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New Bcr-Abl-independent mechanisms of resistance to imatinib treatment in chronic myelogenous leukaemia patients

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Riassunto

La leucemia mieloide cronica (LMC) è un disordine neoplastico della linea mieloide caratterizzato da una singola alterazione genetica, il gene di fusione BCR/ABL, che si origina a seguito di una traslocazione bilanciata tra i cromosomi 9 e 22 e che codifica per un proteina ad attivita' tirosin chinasica costitutivamente attivata [1-3]. Attualmente, l'unico trattamento capace di eradicare in maniera definitiva questa malattia è il trapianto di midollo allogenico, con piu' del 70% di successo nei pazienti in fase cronica [44]. Tuttavia, alcune limitazioni come la mancanza di donatori compatibili o l'eta' avanzata del paziente non rendono sempre possibile questa metodica.
Per questa ragione, è stato necessario sviluppare possibili vie alternative. Per piu' di 15 anni molti dei pazienti con LMC sono stati trattati con interferone-alfa (INF) capace di indurre remissione citogenetica in piu' del 30% dei casi. Successivamente, la scoperta del ruolo chiave dell'oncoproteina Bcr/Abl, costitutivamente attivata nella patogenesi, ha indirizzato verso una terapia mirata che ha portato allo sviluppo di una serie di specifici inibitori. Il capostipite di questa classe di molecole è rappresentato dall'imatinib (anche conosciuto come Glivec o STI571), un inibitore di tipo competitivo delle tirosin chinasi di classe III, che agisce legandosi selettivamente al sito catalitico della proteina chimerica Bcr/Abl, inibendola [46-48]. L'introduzione di questo farmaco ha segnato una profonda svolta nella cura della LMC ed oggi rappresenta il farmaco di prima linea per la cura di questa patologia. Studi clinici hanno dimostrato che il trattamento con imatinib è in grado di indurre una risposta citogenetica completa in piu' dell'80% di nuove diagnosi di LMC. Nonostante la sua introduzione abbia segnato una rivoluzione nella terapia mirata contro la LMC, una parte di pazienti in fase cronica e molti di più in fase avanzata mostrano resistenza primaria al farmaco o sviluppano una resistenza secondaria durante il trattamento [51]. Possiamo distinguere tra due tipi di resistenza, una correlata direttamente a Bcr/Abl ed una indipendente da questo. Tra i meccanismi molecolari di resistenza all'imatinib Bcr/Abl dipendenti, i piu' frequenti e meglio descritti sono sicuramente le mutazioni puntiformi a carico del dominio chinasico di Abl che limitano o impediscono al farmaco di legare ed inibire l'oncoproteina [66-72]. Un altro comune meccanimo di resistenza è rappresentato dall'overespressione della stessa proteina Bcr/Abl che richiede una maggiore concentrazione intracellulare di farmaco per una sua completa inibizione [54,74]. Vi sono poi meccanismi indipendenti dall'oncoproteina quali l'aumento dell'espressione di geni come MDR1 che codifica per la glicoproteina P (Pgp), una pompa di membrana, che agisce espellendo in maniera dinamica il farmaco, limitandone la concentrazione intracellulare [55,58,75-77]. Altri meccanismi indipendenti da BCR/ABL e di piu' recente scoperta sono l'overespressione di alcune proteine appartenenti alla famiglia delle Src chinasi come Lyn e Hck [64-65]. Nonostante i notevoli passi fatti a proposito, molti restano ancora i punti da chiarire sui complessi meccanismi molecolari che sono alla base della resistenza. Lo scopo del mio progetto di dottorato è stato proprio lo studio delle complesse vie intracellulari di trasduzione del segnale e l'espressione genica coinvolta nei meccanismi di resistenza all'imatinib, indipendenti da BCR/ABL. A tale scopo, abbiamo prima utilizzato un sistema modello ideale ed i dati cosi' ottenuti sono stati traslati sui pazienti. Il sistema modello scelto da noi è rappresentato da una coppia di linee cellulari, le KCL22, isolate da un paziente affetto da LMC. In particolare, una linea, le KCL22s, risulta sensibile al trattamento con l'inibitore, mentre un'altra, le KCL22r, è

stata isolata come subclone dalla parentale linea sensibile dal gruppo della professoressa Junia Melo dell'Imperial College di Londra che collabora in questo progetto, e che c'è l'ha gentilmente fornita. Il sistema sperimentale delle linee Kcl22 è molto interessante per due motivi: nessuno dei meccanismi di resistenza ad oggi conosciuti vi sono stati individuati; inoltre anche la linea sensibile è intrinsecamente resistente al farmaco in quanto questo induce arresto della crescita cellulare, piu' che apoptosi ed ha quindi un comportamento simile a quello postulato per le cellule staminali Ph+ [55]. In studi preliminari, combinando un approccio di natura proteomica ad uno di *"gene profil*e", abbiamo identificato una serie di geni e proteine differenzialmente espressi tra le due linee. Tra questi abbiamo focalizzato la nostra attenzione sulla tirosin fosfatasi non recettoriale SHP-1. Mediante real time PCR e western blot abbiamo riscontrato bassi livelli di SHP-1 sia a livello di trascritto che di proteina nella linea resistente KCL22r quando confrontata con quella sensibile KCL22s [Fig.7]. SHP-1 è altamente espressa nelle cellule della linea ematopoietica mentre i suoi livelli di espressione risultano piu' bassi in altri tipi cellulari [26]. Nell'uomo, riduzione dell'espressione di questo gene sono stati osservati in linfomi e altri tipi di leucemie [82-83]. Inoltre, ridotti livelli di SHP-1 sembrano essere associati con la progressione della malattia dalla fase cronica a quella accelerata nella LMC [85]. Shp-1 e stata anche mostrata essere fisicamente associata con Bcr/Abl con conseguenti risvolti funzionali [86-87]. Recenti studi hanno evidenziato che SHP-1 risulta assente nella linea cellulare di leucemia mieloide cronica in crisi bastica K562, e che la sua riespressione è stata accompagnata dal differenziamento di queste cellule [87-88]. Inoltre, l'overespressione di Shp-1 blocca la trasformazione neoplastica indotta da Bcr/Abl [89]. Tutte queste osservazione sembrano indicare che SHP-1 possa svolgere un importante ruolo nella regolazione negativa di BCRABL e che la sua deregolazione possa concorrere alla progressione neoplastica della malattia. Per capire se le cause dei bassi livelli di SHP-1 nella linea cellulare resistente (KCL22r) fosse da ascrivere a meccanismi epigenetici, quali ipermetilazione del suo promotore, abbiamo approcciato un'analisi di *methylation specific PCR* (MSP) su una specifica sequenza del promotore dello stesso gene in entrambe le linee cellulari. Tale analisi ha mostrato l'ipermetilazione del promotore di SHP-1 solo nella linea resistente, mentre nella linea sensibile lo stesso promotore risulta del tutto non metilato (Fig.8). Tale analisi è stata successivamente confermata mediante sequenziamento (Fig.9). La metilazione del DNA catalizzata da specifici enzimi ad attivita' metiltransferasica, riguarda l'aggiunta di un gruppo metilico a carico di specifiche citosine inserite nel dincleotide CG, tali sequenze sono anche conosciute come isole CpG. Questo rappresenta un meccanismo alternativo di regolazione genica negativa utilizzato soprattutto per regolare geni coinvolti nello sviluppo. Vi sono molti casi in cui fenomeni di aberrante metilazione di alcuni geni sono correlati con fenomeni neoplastici. Alcuni studi hanno rivelato come la deregolazione dell' espressione di SHP-1 in alcune patologie come Leucemie Acute, Mielomi e Linfomi sia da attribuirsi ad un'aberrante ipermetilazione del promotore [88,98]. Per capire il significato biologico dell'ipermetilazione del promotore di SHP-1 nel sistema modello in esame, abbiamo trattato queste linee con diverse concentrazioni dell'inibitore di metiltransferasi 5-Azacitidine (5-AC). In seguito al trattamento, dal punto di vista molecolare abbiamo osservato un aumento dei livelli sia del trascritto che della proteina di SHP-1 nella linea cellulare resistente (KCL22r). La riespressione di SHP-1 è stata anche accompagnata da una diminuzione dello stato di fosforilazione di ERK1/2 e STAT3 (Fig. 10). Nella linea sensibile (KCL22s) non sono state riscontrate variazioni sugli stessi *pathway* molecolari (Fig.10).

Parallelamente all'analisi molecolare, abbiamo vagliato anche l'effetto del trattamento con 5-AC sulla proliferazione ed il ciclo cellulare di queste cellule. Utilizzando la bromodeossiuridina, un analogo della timida capace di essere incorporato nel DNA di cellule in fase di sintesi, abbiamo analizzato la proliferazione delle KCL22 trattate. I risultati hanno mostrato una diminuzione della proliferazione cellulare sia nelle KCL22s che nelle KCL22r (Fig.11). Con lo ioduro di propidio, un intercalante del DNA, e successiva analisi citoflorimetrica, abbiamo riscontrato un accumulo di cellule in fase Go/G1 solo nella linea cellulare resistente (Fig.11). Quindi il trattamento con 5-AC ha confermato che l'aberrante metilazione del promotore è alla base della down-espressione di SHP-1 nella linea resistente KCL22r e questo puo' attivare una serie di vie di sopravvivenza alternative come MAPKs e JAK/STAs, indipendenti da Bcr/Abl, che permettono a queste cellule di sopravvivere anche in presenza di imatinib. Il passo successivo di questo lavoro è stato identificare i possibili interattori di SHP-1 e capire il significato biologico che questo comportasse. Esperimenti di co-immunoprecipitazione e western blot hanno mostrato l'interazione di SHP-1 con un'altra tirosin fosfatasi non recettoriale SHP-2 solo nella linea sensibile (KCL22s) mentre nella linea resistente non è stata riscontrata l'interazione (Fig.12). E' da dire che queste due proteine mostrano un'elevata omologia di sequenza sono implicate nelle stesse vie di traduzione del segnale ma sembrano svolgere ruoli opposti. Infatti, mentre SHP-1 è generalmente considerato un regolatore negativo, SHP-2 è invece riconosciuto come modulatore positivo nelle stesse vie (Fig. 6) [96]. Real time PCR e western blot, hanno evidenziato come SHP-2 sia ugualmente espressa tra le due linee cellulari in esame (Fig7). L'interazione tra' SHP-1 e 2 è stata gia' riscontrata nella linea cellulare di adenocarcinoma colorettale Caco-2, ma il significato di questa interazione resta ancora non chiaro [101]. Per capire se i bassi livelli di SHP-1 e la sua interazione con SHP-2 potessero essere coinvolti nei meccanismi molecolari di resistenza al trattamento con imatinib, abbiamo avviato ulteriori indagini funzionali. In particolare, avvalendoci della metodica dell'*RNA interfirence*, abbiamo abbassato i livelli di SHP-1 nella linea sensibile (KCL22s), mimando quelle che erano le condizioni fisiologiche osservate nella linea resistente (KCL22r), mentre abbiamo spento l'espressione di SHP-2 nella linea resistente per capire l'effetto che questo potesse avere sulla risposta al trattamento con il farmaco.
Da un punto di vista molecolare, il *knock-down* di SHP-1 nella linea sensibile (KCL22s), è stato accompagnato da un incremento dei livelli di fosforilazione di ERK1/2 (40%) e STAT3 (50%), mentre a seguito del *knock-down* di SHP-2 nella linea resistente (KCL22r), abbiamo osservato una diminuzione dello stato di fosforilazione di ERK1/2 (80%) e STAT3 (70%) (Fig.13). Questi dati sono molto simili a quelli osservati con cellule overesprimenti mutanti negativi per SHP-2 [102-103]. Allo stesso modo, le KCL22s interferite per SHP-1 e trattate con imatinib 1µM hanno mostrato una ridotta sensibilita' al trattamento col farmaco. Il test di vitalita' trypan blue ha evidenziato, dopo quattro giorni di trattamento, una percentuale di cellule vitali (25%) piu' alta nelle cellule interferite per SHP-1 rispetto alle stesse cellule non interferite prese come controllo e trattate allo stesso modo (Fig.14). Di contro, le KCL22r interferite per SHP-2 e trattate con imatinib 1µM hanno mostrato un aumento della sensibilita' al trattamento col farmaco. Il test di vitalita' trypan blue ha evidenziato, dopo quattro giorni di trattamento, un decremento della percentuale di cellule vitali (50%) nelle cellule interferite per SHP-2 rispetto alle stesse cellule non interferite (Fig.14). E' oggi ben accetto che SHP-2 abbia un ruolo critico nel sostenere l'attivazione dalla via di Ras/MAPK in risposta a diversi stimoli. Inoltre, la via di Ras/MAPK è ben descritta essere alla base di processi quali proliferazione e

differenziamento cellulare in risposta a fattori di sviluppo [96]. Per esempio, nelle cellule in cui è stato inattivato la funzione di SHP-2 è stato riscontrato una diminuzione dell'attivazione delle MAPK. Quindi questi ulteriori esperimenti funzionali hanno mostrato l'implicazione di SHP-1 e 2 nella risposta al trattamento con imatinib. Dunque, noi ipotizziamo che SHP-1 possa intervenire con la sua attivita' fosfatasica a defosforilare specifici residui su SHP-2 mantenendola nella sua conformazione inattiva. A riguardo, è documentato in letteratura come la specifica defosforilazione dei residui tirosinici in posizione 542 e 580 posti al carbossiterminale di SHP-2 possano concorrere a mantenere questa proteina nel suo stato inattivo [96]. Quindi, il giusto equilibrio tra SHP-1 e 2 potrebbe essere implicato nella risposta all'imatinib. In piu', la deregolazione di questo sistema potrebbe sostenere alternative vie di sopravvivenza come Ras/MAPK e JAK/STATs che permettono a queste cellule di eludere l'azione del farmaco e continuare a proliferare. I dati raccolti nelle linee cellulari sono poi stati traslati nei pazienti. Mediante l'utilizzo di metodica RT-PCR abbiamo misurato i livelli del trascritto di SHP-1 nei leucociti, isolati dal midollo osseo mediante gradiente di Ficoll, di 60 pazienti affetti da LMC alla diagnosi. I pazienti sono stati suddivisi in tre sottogruppi in funzione della risposta che questi hanno ottenuto dopo 18 mesi di trattamento. I criteri di risposta utilizzati sono quelli descritti dall'*Euroupean Leukemia Net* (Tab.1) [54]. Abbiamo analizzato 35 pazienti definiti *Optimal responder,* 17 *Suboptimal responder* , ed 8 *Failure responder*. I livelli di SHP-1 sono stati normalizzati utilizzando il gene ABL ubiquitariamente espresso nella linea ematopoietica. I livelli di trascritto per SHP-1 sono risultati significativamente piu' alti nei pazienti *Optimal responder* [rapporto SHP1/ABL 5.8±1.77, (media±deviazione standard)] quando confrontati con i *Suboptimal responder* (3.8±1.3, $^{*}p$=0.001) ed i *Failure* (3.2±1.04, $^{*}p$=0.002) (Fig.15). Come controllo abbiamo misurato lo stesso trascritto in 21 pazienti affetti da sindrome mieloproliferativa cronica (CMDP), Ph negativi, e wild type per il gene JAK2, (6.0±2.2, **p*=0.6). Con la stessa metodica, abbiamo misurato l'espressione di SHP-1 nelle cellule primarie CD34+ isolate mediante *cell sorting* da 6 pazienti *Optimal responder* [1.7±0.25] *,* e 6 *Failure responder* [0.9±0.15, $^{*}p$=0.017] (Fig.16). Anche in questo caso i livelli di SHP-1 sono risultati piu' bassi nei pazienti resistenti al trattamento. L'analisi sui pazienti ha quindi mostrato come pazienti resistenti al trattamento con imatinib presentino livelli piu' bassi di SHP-1, statisticamente significativi, alla diagnosi. Nell'ultima parte di questo studio abbiamo cercato di capire se l'espressione di questa fosfatasi possa avere un ruolo predittivo ed essere utilizzato come marker di risposta al trattamento con imatinib nei pazienti affetti da LMC. Per questo scopo, abbiamo misurato sempre mediante RT-PCR l'espressione di SHP-1 nei leucociti, isolati dal sangue periferico, di 48 pazienti con LMC alla diagnosi. Questi pazienti sono arruolati nel trial clinico TOPS (*Tyrosine kinase inhibitor Optimization and Selectivity*), uno studio randomizzato di fase III che confronta l'effetto della dose giornaliera di imatinib 800mg contro 400mg. Il fine di questo trial è confrontare l'effetto che questo produce su quella che viene indicata come risposta molecolare maggiore (MMR) [53], che sembra avere un ruolo predittivo nell'aspettativa di vita dei pazienti affetti da LMC e trattati con imatinib. I risultati sembrano indicare una significativa differenza nell'espressione di SHP-1 tra quei pazienti che ottengono o non ottengono la MMR dopo 12 mesi dall'inizio del trattamento [SHP1/ABL 7.4 ± 3.8 vs 6.0 ± 3.7, **p* = 0.017] (Tab.2). Ulteriori analisi statistiche hanno anche mostrato come l'espressione di SHP-1 possa essere utilizzato come marker molecolare per la predizione della risposta molecolare maggiore (MMR) a 12 mesi nei pazienti con LMC e trattati con imatinib (Fig.17).

1 Introduction

1.1 The *BCR-ABL* oncogene

Chronic myelogenous leukaemia (CML) results from the neoplastic transformation of a haematopoietic stem cell (fig. 1). The hallmark genetic abnormality of CML is a t(9;22)(q34;q11) translocation, which was first discovered as an abnormal, small chromosome, named the 'Philadelphia chromosome' (Ph+). The Ph+ chromosome was originally described as a chromosomal abnormality in 1960 by Nowell and Hungerford [1]. In 1973, Rowley reported that this abnormal chromosome, found in most patients with chronic myeloid leukemia (CML), has an apparent loss of the long arm of chromosome number 22 and is the result of reciprocal translocation involving the long arms of chromosomes 9 and 22, t(9;22) [2]. The molecular genetics of the Ph+ chromosome showed the *ABL* gene to be on the segment of chromosome 9 that is translocated to chromosome 22 [3]. Breakpoints in chromosome 22 were found to occur over a very short stretch of DNA (5–6 kb), termed the *breakpoint cluster region* (*BCR*) gene. The native c-ABL tyrosine kinase is located partially in the nucleus and has tightly regulated kinase activity. The *BCR–ABL* fusion results in the production of a constitutively active cytoplasmic tyrosine kinase that does not block differentiation, but enhances proliferation and viability of myeloid lineage cells. BCR–ABL is likely sufficient to cause CML, but over time other genetic events occur and the disease progresses to an acute leukemia. CML is typically characterized by phases of variable duration, starting with an initial chronic phase (CP), followed by progression to accelerated phase (AP) and finally resulting in blast crisis (BC) [4]. The initial chronic phase of this biphasic disease is characterized by a massive expansion of the granulocytic cell lineage, even though most, if not all, haematopoietic lineages can be produced from the CML stem cell. The median duration of the chronic phase is 3–4 years. Acquisition of additional genetic and/or epigenetic abnormalities causes the progression of CML from chronic phase to blast phase. This phase is characterized by a block of cell differentiation that results in the presence of 30% or more myeloid or lymphoid blast cells in peripheral blood or bone marrow, or the presence of extramedullary infiltrates of blast cells.

1.2 Structure of the BCR-ABL fusion genes and their transcripts Breakpoints in ABL

Breakpoints within the *ABL* gene can occur anywhere within a 5′ segment that extends for over 300 kilobases (kb) [5]. Typically, breakpoints form within intronic sequences, most frequently between the two alternative first exons of *ABL*. Thus, *BCR-ABL* fusion genes may contain both exons 1b and 1a, exon 1a alone, or neither of the alternative first exons. *BCR-ABL* mRNA lacks exon 1, regardless of the structure of the fusion gene, with the transcript consisting of *BCR* exons fused directly to *ABL* exon a2 (Fig.2).

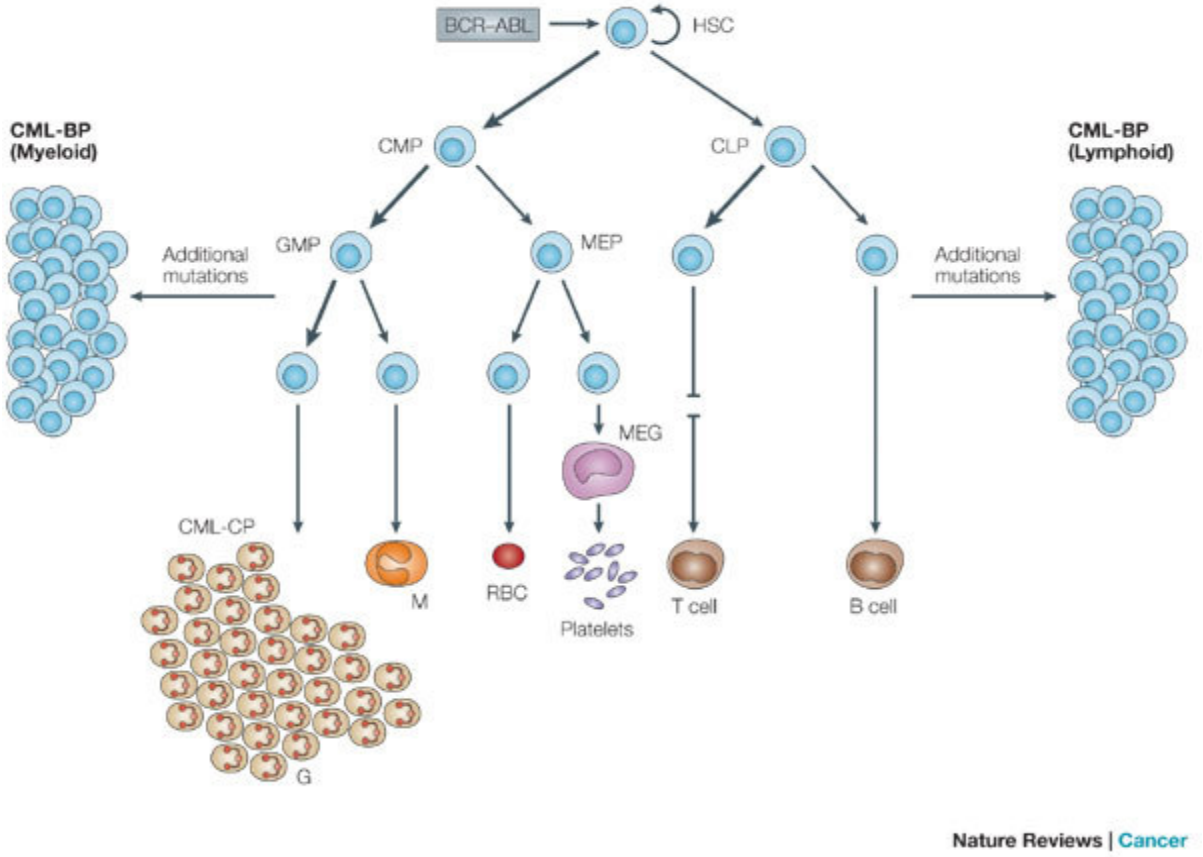

Fig.1 Chronic myelogenous leukaemia (CML) is a biphasic disease, initiated by expression of the *BCR–ABL* fusion gene product in self-renewing, haematopoietic stem cells (HSCs). HSCs can differentiate into common myeloid progenitors (CMPs), which then differentiate into granulocyte/macrophage progenitors (GMPs; progenitors of granulocytes (G) and macrophages (M)) and megakaryocyte/erythrocyte progenitors (MEPs; progenitors of red blood cells (RBCs) and megakaryocytes (MEGs), which produce platelets). HSCs can also differentiate into common lymphoid progenitors (CLPs), which are the progenitors of lymphocytes such as T cells and B cells. The initial chronic phase of CML (CML-CP) is characterized by a massive expansion of the granulocytic-cell series. Acquisition of additional genetic mutations beyond expression of BCR–ABL causes the progression of CML from chronic phase to blast phase (CML-BP).

1.2.1 Breakpoints in BCR

The breakpoints within the *BCR* gene on chromosome 22 are found within three defined regions [5]. In 95% of patients with CML and approximately one third of patients with ALL, the *BCR* gene is truncated within a 5.8-kb region known as the major breakpoint cluster region (M-bcr) (Fig.2). This region contains five exons, originally named b1 to b5, but now referred to as e12 to e16, according to their true positions in the gene [6]. Most breakpoints form within introns immediately downstream of exon 13 (b2) or exon 14 (b3). Because processing of *BCR-ABL* mRNA results in the joining of *BCR* exons to *ABL* exon a2, hybrid transcripts are

produced that have an e13a2 (b2a2) or an e14a2 (b3a2) junction. In both cases, the mRNA consists of an 8.5-kb sequence that encodes a 210-kd fusion protein, $p210^{Bcr-Abl}$ (Fig. 2). In two-thirds of patients with Ph-positive ALL and in rare cases of CML and acute myelogenous leukemia, the breakpoint in *BCR* occurs in a region upstream of the major breakpoint cluster region known as the minor breakpoint cluster region (m-bcr) (Fig.2). This region consists of the 54.4-kb intron between the two alternative second exons of the *BCR* gene, e2′ and e2. *BCR-ABL* fusion genes that have breakpoints within the minor breakpoint cluster region contain both *BCR* alternative first exons (e1 and e1′) together with the alternative second exon (e2′). Chimeric RNA derived from these fusion genes evidently is subject to splicing because the mature transcripts lack exons e1′ and e2′. The hybrid mRNA consists of sequences that are approximately 7 kb in length in which exon e1 from *BCR* is joined to exon a2 of *ABL*. The translated product is a 190-kd fusion protein, $p190^{Bcr-Abl}$ (also referred to as $p185^{Bcr-Abl}$) [7]. Interestingly, transcripts with an e1a2 junction are detectable at very low levels in patients with a major breakpoint cluster region rearrangement. Initially thought to be indicative of disease progression [8], these transcripts subsequently were detected irrespective of the phase of disease [9-10]. Their low level of expression in comparison to the predominant $p210^{Bcr-Abl}$ message suggests that the e1a2 transcripts most likely are the result of alternative splicing of e13a2 or e14a2 transcripts [10-11]. The third defined breakpoint cluster region within the *BCR* gene was named “micro” breakpoint cluster region (μ-bcr),(Fig.2) [12]. In this case, the breaks occur within a 3′ segment of the *BCR* gene between exons e19 and e20 (known as c3 and c4 in the original nomenclature). Transcription of the hybrid gene yields an e19a2 *BCR-ABL* fusion transcript that encodes a 230-kd protein, $p230^{Bcr-Abl}$.

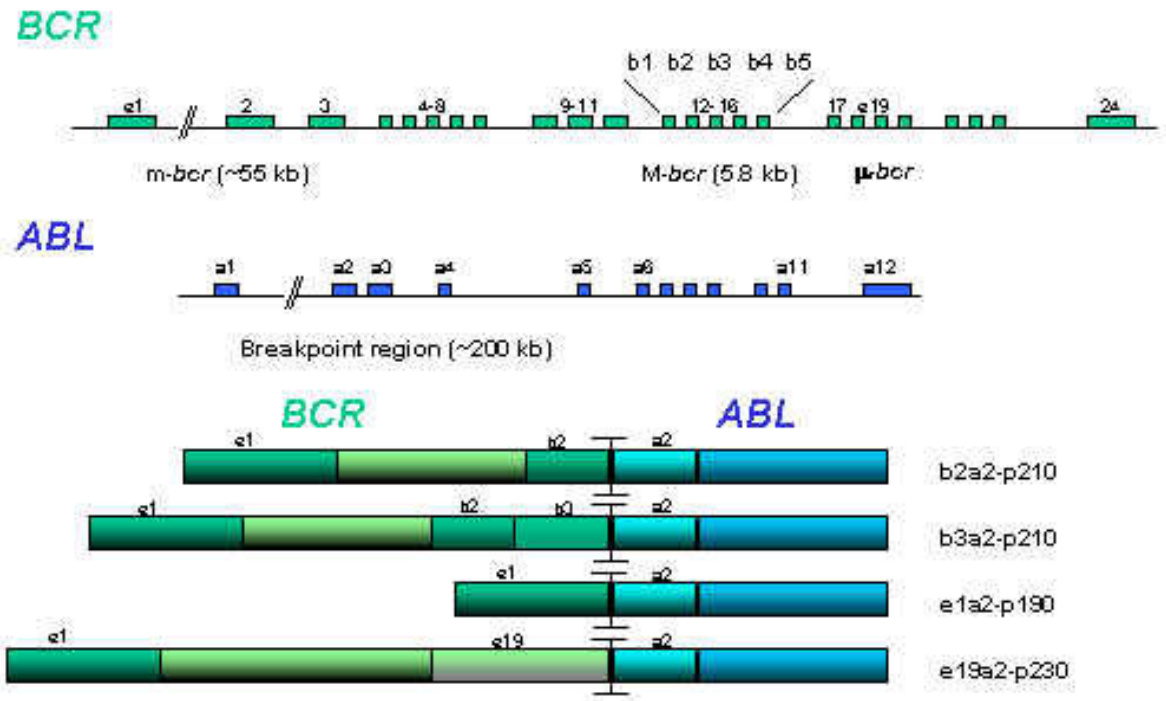

Fig. 2 The t(9;22) translocation and its products with breakpoint locations at the BCR and ABL loci.

1.3 Molecular pathophysiology

Cells expressing the chimeric BCR–ABL oncoprotein show signs typical of malignant transformation, including excessive cell growth of immature myeloid cells with lack of differentiation and inhibition of apoptosis [13]. The mechanisms through which BCR–ABL contributes to malignant transformation have been extensively studied by using BCR–ABL expressing murine or human cell models. Many of the activated signaling pathways are normally regulated by hematopoietic growth factors, such as c-kit ligand, thrombopoietin, interleukin-3, or granulocyte/macrophage-colony stimulating factor. Not surprisingly, it has been demonstrated that BCR–ABL activates a variety of signaling pathways and downstream targets like RAS, STATs, phosphatidylinositol-3′-kinase, production of reactive oxygen species (ROS) and others that can also be activated by hematopoietic growth factors. A major difference between activated growth factor receptors in normal signaling and BCR–ABL is the dysregulated activation of signaling pathways by BCR–ABL, inducing abnormal proliferation and neoplastic expansion. In addition, the BCR–ABL oncoprotein decreases apoptotic reaction to mutagenic stimuli, which results in a survival advantage to the neoplastic clones [14]. In many model systems, BCR–ABL completely abrogates growth factor dependence, and has been associated with reduced requirement for growth factors in primary hematopoietic cells. It is clear, however, that there are other activities of BCR–ABL that remain poorly understood, in particular, the propensity for CML to evolve into blast crisis.

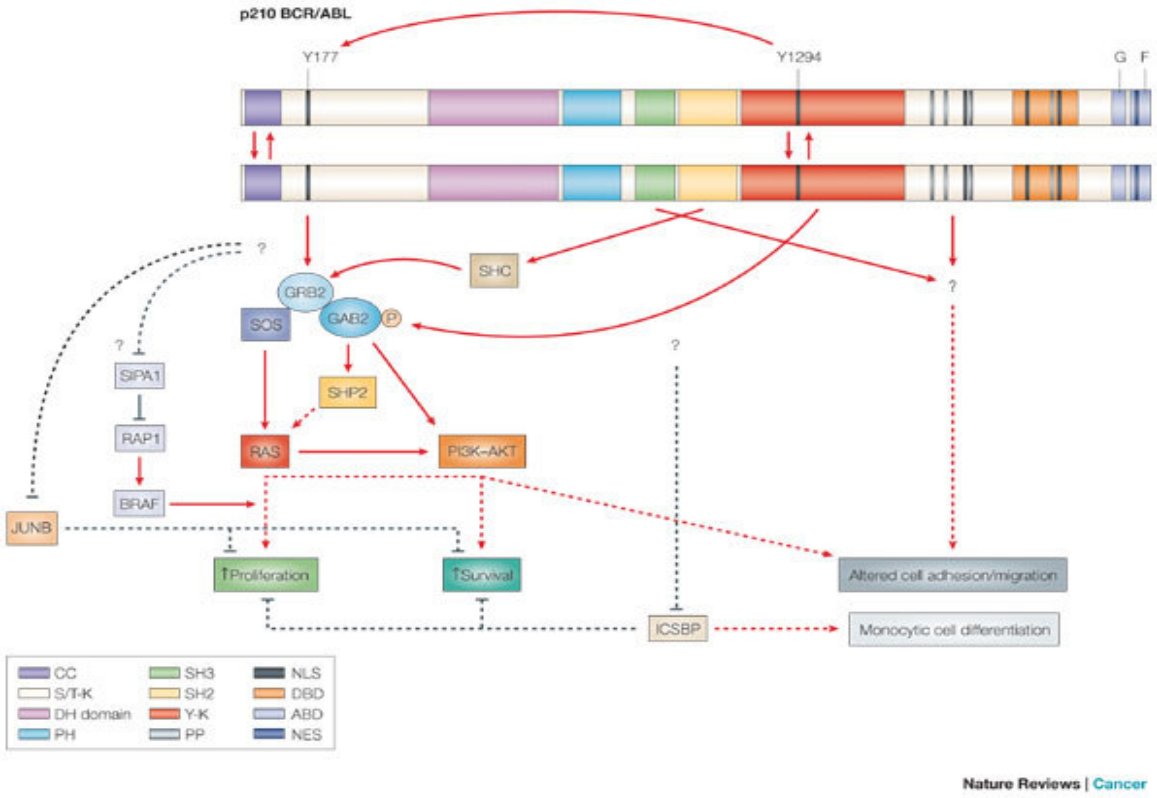

Fig.3 Leukaemogenic signallilg of BCR/ABL. The BCR–ABL proteins can form dimers or tetramers through their CC domains, and trans-autophosphorylate (indicated by up and down arrows between protein structures). Phosphorylation at the Y177 residue generates a high-affinity binding site for growth factor receptor-

bound protein 2 (GRB2). GRB2 binds to BCR–ABL through its SH2 domain and binds to SOS and GRB2-associated binding protein 2 (GAB2) through its SH3 domains. SOS in turn activates RAS. Following phosphorylation (P) by BCR–ABL, GAB2 recruits phosphatidylinositol 3-kinase (PI3K) and SHP2 proteins. The SH2 domain of ABL can bind SHC, which, following phosphorylation can also recruit GRB2. The ABL SH3 domain and the SH3 binding sites in the carboxy-terminal region can bind several proteins that involve regulations of cell adhesion/migration. Interferon consensus sequence binding protein (ICSBP), also known as interferon regulatory factor 8, negatively regulates proliferation and survival of myeloid cells by inducing differentiation of monocytic cells. JUNB inhibits cell proliferation and survival, partly by antagonizing the RAS downstream target JUN. SIPA1 (signal-induced proliferation-associated gene-1) is a RAP1 GAP that keeps RAP1 inactive. BCR–ABL can promote cell proliferation and survival partly by activating the RAS, SHP2 and PI3K–AKT signalling pathways. It can also downregulate transcription of ICSBP and JUNB, and might also inhibit SIPA1. Red arrows indicate direct interactions and/or activations. Black arrows indicate negative regulations. Broken arrows indicate multiple steps. ABD, actin-binding domain; CC, coiled-coil; DBD, DNA-binding domain; DH, Dbl/CDC24 guanine-nuleotide exchange factor homology; NES, nuclear exporting signal; NLS, nuclear localization signal; PP, proline-rich SH3 binding site; S/T-K, serine/threonine kinase; Y-K, tyrosine kinase.

1.4 Pathway downstream of BCR-ABL

Many signalling proteins have been shown to interact with BCR–ABL through various functional domains/motifs (for example, GRB2, CRKL, CRK, SHC, 3BP2, ABL-interacting protein 1 and 2, and CRK-associated substrate (CAS)), and/or to become phosphorylated in BCR–ABL-expressing cells (for example, CRKL, CRK, SHC, docking protein 1, GAB2, CBL, CAS, signal transducer and activator of transcription 5 (STAT5), the p85 subunit of PI3K, phospholipase Cγ, synaptophysin, VAV1, RAS GTPase-activating protein, focal adhesion kinase, FES, paxillin and talin [15-16]. These proteins in turn activate a range of signalling pathways that activate proteins such as RAS, PI3K, AKT, JNK, SRC family kinases, protein and lipid phosphatases, and their respective downstream targets, as well as transcription factors such as the STATs, nuclear factor-kB and MYC [16]. BCR–ABL also induces expression of cytokines such as interleukin-3 (IL-3), granulocyte colony-stimulating factor and granulocyte–macrophage colony-stimulating factor (GM-CSF) [17]. Most of these findings were observed from experiments in *in vitro* systems, or from studies of the properties of cells derived from leukaemia patients with particular stages of disease. The importance of these signalling proteins and pathways in leukaemogenesis and their viability as therapeutic targets needs to be validated by *in vivo* model systems. Mouse models can be used to determine the involvement of these signalling pathways in CML pathogenesis and progression. The leukaemogenic role of various factors that are activated by BCR–ABL expression has been effectively examined using knockout mice. Expression of BCR–ABL in bone-marrow cells, through retroviral transduction, still induced CML-like MPD in *Stat5a*$^{-/-}$*Stat5b*$^{-/-}$, *Cbl*$^{-/-}$ and *Il-3*$^{-/-}$*GM-CSF*$^{-/-}$ mice [18-20], so these proteins are not required for BCR–ABL-mediated leukaemogenesis. BCR–ABL expression also induced CML-like MPD in mice that lacked the SRC family kinases LYN,

haematopoietic cell kinase and FGR, although it failed to induce B-ALL in these mice [21]. Interestingly, downregulating LYN expression by small interfering RNA induces apoptosis of human blast-phase CML cells, particularly the lymphoid blast cells [22]. Together these results indicate that LYN is important in the development of blast-phase CML particularly for lymphoid blast crisis but not for chronic-phase CML. BCR–ABL also recruits the scaffold adapter GAB2 through GRB2. The major GRB2-binding site at Y177 of BCR–ABL was shown to regulate the tyrosine phosphorylation of GAB2 [23], indicating that GAB2 is a substrate of BCR–ABL. Consistent with the importance of Y177 function in BCR–ABL-mediated leukaemogenesis, BCR–ABL was unable to confer cytokine-independent growth (a characteristic of leukaemia cells) in primary myeloid cells isolated from *Gab2*$^{-/-}$ mice *in vitro* [23]. So, the inability of the Y177F mutant of BCR–ABL to induce leukaemia might be partially due to a failure to transmit appropriate signals through GAB2. GAB2 contains binding sites for the SH2 domains of the p85 subunit of PI3K and for SHP2 [23]. The PI3K pathway has been implicated in a wide range of human cancers [24]. Mutations of the *SHP2* gene (also known as PTPN11) have also been found in approximately 50% of individuals with Noonan syndrome, a common human autosomal-dominant birth defect characterized by short stature, facial abnormalities, heart defects and possibly increased risk of leukaemia [25]. The PI3K and SHP2 signalling pathways could be required for BCR–ABL leukaemogenesis and therefore be effective therapeutic targets for CML. SHP2 is required for normal activation of the RAS–ERK (extracellular signal-regulated kinase) pathway that most receptor tyrosine kinases and cytokine receptors signal through [26]. The mechanism of the activation of RAS–ERK pathway by SHP2 is not completely known. In addition to SHP2, RAS can be activated directly by BCR–ABL through the GRB2–SOS complex [27,28]. (Fig. 3). 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 [29]. . Recently, it was shown that expression of an oncogenic KRAS using a conditional knock-in line of mice efficiently induced an MPD that resembled human chronic myelomonocytic leukaemia (CMML) [30]. We found that expression of oncogenic NRAS in mice, through BMT, efficiently induced CMML- or AML-like disease in mice (R. Subrahmanyam, C. Parikh and R.R., unpublished observations). We also found that co-expression of oncogenic NRAS and the Y177F mutant of BCR–ABL could rapidly and efficiently induce CML-like myeloproliferative disorder (R. Subrahmanyam and R.R., unpublished observations). So RAS seems to be a crucial downstream target of BCR–ABL, yet other signalling pathways activated by BCR–ABL restrict BCR–ABL-mediated leukaemogenesis in the granulocytic lineage. Gene-knockout studies also revealed several key negative regulators of myelopoiesis. Mice with disruptions of the gene encoding interferon consensus sequence binding protein (ICSBP), also known as interferon regulatory factor 8, developed a CML-CP-like disease at 10–16 weeks of age, in addition to defects of viral and intracellular parasite immunity [31]. One-third of the mice also underwent blast crisis by 50 weeks of age. Consistent with the myeloid phenotype of the *Icsbp*-null mice, it was shown that ICSBP controls the development of myeloid cells by stimulating macrophage differentiation while inhibiting granulocyte differentiation, in both cases inhibiting cell growth [32] (Fig. 3). Expression of the ICSBP protein is significantly decreased in mice with BCR–ABL-induced CML-like disease, and forced expression of ICSBP inhibited the BCR–ABL-induced colony formation of bone-marrow cells *in vitro* and BCR–ABL-induced CML-like disease *in vivo* [33]. Downregulation of *ICSBP* transcripts was also found in

patients with CML, and this reduction could be reversed by treatment with interferon-α [34]. These data indicate that ICSBP is a tumour suppressor and that downregulation of ICSBP is important for the pathogenesis of CML. Consistent with the importance of the RAS signalling in promoting growth of myeloid cells, JUNB, an antagonist of the RAS downstream target JUN and negative regulator of cell proliferation and survival, was shown to act as a tumour suppressor in myeloid cells. *Junb*-null mice have severe vascular defects in the placenta, leading to early embryonic lethality [35]. However, inactivation of JUNB specifically in haematopoietic cells led to the development of a CML-like disease in mice, beginning at 4 months of age [36]. About 16% of mice further developed a syndrome that resembled blast crisis. More recent studies showed that JUNB inactivation in the long-term self-renewing haematopoietic stem cells of mice, but not in committed myeloid progenitor cells, could induce a CML-like myeloproliferative disorder. Furthermore, only JUNB-deficient long-term self-renewing haematopoietic stem cells from the diseased mice could induce CML-like disease in recipient mice after transplantation [36]. These results support the hypothesis that CML originates from haematopoietic stem cells. Downregulation of JUNB has also been observed in CML cells isolated from patients [37]. These results indicate that inactivation of JUNB could be important for CML development. There is further support for the importance of RAS downstream signalling pathways in myeloid proliferation and survival. Targeted inactivation of SIPA1 (signal-induced proliferation-associated gene-1) a principal RAP1 GTPase-activating protein in haematopoietic progenitors led to a spectrum of myeloid disorders that resembled chronic-phase CML, blast-phase CML and myelodysplastic syndrome by 1 year after birth in mice [38]. In pre-leukaemic SIPA1-deficient mice, there is a selective expansion of pluripotential haematopoietic progenitors. RAP1 is a close member of RAS-family GTPases and, like RAS, can activate the mitogen-activated kinase/ERK kinase (MEK)–ERK signalling pathway through activation of BRAF [39]. It is not clear whether expression or activity of SIPA1 is altered in CML cells, but it was shown that expression of BCR–ABL in a growth-factor-dependent haematopoietic cell line activated RAP1 and BRAF [40]. These results indicate that in addition to the well-established RAS signalling pathway, BCR–ABL might activate MEK–ERK signalling through a pathway involving RAP1 and BRAF (Fig. 3). In mice, disruption of the genes encoding SH2-containing inositol-5-phosphatase (SHIP) also leads to a massive expansion of myeloid cells in the lung, bone marrow and spleen [41]. About half of these mice die at 14 weeks of age. Expression of BCR–ABL in growth-factor-dependent haematopoietic cells downregulates SHIP, so inactivation of SHIP somehow contributes to BCR–ABL leukaemogenesis [42]. However, in CML cells isolated from patients, SHIP expression seems to be differently altered in the early and late stages of differentiation [43]. In addition, SHIP-null primitive haematopoietic cells showed modestly reduced growth-factor independence. These results indicate that SHIP could have both positive and negative roles in haematopoiesis, depending on the cell context.

1.5 CML therapies

Allogeneic stem-cell transplantation is the only known curative therapy for CML. However, most patients are not eligible for this therapy, because of advanced age

(making them unable to tolerate the serious side effects of the treatment) or lack of a suitable stem-cell donor [44]. The discovery that BCR–ABL is required for the pathogenesis of CML, and that the tyrosine-kinase activity of ABL is essential for BCR–ABL-mediated transformation, made the ABL kinase an attractive target for therapeutic intervention [45]. Imatinib mesylate (Glivec, previously known as STI571) a potent inhibitor of the tyrosine kinases ABL, ARG, platelet-derived growth factor receptor and KIT has been shown to selectively induce apoptosis of BCR–ABL$^+$ cells [46,47], and is remarkably successful in treating patients with CML [48] (Fig. 4). In newly diagnosed patients with CML in chronic phase, imatinib induces complete cytogenetic response in more than 80% patients. Patients with more advanced phases of CML also respond to imatinib, but this occurs much less frequently and treatment is less durable [48].Today Imatinib is the front line terapy in the CML treatment. However, there are two major obstacles to imatinib-based therapies for patients with CML. One is the persistence of *BCR–ABL*-positive cells this is known as 'residual disease', and is detected by a sensitive nested reverse-transcriptase PCR assay [49,50]. Suppression of the disease therefore relies on continuous imatinib therapy. The other major problem is relapse of the disease due to the emergence of resistance to imatinib [51]. Several mechanisms of resistance have been described, the most frequent of which are the appearance of point mutations in the *BCR–ABL* gene that impair the drug binding [51,52].

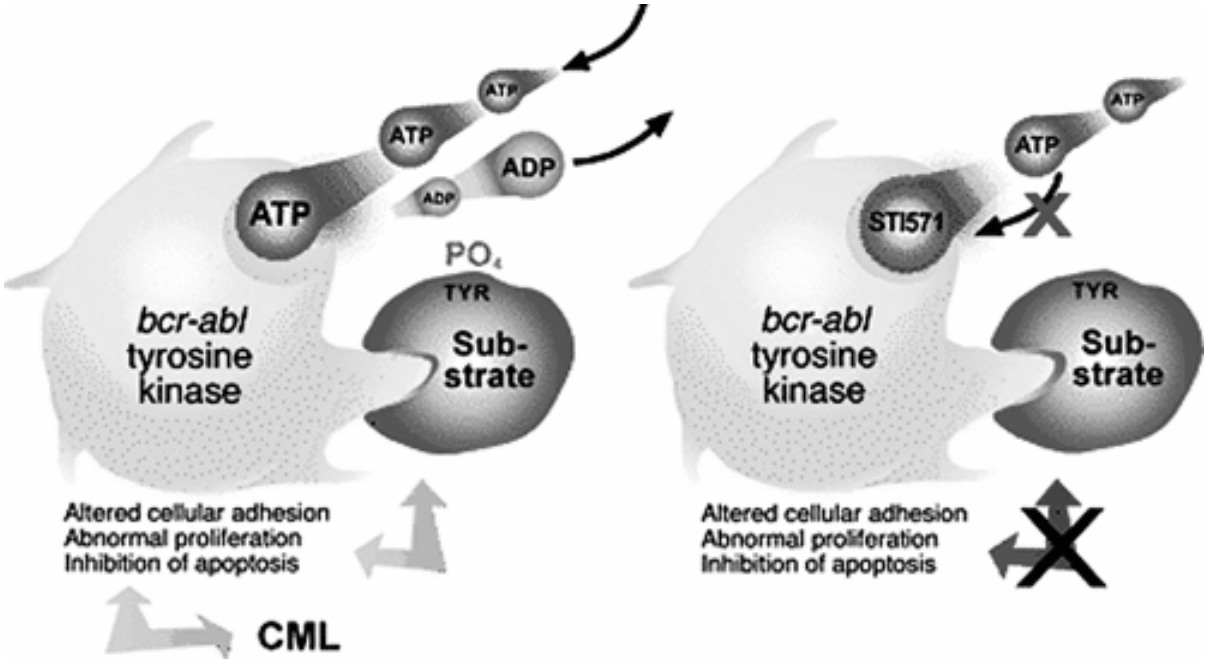

Fig.4. **Imatinib Action**. Imatinib works by binding to the ATP binding site of Bcr/Abl and inhibiting the enzyme activity of the protein competitively. Inibition of Bcr/Abl activity blocks deregulated signaling as alterated cellular adhesion, abnormal proliferation and apoptosis inhibition.

1.6 Clinical resistance to imatinib

The efficacy of imatinib in CML is remarkable, but the development of resistance and the persistence of minimal residual disease have dampened the initial enthusiasm for this much heralded 'magic bullet'. Resistance can be defined on the basis of its time of onset. Primary resistance is a failure to achieve a significant haematological or cytogenetic response, whereas secondary or acquired resistance is the progressive reappearance of the leukaemic clone after an initial response to the drug. Resistance is also defined on the basis of clinical and laboratory criteria used for detection of leukaemia, which includes haematological, cytogenetic and molecular resistance. Haematological resistance is a lack of normalisation of peripheral blood counts and spleen size; cytogenetic resistance is a failure to achieve a major cytogenetic response, i.e. less than 35% Philadelphia (Ph) chromosome positivity; and molecular resistance represents the failure to achieve or the loss of complete or major molecular response (MMR). MMR can be defined as a 3 or more log-reduction of *BCR-ABL*/control gene ratio from a laboratory standardised baseline or an international scale converted *BCR-ABL*/control gene ratio of <0.1% [53]. Recently, the European Leukaemia Net refined response criteria allowing resistance to be categorised into 2 groups, 'suboptimal response' and 'failure to respond' (Table 1). Continuing imatinib treatment is unlikely to be beneficial in those with failure to respond, while the suboptimal responders may still have a benefit in continuing, although with a less favourable long term outcome [54].

Duration of treatment (months)	**Failure**	**Suboptimal response**
3	• No HR	• Less than CHR
6	• Less than CHR	• Less than PCyR
	• No cytogenetic response	
12	• Less than PCyR	• Less than CCyR
18	• Less than CCyR	• Less than MMR
Any time	• Loss of CHR	• Ph-positive clonal evolution
	• Loss of CCyR	
	• Disease progression	• Loss of MMR
	• Mutations with high level imatinib-insensitivity	• Mutations with low level imatinib-insensitivity

Table 1. Definition of failure and suboptimal response for previously untreated CML-early chronic phase patients treated with imatinib 400 mg daily. *Abbreviations*: HR, haematological response; CHR, complete haematological response with recovery of peripheral blood counts; CCyR, complete cytogenetic response (0% Ph chromosome

positive marrow metaphases); PCyR, partial cytogenetic response (1–35% Ph chromosome positive marrow metaphases).

1.6.1 Molecular basis of resistance

Ever since the first reports of resistance were described in 2000, the mechanisms of resistance to imatinib have been extensively studied and three major mechanisms of resistance have been identified. The two most common affect the *BCR-ABL* gene itself, namely mutations in its tyrosine kinase domain and overexpression of the Bcr-Abl protein due to amplification of the *BCR-ABL* gene [55-57]. The third mechanism is less well characterised and understood, and is represented by phenomena which lead to resistance independent of Bcr-Abl. These include upregulation of the drug efflux pumps [55], [58-60], downregulation of drug influx transporters [61-62], binding of the α1-acid glycoprotein (AGP) [63], overexpression of Lyn, a Src kinase [64], and other Bcr-Abl-independent mechanisms [65].

1.6.2 Mutations in the Abl kinase domain

The emergence and selection of clones exhibiting point mutations in the Abl kinase domain is the most frequently identified mechanism of resistance in patients treated with imatinib and is more common in acquired than in primary resistance [66]. These mutations are not *induced* by imatinib, but rather, just like antibiotic resistance in bacteria, arise through a process whereby the drug itself *selects* for rare *pre-existing* mutant clones, which gradually outgrow drug-sensitive cells [67]. Mutations can be categorised into 4 groups: (i) those which directly impair imatinib binding; (ii) those within the ATP binding site; (iii) those within the activation loop, preventing the kinase from achieving the conformation required for imatinib binding; and (iv) those within the catalytic domain (Fig. 5). The substitution of the amino acid threonine with isoleucine at position 315 of the Abl protein was the first mutation to be detected in resistant patients [68]. Based on the crystal structure of the catalytic domain of Abl complexed to a variant of imatinib [69], this substitution was predicted to reduce the affinity for the drug in two ways. Firstly, the oxygen atom provided by the side chain of threonine 315 is not present, and this prevents the formation of a hydrogen bond with the secondary amino group of imatinib. Secondly, isoleucine contains an extra hydrocarbon group on its side chain and this sterically inhibits the binding of the inhibitor [68]. Another amino acid that makes contact with imatinib is phenylalanine 317, and its mutation to leucine also leads to resistance. Mutations can also cluster within the ATP-binding loop (phosphate or P-loop). This domain is a highly conserved glycine-rich sequence that spans amino acids 248–256 and interacts with imatinib through hydrogen and van der Waals bonds [66]. These mutations modify the flexibility of the P-loop and destabilise the conformation required for imatinib binding [70]. Apart from imatinib insensitivity, a feature of clinical relevance is that imatinib-treated patients who harbour P-loop mutations have been suggested to have a worse prognosis than those with non-P-loop mutations [56]. However, this has not yet been confirmed in larger series. The activation loop of the Abl kinase begins at amino acid 381 with a highly conserved motif of 3 amino acid residues

(aspartate–phenylalanine–glycine). This region of the kinase can adopt a closed (inactive) or an open (active) conformation. Imatinib forces Abl into the inactive conformation and is incapable of binding to the active configuration [71]. Mutations in the activation loop may disturb the energetic balance required to stabilise the closed conformation of the loop and thus favour the open, active conformation [70]. Finally, some amino acid substitutions cluster in the catalytic domain, a region that has a close topologic relation to the base of the activation loop. Mutations in this region can also influence the binding of imatinib [70]. At least 73 different point mutations leading to substitution of 50 amino acids in the Abl kinase domain have been isolated from CML patients resistant to imatinib so far, and this number is likely to increase with more sensitive methods of detection (Fig.5).

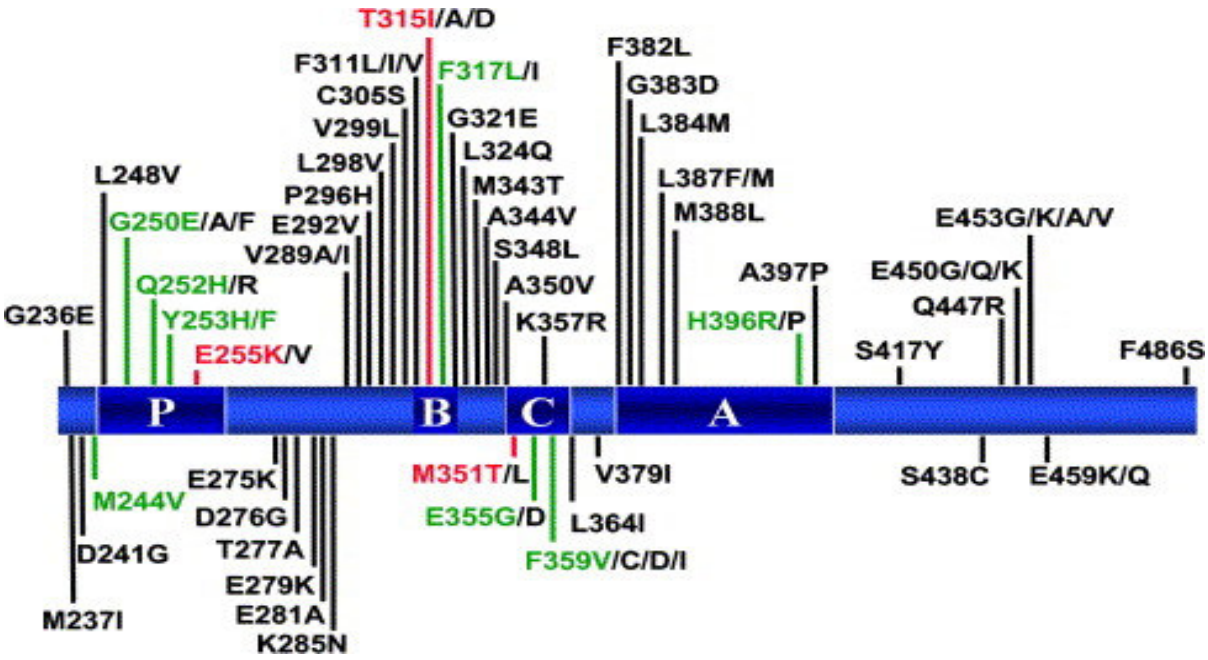

Fig. 5 Map of Bcr-Abl kinase domain mutations associated with clinical resistance to imatinib. *Abbreviations*: P, P-loop; B, imatinib binding site; C, catalytic domain; A, activation loop. Amino-acid substitutions in green indicate mutations detected in 2–10% and in red in >10% of patients with mutations.

The detection of a Ph-positive clone harbouring an Abl kinase domain mutation is associated with resistance to imatinib and may be associated with progression to a more advanced phase disease [56] and [57]. Using highly sensitive assays, mutations have also been detected in patients in complete cytogenetic response, and in imatinib-naïve advanced phase but not chronic phase patients [72]. However, detection of mutations in these patients did not always result in progressive disease while on imatinib. It is likely that mutant clones in the presence of low leukaemic

burden or low levels of mutant clones do not have a similar clinical impact as clones which are detected when disease burden is rising or high [72]. Furthermore, the probability of detecting a clone is low when BCR-ABL transcript levels are stable or declining [73].

1.6.3 Bcr-Abl overexpression

Overexpression of the Bcr-Abl protein due to amplification of the BCR-ABL gene was first observed in vitro when resistant CML cell lines were generated by exposure to gradually increasing doses of imatinib [55], [74]. This phenomenon has been reported in a relatively small proportion of patients, with an overall percentage of 18% [68], [66], but this may be an underestimate if its detection is only based on the cytogenetic findings of Ph chromosome duplication. In one study, 3 out of 11 CML patients in blast crisis who relapsed after initially responding to imatinib were shown to have multiple copies of the BCR-ABL gene by fluorescence in situ hybridisation (FISH) [68]. In another study, 7 out of 55 patients showed a more than 10-fold increase in BCR-ABL transcript levels and 2 out of the 32 patients evaluated were found to have genomic amplification of BCR-ABL by FISH [66]. In the latter 2 patients, resistance was primary and not acquired. Overexpression of Bcr-Abl leads to resistance by increasing the amount of target protein needed to be inhibited by the therapeutic dose of the drug. It is also possible that a transient overexpression of Bcr-Abl may be an early phenomenon in the establishment of imatinib resistance, preceding the emergence of a dominant clone with a mutant kinase domain, as suggested by kinetic studies in cell lines [74].

1.6.4 Drug efflux and influx transporters

Multidrug resistance (MDR) due to cross-resistance of mammalian cells to a number of anticancer agents following exposure to one such drug is a well described mechanism of resistance in cancer therapy. This is mediated by an increased expression at the cell surface of the *MDR1* gene product, Pgp, an energy dependent efflux pump, which reduces intracellular drug concentrations and leads to ineffective levels of the drug reaching its target [75]. Imatinib and other tyrosine kinase inhibitors are substrates of Pgp, and the intracellular levels of imatinib were shown to be significantly lower in Pgp-expressing cells [61], [76-77]. An imatinib-resistant CML cell line generated by gradual exposure to increasing doses of the drug was shown to exhibit Pgp overexpression, and *MDR1* overexpression in CML cell lines also confers resistance to imatinib [55] and [58]. Pgp overexpression has not been reported in patients who are resistant to imatinib. However, the addition of a Pgp pump inhibitor, PSC833, to cultures of imatinib-treated cells from drug-resistant CML patients produced a significant decrease in colony formation, thus suggesting that *MDR1* overexpression may play a role in clinical imatinib resistance [58]. Recently, two other drug transporters, breast cancer resistance protein (BCRP)/ABCG2 and human organic cation transporter 1 (hOCT1), have been implicated as possible mechanisms for promoting imatinib resistance. Imatinib has been variably reported to be a substrate and/or an inhibitor for the BCRP/ABCG2 drug efflux pump which is overexpressed in many human tumours and also found to be functionally expressed in CML stem cells [59-60]. The drug transporter, hOCT1 mediates the active

transport of imatinib into cells, and inhibition of hOCT1 decreases the intracellular concentration of imatinib, which may predict for a less favourable molecular response [61] and [62]. The hOCT1 gene was also found to be expressed in significantly higher levels in patients who achieved a complete cytogenetic response to imatinib than in those who were more than 65% Ph chromosome positive after 10 months of treatment. This would suggest that patients with low baseline expression of *hOCT1* may not achieve a complete cytogenetic response because of insufficient intracellular levels of imatinib.

1.6.5 Bcr-Abl-independent mechanisms

The Src family kinases, Lyn and Hck, are activated in *BCR-ABL*-expressing cell lines. Lyn is overexpressed and activated in an imatinib-resistant CML cell line generated by incubation of the parental line in increasing concentrations of imatinib and in samples from CML patients who were resistant to imatinib [64]. Lyn suppression by a Src kinase inhibitor resulted in reduced proliferation and survival of the imatinib-resistant but not the sensitive cell line [64]. Microarray analysis have shown that transcripts from genes with anti-apoptotic or malignant transformation properties and with involvement in signal transduction/transcriptional regulation are overexpressed in CML cells innately resistant to imatinib. This would suggest that pathways downstream of Bcr-Abl and independent of its kinase activity may be important factors which confer resistance to imatinib [65].

1.7 SHP-1 and SHP-2 phosphatases

Although rare in chronic phase myeloid leukemia (CML), primary or acquired resistance to the treatment with tyrosine kinase inhibitors (TKI) Imatinib may be observed in the advanced phases of disease. Bcr/Abl related mechanisms of resistance, i.e. K. D. mutations have been well described, while the other mechanisms of resistance are poorly understood. Many studies have been focused on the role of other tyrosine kinases in BCR/ABL transformed cells as modulation of leukemic phenotype and as determinants of resistance to the treatment with Imatinib. Howewar, very few data are available on the possible role of tyrosine phosphatases in Ph+ cells and in patients, who lack or loose response to the Imatinib treatment. In this study, we investigate the role of two SH2-containing, non-receptor protein tyrosine phosphatases (Shp1 and Shp2) in the resistance to Imatinib (Ima). SHP-1 is generally considered as a negative signal transducer and SHP-2 as a positive one. However, the precise role of each enzyme in shared signaling pathways is not well defined. SHP-1 and SHP-2 are Src homology 2 (SH2) domain-containing tyrosine phosphatases. High expression of SHP-1 and SHP-2 in the hematopoietic system suggests that these two tyrosine phosphatases play important roles in hematopoietic cell functions. SHP-1 is highly expressed in hematopoietic cells and, at a lower level, in various nonhematopoietic cells, whereas SHP-2 is ubiquitously expressed [78]. Both SHP-1 and SHP-2 have important physiological roles and may be implicated in neoplastic transformation of hematopoietic cells. SHP-1 generally exerts a negative effect upon the hematopoietic differentiation of

embryonic stem cells (ESC) and may act at different stages of embryonic stem cell differentiation [79]. In mice, mutation of the SHP-1 gene is responsible for the motheaten and viable motheaten phenotypes [80-81]. In humans, reduction of SHP-1 gene expression is observed in natural killer cell lymphomas as well as other types of lymphomas/leukemias [82-83]. Methylation of the SHP-1 promoter causes loss of SHP-1 expression in malignant T-lymphoma cells [84]. Interestingly, decreased expression level of SHP-1 is associated with progression of Chronic myeloid leukaemia (CML) [85]. Moreover, Shp1 was shown to be physically associated with Bcr-Abl with evidence supporting their functional interaction [86-87]. More recent studies showed that Shp1 protein and mRNA are absent in the CML blast cell line K562 [87-88], whereas differentiation of K562 cells was associated with regulation of Shp1 expression [87]. On the other hand, forced expression of Shp1 blocks transformation by Bcr-Abl [89]. These observations indicate that Shp1 plays a significant role in the negative regulation of Bcr-Abl and that lack of Shp1 is important for CML transformation. On the other hand, SHP-2 has been shown to be required for development of hemangioblast and progenitor hematopoietic cell development [90]. Down modulation of SHP-2 expression, through SHP-2 siRNA transfection, significantly decreases the formation of hematopoietic progenitor formation and also blocks fibroblast growth factor induced hemangioblast formation [91]. Development of embryonic cells with a homozygous SHP-2 mutation leads to the suppression of hematopoietic cell differentiation. Activation mutation of SHP-2 causes Noonan syndrome [92], an autosomal dominant disorder characterized by dysmorphic facial features, proportionate short stature, and heart disease (most commonly pulmonic stenosis and hypertrophic cardiomyopathy). This gain-of-function mutation of SHP-2 is also associated with sporadic juvenile myelomonocytic leukemia, myelodysplasic syndrome, acute lymphoblastic leukemia, and acute myelogenous leukemia [93-94]. Furthermore, SHP-2 is an intracellular target of *Helicobacter pylori* CagA protein [95] that is associated with severe gastritis and gastric cancer. SHP-1 and SHP-2 share over 55% sequence identity and are regulated by similar mechanisms. However, their biological functions seems to be quite different (Fig.6) [96]. Since, motheaten and viable motheaten mice, which have germiline mutation of the SHP-1 gene, develop a severe auto-immune and immunodeficiency syndrome characterized by increased numbers of many hematopoietic cells [80-81]; SHP-1 is generally considered a negative regulator of cell proliferation. At molecular level SHP-1 dephosphorylates receptors of growth factors, cytokines, and antigens, and tyrosine-phosphorylated proteins associated with these receptors, and therefore, it is often defined as a negative regulator of signal transduction and of cytokine signals acting on hematopoietic cells. On the other hand, SHP-2 is generally considered as a positive signal transducer. Cells expressing a catalytically inactive cysteine-to-serine mutant of SHP-2 and those derived from SHP-2 knock-out mice show reduced activation of signal transduction pathways induced by growth factors and cytokine [78,97].

1.8 Aim of work

The aim of this work is the study of the Bcr-Abl-independent signaling pathways involved in imatinib-resistance in chronic myeloid leukemia (CML) patients. To this aim, we have first used, as model system a couple of, Ima-sensitive (KCL22s) and

Ima-resistant (KCL22r) KCL22, CML cell lines. The Imatinib-resistant cell lines KCL22r and its sensitive counterpart KCL22s were established by Junia Melo and co-workers [55] to analyse primary Imatinib resistance *in vitro.* The parental cell line KCL22s is initially resistant to Imatinib and consist of sensitive and resistant subpopulations, witch can be isolated from the original cell line using methylcellulose. In KCL22r cells the resistance is innate and is not associated to mutations in the ATP-binding site of BCR/ABL or BCR/ABL overexpression [55]. Moreover, this cell couple exhibits same of the features typical of Ph+ $CD34^{+}/CD38^{-}$ $/Lin^{-}$ cells when exposed to Ima. Indeed, Ima exposure induces growth arrest but not apoptosis in KCL22s cells. Therefore, they represent an optimal *in vitro* system to analyse Imatinib resistance witch is based on a BCR/ABL-independent mechanisms.

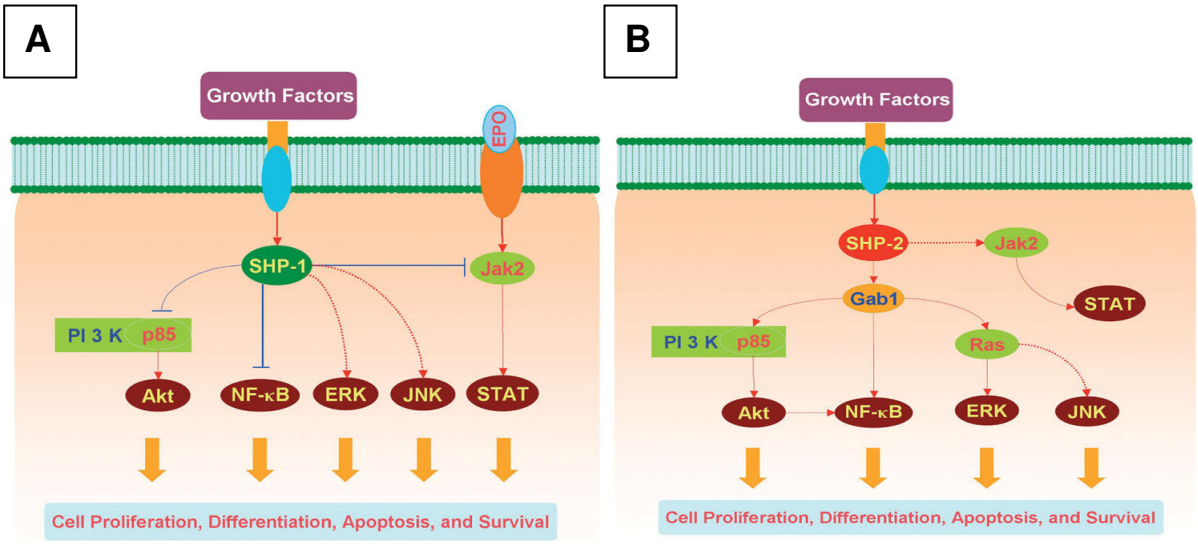

Fig.6. A. Illustration of potential signaling pathways controlled by Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 1 (SHP-1). SHP-1 plays an important role in the regulation of growth factor and cytokine signal transduction to modulate cell proliferation, differentiation, survival, and apoptosis. SHP-1 can regulate growth factor induced activation of phosphatidylinositol 3-kinase (PI 3-K)/Akt and nuclear factor-kappa B (NF-κB). SHP-1 may either negatively or positively regulate the activation of the extracellular signal-related kinases (ERKs) and the c-Jun-amino terminal kinases (JNKs). In addition, SHP-1 can bind to the erythropoietin (EPO) receptor via its SH2 domain and modulate EPO activation of the Janus kinase 2 (Jak2)/signal transducer and activator of transcription (STAT). **B.** Illustration of potential signaling pathways controlled by Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP-2). Similar to SHP-1, SHP-2 has a critical role in a host of cellular signal transduction pathways that involve cell proliferation, differentiation, survival, and apoptosis. Through the association with Grb2-associated binder-1 (Gab1), a docking protein containing an N-terminal pleckstrin homology domain and several proline-rich SH3 domain-binding sequences, SHP-2 promotes growth factor induced activation of phosphatidylinositol 3-kinase (PI 3-K)/Akt, the extracellular signal-related kinases (ERKs), and nuclear factor-kappa B (NF-κB).

SHP-2 can either negatively or positively regulate the activation of Janus kinase 2 (Jak2)/signal transducer and activator of transcription (STAT) and the c-Jun-amino terminal kinases (JNKs) depending on different circumstances.

2 Materials and methods

2.1 Drug

STI571 was kindly provided by Novartis Pharma (Basel, Switzerland). A 10 mM stock solution was prepared by dissolving the compound in sterile phosphate-buffered saline (PBS) or dimethylsulfoxide (DMSO).

2.2 Cell lines

The K562 and KCL22s cells ($5x10^5$ cells/mL) were grown in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) and 1 mM L-glutamine, 100 U/ml penicillin, and 50 µg/ml streptomycin (herein referred to as RP-10) in a humidified 95% O_2 and 5% CO_2 atmosphere at 37°C. For KCL22r imatinib-resistant cell line, we used same conditions but the media was further supplemented with 1µM Imatinib. This cell line has been supplied from professor Junia V. Melo of the Department of Haematology, Imperial College School of Science, Technology and Medicine, Hammersmith Hospital, London, UK.

2.3 DNA and RNA extraction

Mononuclear cells were isolated from BM aspirates by density gradient centrifugation, according to standard procedures. High-molecular-weight genomic DNA was performed with a commercially available kit (QIAamp DNA, Qiagen, Milan, Italy). Total RNA was extracted using the RNeasy kit (Qiagen, Milan, Italy) and treated with RNase-free DNase (Qiagen), according to the manifacturer direction.

2.4 Methylation-Specific Polymerase Chain Reaction (MSP)

The methylation-specific polymerase chain reaction (MSP) for promoter methylation was performed as described [104-105]. Briefly, treatment of DNA with bisulfite (which resulted in conversion of unmethylated cytosine to uracil, but unaffecting methylated cytosine) was performed with a commercially available kit (EpiTect Bisulfite Kit, Qiagen,Milan,Italy). MSP primers were designed to amplify the methylated (M-MSP) and unmethylated (U-MSP) alleles. Primers for *PTPN6* were: 5'-GTG AAT GTT ATT ATA GTA TAG TGT-3' (forward) and 5'-TTC ACA CAT ACA AAC CCA AAC AAT-3' (reverse) for the unmethylated reaction; 5'-GAA CGT TAT TAT AGT ATA GCG TTC-3' (forward) and 5'-TCA CGC ATA CGA ACC CAA ACG-3' (reverse) for the methylated reaction [88]. The annealing temperature for unmethylated reactions was 59°C and for methylated reaction was 60°C. All MSP reactions were performed with positive and negative controls for both unmethylated and methylated alleles. DNA from normal samples was used as negative control, and methylated DNA (CpGenome Universal Methylated DNA; Intergen) was used as positive control. Control experiments without DNA were performed for each set of PCR.

2.5 DNA sequencing

The identity of the methylated and unmethylated sequences was confirmed by automated DNA sequencing. PCR products were gel purified, sequenced

bidirectionally, and analyzed on an automated DNA sequence analyser (3730 ABI Prism; Applied Biosystems).

2.6 5-Azacytidine (5-AC) treatment of the Leukemic Kcl22 cell lines

The human leukemic cell lines Kcl22s/r were seeded in 25 cm^2 culture flasks at a density of $5*10^5$/mL and treated with 1-2 µM 5-AC (Sigma, St Louis, MO) for 5 days, with fresh medium containing 5-AC replenished on day 2. Kcl22s/r cells were harvested every day for genomic DNA, total cellular RNA, and whole cell protein extractions.

2.7 Reverse transcription polymerase chain reaction (RT-PCR)

One microgram of total RNA extracted from the patient samples or cell lines, was pre-warmed for 10 minutes at 70 °C; the RNA solution was then incubated for 10 min at 25 °C, 45 min at 42 °C and 3 min at 99 °C in a 20 µL reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5.5 mM $MgCl_2$, 1 mM of each deoxyribonucleotide, 20 U of RNAsin (Pharmacia, Upsala, Sweeden), 25 mM random examers (Pharmacia), 10 mM of DTT (Pharmacia), and 100 U of MoMLV reverse transcriptase (Invitrogen).PCR amplification of SHP-1 and SHP-2 encoding cDNAs were separately carried out in a reaction mixture consisting of 1 × Master Mix (Applied BioSystem, Foster City, CA USA), 300 nM of the appropriate primer pair and 200 nM of the appropriate probe in a final volume of 25 µL using the following time/temperature profile: 95 °C, 15 s, and 60 °C, 1 min, for 50 cycles. All amplification reactions were carried out in triplicate. The primers and probes sequences were as follows: SHP1: 139bp; Forward: CGAGGTGTCCACGGTAGCTT, Reverse:CCCCTCCATACAGGTCATAGAAAT, Probe: Fam-TGACCCATATTCGGATCCAGAACTCAGG-Tamra; SHP2: were purchased assay on the made (Applied BioSystem, Foster City, CA USA). ABL was used as an internal control. SHP1 and SHP2 mRNA was normalized to ABL to obtain the relative threshold cycle (ΔCT) and then related to the ΔCT of normal cases to obtain the relative expression level ($2^{-\Delta CT}$). All reaction were performed using an ABI-7900 sequence detector (Applied BioSystem, Foster City, CA USA).

2.8 SHP1 and SHP2 shRNA

To knock-down of SHP1 and SHP2 we used a vector that direct the transcription of a short-hairpin RNA (shRNA). In particular, shRNAs transcribed by RNA polymerase III (Pol III) promoters can trigger sequence-selective gene silencing in culture and in vivo. The vectors shSHP1, shSHP2 as well as a shRNA Non-silencing (NS) control vector were purchased from Open Biosystem (Huntville, AL, USA) as glycerol stocks of transformed *E. coli*. The base vector for both plasmids is a retroviral pShag Magic version 2 (pSM2). The pSM2-SHP1 and pSM2-SHP2 are designed for high level expression of shRNA for the human SHP1 and SHP2 under the control of the mouse U6 promoter. The pSM2-NS represents a negative control shRNA vector. *E. coli* were grown according to the manufacturer`s recommended protocols. The plasmids were purified using the Plasmid kit (Qiagen, Milan, Italy), according to the manifacturer direction. The sequence of the shRNA insert was confirmed using a primer developed for the U6 promoter region ( 5` TGT GGA AAG GAC GAA ACA

CC 3`). Sequence analysis was performed using an automated DNA sequence analyser (3730 ABI Prism; Applied Biosystems).

2.9 Cell transfection

We seeded $5x10^5$ KCL22 cells in 500 µl of growth medium with serum but without antibiotics, in a well of a 24-well plates and transfected them 24 h later with 1 µg for well of the appropriate plasmid. The transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) in accordance with the manufacturer's protocols. We determined transfection efficiency by parallel transfection of a GFP-expressing plasmid and assayed the percentage of fluorescent cells by flow cytometry. Forty-eight hours after transfection we selected for stably transfected cells. We transferred the transfected cells to medium containing puromycin (1µg/mL) for 2 days. For analysis of target gene mRNA knock-down, we collected stably transfectes cells lysates and prepared total RNA. Expression of SHP1 and SHP2 (mRNA and Proteins) was monitored by real time and western blot.

2.10 Cell viability assay

Cells were plated at a density of 5×10^5 cells/mL in RP-10 with or without the inhibitor. Aliquots were taken out at 24-hour intervals for assessment of cell viability by trypan blue exclusion.

2.11 Immunoblotting and immunoprecipitation analysis

Cell lines were washed with cold phosphate-buffered saline three times. They were incubated with cold lysis buffer, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1mM NaF, 1 mM PMSF, 1% Nonidet P-40, 1mM EDTA, 1mM sodium orthovandate, Protease inhibitor cocktail (Complete mini EDTA-free Roche Applied Science) for 30 min and then cleared by centrifugation at 15,000xg for 20 min at 4°C. Protein concentration was measured by using the 2-D Quant kit (GE Healthcare). For immunoblot analysis KCL22r and KCL22s protein extracts (20 µg) were resolved on a 10% SDS-PAGE gel and than transferred onto nitrocellulose membrane (GE Healthcare). The membrane was blocked in 5% non-fat milk in PBS for 2 h and incubated with 1% milk/PBS1X /0,05% TWEEN containing a monoclonal antibody against SHP2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at a diluition of 1:500 or a polyclonal antibody against SHP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at a diluition of 1:500. An anti-GAPDH antibody was used as control, at a diluition of 1:1000. Immunoblot detections were carried out using HRP-conjugated anti-rabbit or anti–mouse secondary antibodies (Ge Healthcare) used respectively at a diluition of 1:10000 and 1:5000. Detection was made by using the ECL-Advance Western Blotting Detection kit (GE Healthcare) by chemioluminescence. The resulting western blot image were scanned and analyzed by PDquest 7.1 software (Biorad). Protein bands were defined , background was substracted and volumes were measured. Band volumes were normalized by using actin as control, visualized on the same membrane. For immunoprecipitation assays, cell lysates (500 µg) were precleaned with the appropriate control, normal mouse or rabbit IgG (Santa Cruz

Biotechnology, Santa Cruz, CA, USA), corresponding to the host species of the primary antibody used for the immunoprecipitation, for 2h at 4°C and then incubated with a resuspended volume of Protein A/G-PLUS-Agarose beads (Santa Cruz Biothecnology Santa Cruz, CA, USA) for 1h at 4°C. Beads were then centrifugated at 8000xg for 5 min at 4°C. The supernatant was incubated with primary polyclonal antibodies (20 ul) of anti–SHP1 or anti–SHP2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C and then incubated with a fresh volume of Protein A/G-PLUS-Agarose beads for 5h at 4°C. Beads were then centrifugated at 8000xg for 5 min at 4°C. After extensive washing of the pellet beads with IP buffer (Tris-HCl 50 mM pH 7.5, NaCl 150 mM, NaF 1mM, PMSF 1 mM, Nonidet P-40 1%, 1mM EDTA, 1mM sodium orthovandate, Protease inhibitor cocktail (Complete mini EDTA-free Roche Applied Science) the resulting immune complexes were eluted from the beads with 2X electrophoresis sample buffer at 90°C for 10 min. The supernatants were then separated by electrophoresis in 10% SDS/polyacrylammide gels and then transferred to nitrocellulose paper for immunoblotting analysis, performed as described above.

2.12 Cell Cycle Analysis

Cells were washed once in PBS then incubated for 15 min with the DNA binding dyes propidium iodure (10 µg/ml,Immunotech SA, France). Thereafter, the cells were analysed by flow cytometry using forward and side scattering to exclude any cell debris of the analysis. The percentage of apoptotic cells was determined in the hypodiploid peak resulting of PI fluorescence.

2.13 Proliferation assay

Cells were plated at a density of 5×10^5 cells/mL in RP-10 with or without the drug. The proliferation was assayed by incubation with 10 µM bromodeossiuridine (BrdU) (Becton Dickinson, USA) for 30 min at 37°C; cells were then treated with 0.5% Tween 20 (Merck) centrifuged at 2000 rpm for 5 min, resuspended in 0.5 ml 0.5% Tween 20 in PBS and in 0.5 ml 4 N HCl and incubated for 30 min at room temperature. After centrifugation, cells were suspended in 1 ml 0.1 M Borax (Riedel-de Haen), centrifuged, incubated for 1 h at 4°C in 200 µl 0.5% Tween 20 in PBS containing 5 µl anti-BrdU monoclonal antibody (Becton Dickinson, USA), centrifuged, incubated for 30 min at 4°C in 200 µl 0.5% Tween 20 in PBS and 5 µl of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulins (Becton Dickinson, USA), centrifuged, incubated for 15–30 min at 4°C in 200 µl 0.5% Tween 20 in PBS and in 200 µl propidium iodide (Sigma) and finally analyzed on a fluorescence activated cell sorter FACScan (Becton Dickinson, USA).

3 Results and Discussion

Although rare in chronic phase myeloid leukemia (CML), primary or acquired resistance to the treatment with tyrosine kinase inhibitors (TKI) may be observed in the advanced phases of disease. Bcr/Abl related resistance has been well described, while the other mechanisms of resistance are poorly understood. In this study, we investigated the role of two SH2-containing, non-receptor protein tyrosine phosphatases (Shp1 and Shp2) in the resistance to Imatinib (Ima). To this aim, we have first used, as model system, a couple of Ima-sensitive (KCL22s) and Ima-resistant (KCL22r) KCL22, CML cell lines. The Imatinib-resistant cell lines KCL22r and its sensitive counterpart KCL22s were established by Junia Melo and co-workers [55] to analyse primary Imatinib resistance *in vitro.* The parental cell line KCL22s is initially resistant to Imatinib and consist of sensitive and resistant subpopulations, witch can be isolated from the original cell line using methylcellulose. In KCL22r cells the resistance is innate and is not associated to mutations in the ATP-binding site of BCR/ABL or BCR/ABL overexpression [55]. Moreover, this cell couple exhibits some of the typical features of Ph+ $CD34^{+}/CD38^{-}/Lin^{-}$ cells when exposed to Ima. Indeed, Ima exposure induces growth arrest but not apoptosis in KCL22s cells. Therefore, they represent an optimal *in vitro* system to analyse Imatinib resistance witch is based on a BCR/ABL-independent mechanisms. In particular, in this study, we investigate the role of two SH2-containing, non-receptor protein tyrosine phosphatases (Shp1 and Shp2) in the resistance to Imatinib (Ima). In a preliminary evaluation, using in parallel both a microarray and a proteomic approach, we found that the cell lines examined had a great divergence in SHP-1 expression and that SHP-1 was down-regulated in resistant KCL22r cell line. Real time PCR and western blot analysis, confirmed a very low level of Src homology 2 domain-containing tyrosine phosphatases SHP-1 (both mRNA and protein), a protein with a tumour suppressor activity, in the KCL22r resistant cells, when compared to KCL22s sensitive cells (Fig.7). While, SHP-2 seems to be equally expressed between two cell lines (both mRNA and protein),(Fig.7).

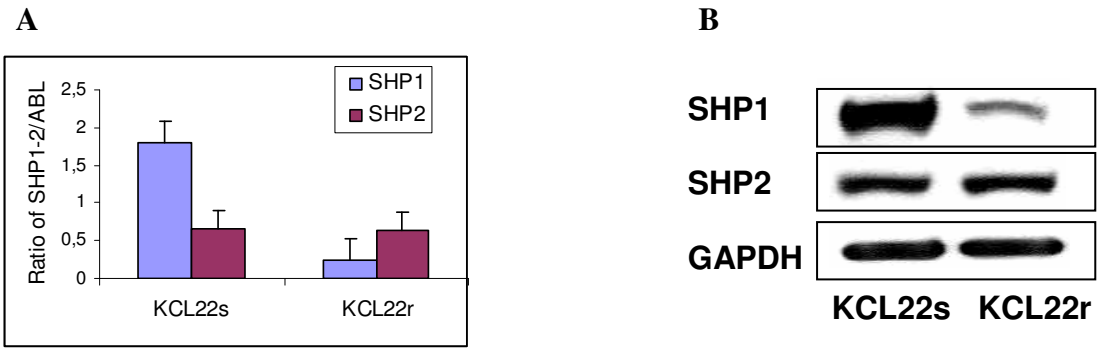

Fig.7. A Levels of SHP-1 and SHP-2 mRNA in KCL22 cell lines performed by real time PCR. All experiment were performed in triplicate and results were indicate as mean±SD. **B.** Western blot analysis on total protein lysates of KCL22r cells and KCL22s cells. Proteins were separated on 10% SDS-PAGE and immunoblotted respectively with antibodies against SHP2 and SHP1 proteins GAPDH was used as control.

SHP-1 is highly expressed in hematopoietic cells and, at a lower level, in various nonhematopoietic cells [26]. In humans, reduction of SHP-1 gene expression is observed in natural killer cell lymphomas as well as other types of lymphomas/leukemias [82-83]. Decreased expression level of SHP-1 seems to be associated with progression of Chronic myeloid leukaemia (CML) [85]. Moreover, Shp1 was shown to be physically associated with Bcr-Abl with evidence supporting their functional interaction [86-87]. More recent studies showed that Shp1 protein and mRNA are absent in the CML blast cell line K562 [87-88], whereas differentiation of K562 cells was associated with Shp1 expression [87]. Furthermore, overexpression of Shp1 blocks transformation by Bcr-Abl [89]. These observations indicate that Shp1 might plays a significant role in the negative regulation of Bcr-Abl and that lack of Shp1 is important for CML transformation. To identify the reason of the SHP-1 down-regulation in KCL22r cell line we approached a methylation-specific PCR (MSP) analysis on SHP-1 promoter. This analysis showed that the down-regulation of this gene is related to the methylation level of its promoter. In fact, only KCL22r showed the SHP-1 promoter methylated respect to KCL22s cell line (Fig.8). The identity of the methylated and unmethylated sequences was confirmed by automated DNA sequencing (Fig.9).

Fig.8 Methylation-specific polymerase chain reaction (MSP) of KCL22 cell lines. MSP for SHP1 showing that SHP1 Methylation is present in KCL22r and absent in KCL22s. B indicates blank; P, positive control; U, Unmethylated allele; M, Methylated allele; MW molecular weight.

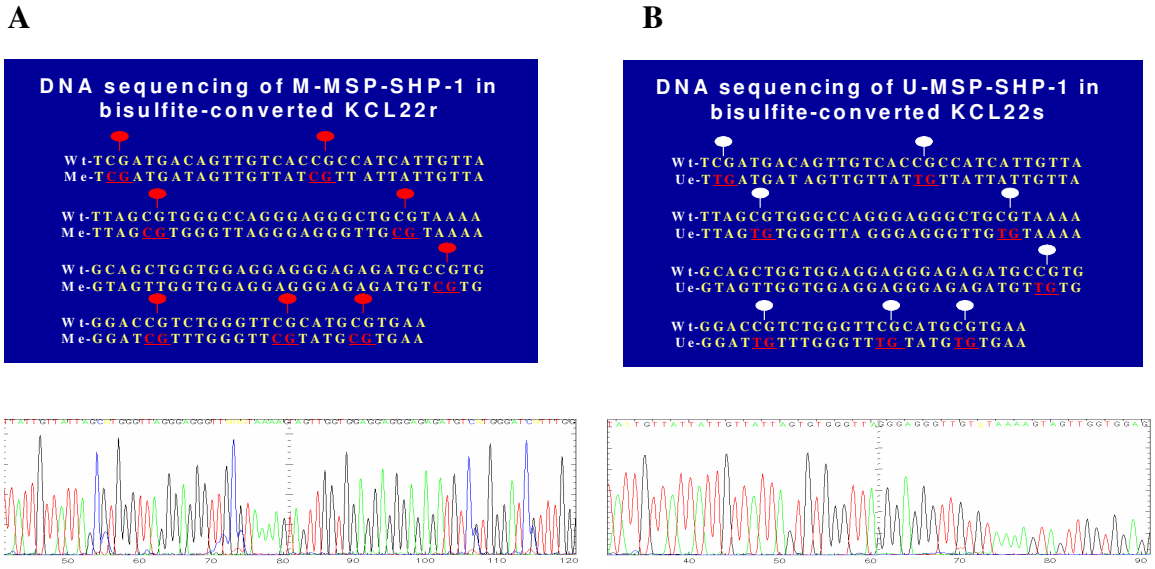

Fig.9 Sequencing of MSP SHP1. (A) Sequencing of M-MSP SHP1 in bisulfite converted KCL22r. The DNA sequence of the "methylated" Me PCR product was aligned and compared with the germ line sequence of the wild-type DNA (Wt). Methylated cytosine residues in CpG dinucleotide remained as C, whereas unmethylated cytosine read as T after bisulfite conversion. (B) Sequencing of U-MSP SHP1 in bisulfite converted KCL22s. The DNA sequence of the "unmethylated" Ue PCR product was aligned and compared with the germ line sequence of the wild-type DNA (Wt).

DNA methylation, catalyzed by DNA methyltransferase, involves the addition of a methyl group to the carbon 5 position of the cytosine ring in the CpG dinucleotide, leading to a conversion to methylcytosine. In many cancers, the CpG islands of selected genes are aberrantly methylated (hypermethylated), resulting in transcriptional repression of these genes. This may serve as an alternative epigenetic mechanism of gene inactivation. DNA methylation has been thought to be one of the important mechanisms of gene silencing and development of cancer. Some studies revealed that reduced expression of the SHP-1 gene in various types of leukemias, multiple myeloma and lymphomas mainly occurred by promoter methylation [88, 98]. To study the biologic significance of SHP-1 methylation, we treated the KCL22 cell lines with a DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-AC) that induces DNA demethylation and re-expression of epigenetically silenced genes. Treatment with 5-AC led to a progressive demethylation of SHP-1 in KCL22r that started from day 2 onward, as shown by positive U-MSP with increasing amplification intensity (Fig.10). The progressive demethylation of SHP-1 was associated with a parallel re-expression of SHP-1 mRNA and protein (Fig.10). Interestingly, the re-expression of Shp1 resulted in a corresponding down-regulation of phosphorylated STAT3 and Erk1/2 (Fig.10), two important signal mediator involved in proliferation and differentiation of myeloid lineage. The level of nonphosphorylated STAT3 and Erk1/2 remained unchanged, showing that SHP-1 re-expression interfered with phosphorylation of STAT3 and Erk1/2. In sensitive KCL22s cell line treated with 5-AC, we didn`t find variations in the same molecular pathway after treatment (Fig.10). Therefore, the biologic consequence of SHP-1 gene methylation in KCL22r, was repression of SHP1 expression and, hence, unopposed STAT3 and Erk1/2 phosphorylation. On the other hand, demethylation leading to re-expression of SHP-1 resulted in down-regulation of phosphorylation of STAT3 and Erk1/2. These results implied that the epigenetic control of SHP-1 expression might be critically involved in the regulation of STAT3 and Erk1/2 phosphorylation in KCL22r cell line. The proliferation and cell cycle of the same cell lines, exposed to 1 and 2 µmol/L 5-AC, have been analyzed by bromodeossiuridine (BrdU) and propidium iodide (PI) assay to evaluate the effects of the SHP-1 re-expression in resistant KCL22r cell line. In particular, KCL22 cell lines were exposed to 1 or 2 µM 5-AC for 4 days. Everyday we collected and harvested cell aliquots that were treated with PI or BrdU as described in materials and methods. After all samples were analyzed by flow cytometry. The results, showed that the 5-AC treatment reduces the proliferation capacity both KCL22s and KCL22r cell lines. Moreover, we found a increase percentage of cells in G_0/G_1 phase only in resistant KCL22r cell line (Fig.11).

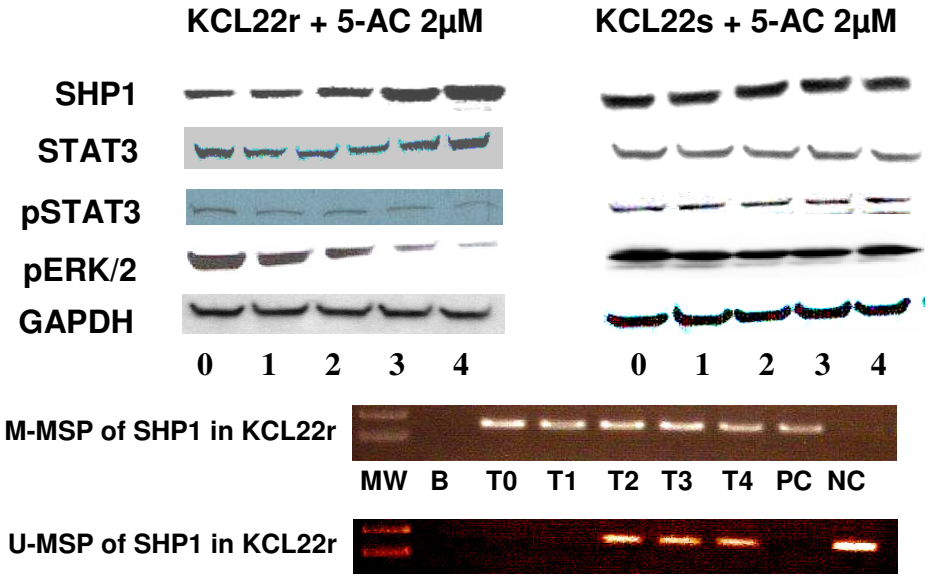

Fig.10. 5-Azacytidine (5-AC) treatment of the KCL22r and KCL22s cell lines. The KCL22r cell line was totally methylated at SHP1, as shown by positive amplification only in M-MSP and not U-MSP at day 0 (T0) before treatment with 5-AC. On treatment with 5-AC, positive amplification appeared in U-MSP on day 2 (T2), indicating SHP1 demethylation. The progressive demethylation of SHP1 was associated with increasing re-expression of SHP1, as shown by Real time PCR and Western blot. Following treatment we observed an increase in the expression of SHP-1 in KCL22 resistant cell line. This data supports the hypothesis that SHP-1 promoter hypermethylation is related to the down-regulation of SHP-1 in resistant cell line. In addiction we observed a reduction of pSTAT3 and pERK1/2 expression in KCL22 resistant cell line in contrast of KCL22 sensitive cell line. Comparable protein loading was shown by GAPDH. MW indicates molecular weight marker; B, blank; TO to T4, days 1 to 4 after 5-AC treatment; NC, normal control; PC, positive control with methylated DNA.

Therefore, 5-AC treatment confirm that aberrant hypermethylation of SHP-1 promoter lead to a decreased of its expression in KCL22r cell line and this assets proliferation and survival alternative pathway, BCR-ABL independent, as JAK-STATs and MAPKs. Subsequently, we found the SHP-1 binding proteins. By immunoprecipitation and western blot analysis we found the interaction of SHP-1 with another Src homology 2 domain-containing tyrosine phosphatases SHP-2 only in sensitive KCL22s but not in resistant KCL22r cell line that showed also low level of SHP-1 (Fig.12). As control we analyzed the SHP-1 binding proteins also in the K562 CML cell line that is sensitive to Imatinib treatment and also in this case we found the interaction between SHP-1 and SHP-2. Previous experiments of real time PCR and western blot, showed that SHP-2 is equally expressed in sensitive and resistant KCL22 cell lines (Fig.7). It has been well accepted that signal transduction is carried through formation of multiple signaling complexes.

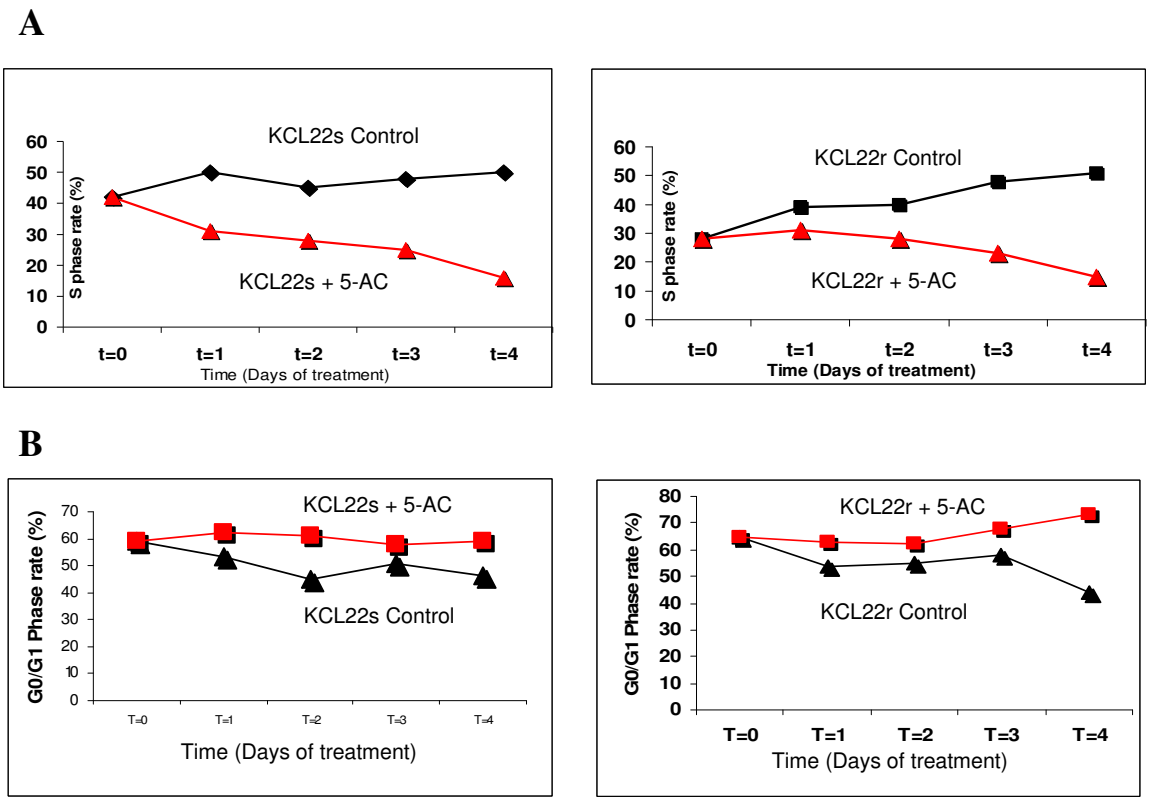

Fig.11. A Proliferation assay by BrdU test on KCL22 cell lines exposed to 1 µmol/L 5-AC for 4 days **B.** Cell cycle assay by (PI) assay on KCL22 cell lines exposed to 1 µmol/L 5-AC for 4 days. As control we used untreated cell lines.

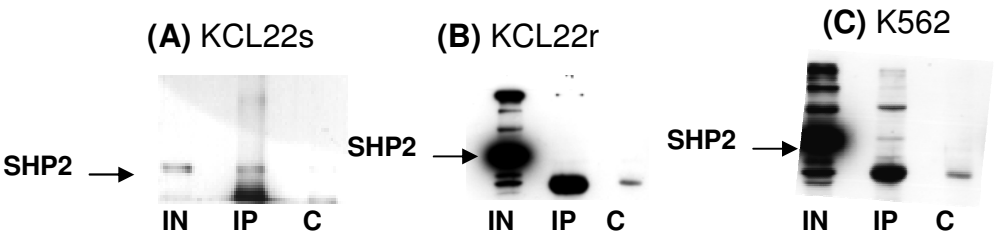

Fig.12. KCL22 sensitive **(A)** and resistant **(B)** cellular extracts were immunoprecipitated (IP) with anti-SHP1 antibody and the immunoprecipitates were subjected to Western blotting analyses with an anti-SHP2 antibody as indicated. K562 **(C)** cellular extracts were immunoprecipitated (IP) with anti-SHP1 antibody and the immunoprecipitates were subjected to Western blotting analyses with an anti-

SHP1 antibody as indicated. An irrelevant rabbit IgG was used as control. IN = input, IP = immunoprecipitate, C = control.

This is particularly true for SHP-1 and SHP-2 because both enzymes stay largely inactive in cytosol because of their internal suppressed structures [99-100]. Furthermore, SHP-1, and SHP-2 formed a signaling complex with GAB1, and SHP-1 and SHP-2 interact with each other in colorectal adenocarcinoma Caco-2 cell line, but the functional role of this interaction in growth factor signaling is still unknown [101]. In order, to understand if the down-regulation of SHP-1 and its interaction with SHP-2 could be involved in KCL22 Imatinib resistance we approached other functional analysis. We used the RNA interference technique, that has been widely used to study the loss-of-function phenotype of genes, to knock down the expression of SHP-1 in sensitive KCL22s and SHP-2 in resistant KCL22r cell lines. For this reason we employed commercially available short hairpin RNA (shRNA). Using Lipofectamine 2000 (Invitrogen) as described in materials and methods, we transfected KCL22r cell line with a specific retroviral plasmid containing a specific sequence that originates a small hairpin RNA (ShRNA) that interfering SHP-2 expression. We done the same way in KCL22s but in this case we interfering SHP-1 expression. As control we transfected the cell lines with a empty plasmid. Results are shown in (Fig.13) SHP-1 shRNA specifically suppressed the expression of SHP-1 in KCL22s by 70% but had no effect on that of SHP-2. Conversely, the shRNA of SHP-2 specifically knocked down the expression of SHP-2 in KCL22r by 75% but left SHP-1 intact. We also examined the protein levels of GAPDH to confirm that they were not affected by the siRNAs. The data suggest that these shRNAs worked very efficiently to suppress the expression of SHP-1 and SHP-2. This provides an excellent system to study the function of these enzymes in KCL22 Imatinib resistance. We employed phospho-specific antibodies to determine the activation of ERK1/2 and STAT3 in knockdown SHP-2 KCL22r cells. As shown in (Fig.13), knockdown of SHP-2 in KCL22r cells significantly reduced the activation of ERK1/2 and STAT3 . The magnitude of ERK1/2 activation was reduced by about 80% and STAT3 by about 70%. These data are very similar to those observed with cells overexpressing dominant negative mutants of SHP-2 [102-103].

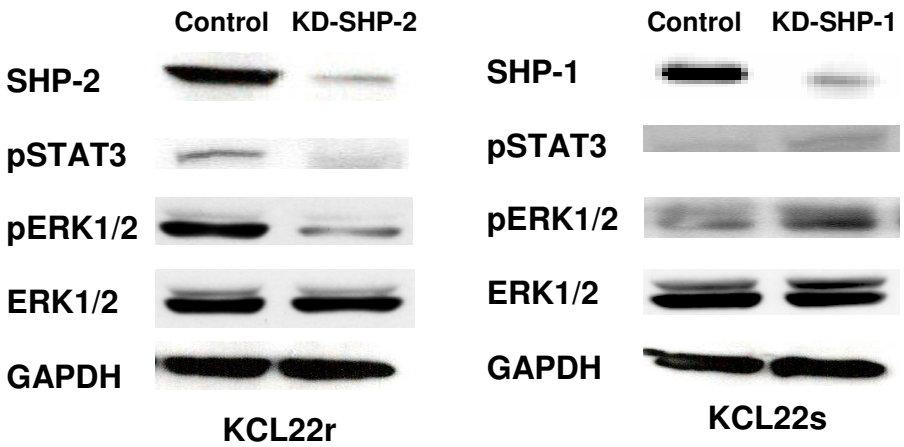

Fig. 13. Effects of knockdown of SHP1 on KCL22s and SHP2 on KCL22r by shRNA. The cell lines were transfected with the indicated ShRNA in the presence of Lipofectamine 2000 as described under “Materials and methods“. Cell extracts were

subjected to western blot analysis with specific antibodies to detect the indicated proteins. KD = Knockdown, Control= Cell line transfected with plasmid empty as described in materials and methods.

It is now well accepted that the critical role of SHP-2 in development is due to its ability to promote Ras/MAPK stimulation in response to diverse agonists. Ras/MAPK is a major signalling cascade mobilized by a broad range of membrane receptors to modulate cell fate (proliferation, differentiation), notably in response to growth factors. Abundant genetic and biochemical evidence demonstrate that Shp2 has a positive (signal-enhancing) action on this pathway [96]. For example, in cells with inactivated SHP-2, MAPK activation induced by EGF, PDGF or FGF is lower, or at least less sustained, than in normal cells. Similarly, overexpression of a catalytically inactive form of Shp2 exerts a strong dominant negative effect on Ras/MAPK stimulation in a number of different cellular models [96]. In the same way, knockdown SHP-2 KCL22r cells treated with Imatinib 1µmol/L showed a greater sensibility to treatment. To compare the cell viability respect to imatinib treatment between knockdown SHP-2 KCL22r and KCL22r control cells, trypan blue exclusion assay were performed on the same cells treated with Imatinib 1µmol/L for 4 days. The results in Fig.14. showed after 4 days of treatment, a 50% decrease of cell viability in knockdown SHP-2 KCL22r respect to KCL22r control. Knockdown of SHP-2 in KCL22r seems restore the imatinib sensitivity in these cells. Knockdown of SHP-1 in KCL22s cells increased the activation of ERK1/2 and STAT3. The magnitude of ERK1/2 activation was increased by about 40% and STAT3 by about 50% (Fig.13). In addition, knockdown SHP-1 KCL22s cells treated with Imatinib 1µmol/L showed reduced sensitivity to the treatment. The results in fig.14. after 4 days of treatment showed an higher percentage of 25% of cell viability in knockdown SHP-1. Knockdown of SHP-1 in KCL22s seems increase the imatinib resistance in these cells. This data confirm that SHP-1 and SHP-2 might be implicated in Imatinib response. Experiments with knockdown SHP1 and 2 seems moreover show that SHP-1 might be implicated in negative control of SHP-2. Therefore, a deregulated balance between the expression levels of Shp1 and 2 in resistant KCL22 cell line may account for the Bcr-Abl independent activation of alternative pathway as Ras/MAPKs, JAK/STATs and for the resistance to Imatinib of these cells. The data obtained in KCL22 model system were next translated in patients. By real time PCR we misured the expression levels of SHP-1 and SHP-2 in bone marrow of 60 patients with CML at base line. Patients were divided into three different subgroups on the basis of their response to imatinib treatment at 18 months (Tab.1) according to the European Leukemia Net (ELN) criteria. We analysed 35 optimal, 17 suboptimal and 8 failure responder to Imatinib treatment. The levels of SHP-1 mRNA were significantly more high in optimal responders [ratio of SHP1/ABL 5.8±1.77, (mean±SD)] when compared to the suboptimal (3.8±1.3, $^{*}p$=0.001) and failure (3.2±1.04, $^{*}p$=0.002) (Fig.15). As control we analysed also SHP-1 mRNA in 21 Ph-negative chronic myeloproliferative disorders (CMDP), JAK2 wild type (ratio of SHP1/ABL 6.0±2.2, *p=0.6). Therefore, our data showed that imatinib resistance patients have low levels of SHP-1 respect to the imatinib sensitive and CMDP patients. With the same methods we analyzed also SHP-1 expression in CD34+ cells, selected by cell sorting, as described in materials and methods, from 6 optimal [ratio of SHP1/ABL 1.7±0.25, (mean±SD)] and 6 failure responders [ratio of SHP1/ABL 0.9±0.15, (mean±SD) $^{*}p$=0.017] (Fig.16). In this case, we analyzed as controls 3 CMDP samples [ratio of SHP1/ABL 2,5±0.25, (mean±SD)] and 3 normal

samples [ratio of SHP1/ABL 2,1±0.22, (mean±SD)]. Therefore, also in CD34+ cells isolated from patients with CML, we found a statistically significative differences between failure and optimal responders patients.

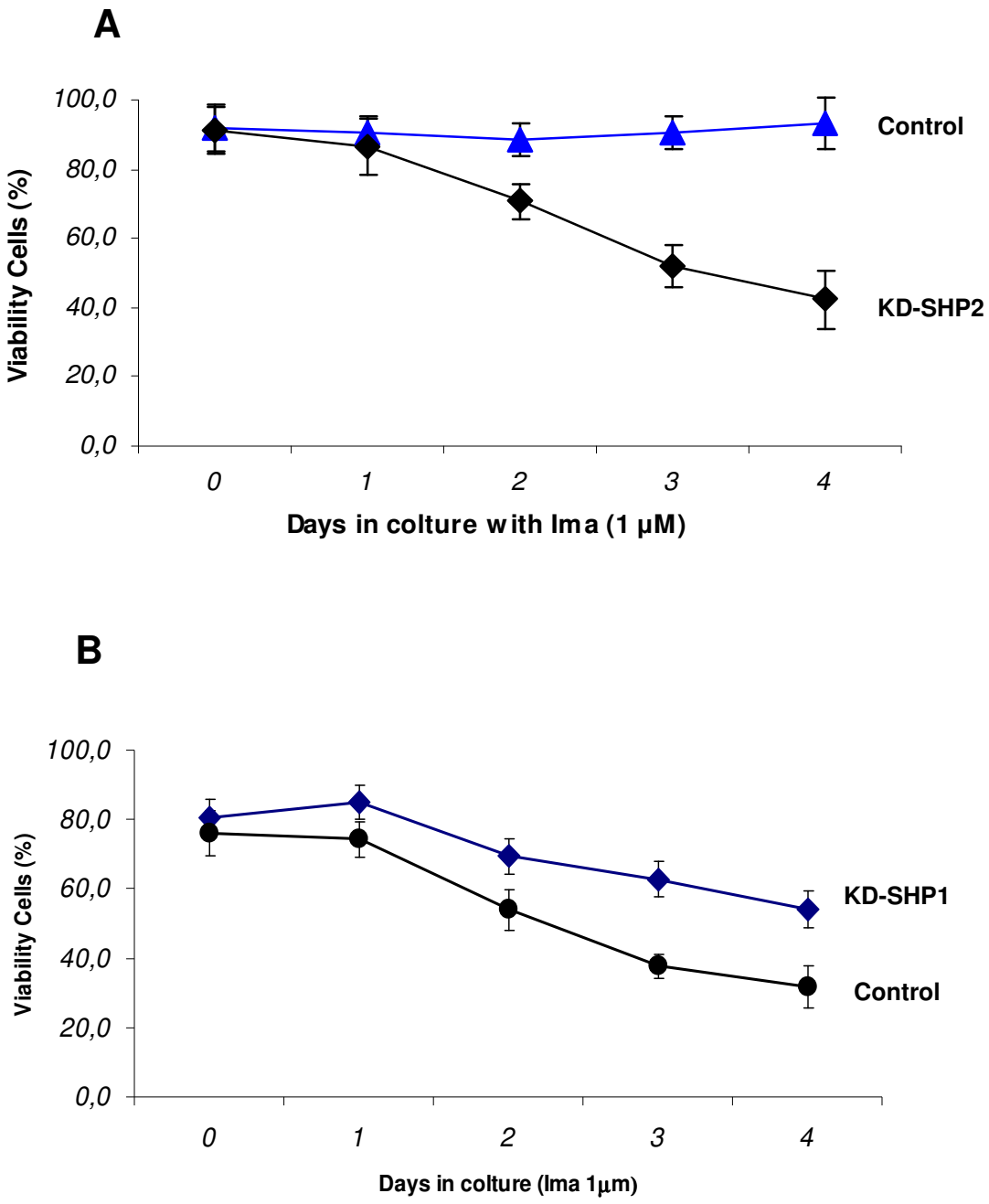

Fig.14. A. Cell viability assessed by trypan blue exclusion of Knockdown SHP-2 KCL22r cell line respect to KCL22r control. All experiment were performed in triplicate and results were expressed as means±SD. **B.** Cell viability assessed by trypan blue exclusion of Knockdown SHP-1 KCL22s cell line respect to KCL22s control. All experiment were performed in triplicate and results were expressed as

means±SD. KD= knockdown, Control= Control= Cell line transfected with plasmid empty as described in materials and methods.

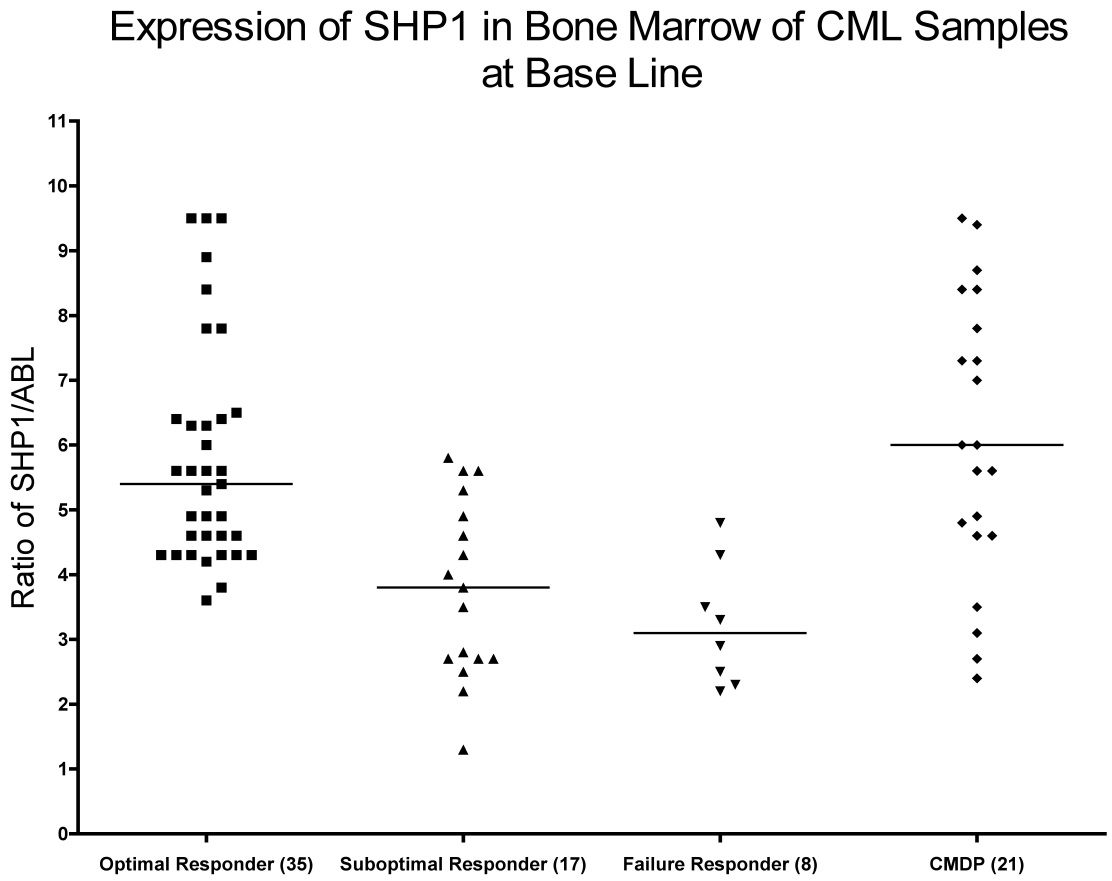

Fig 15. Levels of SHP1 mRNA in bone marrow of CML patients at base line. CML patients were classified, according to the ENL definitions and compared with CMDP. Dotted lines indicate median values of the four groups. Statistical analysis performed by Tukey`s multiple comparison test revealed a significant difference in SHP1 expression between optimal responder and both suboptimal (*p<0.001) and failure responder (*p<0.002).

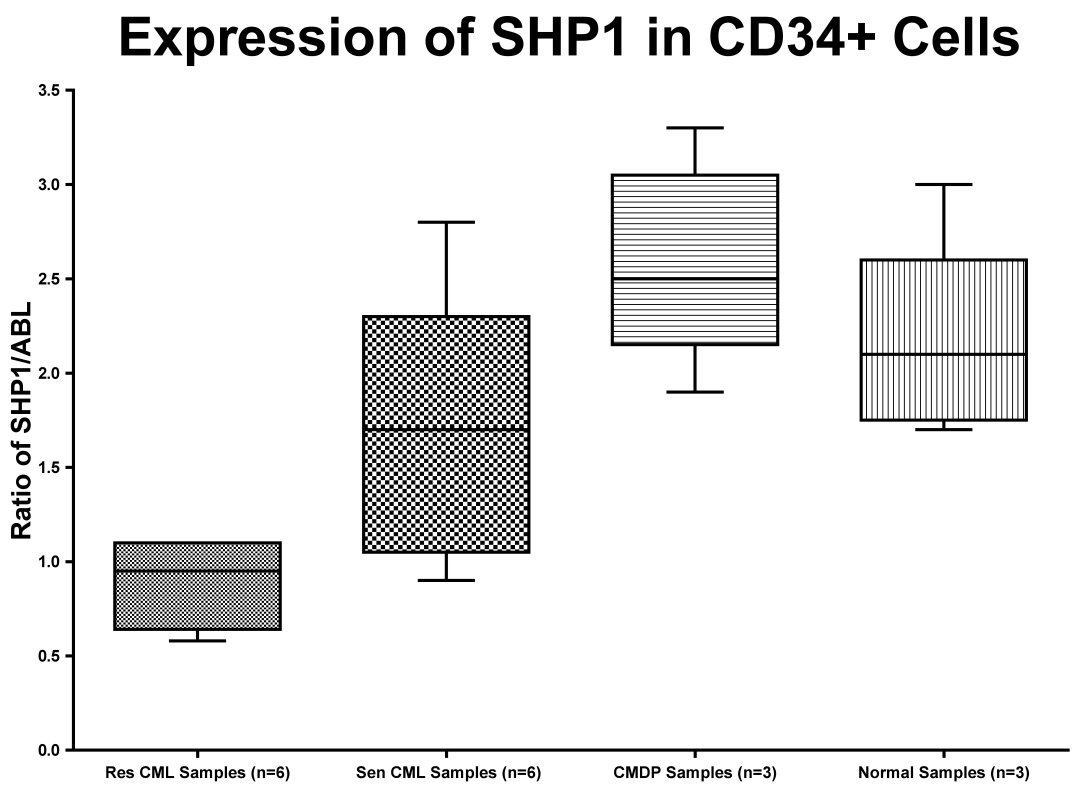

Fig.16. Levels of SHP1 mRNA in CD34+ cells of CML patients at base line. CML patients were classified according to the ENL definitions and compared with CD34+ of CMDP patients and CD34+ of normal samples (NC). The results are shown as means±SD. .Statistical analysis performed by by Tukey`s multiple comparison test revealed a significant difference in SHP1 expression between optimal responder and failure responder (*p=0.017).

In next step of this study we investigated also if the SHP-1 expression might have a predictive role in Imatinib treatment response. To this aim, we measured, by real time PCR, SHP-1 mRNA levels in leukaemia cells isolated from peripheral blood of 48 newly diagnosed CML patients enrolled into the TOPS (Tyrosine kinase inhibitor Optimization and Selectivity) trial. TOPS is a prospective, open-label, randomized (2:1) Phase III trial that compared Ima 800mg/d to 400mg/d in CP-CML. The findings end point of the trial is the rate of major molecular response (MMR) indicated by several reports as a parameter that predict a benefit for progression free survival (PFS). MMR can be defined as a 3 or more log-reduction of *BCR-ABL*/control gene ratio from a laboratory standardised baseline or an international scale converted *BCR-ABL*/control gene ratio of <0.1% [53]. Results indicate that the SHP1 mRNA levels is significantly different between those patients who do and do not achieved MMR by 12 months [ratio of SHP1/ABL 7.4 ± 3.8 vs 6.0 ± 3.7, mean±SD, *p = 0.017] (Tb.2). There is not statistical evidence that patients who achieved MMR earlier than 12 months i.e. at 6 and 9 months, have different baseline levels of SHP1, however the data are suggestive of a difference which might become statistically significant with a larger sample size. Complete cytogenetic response, CCyR, was a secondary end point of the TOPS study. CCyR is the complete absence of leukemic (Ph+) cells

in the bone marrow of CML patients by either conventional or FISH cytogenetic testing. Overall, 65% have achieved CCyR by 6 months, and 85% by 12 months, and although not statistically different, results indicate that SHP1 levels tended to be higher in patients who obtained CCyR, and the our further study with a larger sample size will show if the differences might become significant. Therefore, SHP1 expression may acts as predictor of MMR at 12 months. Statistical analysis have al shown that SHP-1 may be used as molecular marker for prediction of imatinib response (Fig.17).

	n° Patients	**SHP-1/ABL Mean**	**S.D.**	**Median**	**p-value***
No MMR	27	5.0	3.2	4.3	0.017
MMR	21	7.4	3.8	6.9	
Total	48	6.0	3.7	5.3	

Tab. 2. Table of 48 TOPS patients analyzed for SHP-1 expression. Statistical analysis confirm a statistically significative variation between SHP-1 expression level of patients that by 12 months obtained Major Molecular Response (MMR) and those that at same time didn`t obtained Major molecular response (No MMR). SD = Standard deviation. * Test for difference in location by Wilcoxon rank-sum test

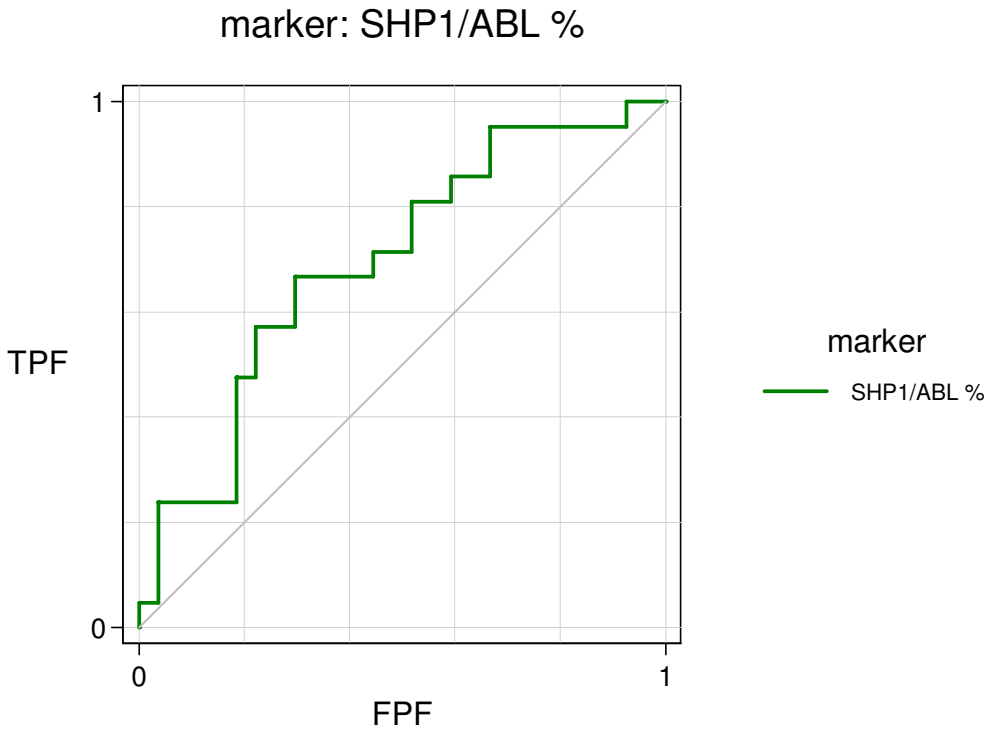

Fig. 17. ROC curve for SHP1 expression as a marker for MMR at 12 months. AUC = 0.69 (0.54, 0.84).

4 Conclusions

To identify new Bcr-Abl-independent mechanisms involved in imatinib-resistance in chronic myeloid leukemia (CML) patients, we first analyzed as model system, a couple of Ima-sensitive (KCL22s) and Ima-resistant (KCL22r) KCL22 cell lines. In these cells, Ima resistance is independent by the oncogenic Bcr/Abl activity. Expression and proteomic analysis showed a very low level of Shp1 (both mRNA and protein), a protein with a tumour suppressor activity, in the KCL22r resistant cells, when compared to KCL22s sensitive cells. We have al shown the down-regulation of this gene to be related to the methylation level of SHP1 promoter. Indeed, 5-Azacytidine (5-AC) treatment, along with demethylation of the promoter region, re-induced expression of SHP-1 in KCL22r. That treatment also re-established the Ima sensitivity, i.e. Ima growth inhibition, in these cells. At molecular level, the restored Ima sensitivity was associated to a significant reduction of phosphorylation of both STAT3 and ERK1/2. Therefore, 5-AC treatment confirm that aberrant hypermethylation of SHP-1 promoter lead to a decreased of its expression in KCL22r cell line and this assets proliferation and survival alternative pathway, BCR-ABL independent, as JAK-STATs and MAPKs. We also found a interaction between SHP-1 and SHP-2 another SH2-containing, non-receptor protein tyrosine phosphatases, well known as positive regulator of oncogenic pathways, including the Ras/MAPK pathway. We found the interaction only in sensitive KCL22s but not in resistant KCL22r cell line that showed also low level of SHP-1 (Fig.12). SHP-1 and SHP-2 share over 55% sequence identity and are regulated by similar mechanisms. However, their biological functions seems to be quite different (Fig.6) [96]. In Ph+ cells, oncogenic Bcr/Abl protein activates Shp2 through Gab2, an adaptor protein that, once phosphorylated is able to bind SH2 domain of Shp2. Through complex interactions that may involve the two carboxy-terminal tyrosine residues (542 and 580) Shp2 is also a signal transducer of growth factor receptor. We hypothesized that, SHP-1, through dephosphorylation, might modulate the activity of SHP-2 and constitute an important mechanism of Ima resistance. Knock-down of SHP-1 in KCL22s cell line resulted in increase phosphorylation of ERK1/2 and STAT3 and in its reduced sensitivity to the drug, thus supporting the role of this protein in Ima sensitivity. On the other hand, knock-down of SHP-2 in KCL22r, that shows low SHP-1 level, resulted in growth inhibition, restored Ima sensitivity and is associated to a significant reduction of phosphorylation of both STAT3 (60%) and ERK1/2 (70%). The data on primary cells support the role of Shp1 in Ima resistance in patients. The levels of Shp-1 mRNA were significantly reduced in resistant patients [ratio of SHP1/ABL 3.2 ± 1.04, (mean±SD), $^{*}p<0.002$] when compared to the suboptimal (3.8±1.54) and optimal responders (5.8±1.77). Moreover, the SHP-1 decrease was observed also in CD34+ cells isolated from 6 resistant patients in comparison to 6 optimal responders. In conclusion, our study suggests that an aberrant balance between the Shp1 and 2 levels play a role in the Bcr-Abl independent resistance to Ima through activation of Ras/MAPK pathway and that lower levels of Shp1 are associated with non responsive patients. Therefore, expression and statistical analysis on set of patients enrolled in TOPS trials showed

that SHP1 expression may acts as predictor of MMR at 12 months and that this gene might be used as a molecular marker to imatinib response.

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