA**	2
50 mM $MgCl_2$	2	2 mM		2		2
Primer B2A [100 pmol/µl]	0.2	20 pmol	**NTPE3+ [100 pmol/µl]**	0.2	**ABL-ATP F [100 pmol/µl]**	0.2
Primer Bcr-Abl R [100 pmol/µl]	0.2	20 pmol	**Bcr-Abl R [100 pmol/µl]**	0.2	**ABL-ATP R [100 pmol/µl]**	0.2
10 mM dNTPs	1	0.2 mM		1		1
10x PCR buffer	5	1x		5		5
TC water	39.12			39.12		39.12
total volume	50			50		50
annealing temperature	56°C			57°C		54°C
size of product [bp]	3374			708		257

The amounts of the stock solutions are given for 1 reaction in µl. dNTPs: deoxyribonucleotides (Gibco); TC water: tissue culture (autoclaved deionized) water

5.2.3. Restriction Analysis of the *BCR-ABL* Kinase Mutations

PCR product of one round PCR as described above with primers B2A and Bcr-Abl R was subjected to restriction analysis with the specific restriction enzyme (Table…). Typical reaction consisted of 17 µl of the specific PCR product, specific buffer and 1 µl (5U) restriction enzyme (New England Biolabs, Beverly, MA). The reaction took place for 4 hours at 37°C. The total amount of the mixture was detected by 1.8% agarose gel electrophoresis.

5.3. Detection of *JAK2* V617F Mutation

JAK2 V617F mutation was detected on the level of genomic DNA isolated from leukocytes from peripheral blood by allele-specific (AS) PCR and by restriction analysis [Baxter et al., 2005], on the level of RNA isolated from platelets, granulocytes and mononuclear cells from peripheral blood by allele-specific (AS) reverse transcriptase (RT-) PCR as described by Cambell et al., 2005. In addition, *JAK2* V617F mutation was analyzed also on the level of single myeloid colonies see 3.3.2.

5.3.1 Detection of *JAK2* V617F Mutation on Genomic DNA from Peripheral Blood

5.3.1.1 Allele-Specific PCR

The leukocytes were isolated from 8 – 10 ml of peripheral blood with EDTA as described in 3.2.1. The pelleted leukocytes were washed in PBS and digested overnight at 37ºC in 4.5 ml of STE buffer (100 mM NaCl, 50 mM TRIS, 1 mM EDTA . $2H_2O$, pH = 7.4), 250 µl of 0.2% proteinase K (Invitrogen), 250 µl of 10% w/v sodium dodecyl sulphate (SDS) (all chemicals from Serva, Heidelberg, Germany). The DNA was isolated by phenol-chloroform extraction (phenol pH=8 saturated with 0.2 M TRIS) and precipitated with 2 volumes of 96% ethanol (Lachema, Brno, Czech Republic). The pelleted DNA was washed with 70% ethanol, dried and dissolved in TC water (autoclaved deionized water).

DNA was amplified by AS-PCR using a combination of three primers:

Forward primer_specif: 5'-AGCATTTGGTTTTAAATTATGGAGTATATT-3'
Forward primer_cont: 5'-ATCTATAGTCATGCTGAAAGTAGGAGAAAG-3'
Reverse primer: 5'-GGCGACGAGTGAGACTCCAT-3.'

The reaction uses a common reverse primer. The first forward primer anneals only to *JAK2* V617F mutated allele (JAK2 F mut) with PCR product of 203 bp. The second forward primer (JAK2 F) is an internal control of the PCR reaction, it anneals to the wild type as well as to the mutated allele. Cycle: 94°C for 2 min, 36 x (94°C for 30 sec, 58°C for 30 sec, 72°C for 45 sec), 72°C for 10 min. See Table VII for the details of the PCR reaction. The PCR products were analyzed on a 1.2% agarose gel.

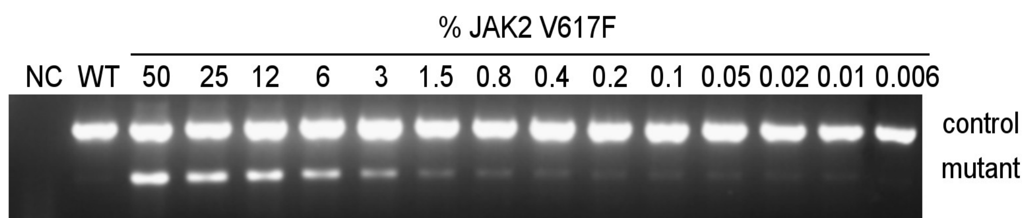
Table VII: AS-PCR for detection of *JAK2* V617F mutation

	1 reaction	final
Taq Poly [5U/ml]	0.3	1.5 U
DNA	2	
50 mM $MgCl_2$	2	2 mM
Primer JAK2 R [100 pmol/µl]	0.25	25 pmol
Primer JAK2 F [100 pmol/µl]	0.125	12.5 pmol
Primer JAK2 F mut [100 pmol/µl]	0.125	12.5 pmol
10 mM dNTPs	1	0.2 mM
10x PCR buffer	5	1x
TC water	43.2	
	50	
Annealing temp	58°C	
Size of product [bp]	mutated: 203, control: 364	

The amounts of the stock solutions are given for 1 reaction, bp: base pairs, TC water: tissue culture/ autoclaved deionized water

In order to detect the sensitivity of this PCR reaction in our hands serial two-fold dilutions of 100% V617F-homozygous clonal control DNA (InVivoScribe Technologies, San Diego, CA) with genomic DNA of a healthy donor were used starting with 50% of mutant DNA. Total 400 ng DNA were put into the PCR reaction. AS-PCR detected a homozygous mutation in up to 0.8% dilution (equivalent to one heterozygous cell in 64 normal cells) see Fig. 19.

Figure 19: Sensitivity of AS-PCR detection of *JAK2* V617F mutation on the level of genomic DNA.



5.3.1.2 *Bsa*XI Restriction Analysis of *JAK2* V617F Mutation

JAK2 V617F mutation abolishes *Bsa*XI restriction site. The corresponding part of the gene was amplified with a set of primers:

Forward primer: 5'-GGGTTTCCTCAGAACGTTGA-3,'

Reverse primer: 5'-TCATTGCTTTCCTTTTTCACAA-3.'

Cycle: 94°C for 2 min, 45x (94°C for 30 sec, 57°C for 30 sec, 72°C for 45 sec), final elongation at 72°C for 10 min. See Table VIII for the details of the PCR reaction. The PCR products (460 bp) were digested with 1 U *Bsa*XI restriction endonuclease (New England Biolabs, Beverly, MA) in corresponding buffer for 24 hours at 37°C. The mutated allele was not digested, the wild type allele was digested with resulting products of 241 bp, 189 bp, and 30 bp. The PCR products were analyzed on a 2% agarose gel.

Table VIII: PCR reaction for *Bsa*XI restriction analysis of *JAK2* V617F mutation

	1 reaction	**final**
Taq Poly [5 U/µl]	0.3	1.5 U/ rci
DNA	2	
50 mM $MgCl_2$	2	2mM
reverse primer [100 pmol/µl]	0.25	25 pmol
forward primer [100 pmol/µl]	0.25	25 pmol
10 mM dNTPs	1	0.2 mM
10x PCR buffer	5	1x
TC H_2O	39.2	
total volume	50	
annealing temp	57°C	
size of product [bp]	460	

The amounts of the stock solutions are given for 1 reaction in µl.
TC water: tissue culture (deionized autoclaved) water

5.3.2 Detection of *JAK2* V617F on Transcript Level by Allele-Specific (AS) Reverse Transcriptase (RT-) PCR

In order to detect *JAK2* V617F mutation on the level of RNA, platelets, granulocytes, and mononuclear cells were isolated from 8-10 ml of peripheral blood with EDTA of each patient.

5.3.2.1 Isolation of Platelets, Granulocytes, and Mononuclear Cells from Peripheral Blood

Isolation of platelets:

Full blood was fuged at 700 rpm for 10 min. The pellet containing erythrocyte mass was kept on ice until later. The supernatant, which contained plasma was centrifuged at 2000 rpm for 10 min. After the centrifugation the supernatant was put back into the original tube with erythrocyte mass. The pellet now contained the platelets with some contaminations of

erythrocytes. The erythrocytes were lysed with NH_4Cl solution (StemCell Technologies, Vancouver, BC, Canada) for 10 min at room temperature with occasional vortex. Centrifugation at 2000 rpm for 10 min was performed. The purified platelets were lysed in 500 µl Trizol (Invitrogen).

Granulocytes and mononuclear cells:

1 ml of 6% Dextran 70 in 0.9% NaCl (Infusia, Hořátev, Czech Republic) was added to the original tube of erythrocyte mass. The tube was inverted several times and let stand for 60 min at room temperature. The supernatant was put into clean tube and centrifuged at 1600 rpm for 10 min. The pellet was resuspended in 5 ml of PBS and centrifuged on top of 2 ml of Histopaque (1077 Hybri-Max, Sigma-Aldrich) at 1500 rpm for 20 min at room temperature.

The ring of mononuclear cells on the top of histopaque was put into new tube and washed with 1 volume of PBS. The mononuclear cells were pelleted at 1600 rpm for 7 min and resuspended in 500 µl Trizol (Invitrogen).

After the ring of mononuclear cells was isolated the entire volume of the rest of the tube was discarded and the remaining pellet contained mononuclear cells. The contaminations of erythrocytes in the pellet were lysed in 6 ml od DEPC water (deionized water treated with 1% of diethylpyrocarbonate, Sigma) for 30 sec. The lysis was stopped by addition of 2 ml of 3.5% NaCl (Litolab, Litovel, Czech Republic). The purified mononuclear cells were pelleted at 1600 rpm for 7 min and resuspended in 500 µl Trizol (Invitrogen). All Trizol lyzates were stored at -80ºC.

5.3.2.2 Allele-Specific (AS)-Reverse Transcriptase (RT-) PCR

Total RNA was isolated from Trizol lysates of platelets, granulocytes, and mononuclear cells according to the manufacturer's manual (Invitrogen). The first step in RT-PCR was the hybridization of random primers (Promega) to the total RNA. 1-5 µg of RNA was incubated with 500 µg of random primers in total volume of 12 µl at 75ºC for 10 min. The sample was then quickly chilled on ice (4 ºC) for 15 min. The RNA with annealed random primers was either directly used for reverse transcription or stored at -80ºC. Reverse transcription was performed with AMV Reverse Transcriptase (Finzyme) at 42ºC for 40 min. For details of this reaction see Table IX.

Table IX: Reverse Transcription

	1 reaction	Final
AMV Reverse Transcriptase [20 U/µl]	1	20 U
RNA with random primers	5	
10x AMV buffer	1.5	1x
RNasin [2 U/µl]	0.4	0.8 U
10 mM dNTPs DEPC	1	0.67 mM
DEPC water	6.1	
total volume	15	

The amounts of the stock solutions are given for 1 reaction in µl. DEPC: diethylpyrocarbonate treated. RNasin (Promega).

The PCR reaction used a common reverse primer. The first forward primer anneales only to *JAK2* V617F mutated allele with PCR product of 294 bp. The second primer was an internal control of the PCR reaction, it annealed to the wild type as well as to the mutated allele producing 488bp DNA fragment. Cycle: 94°C for 11 min, 38 x (94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec), final elongation at 72°C for 6 min. See Table X for the details of the RT-PCR reaction. The RT-PCR products were analyzed on a 1.5% agarose gel.

Table X: AS-RT-PCR for detection of *JAK2* V617F mutation

	1 reaction	Final concentration
Taq Poly [5 U/µL]	0.3	1.5 U
10x PCR buffer	5	1x
50 mM $MgCl_2$	1.5	1.5 mM
10 mM dNTPs	1	0.2 mM
JAK2-CMF [100 pmol/µL]	0.25	25 pmol
JAK2-CF [100 pmol/µL]	0.25	25 pmol
JAK2-CR [100pmol/µL]	0.5	50 pmol
cDNA	1	
TC water	40.2	
	50	
Annealing temp	**58°C**	
Size of product [bp]	**mutated: 294, control: 488**	

The amounts of the stock solutions are given for 1 reaction in µl.

Common reverse primer

JAK2-CF: 5'-GTAATACTAATGCCAGGATCACTAAGTTT-3'

Mutation-specific forward primer

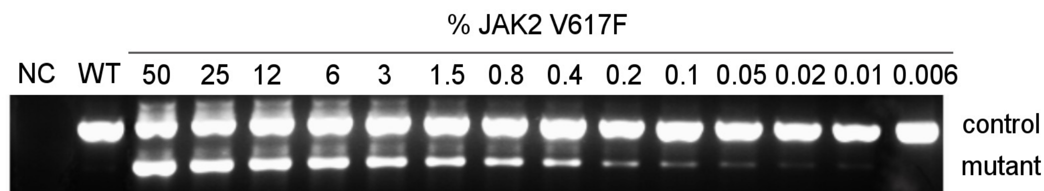
JAK2-CMF: 5'-AGCATTTGGTTTTAAATTATGGAGTAGGTT-3'

Control forward primer

JAK2-CR: 5'-GAAGATTTGATATTTAATGAAAGCCTTG-3'

The sensitivity of AS-RT-PCR was determined by serial two-fold dilutions of plasmids obtained by cloning of AS-RT-PCR product of a *JAK2* V617F-positive patient and a healthy donor into pCR4Blunt-TOPO vector (Invitrogen). The plasmids were sequenced on ABI-PRISM 310 (Applied Biosystem). Total 13.5×10^9 of *JAK2* gene copies were used for RT-PCR reaction. AS-RT-PCR detected the mutation in up to 0.05% dilution (1.3×10^6 copies of mutant *JAK2* in PCR reaction) see Fig. 20.

Figure 20: The sensitivity of AS-RT-PCR in detection of *JAK2* V617F mutation on transcript level.



5.4. Colony-Forming Assay of Hematopoietic Progenitors

As described in Introduction (chapter 1.6.1), the colony-forming assay was used for detection of proliferative and differentiation capacity *in vitro* of hematopoietic progenitors isolated from bone marrow or peripheral blood [Prchal JF et al., 1976].

Mononuclear cells were isolated from peripheral blood/ bone marrow by centrifugation on density gradient (Fig. 21). 16- 20 ml of heparinized blood/ bone marrow were mixed with washing medium (IMDM, 2% fetal-bovine serum (FBS), penicillin- streptomycin (P/S), Sigma-Aldrich) in 1:1 ratio. 4 ml of diluted sample was carefully put on top of 4 ml of histopaque (Histopaque 1077 Hybri-Max, Sigma-Aldrich) and centrifuged at 1400 rpm for 20 min. The ring of mononuclear cells on top of histopaque was collected and washed in 10 volumes of washing media and then in PBS. The cells were resuspended in washing

medium and counted. Finally the isolated cell were either frozen at -80ºC or used directly for experiment.

2.5 ml of methylcellulose medium (MethoCult H4531, StemCell Technologies) was prepared for two 35-mm Petri dishes (1.1 ml per one Petri dish) with 100 μL of mononuclear cell suspension (2.5 x 10^5 cells per dish for peripheral blood and 2.5 x 10^4 per dish for bone marrow) and with addition of erythropoietin (Epo, Epogen Epoetin Alfa, Amgen Inc., Thousand Oaks, Ca) or with other substances e.g. IM. Standard Epo concentration was 1 U/ml media. Epo concentrations of 0.03, 0.06, 0.12, 0.24, 0.5 U/ml media were used for test of hypersensitivity to Epo. Cultures were maintained in humidified atmosphere at 5% CO_2 and 21% O_2 at 37°C. The numbers and morphology of myeloid colonies were evaluated at day 7 (CFU-Es: colony-forming unit erythroid) and 14 (BFU-Es: burst-forming unit erythroid and CFU-GMs: colony-forming unit granulocytes, macrophages) (Fig. 22).

5.4.1 *BCR-ABL* Detection on the Level of Single Hematopoietic Colonies

In order to obtain total RNA from individual colonies, BFU-E and CFU-GM colonies containing approximately 500 to 2,000 cells were harvested under the control of a microscope and lysed in Trizol Reagent (Invitrogen) [Chomczynski P et al., 1987]. Reverse transcription with nested PCR was performed in order to detect the fusion region of *BCR-ABL* with Superscript™ One-step RT-PCR Platinum® Taq kit (Invitrogen) (details of PCR procedure see Chapter 3.2. Mutation Analysis). The control one-step one-round RT-PCR was performed for *ABL* gene (with primers ABL-ATP F and ABL-ATP R, see Chapter 5.2.).

5.4.2 *JAK2* V617F Detection on the Level of Single Hematopoietic Colonies

The *JAK2* V617F detection was based on real-time allelic discrimination on the level of single hematopoietic colonies and was performed in collaboration with Dr. Soňa Peková from Department of Clinical Biochemistry, Hematology and Immunology, Na Homolce Hospital, Prague on RotorGene 300 instrument (Corbett Research, Sydney, Australia). Later on, this technique was used also in our laboratory and performed by Jana Kučerová on LightCycler 480 (Roche Applied Science, Mannheim, Germany).

Figure 21: Colony-forming assay of hematopoietic progenitors. The mononuclear cells (MNCs) were isolated from blood or bone marrow by desity centrifugation on histopaque. The isolated cells were cultured in methylcellulose media on Petri dishes with growth hormones. After 14 days the number, type, and morphology of the colonies were evaluated.

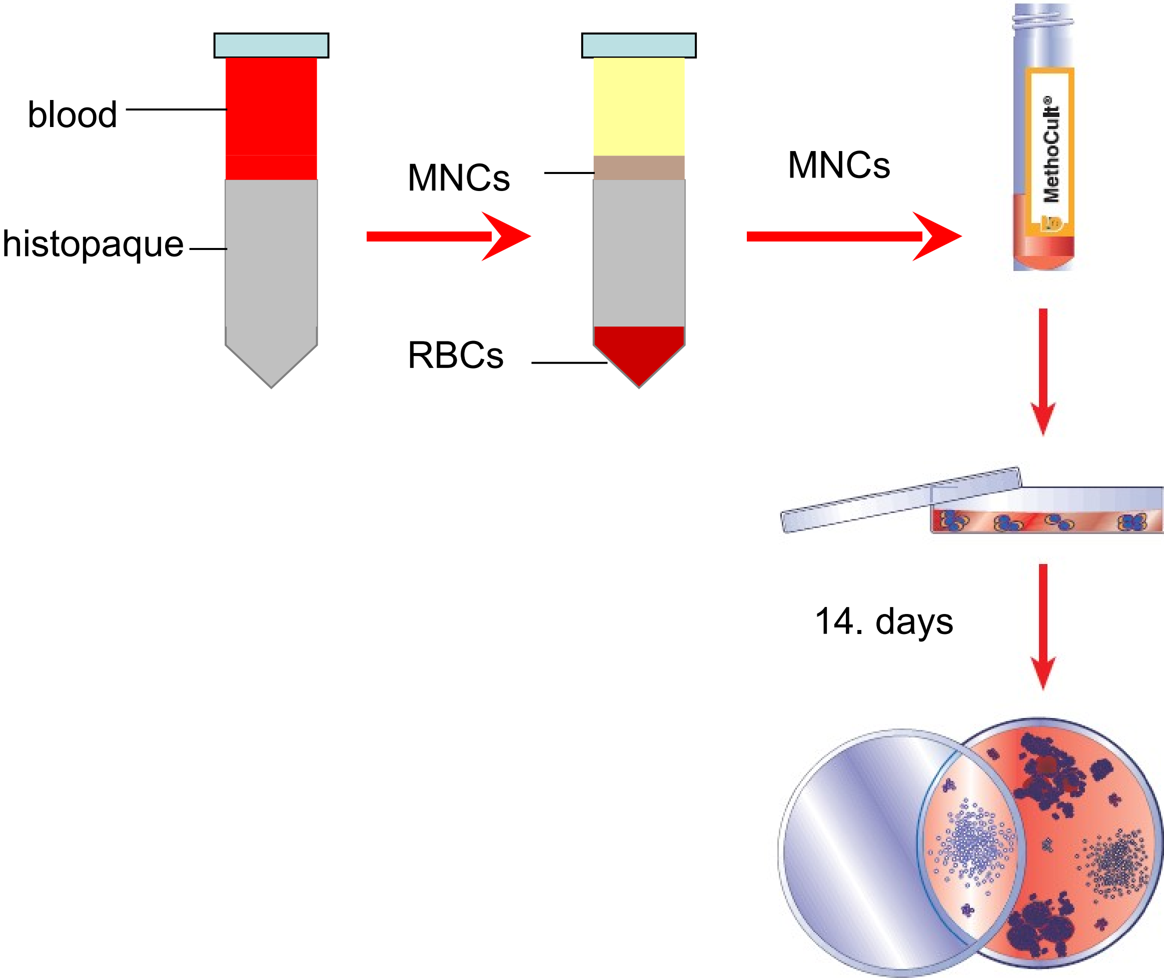
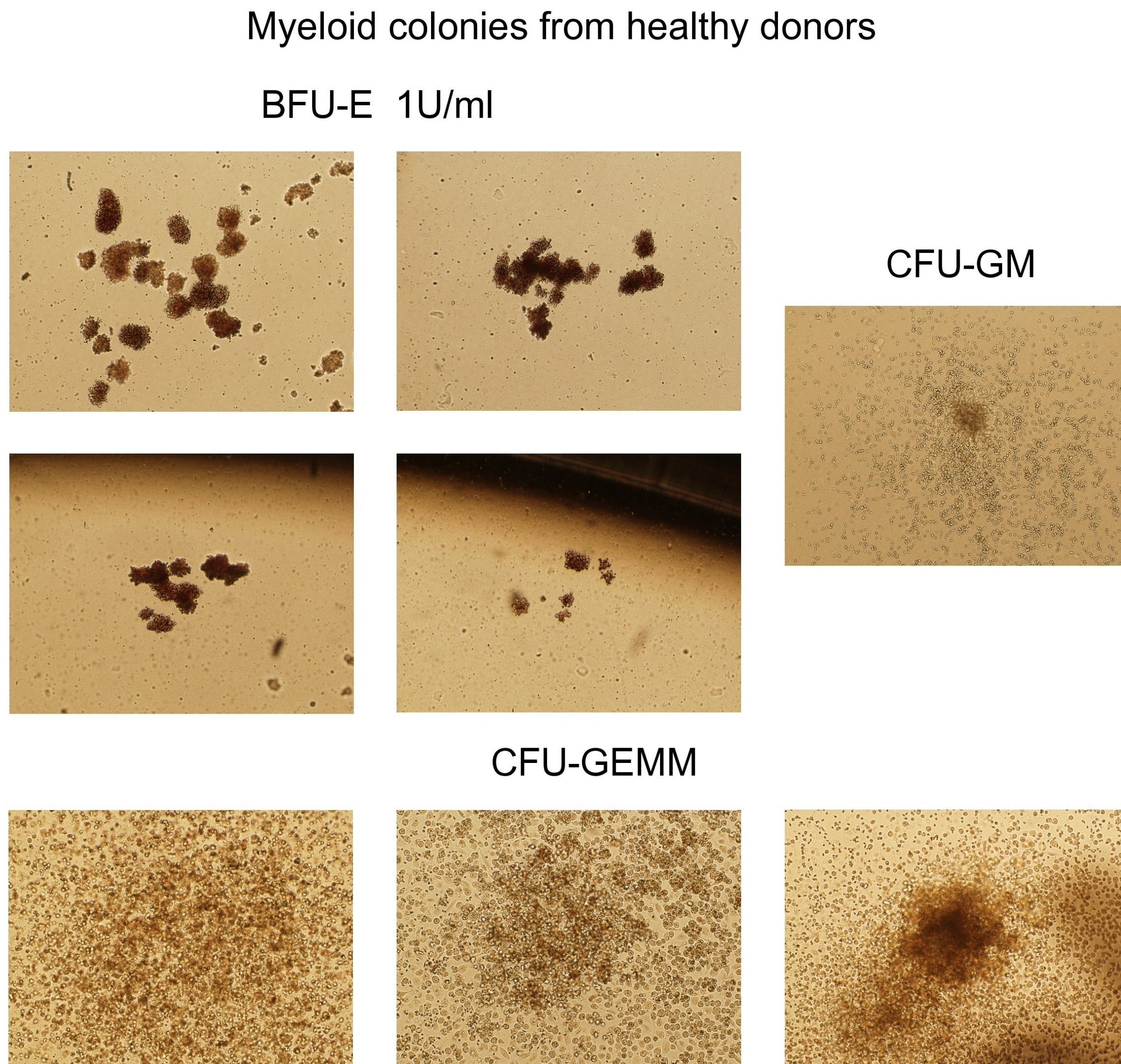


Figure 22: Myeloid hematopoietic colonies in colony forming assay from healthy donors. On day 14 of the culture BFU-E (burst-forming unit erythroid), and CFU-GM (colony-forming unit granulocyte macrophage) were evaluated. On day 21 CFU-GEMM (colony-forming unit granulocyte erythrocyte macrophage megakaryocyte) were observed. 1U Epo/ml: one unit of erythropoietin per ml of media.



The individual colonies were harvested as described in chapter 3.3.1 into 500 µl Trizol Reagent (Invitrogen). DNA was precipitated overnight by the addition of 300 µl of DNase free 96% ethanol. The DNA was centrifuged at 14 000g for 30 min, and washed twice in 10% ethanol in 1x PBS, air-dried and dissolved in 10 µl of tissue culture water (deionized, autoclaved).

The assay relied on the usage of one set of primers to PCR amplify the fragment of *JAK2* gene potentially carrying the V617F mutation, and two fluorescently labeled LNA-modified probes to discriminate between both *JAK2* genotypes see Fig. 23. The sequences of primers and probes were as follows:

JAK2 forward: 5'-GAAGCAGCAAGTATGATGAGCAA-3'

JAK2 reverse: 5'-ACTGACACCTGCTGTGATCC-3'

wild type JAK2 probe: FAM-tcCacAga**C**aCatAc-BHQ1

mutant JAK2 V617F probe: HEX-ctcCacAga**A**acAtaCtc-BHQ1.

The spiking pattern of the LNA-modified probes was designed using the web-based algorithms by Exiqon, Denmark (www.exiqon.com). LNA nucleotides within the probes are highlighted by capital letters; the position of the V617F SNP is depicted in red and underscored. Primers were obtained from MWG Biotech, Germany; probes were purchased from Exiqon, Denmark. The PCR reaction is described in details in Table XI.

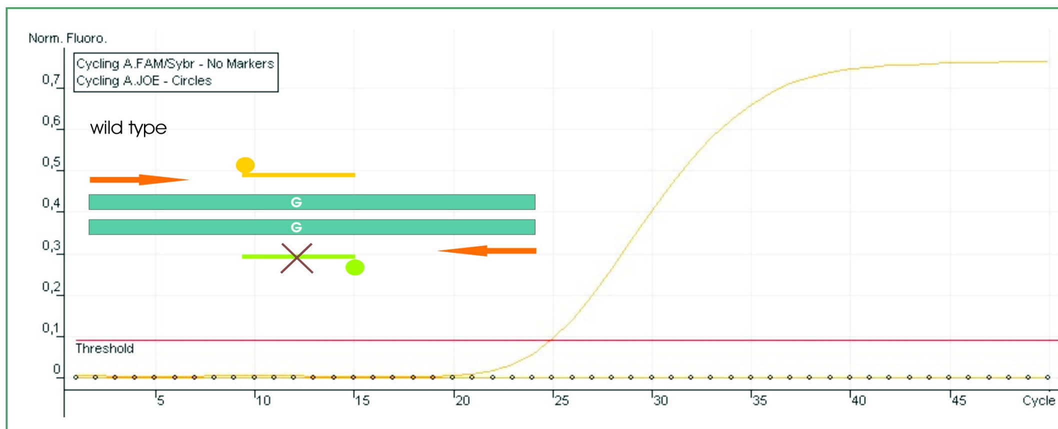
Table XI: Real-Time Allelic Discrimination of *JAK2* V617F Mutation

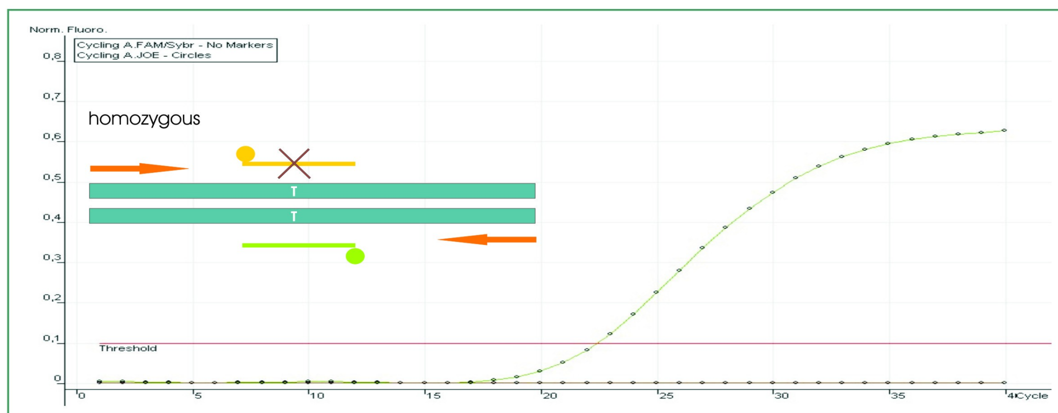
	1 reaction	**Final**
ThermoStart DNA Poly [5 U/µl]	0.2	1 U
10x PCR buffer	2	1x
25 mM $MgCl_2$	3.2	4 mM
10 mM dNTPs	0.4	0.2 mM
forward primer [10 pmol/µl]	1	10 pmol
reverse primer [10 pmol/µl]	1	10 pmol
FAM-wild type probe [10 pmol/µl]	0.4	4 pmol
JOE-mutant probe [10 pmol/µl	0.4	4 pmol
DNA	2	
TC water	9.4	
total volume	**20**	

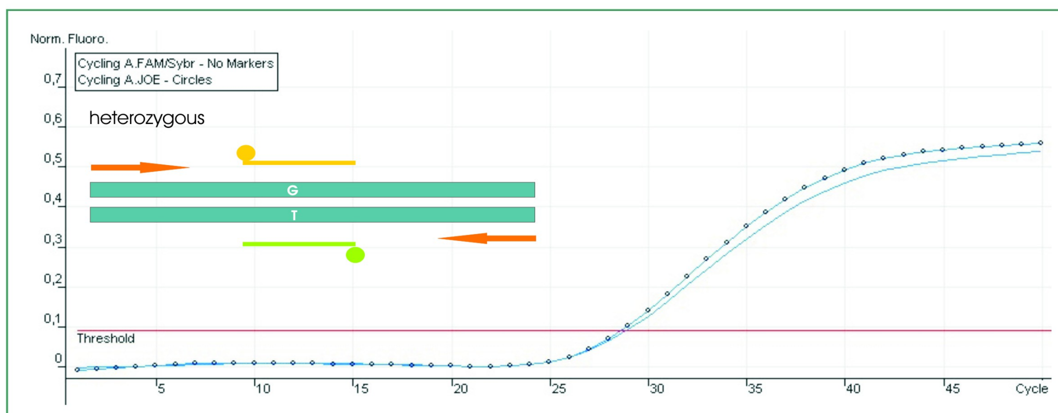
The amounts of the stock solutions are given for 1 reaction in µl.

The PCR cycle: initial denaturation at 95°C for 8 min, with subsequent 50 cycles of 95°C for 20 sec and 62°C for 1 min, with fluorescence acquisition at the annealing and polymerization step (62°C).

Figure 23: The principle of real-time allelic discrimination of *JAK2* V617F mutant versus wild type gene. A set of specific primers were used (red arrows) to amplify the corresponding part of the *JAK2* gene (green lines). Two different fluorescently-labeled probes were used. One specific to wild type gene (yellow, FAM-labeled) and the other only to mutant (light green, JOE-labeled). If the template was 100% wild type only the wild type probe gave the FAM signal (straight line). If the tamplate was 100% mutant only the mutant-specific probe gave the JOE signal (dotted line). In case of heterozygosity both signals appeared with equal strength.







5.4.3 DNA Isolation from Harvested Plates with Hematopoietic Colonies

Two ml of washing medium was added to each Petri dish with methylcellulose media on day 14 and left for 1 hour in the incubator. The plate was harvested into 46 ml of washing medium (see above) and centrifuged (2500 rpm, 20 min, 4°C). Pellet was resuspended in 1ml of PBS and centrifuged at13 000 rpm for 14 min. The pellet was dissolved overnight in water bath at 37°C in solution, which consisted of 450 µL STE solution (100 mM NaCl, 50 mM TRIS, 1 mM EDTA . 2 H_2O), 25µL 10% SDS (w/v water solution, sodium dodecyl sulphate), and 25 µL proteinase K solution.

The preparation of proteinase K solution: 40 mg proteinase K, 2 ml of 10% SDS, 40 µl of EDTA.Na_2, pH=8, TC water (prepared by reverse osmosis, autoclaved) to the total volume of 20 ml. The resulting clear cell lysate was either kept in -80°C or directly subjected to phenol-chloroform DNA extraction according to Chomczynski P et al., 1987.

4 RESULTS: CHRONIC MYELOGENOUS LEUKEMIA

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ABSTRACTS

Faber E, Jarosova M, **Nausova J,** et al. Intermittent dosage of imatinib—a feasible strategy for patients with significant hematologic toxicity during standard therapy. **Blood.** 2004; 104: Abstract #4657.1.

6.1. Analysis of IM resistance in CML patients

In collaboration with Hemato-Oncology Clinic of Faculty Hospital Olomouc, CML patients were selected for examination in our laboratory on the basis of clinical resistance to IM therapy. The patients were regularly examined in Hemato-Oncology clinic by standard procedures i.e. classical cytogenetic analysis, FISH (percentage of *BCR-ABL*-positive cells), real-time RT-PCR (log reduction of *BCR-ABL* transcript). In order to determine if the mechanisms of the patients' resistance are dependent or independent of the activity of Bcr-Abl kinase, *in vitro* incubations of patients' leukocytes with kinase inhibitors was performed. The activity of Bcr-Abl kinase can be measured by detection of its autophosphorylation. However, this protein is very difficult to detect in patients samples because it possesses large molecular weight plus it is very easily degraded. Therefore a small downstream molecule Crkl could serve as a surrogate for Bcr-Abl detection. This molecule is selectively phosphorylated by Bcr-Abl. Inhibition of Bcr-Abl activity leads to inhibition of phosphorylation of Crkl. Das is a dual inhibitor of Bcr-Abl and Src family of kinases. Therefore in addition to Crkl phosphorylation, the efficacy of Das was proved by the extent of inhibition of phosphorylation of Src kinases. If the inhibitor (IM, Das) was not able to inhibit the phosphorylation of Crkl (thus Bcr-Abl), direct sequencing of *BCR-ABL* kinase domain was performed in order to detect potential mutations responsible for the resistance.

6.1.1. Detection of Inhibition of Phosphorylation of Crkl and Src Kinases

In the year 2004 we have introduced the methodology of detection of Crkl phosphorylation on a cellular model (*BCR-ABL*-positive K562 cell line) (Fig. 21) and adjusted it in order to examine clinical patient samples. In the year 2006 Das was introduced to the Czech Republic. We have added, therefore, the examination of downstream target: the Src family of kinases (SFK). From 2004 to 2008, 116 different tests were performed (102 detections of phosphorylated Crkl: P-Crkl, 14 detections of phosphorylated SFK i.e. P-SFK) from clinical samples of overall 63 patients. Some patients were re-evaluated in the course of the disease.

The Crkl molecule is of a low molecular weight, therefore if we use antibody against total Crkl protein, we can observe the phophosrylated form of Crkl as a shift on a gel, forming a double band representing the non-phosphorylated and phosphorylated forms. The upper band corresponds to the phophosrylated form of Crkl, which is 1kDa larger than the non-

phosphorylated form. In the case of SFK various different kinases can be expressed in the blood cells, therefore we used a phospho-specific antibody, which crossreacts with all the family members.

In the cellular model (K562 cell line) the P-SFK was detected in the mock treated cells (Fig. 24). After incubation with either IM or Das the P-SFK was not detected, suggesting that phosphorylation of SFK was eliminated by the inhibitors (Fig. 24). This proves the sensitivity of the Bcr-Abl kinase to both inhibitors. In addition, IM partialy inhibited some of the phosphorylated members of Src kinase, while Das completely inhibited the phosphorylation of all family members.

In samples from normal healthy subject, neither phosphorylation of Crkl nor of SFK was detected. In contrast, phosphorylation of Crkl and SFK was detected in newly diagnosed CML patients. The case given in Fig. 24 is an example of a patient, who was sensitive to IM *in vitro*, because we observed the inhibition of phosphorylation of both Crkl and SFK.

In Fig. 24 is an example of a CML patient in myeloid blast crisis. The Bcr-Abl activity was partially inhibited by IM *in vitro*, because the ratio of P-Crkl to Crkl was moved in the favor of Crkl. In contrast, the phosphorylation of SFK was not inhibited at all. This is a case of activation of Src kinases independently of Bcr-Abl kinase.

Fig. 25 shows two typical examples of CML patients with IM-resistance dependent on Bcr-Abl kinase. After incubation with IM *in vitro*, in both cases the ratio of P-Crkl to Crkl did not change. The sequencing analysis revealed in both cases mutation in Abl kinase domain (T315I, Y253H see 2.3.2). Both these mutations are associated with severe IM-resistance and bad prognosis. In these cases the activation of Src kinases seems to be downstream of Bcr-Abl kinase.

Figure 24: The detection of phosphorylation of Crkl and Src family of kinases (SFK) by western blot analysis. See the text for detailed explanation. P-Crkl: phosphorylated form of Crkl (downstream molecule of Bcr-Abl kinase); P-SFK: phosphorylated form of SFK. IM: imatinib i.e. lysate of cells incubated with 10µM IM *in vitro*. 0: cells incubated without IM. PC: positive control (CML in blast crisis).

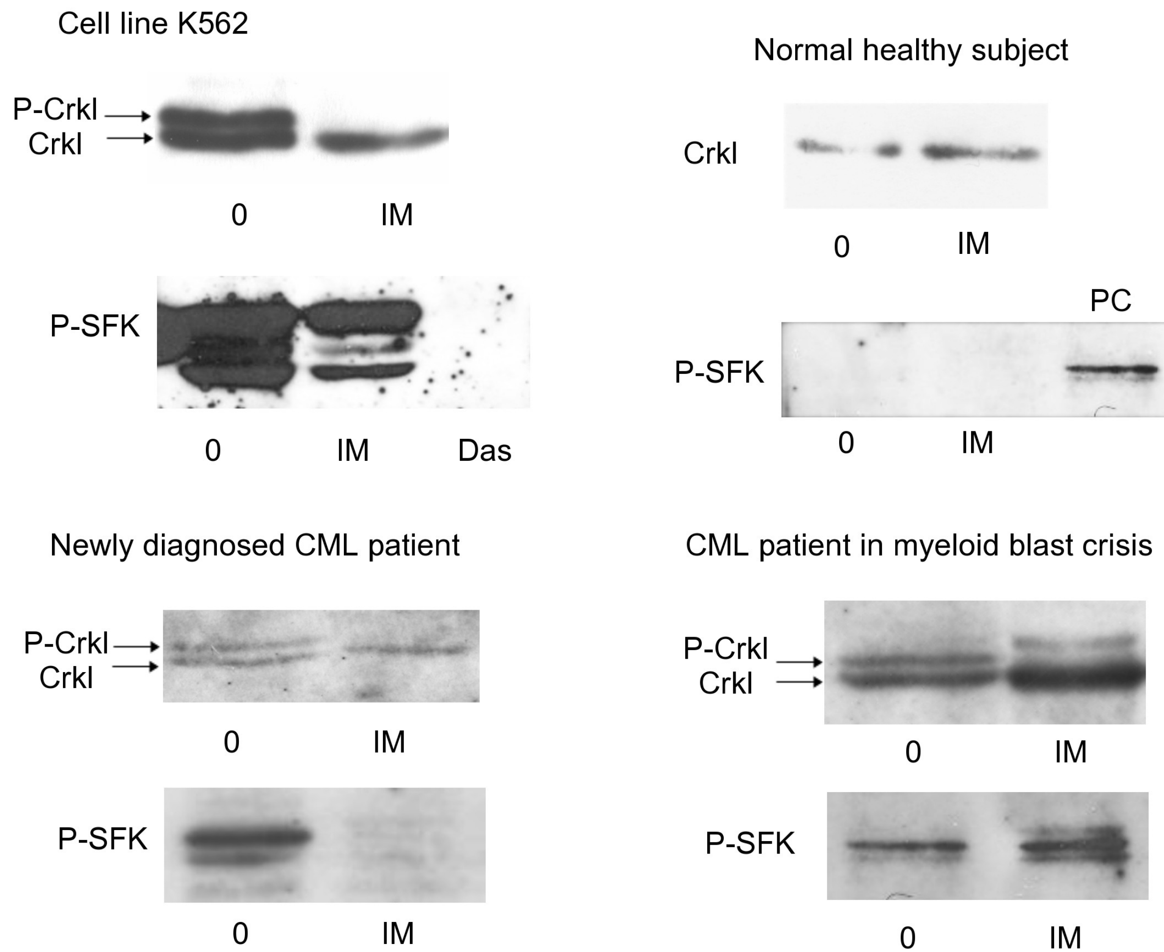
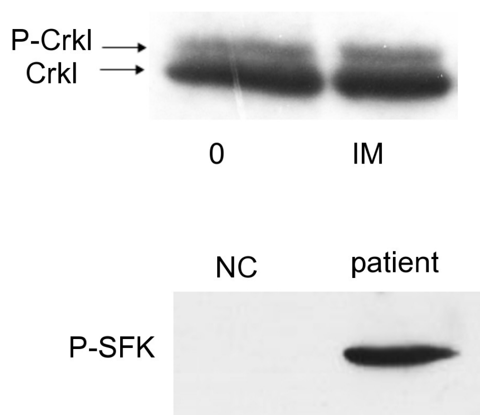
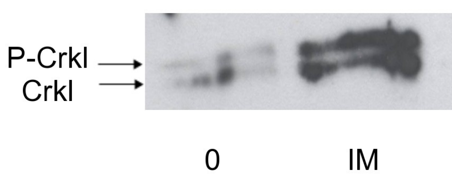


Figure 25: Detection of phosphorylation of Crkl and SFK in two typical examples of CML patients with Bcr-Abl-dependent resistance. The direct sequencing of Abl kinase domain was performed. In patient No. 3 a point mutation (nucleotide exchange C1308T leading to amino acid substitution of threonine to isoleucine in the 315th position: T315I) was detected in 100% of cell population. This mutation abolishes the restriction site for *Dde*I. PC: positive control; NC: negative control. In the case of patient No. 2 a point mutation (nucleotide exchage T1121C) leading to substitution of tyrosine (Y) to histidine (H) at the 253rd position of the kinase.





6.1.2. Mutation Analysis of ATP Binding Pocket of Bcr-Abl Kinase

From the year 2003 to the year 2006 151 sequencing experiments were performed in our laboratory in order to detect mutations of Bcr-Abl kinase domain from clinical samples of 35 CML patients. Nine mutations were detected in 8 CML patients (two simultaneous mutations in one case, see Table XII). Whenever possible, the presence of mutation was re-evaluated by enzyme restriction analysis.

Table XII: Detection of mutations of Bcr-Abl kinase in clinically resistant CML patients

Patient No.	date	Nucleotide change	Amino-acid change	restriction analysis
1	7.10.2003	A1191G, C1315G	D276G, F317L	BanI, MseI
2	8.11.2004	T1121C	Y253H	-
3	2.11.2005	C1308T	T315I	DdeI
4	13.3.2006	T1428G	E355G	-
5	31.3.2006	A1233G	M290V	-
6	9.11.2006	C1308T	T315I	DdeI
7	13.4.2006	C1290T	P309L	-
8	24.1.2007	C1315G	F317L	MseI

6.2. Analysis of Persistence of *BCR-ABL*-Positive Cells in CML Patients with Good Response to IM Therapy

So far in this section I have dealt with overt resistance, where the resistant usually *BCR-ABL*-positive clone comprises most of the hematopoietic population and overgrows the wild-type hematopoiesis. In this section results will be presented from our study of molecular background of persistence of minor *BCR-ABL*-positive clone on IM therapy. According to experimental models IM not only stops proliferation of *BCR-ABL*-positive cells but also induces apoptosis [Gambacorti-Passerini C et al., 1997; Fang G et al., 2000]. Theoretically, *BCR-ABL*-positive population of hematopoietic cells should be therefore eradicated on IM treatment *in vivo*. In reality, there are always some CML patients, who even though respond well to IM therapy, have continuously a minor population of *BCR-ABL*-positive cells

in the blood stream. These cells respond to the antiproliferative effect of IM but escape the IM-induced apoptosis. We decided to look at the possible mechanisms underlying this ability to escape IM-induced apoptosis. These molecular changes in the *BCR-ABL*-positive clone may prevent the complete eradication of the *BCR-ABL*-positive population and in addition, may be involved in the disease progression.

CML patients were selected for this experiment according to the following criteria:

- Ph-positive CML patient with no additional chromosomal aberration,
- who has been treated with standard IM dose (400 mg/day) for at least 6 months;
- who had persistant >1-30% of *BCR-ABL*-positive population in peripheral blood (detected by FISH or RT-PCR) on IM treatment.

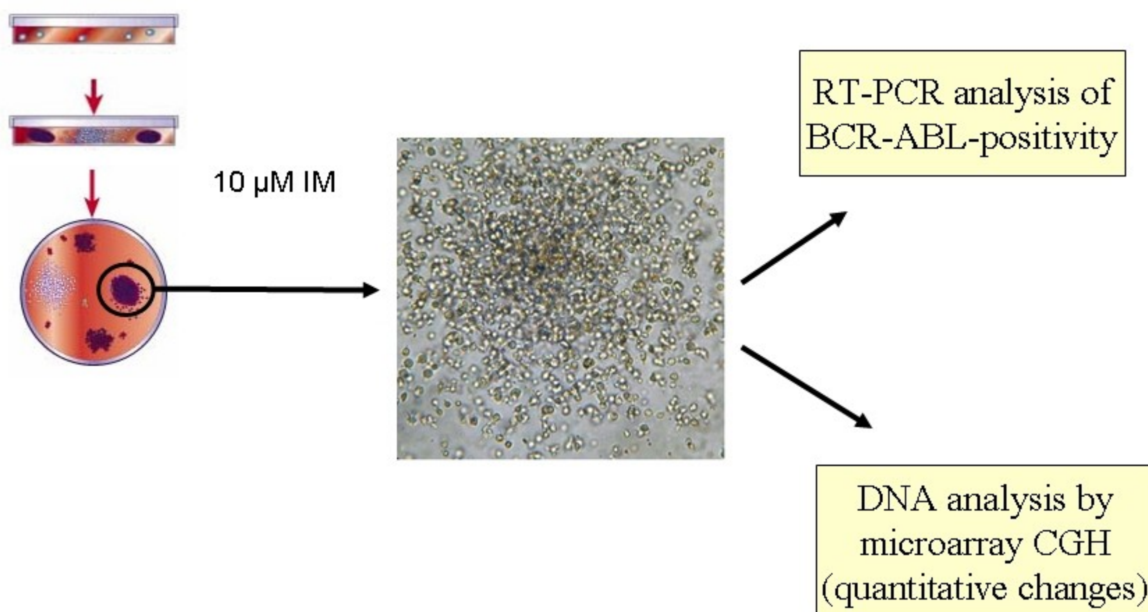
In order to exclude patients with IM-resistance dependent on Bcr-Abl, all patients were sequenced for possible mutations in *BCR-ABL* kinase domain. Hematopoietic progenitor cells were isolated (in the form of mononuclear cells) from patients without any mutations, and plated in the colony-forming assay of hematopoietic progenitors in media with and without 10 μM IM see Fig. 26. On day 14 individual myeloid colonies were picked, and in addition whole plates were harvested. We were interested in *BCR-ABL*-positive population; we evaluated therefore individual colonies for the presence of *BCR-ABL* transcript (see Fig. 27). If the number of *BCR-ABL*-positive colonies was more than 50%, the DNA from harvested colonies was subjected to CGH microarray analysis. (The cut-off level for the CGH microarrays system used was 50%.) The CGH microarray analysis is a powerful tool for detection of DNA microamplifications and/or deletions. The CGH microarray analysis was performed at the laboratory of Hemato-Oncology Clinic (Dr. Jarosova, Faculty Hospital Olomouc) and detected 300 or 1700 fragments of genes implicated in cancer, including possible tumor suppressor genes and oncogenes [CGH Microarrays: Vysis, Downers Grove, IL; Affymetrix, Santa Clara, CA with GenoSensor reader and analytic software]. The control DNA harvested from plate without IM was compared to the DNA harvested from colonies, which grew in media with IM.

Total 15 cultivations were performed with cells from 10 selected CML patients. Later on one of them developed mutation (T315I) and therefore was excluded from the cohort. 10 μM IM used in cultures is a rather toxic concentration even for wild-type colonies, therefore the colony growth was usually very poor. In most cases the cultivations had to be repeated with freshly isolated cells (which were not previously frozen). In addition, it was difficult to obtain

enough DNA from the colonies for the experiment. Enough material was obtained from 5 patients and this was subjected to array CGH. Three arrays did not show any significant difference between DNA from plates without IM and with IM. Two arrays gave interesting data.

When the DNA from IM culture from patient No. 1 was compared to DNA from culture without IM, subtelomeric deletions 2qtel and 7qtel, in addition to amplifications of *MSH2* and *KCNK12* genes were detected. Deletions and rearrangements of small chromosomal regions in the proximity of telomeres (subtelomeric DNA) are associated with various hematological malignancies and other cancers. The protein MSH2 is involved in DNA mismatch repair in postreplication phase. Overexpression or deficiency of this protein is associated with drug resistance in many cancers, differing in the biology of the cancer and the type of therapy. The role of these proteins in the IM-resistance is still to be evaluated.

Figure 26: The design of the study of persistence of *BCR-ABL*-positive clone on IM therapy. Hematopoietic cells isolated from CML patient with good response to IM therapy and with up to 30% *BCR-ABL*-positive clone in peripheral blood were cultured in the colony-forming assay with and without IM. On day 14 individual colonies were analyzed for presence of *BCR-ABL* fusion gene and simultaneously the plates were harvested for CGH analysis of microamplifications and microdeletions in genes implicated in cancer.

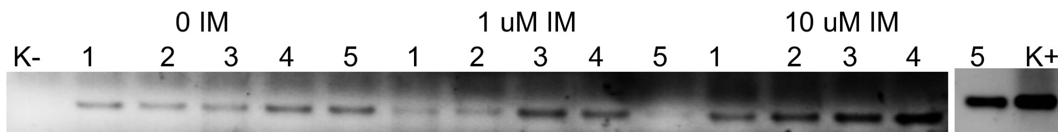


The most significant result was obtained from CGH microarray analysis of cultures derived from patient No. 2. When the results from CGH on DNA from cultures with 10 μM IM were

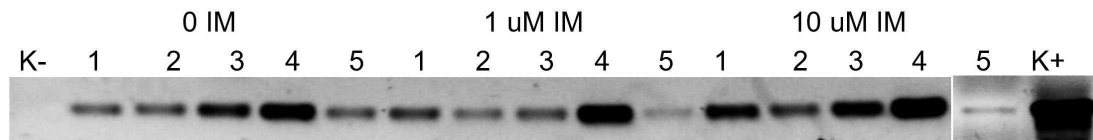
compared to results from CGH on DNA from cultures without IM, a deletion of one locus on chromosome 9 (9p12 to 9p21.1) was detected (see Fig. 28). This region contains several tumor suppressor genes. This region is now being more precisely evaluated by BAC clones – RP11-182N22 and RP11-112J2 localized in region 9p13.3. We hypothesize that this locus contains a candidate gene, loss of which is responsible for survival of *BCR-ABL*-positive cells in IM media (i.e. their escape from IM-induced apoptosis).

Figure 27: Analysis of myeloid hematopoietic colonies derived from patient No.1. **A)** The detection of *BCR-ABL* transcript on 5 colonies for each IM concentration. K-: negative control (only water in PCR reaction no template RNA), K+: positive control (total RNA from peripheral blood). **B)** Control RT-PCR on single colonies i.e. detection of ABL transcript on the same colonies as in A). In this case all colonies which grew in all concentrations of IM were *BCR-ABL*-positive. **C)** 1µL of DNA out of 20 µL isolated from harvested plates of hematopoietic colonies derived from patient No. 1. This DNA was used for CGH microarray analysis. M: weight marker.

A) RT-PCR detection of BCR-ABL on the level of single colonies



B) Control RT-PCR detection of ABL on the level of single colonies



C) DNA isolated from harvested plates

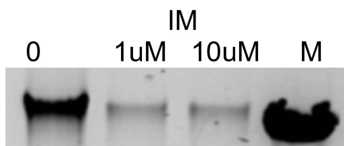
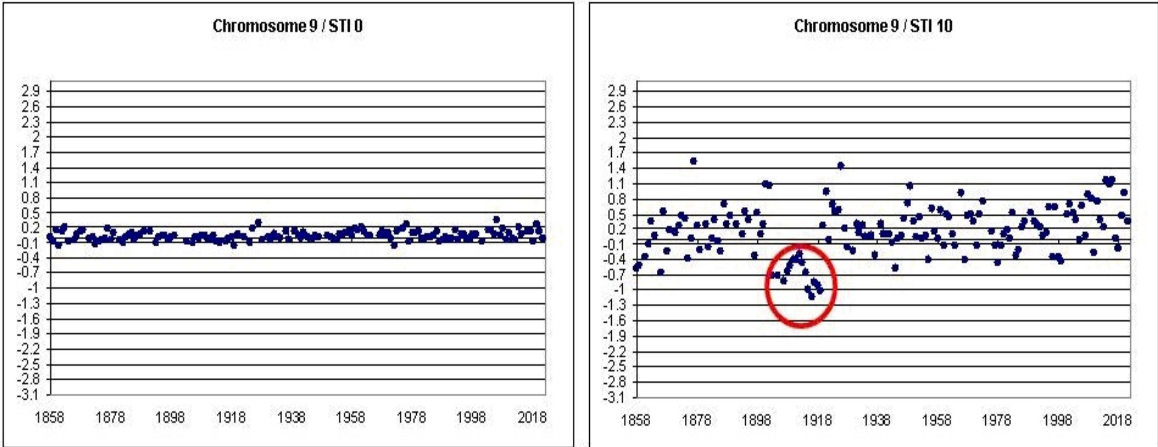


Figure 28: CGH microarray analysis of DNA isolated from harvested plates with myeloid colonies derived from patient No. 2. The red circle marks the deleted region 9p12 to 9p21.1 in material isolated from colonies grown in 10 µM IM. STI 0: material from colonies in media without IM (STI571), STI 10: material from colonies in media with 10 µM IM. Positive values signify amplifications and negative values deletions. Numbers on horizontal axis denominate precise locations on chromosome 9.



5 RESULTS: *JAK2* MUTATION

7.5.1 Pediatric Patients with Myeloproliferative Disorders

Veselovska J, Pospisilova D, Pekova S, Horvathova M, Solna R, Cmejlova J, Cmejla R, Belickova M, Mihal V, Stary J, Divoky V. Most pediatric patients with essential thrombocythemia show hypersensitivity to erythropoietin in vitro, with rare JAK2 V617F-positive erythroid colonies. **Leukemia Research.** 2008; 32: 369-77. **IF= 2.483**.

Pospíšilová D., **Veselovská J**., Horváthová M., Solná R., Kučerová J., Čmejlová J., Čmejla R., Běličková M., Peková S., Petrtýlová K, Hadačová I., Mihál V., Votava T., Hak J., Zapletal O., Starý J., Divoký V. Esenciální trombocytémie v dětském věku. [Essential thrombocythemia in childhood] **Transfuze a hematologie dnes** 2008; 14: 63-70.

Pospisilova D, **Veselovska J**, Pekova S, Horvathova M, Solna R, Cmejlova J, Cmejla R, Belickova M, Stary J, and Divoky V. *JAK2* Genotyping (Real-Time Allelic Discrimination) Reveals Rare *JAK2* V617F-Positive Erythroid Colonies in Pediatric Patients with Essential Thrombocythemia. **Blood.** 2007; 110: 748A **(**ASH Annual Meeting Abstracts: poster).

Pospisilova D, Kucerova J, Horvathova M, **Veselovska J**, Sedlacek P, Stary J, Divoky V. Primary polycythaemias in the Czech paediatric population. **Pediatric Blood & Cancer.** 2007; 49: 381 (European Society for Paediatric haematology and immunology (ESPHI) biannual meeting, advances in paediatric haematology and immunology: from bench to bedside, Athens, Greece, September 14-16, 2007).

Pospíšilová D., Horváthová M., **Naušová J.,** Čmejlová J., Čmejla R., Běličková M., Mihál V., Ptertýlová K., Hak J., Zapletal O., Černá Z., Starý J. Divoký V. Klinické a molekulárně-genetické aspekty esenciálních trombocytémií u dětí. Zborník abstract: s.69. XVI. konferencia detských hematologov a onkologov SR a ČR, Podbánske, Slovak Republic, November 3-5, 2006.

Pospisilova D, Horvathova M, **Nausova J**, Divoky V, Cmejlova J, Cmejla R, Belickova J, Petrtylova K, Mihal V, Votava T, Hak J, Ptoszkova H, Blatny J, Stary J. Essential Thrombocythemia in Children in the Czech Republic. Second ESH Euroconference on Myeloproliferative Disorders: Molecular Pathogenesis and Therapy, Madeira, Portugal, September 14-16, 2006, Poster 22.

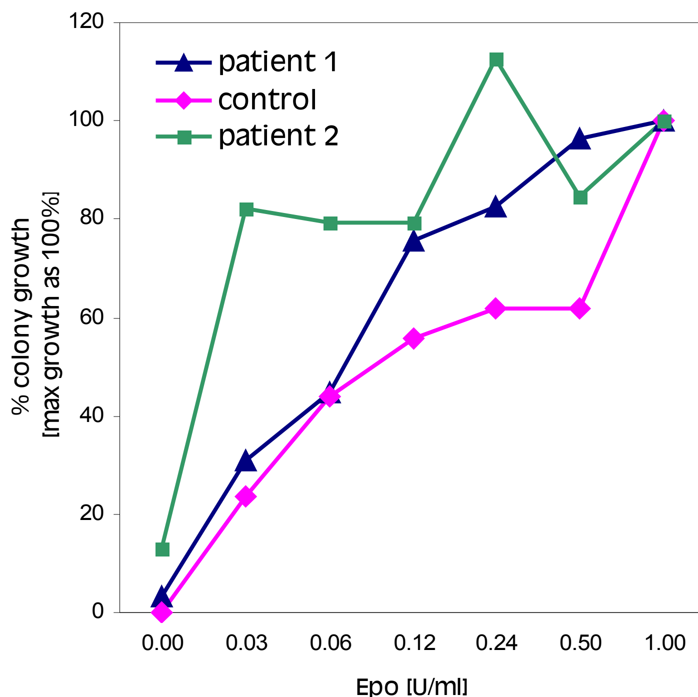
One original article in *Leukemia Research*, one article in a Czech medical journal and four different abstracts presented at international conferences are discussed in this section dealing with childhood myeloproliferative disorders.

I have participated in cellular and molecular characterization of pediatric patients with suspected myeloproliferative disorders (polycythemias, thrombocythemias). More precisely, the functional analysis of hematopoietic progenitors by colony-forming assay (Fig.

29), detection of growth of endogenous erythroid colonies, and detection of *JAK2* V617F mutation were performed in our laboratory.

Thanks to D. Pospisilova, MD, Ph.D, from Pediatric Clinic, Faculty Hospital Olomouc, pediatric patients diagnosed with very rare childhood essential thrombocythemia were collected into one cohort from all hematologic centers in the Czech Republic. The two articles and three abstracts presented at international conferences represent the results of the cellular and molecular characterization of these patients. Our main finding, that these patients possess very rare clones with *JAK2* V617F mutation, brings up new questions to the recently hot debate of the role of this mutation in the pathogenesis of myeloproliferative disorders.

Figure 29: Result of colony assay of hematopoietic progenitors: hypersensitivity to Epo in childhood polycythemia. The number of colonies in different concentration of Epo were expressed in percentage, taking the number of colonies grown in 1 Epo U/ml media as 100%. Both patients are *JAK2* V617F negative. The growth curve of colonies of patient No. 1 mimics the growth curve of normal healthy control. The progenitors of this patient are not hypersensitive to Epo. In contrast, the maximum percentage of colony growth in patient No. 2 in the low concentrations of Epo are much higher than in control. In addition, in this patient the growth of endogenous erythroid colonies was observed. Progenitors from this patient are hypersensitive to Epo.



7.1.1 Pediatric Patients with Essential Thrombocythemia

Our cohort consists of 15 patients diagnosed with essential thrombocythemia (ET) at the age of 6 – 17 (median 11) and one child with thrombocytosis (too young for the diagnosis of ET) and a family history of ET.

7.1.1.1 Clinical and Biochemical Findings

The clinical signs at diagnosis in our cohort of children were milder than are usual in ET adults with only headache, chest pain, joint or rectal bleeding, or syncope in some of them. Splenomegaly was confirmed in less than half of the patients. The diagnosis in majority of patients was obtained usually by chance in the course of regular complete blood count analysis for other purposes such as pre-surgical examination. The platelet number at diagnosis was 681 – 2428 x 10^9/L (median 1468.5 x 10^9/L). The bone marrow morphology of all ET patients showed dominant megakaryocytic proliferation with an increased number of mature polyploid megakaryocytes. No pathology of erythropoiesis and granulopoiesis was found. There were no complications such as major bleeding or thrombosis either at the time of diagnosis or during the follow-up. All patients had normal karyotype. Erythropoietin (EPO) levels were normal in 13 children and slightly decreased in 2 patients. Thrombopoietin (TPO) levels were normal, only in 2 children slightly increased. In collaboration with Institute of Hematology and Blood Transfusion in Prague (Dr. Cmejla, Ph.D and Dr. Cmejlova, Ph.D.) the genes for TPO and its receptor i.e. MPL were sequenced. *MPL* and *TPO* gene mutation are associated with phenotypes of thrombocytosis, extramedullary disease, myelofibrosis, and osteosclerosis. No such mutations were found in this cohort of patients.

7.1.1.2 Hypersensitivity and Independence of Erythroid Progenitors to Epo *in vitro*

The hypersensitivity of hematopoietic colonies to growth factors is a typical sign of myeloproliferative disease. The more malignant disease is characterized by growth of colonies even in media without the addition of the growth factor. Our cohort of ET patients was assessed by the growth of erythroid colonies with and without addition of different concentrations of Epo. The most important Epo concentrations for the assessment of hypersensitivity to Epo are 0.03, 0.06, and 0.12 U/ml of media (Fig. 30, Fig. 31, Fig. 32). In the colony assay of hematopoietic progenitors all samples except two patients (repeatedly with no or poor growth) gave analyzable data see Table XIII. Hematopoietic progenitors from only two children did not show hypersensitivity to Epo. All patients but one

with hypersensitive progenitors to Epo also formed endogenous erythroid colonies (EECs) without the addition of Epo.

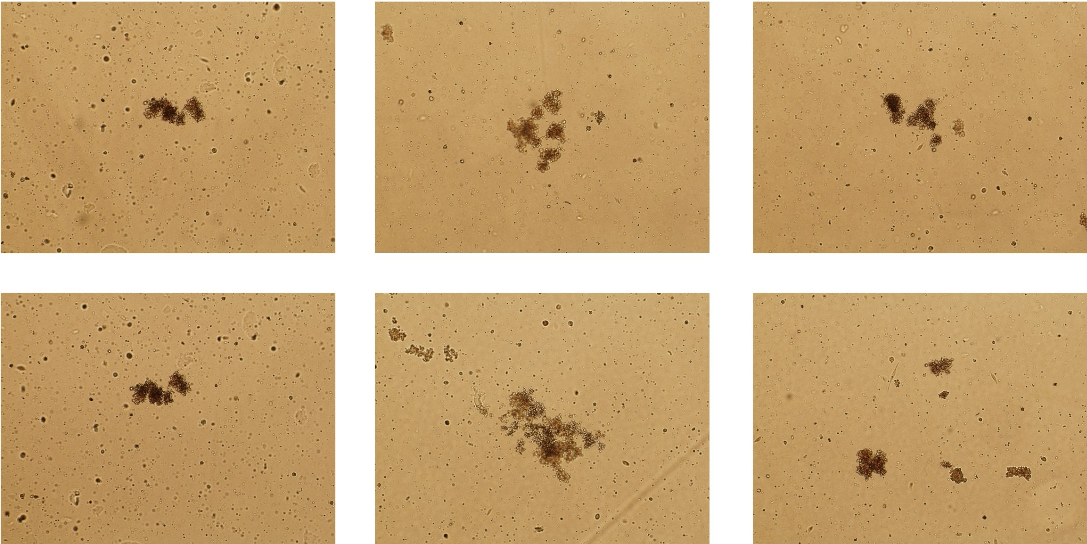
7.1.1.3 Clonality

In collaboration with Institute of Hematology and Blood Transfusion in Prague (Dr. Belickova) DNA samples from our cohort were tested for clonality based on X chromosome inactivation by HUMARA assay. The assay uses human androgen receptor *(HUMARA)* locus, which is differentialy methylated on the active and the inactive X chromosomes (Cermak et al., 2005). All female patients tested were polyclonal except patient No. 2, who was tested already at an adult age.

Figure 30: Erythroid colonies derived from patient No. 13. In the colony assay of hematopoietic progenitors, there was evaluated not only the number but also the morphology of colonies. The pictures of erythroid hematopoietic colonies taken on day 14 of the culture with magnification 40 x by Olympus IX71.

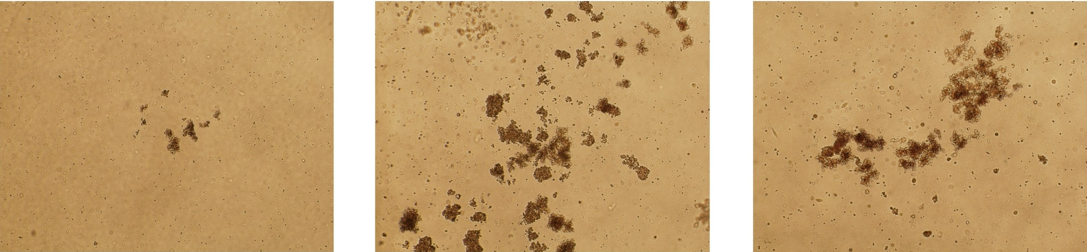
Patient No. 13: Erythroid colonies

0 U Epo/ml (EECs)



0.03 U Epo/ml (BFU-Es)

healthy donor patient No. 13



0.06 U Epo/ml

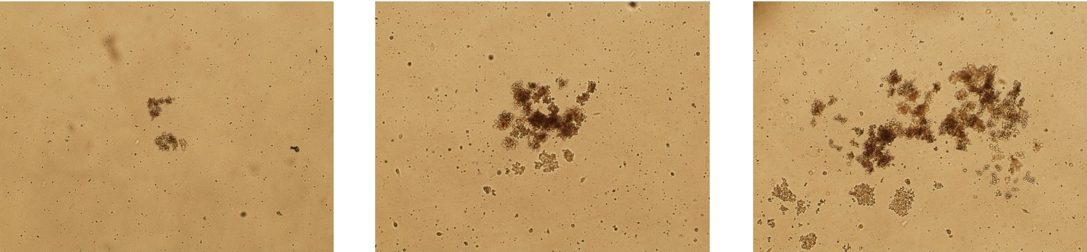


Figure 31: Hypersensitivity to Epo. Erythroid colonies derived from patient No.13 and normal healthy control. Photographs of erythroid hematopoietic colonies taken on day 14 of the culture with magnification 40 x by Olympus IX71.

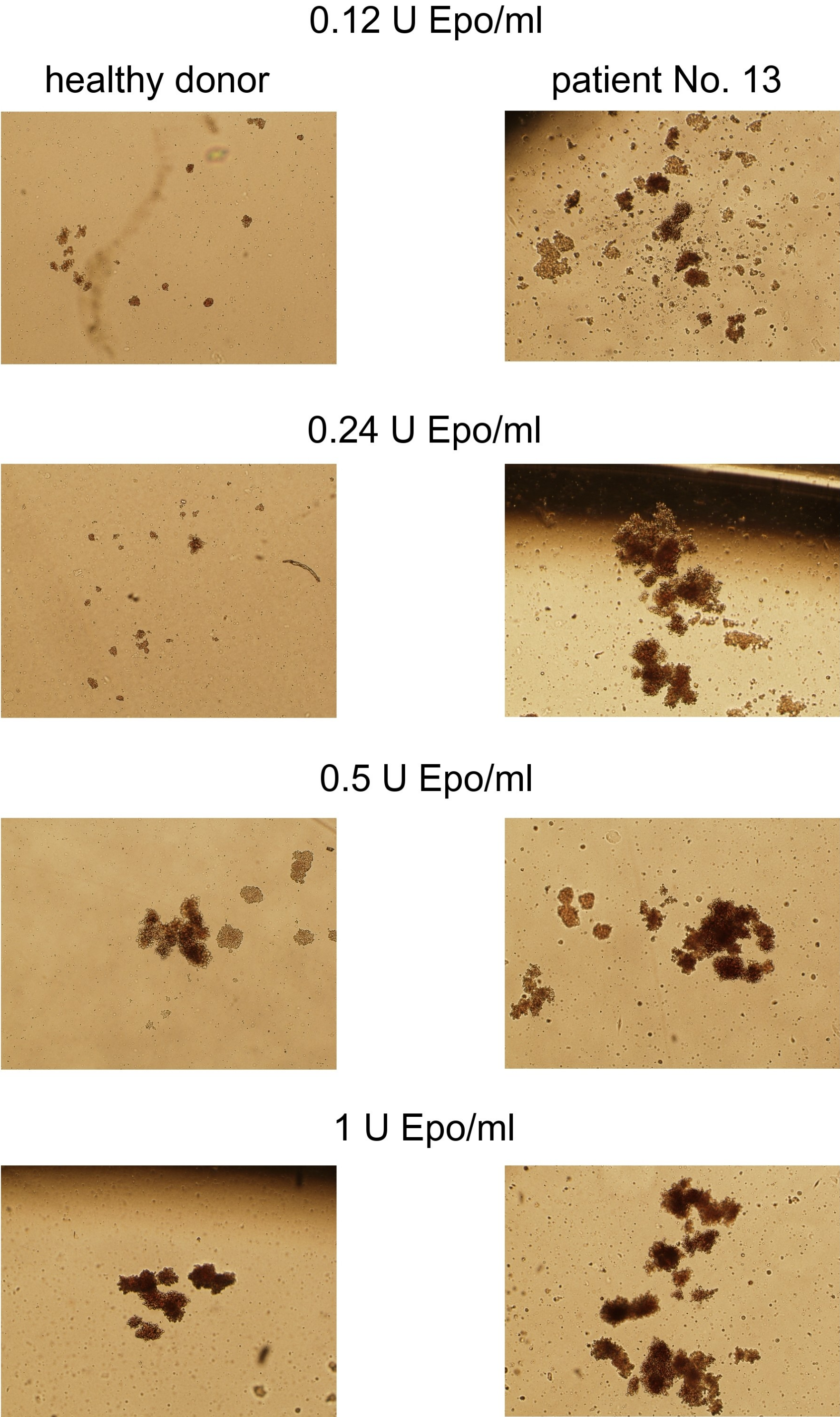
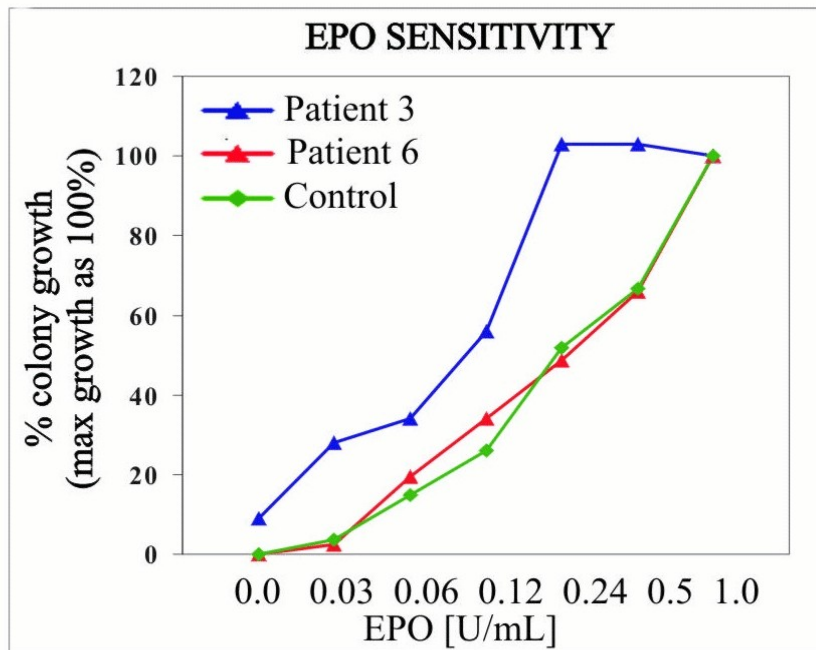


Figure 32: The assay of hypersensitivity of hematopoietic progenitors to erythropoietin (EPO). The green line represents the number of colonies obtained from a healthy donor. The percentage of growth was stated as maximum percentage of the number of colonies grown in 1U Epo/ml media (100%). Patient No. 3 (blue line) shows clear hypersensitivity of hematopoietic progenitors to Epo with formation of endogenous erythroid colonies (EECs) i.e. colonies grown in media without addition of Epo. Patient No. 6 colony growth curve (red line) resembles that of a normal control.



7.1.1.4 Detection of the *JAK2* V617F Mutation

All children from the cohort were tested for the presence of *JAK2* V617F mutation due to its important role in pathophysiology of myeloproliferative disorders. Only child number 13 was positive for this mutation (see Table XIV) on the level of genomic DNA from full blood by AS-PCR. In our hands the AS-PCR detected a homozygous mutation in up to 0.8% dilution (equivalent to one heterozygous cell in 64 normal cells) see Experimental Procedures. In addition, all samples were examined by restriction analysis (see Fig. 33). Further, more sensitive method AS-RT-PCR was used on the transcript level of isolated platelets (see Fig. 34). In our hands AS-RT-PCR detected the mutation in up to 0.05% dilution (1.3 $x10^6$ copies of mutant *JAK2* in PCR reaction) see

Experimental Procedures. Again only patient No. 13 was positive for the mutation in this assay (see Table XIV).

Table XIII: Patients characteristics.

Patient No.	Sex	Age at diagnosis (years)	Platelets at diagnosis (x 10^9/l)	Hb levels (g/l)	Clinical signs at diagnosis	Splenomegaly	Follow up (months)	Treatment overview
1	F	6	1456	128	no	no	6	Anagrelide
2[a]	F	10	1481	135	headache	no	192	ASA, IFN[b]
3	F	11.5	1720	140	syncope	yes	12	Anagrelide
4	M	6.5	2428	136	rectal bleeding	yes	24	ASA
5	M	15.5	1307	156	no	no	18	No
6[a]	F	15	2320	126	headache	yes	92	Anagrelide
7	M	8.5	1230	127	headache	yes	48	Anagrelide
8	M	8	1616	151	no	yes	96	Anagrelide
9[a]	F	10.5	2285	141	no	yes	144	HU[c], IFN[c]
10	F	15	1374	125	no	no	19	Anagrelide, ASA
11	M	15	1690	145	no	yes	36	ASA
12	F	6	1720	129	no	no	72	ASA
13	F	17	906	165	chest pain	no	6	No
14	M	14	1022	136	joint bleeding	no	5	No
15	F	14	681	151	no	no	12	No

Abbreviations: ASA: acetylosalicylic acid; IFN: interferon-α; HU: hydroxyurea. [a]Patients currently at adult age; [b]during pregnancy only; [c]only temporarily in the past.

Figure 33: *JAK2* restriction analysis of the mutation status by *Bsa*XI restriction enzyme.

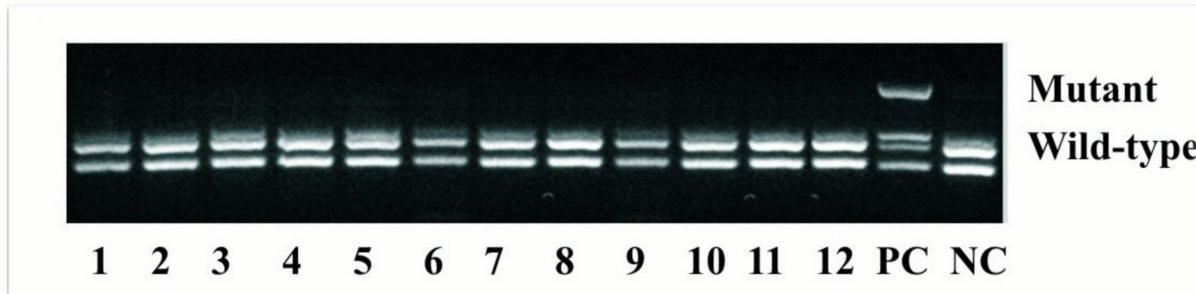
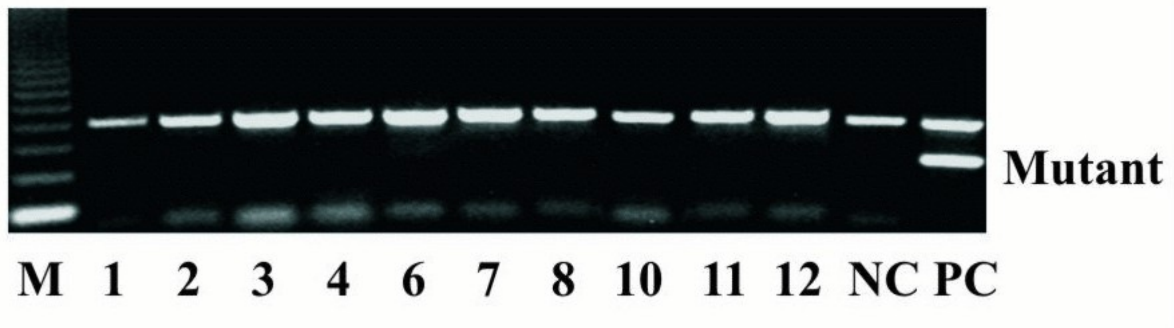


Figure 34: AS-RT-PCR on the isolated platelets from ET children No. 1 to 12.



Taking under consideration the fact, that *JAK2* V617F mutation provides myeloid cells with hypersensitivity to growth factors [Lu X et al., 2005], we assumed that if we analyzed the hypersensitive population of myeloid cells, the probability of detecting this mutation would be greatly enhanced. In addition, in the case of patient No. 13, who was positive for this mutation from peripheral blood, we were interested in the composition of the single clones as far as homozygosity and heterozygosity goes.

Six patients with childhood ET diagnosis were analyzed on the level of single myeloid colonies for the presence of *JAK2* V617F mutation by real-time allelic discrimination (see Fig. 35). Over 300 myeloid colonies were picked and analyzed.

In the case of child No. 13, who was *JAK2* V617F-positive by both AS and AS-RT-PCR, 17 EECs gave analyzable data. Most of the colonies (13) were heterozygous for the mutation, 2 were homozygous and 2 possessed only the wild type allele (see Fig. 36).

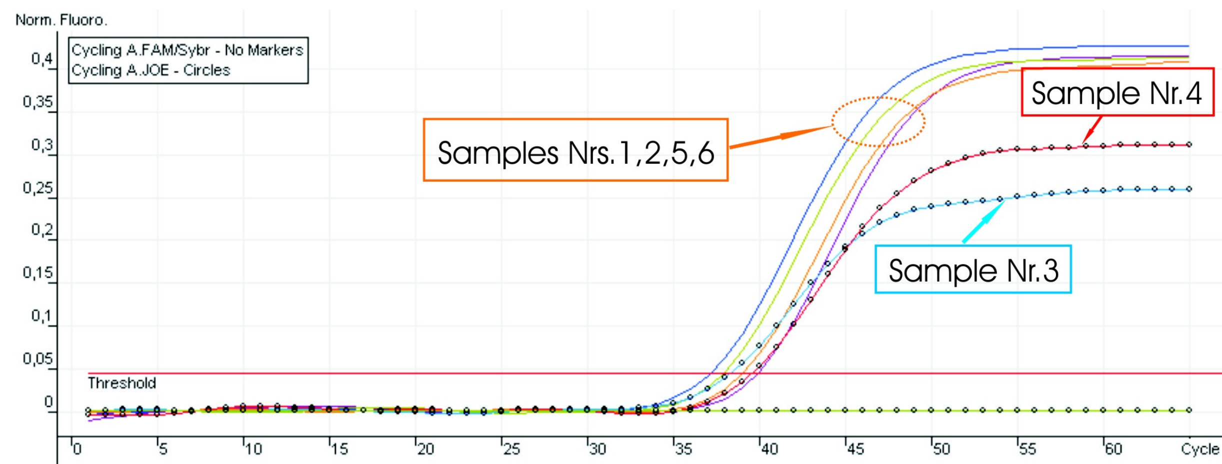
Five children, who were *JAK2* V617F-negative on the level of both DNA and RNA analysis from full blood, were also examined on the level of single myeloid colonies. As expected none of the erythroid colonies grown in media with addition of Epo possessed the mutation. In contrast, in child No. 3 one homozygous and one heterozygous clone was detected out of six EECs with analyzable data. In this case also one homozygous clone was detected in non-erythroid colony out of ten (see Fig. 37). In patient No.4 forty EECs gave analyzable data and one was homozygous for the mutation (see Fig.38). In order to prove the *JAK2* V617F mutation by second independent technique, some positive colonies were directly sequenced (see Fig. 39). In summary, patients with childhood ET even if they are *JAK2* V617F-negative on the level of DNA or RNA standard analysis from full blood, can possess very rare clones, which are homozygous or heterozygous for the mutation

Table XIV: Results of selected analyses.

Patient No.	EPO hypersen-sitivity	EECs	JAK2 V617F DNA	JAK2 V617F RNA	EECs JAK2 V617F neg	EECs JAK2 V617F homoz	EECs JAK2 V617F heteroz	CFU-GMs JAK2 V617F neg.	CFU-GMs JAK2 V617F homoz	BFU-Es with EPO JAK2 V617F neg.	Clonality
1	no growth	-	neg.	neg.	NA	NA	NA	NA	NA	NA	no
2**	yes	yes	neg.	neg.	8	0	0	20	0	7	yes
3	yes	yes	neg.	neg.	5	**1**	**1**	9	**1**	13	no
4*	yes	yes	neg.	neg.	19	0	**1**	10	0	12	NI
5	yes	yes	neg.	NA	NA	NA	NA	NA	NA	NA	NI
6**	no	no	neg.	neg.	NA	NA	NA	NA	NA	NA	no
7	yes	no	neg.	neg.	NA	NA	NA	NA	NA	NA	NI
8	yes	yes	neg.	neg.	12	0	0	10	0	13	NI
9	poor growth	-	neg.	NA	NA	NA	NA	NA	NA	NA	no
10*	no	no	neg.	neg.	NA	NA	NA	NA	NA	NA	no
11	yes	yes	neg.	neg.	8	0	0	11	0	4	NI
12	yes	no	neg.	neg.	NA	NA	NA	NA	NA	NA	no
13	yes	yes	**pos.**	**pos.**	2	**13**	**2**	NA	NA	NA	NA
14	yes	yes	neg.	neg.	NA	NA	NA	NA	NA	NA	NI
15	yes	yes	NA	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviations: EEC: endogenous erythroid colony; JAK2: Janus kinase 2; NA: not analyzed, NI: not informative. * Slightly decreased EPO levels;
** increased TPO levels.

Figure 35: Real-time allelic discrimination of *JAK2* gene on single myeloid colonies picked on the day 14 of the culture. Different colors represent different colonies and straight line represents the signal from FAM-labeled wild type probe and the dotted line represents the signal from JOE-labeled mutant probe (specific for *JAK2* V617F).



No.	Colour	Name	Genotype	Cycling A.FAM/Sybr	Cycling A.JOE
1		daughter of patient Nr. 2 - CFU-GM	Wild Type	Reaction	No Reaction
2		daughter of patient Nr. 2 - CFU-GM	Wild Type	Reaction	No Reaction
3		daughter of patient Nr. 2 - EEC	Mutant	No Reaction	Reaction
4		patient Nr. 3 - CFU-GM	Mutant	No Reaction	Reaction
5		patient Nr. 3 - CFU-GM	Wild Type	Reaction	No Reaction
6		patient Nr. 3 - CFU-GM	Wild Type	Reaction	No Reaction
7		JAK2 negative control		No Reaction	No Reaction

Figure 36: The analysis of endogenous erythroid colonies (EECs) of child No. 13, who is *JAK2* V617F positive on the level of both AS and AS-RT-PCR from full blood. N/A: not available.

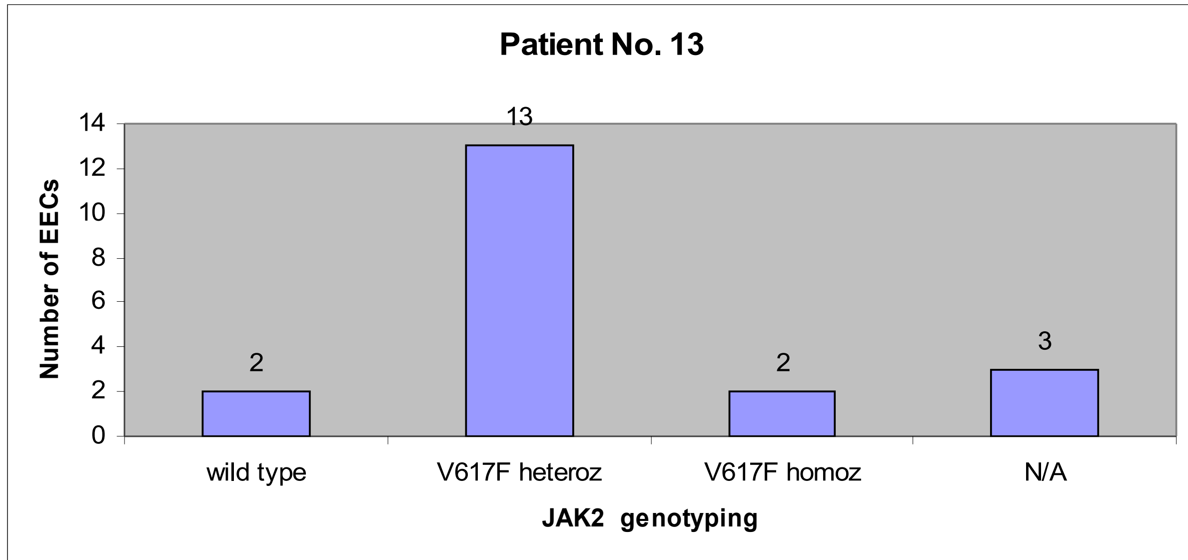


Figure 37: The analysis of endogenous erythroid colonies (EECs) of child No. 3, who is *JAK2* V617F-nagative on the level of both AS and AS-RT-PCR from full blood. N/A: not available.

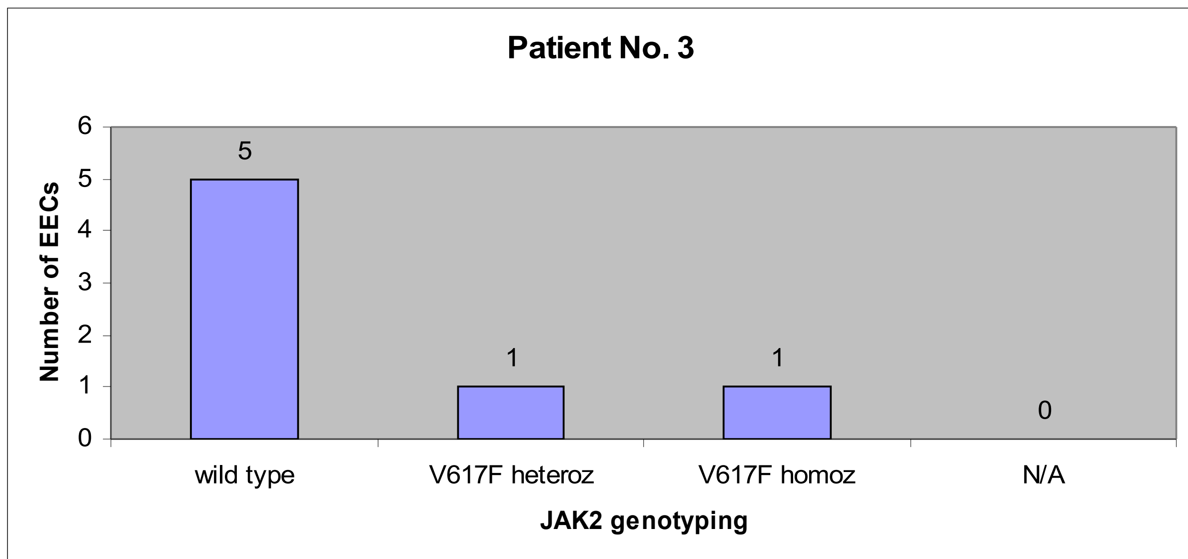


Figure 38: The analysis of endogenous erythroid colonies (EECs) of child No. 4, who is *JAK2* V617F-negative on the level of both AS and AS-RT-PCR from full blood. N/A: not available.

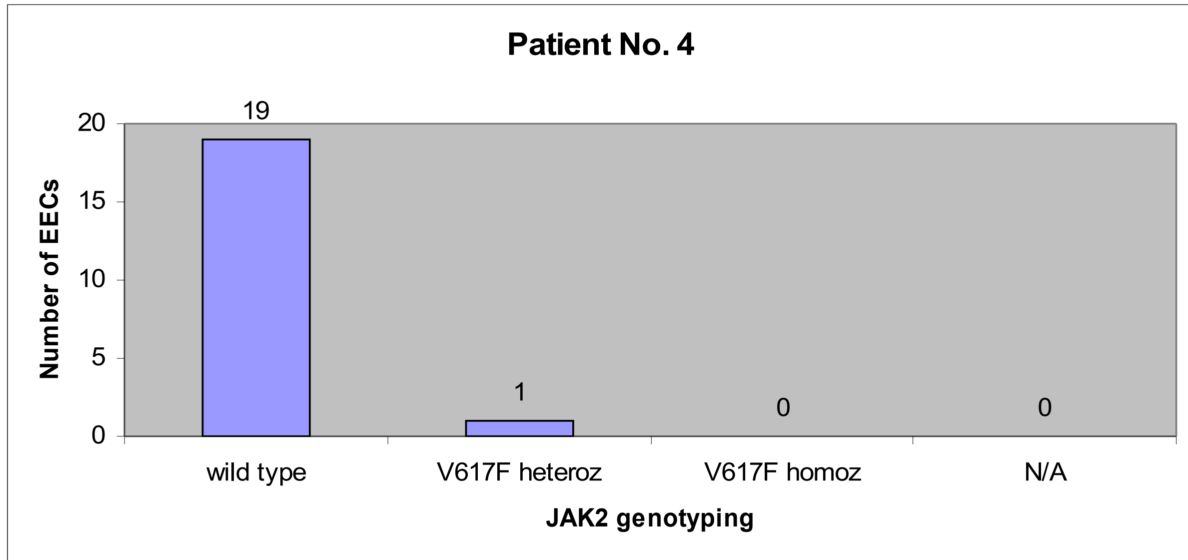


Figure 39: The sequenogram from analysis of *JAK2* V617F-positive EEC from patient No. 3 (a) in comparison with a wild type sequence (b).

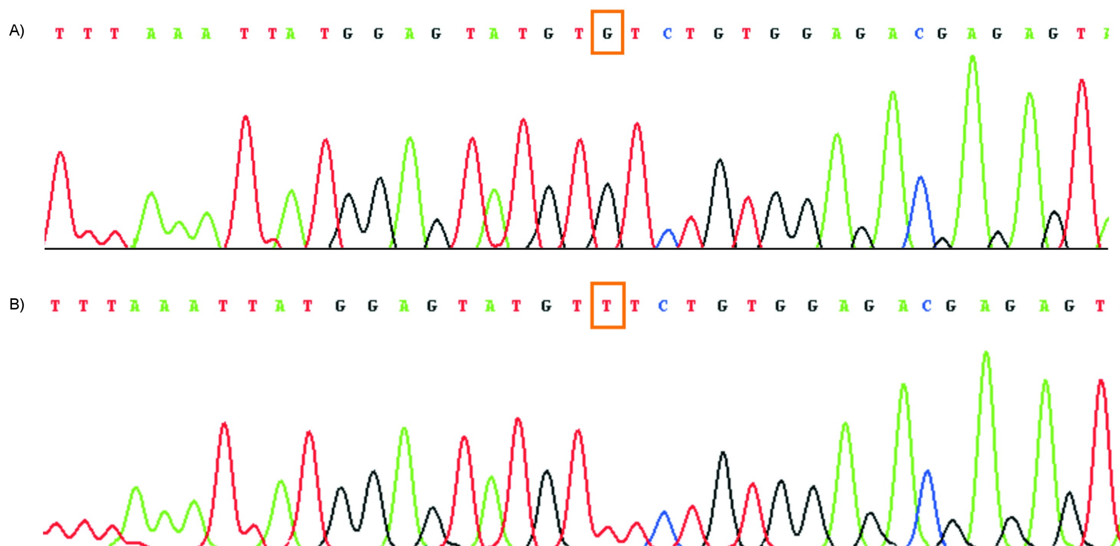
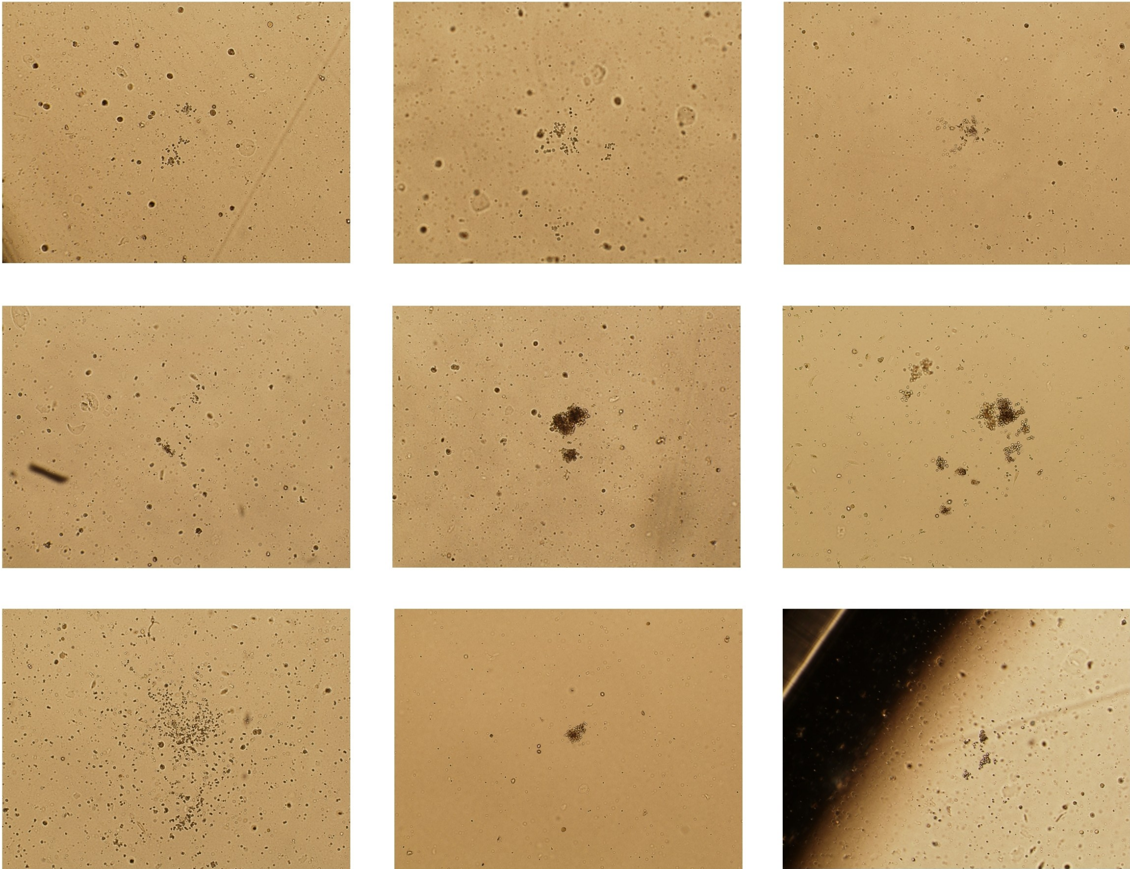


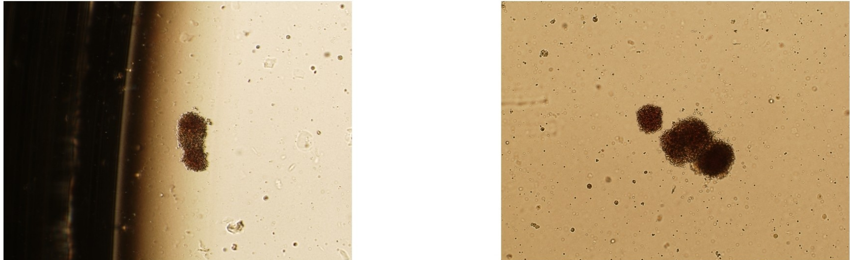
Figure 40: Colony assay of hematopoietic progenitors of child with thrombocythemia (daughter of patient No. 2). Erythroid colonies grown in media without addition of Epo (EECs: endogenous erythroid colonies) and with 1 U Epo/ml media (BFU-Es: burst-forming unit erythroid).

Child with thrombocythemia (child of patient No.2)

EECs



BFU-Es 1U Epo/ml



7.1.1.5 *JAK2* V617F-Positive Child with Thrombocythemia with a Family History of ET

In the course of our study, patient No.2, who was diagnosed with childhood ET came out of age and had a child. This child was followed directly from birth due to the family history. Already at 12 months a thrombocythemia was diagnosed with platelets counts 480 – 620 x 10^9/l. Essential thrombocythemia could not be diagnosed due to the young age of the child. The child was tested for the presence of *JAK2* V617F on the level of DNA from full blood and RNA from isolated platelets. Both tests were negative for the mutation. In contrast the presence of the mutation was confirmed on the level of single hematopoietic colonies. In total, nine EECs (Fig. 40) gave analyzable data including one *JAK2* V617F-homozygous clone and two heterozygous ones. This is so far the youngest published child with *JAK2* V617F positivity in somatic cells.

7.1.1.6 Summary for Pediatric ET Cohort Study

The role of *JAK2* V617F mutation in the ethiopathogenesis of pediatric ET is still elusive. From our cohort of 15 children, only one child was positive for this mutation using standardized methodology of mutation detection on DNA from full blood and RNA from isolated platelets. We have studied the presence of this mutation in single hematopoietic colonies. The patient with *JAK2* V617F detectable by standard procedures possessed clones both homozygous, and heterozygous for the mutation, as well as wild type. Surprisingly, even children, who were *JAK2* V617F-negative in full blood, possessed very rare *JAK2* V617F-positive clones (both homozygous and heterozygous) of hypersensitive progenitors to Epo.

7.5.2 Case Report of *JAK2* V617F-positive PV with Simultaneous B-CLL

The simultaneous occurrence of both chronic myeloid and lymphoid proliferations is a very rare event which offers a possibility to study the origins of these diseases. Do they arise from a common stem cell or are these disorders distinct illnesses? A few cases of this phenomenon have been reported and studied in detail [Hussein K et al., 2006; Jelinek J et al., 2002].

We studied a case of 79-year-old female diagnosed in 1998 with polycythemia vera (PV) and in 2000 with B-cell chronic lymphocytic leukaemia (B-CLL) based on WHO criteria. The diagnosis was supported by molecular-genetic analysis: the peripheral blood mononuclear cells showed heterozygosity for the *JAK2* V617F mutation, detected by AS-PCR

and real time RT-PCR. The malignant lymphocyte clone was characterized by CD19, CD20, CD23 and CD5 immunophenotype. Deletion of *RB1* gene, another characteristic marker for B-CLL, was detected by fluorescence in situ hybridisation (FISH). With respect to *RB1*, there were three different clones in peripheral blood at the time of diagnosis: deletion of one copy of *RB1* gene (62% of analyzed cells), deletion of two copies of *RB1* gene (20%) and cells without the deletion (18%). The clonality of B-lymphocyte population was determined by PCR-based detection of clonal variable heavy chain gene (IgH) rearrangement. (The cytogenetic studies and B-lymphocyte clonality was performed in the laboratory of Hemato-Oncology LF University Hospital Olomouc).

This patient underwent radioactive iodine therapy due to a hyperthyroidism 10 years before the diagnosis of the myeloproliferative disease. The PV was treated with venipuncture. In 2004 the patient received 10 day therapy of Leukeran which lead only to a transient decrease of leukocytosis. In 2005 three cycles of a 3 day therapy of Fludara was administered in May, June and August. After this treatment complete hematologic remission was observed involving both PV, as well as, B-CLL parameters including the decrease of the clone with *RB1* deletion below 10% in peripheral blood, and the patient did not necessitate any therapy since. At the time of sustain hematological remission (in 2006) we performed a detailed analysis of individual blood lineages to determine if the two diseases come from the same hematopoietic stem cell in this particular case.

The granulocytes, monocytes, T and B lymphocytes were separated by flow cytomtery (in collaboration with E. Mejstříková, Department of Pediatric Hematology and Oncology, University Hospital Motol, Prague). The *JAK2* V617F mutation was not detected in T or B lymphocytes (Fig. 41 a,b). In contrast, the monocytes and granulocytes were heterozygous for the *JAK2* V617F mutation (Fig. 41 c,d). In addition, dual-color FISH analysis of involvement of *RB1* deletion (LSI 13 RB1 probes, Vysis Inc., Downers Grove, IL) showed deletional status in B and T lymphocytes (Table XV), RB1 deletional status in granulocytes was not confirmed as it was within the range of “false-positivity” for this probe. X chromosome inactivation showed that after the treatment all cell populations were indeed polyclonal (Fig. 42). Clonal heterogeneity was also observed in the myeloid populations. Using *JAK2* genotyping of individual myeloid colonies, we detected heterozygous, homozygous and wt subclones with respect to *JAK2* V617F mutation (Table VI). There were

some endogenous erythroid colonies (EECs) without the *JAK2* V617F mutation suggesting that *JAK2* mutation was a secondary event in this case (Nussenzveig et al. 2007).

Table XV: *RB1* deletion status in different cell populations. WT: wild type; homo: homozygous cell for *RB1* deletion; hetero: heterozygous cells i.e. with one *RB1* deletion.

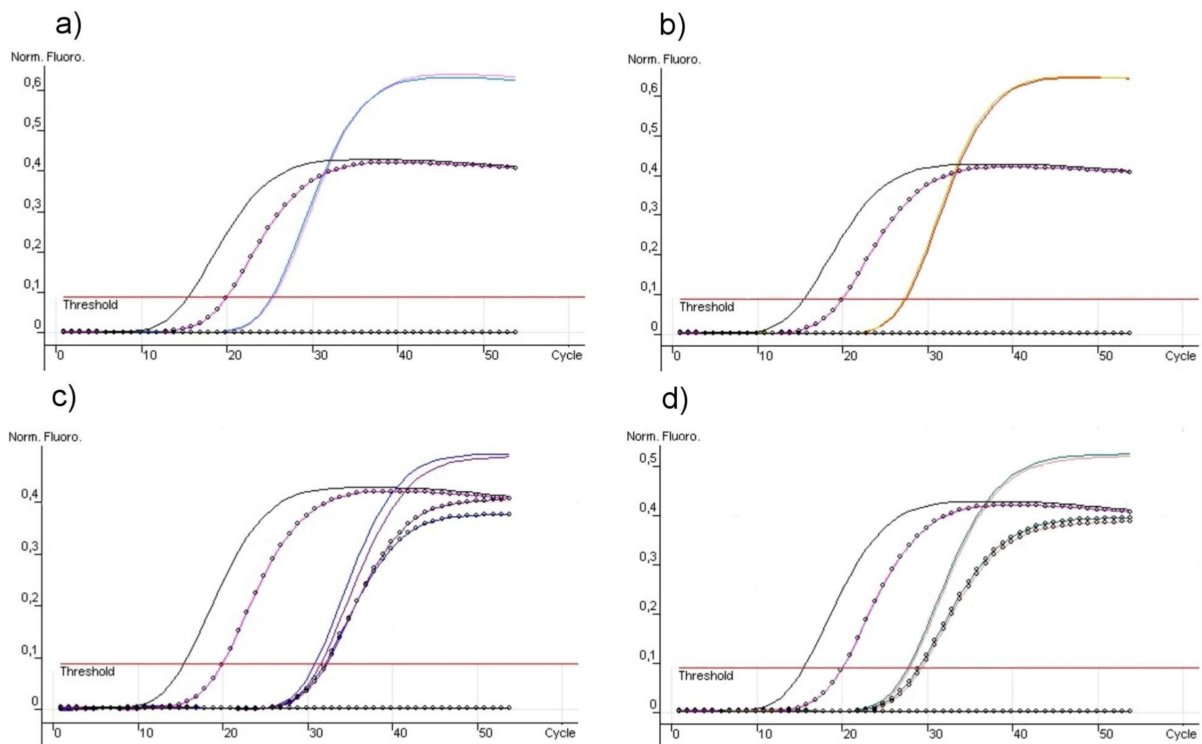
***RB1* - deletion status**			
	WT	homo	hetero
B-lymphocytes	10%	20%	17%
T-lymphocytes	47%	22%	19%

Table XVI: Detection of *JAK2* V617F mutation on the level of single hematopoietic colonies. The table gives the numbers of colonies. EECs: endogenous erythroid colonies; BFU-Es with Epo: erythroid colonies grown in media with addition of exogenous erythropoietin; CFU-GM: non-erythroid myeloid colonies. WT: wild type; homo: homozygous for *JAK2* V617F; hetero: heterozygous cells for *JAK2* V617F.

Myeloid colonies - *JAK2* V617F mutation			
	WT	homo	hetero
EECs	2	1	10
BFU-Es with Epo	2	0	6
CFU-GMs	5	1	20

The fact, that the patient did not need any treatment since the administration of Fludara, lead us to investigate the kinetics of both illnesses on molecular level. A sample before the treatment and a year after the treatment was examined for the quantitative expression of PRV-1 for polycytemia vera clone and for IgVh rearrangement for the lymphoid clone by real-time PCR. While the relative expression of IgVh decreased by 1 log after the treatment, the relative expression of PRV-1 significantly increased (Fig. 43). These results suggest that both illnesses react differently to Fludara treatment on molecular level further supporting the notion of two distinct diseases.

Figure 41: *JAK2* Allelic Discrimination. The granulocytes, monocytes, T and B lymphocytes were separated by flow cytometry using FACSAria (BD San Jose, USA) obtaining high purity populations (> 97%). The real-time allelic discrimination of *JAK2* gene was used to detect *JAK2* V617F mutation as described in 3.3.2. The *JAK2* V617F mutation was not present in T (a) or B lymphocytes (b). In contrast, the monocytes (c) and granulocytes (d) were heterozygous for the *JAK2* V617F mutation.



In summary, we report a patient diagnosed with typical clonal B-CLL and typical PV, who after the treatment with Fludara has not needed any further therapy for more than two years. Moreover, both lymphoid as well as myeloid compartments became polyclonal after the treatment. Typical cytogenetic marker for B-CLL (*RB1* deletion) was detected only in the lymphocytes. PV was characterized by formation of EECs which were both wild type as well as *JAK2* V617F positive. The *JAK2* V617F mutation was not detected in B or T cells.

Our investigation does not surely answer if the two diseases arose from a common stem cell or from distinct progenitors; yet undefined mutation may have predisposed stem cells to both malignancies. Our study suggests that typical well known markers of the two diseases (*RB1* deletion and *JAK2* V617F mutation) develop as secondary events and only in lymphoid or myeloid lineage, respectively.

Figure 42: Clonality analysis of separated cell populations of the patient performed by HUMARA assay based on X-chromosome inactivation in females (performed by Monika Běličková, Institute of Hematology and Blood Transfusion, Prague). DNA was isolated from leukocytes obtained from full peripheral blood and from individual cell populations. All populations were polyclonal. Different clones are depicted by different colors. The ratio of clones 35%:65% in the granulocytes is still considered as a marker of polyclonal cell population (Cermak et al., 2005).

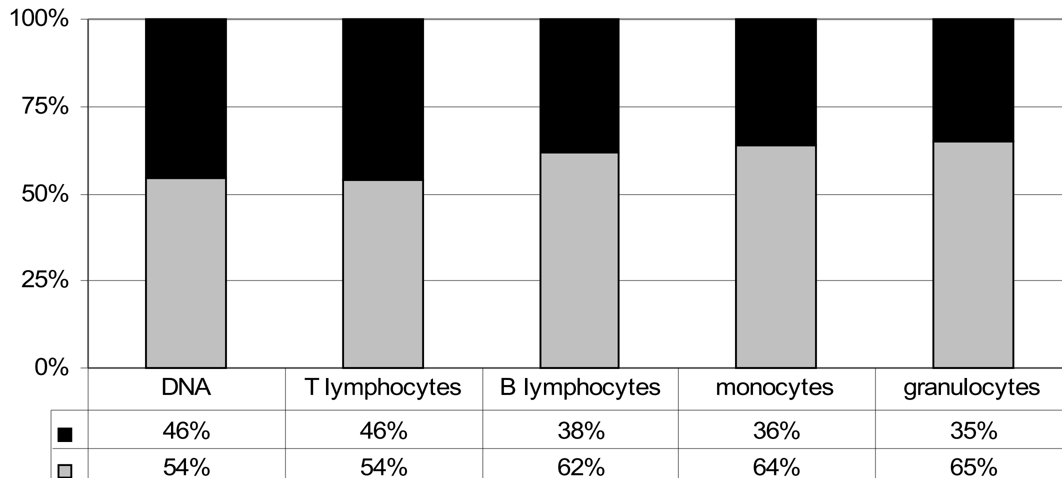
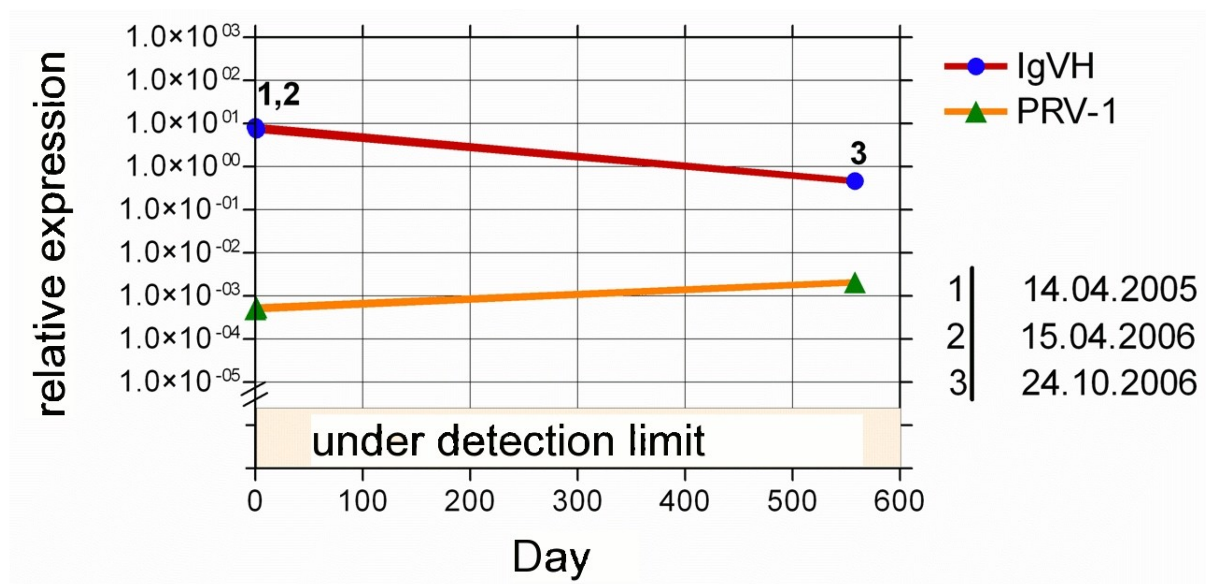


Figure 43: Quantification of relative expression of PRV-1 and IgV_H (performed by Soňa Peková, Institute of Hematology and Blood Transfusion, Prague).



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7 ABBREVIATIONS

ABL	Abelson
AML	acute myeloid leukemia
AS-PCR	allele-specific polymerase chain reaction
BC	blast crisis
BCR	breakpoint cluster region
BFU-E	burst-forming unit erythrocyte
CCR	complete cytogenetic response
CFU-GM	colony-forming unit granulocyte- macrophage
CGH	comparative genomic hybridization
CML	chronic myelogenous leukemia
CMML	chronic myelomonocytic leukemia
CP	chronic phase
CRKL	Crk like protein
Das	dasatinib
DEPC	diethylpyrocarbonate (-treated)
dNTP	deoxynucleotidetriphosphate
EEC	endogenous erythroid colonies
Epo	erythropoietin
ET	essential thrombocytemia
FBS	fetal bovine serum
FISH	fluorescent-in-situ-hybridization
HUMARA	human androgen receptor locus
IM	imatinib mesylate
IMF	idiopatic myelofibrosis
JAK	Just Another Kinase, Janus Kinase
MDS	myelodysplastic syndrome
MMR	major molecular response
MNC	mononuclear cell
MPD	myeloproliferative disease
P-Crkl	phosphorylated form of Crkl
P-SFK	phosphorylated form of SRC kinases
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Ph chromosome	Philadelphia chromosome
PV	polycythemia vera
RB1	retinoblastoma

RBC	red blood cell
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SFK	SRC family of kinases
SNP	single nucleotide polymorphism
T315I	substitution of threonin to isoleucine at the position 315
TPO	thrombopoietin
V617F	substitution of valin to phenylalanine at position 617
WHO	World Health Organization
WT	wild type