EXPRESSION LEVELS OF JAK/STAT SIGNALING GENES IN NEWLY DIAGNOSED, DRUG SENSITIVE AND RESISTANT CHRONIC MYELOID LEUKEMIA PATIENTS

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ABSTRACT

EXPRESSION LEVELS OF JAK/STAT SIGNALING GENES IN NEWLY DIAGNOSED, DRUG SENSITIVE AND RESISTANT CHRONIC MYELOID LEUKEMIA PATIENTS

JAK/STAT signaling pathway has a role in transmission of information carried by cytokines, from outside of the cell to the nucleus. The system is run by the proteins known as Janus kinases (JAK) located on the cell membrane and STAT proteins acting as signal transducer and activator of transcription. JAK proteins activated by cytokines, phosphorylates and initiates the dimerization of STATs, which become active, move into nucleus and regulate expression of target genes. Previous studies demonstrated that there is overexpression of JAK/STAT genes in various types of cancer.

The aim of this study is to examine the relationship between expression levels of JAK/STAT genes and clinical outcome of chronic myeloid leukemia (CML) patients.

In this study expression levels of Jak/STAT pathway genes were analyzed in 23 different patients (1 patient responded positively, 1 only imatinib and 1 both imatinib and nilotinib resistant patients, 1 patient lost molecular response, 5 imatinib treated, and 14 newly diagnosed CML patients).

The results showed that expression levels of *Jak3, STAT1, STAT2, STAT3, STAT4 and STAT5A* genes were overexpressed in TKI resistant patients. Expression levels of *STAT5B, Jak1, Jak2* and *Tyk2* genes were higher in newly diagnosed patients compared to resistant patients while *STAT1* was lower in imatinib-treated patients.

It was demonstrated for the first time that there is a relation between the clinical outcome of CML patients and expression levels of JAK-STAT genes that could make this signaling pathway a new target for more effective treatment of CML.

ÖZET

KRONİK MİYELOİD LÖSEMİ HASTALARINDA JAK-STAT SİNYAL İLETİ YOLAĞI GENLERİNİN EKSPRESYON DÜZEYLERİ VE KLİNİK SEYİRE ETKİLERİ

JAK-STAT gen ailesi, tirozin kinaz aktivitesine sahip 4 adet JAK (JAK1, JAK2, JAK3 ve TYK2) ve sinyal dönüştürücü ve transkripsiyon aktivatörü fonksiyonlarına sahip 7 adet STAT (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B ve STAT6) proteininden oluşmaktadır. JAK-STAT proteinleri, hücreye dışarıdan gelen sitokin aracılı sinyallerin dönüşümü, hücresel yanıtın belirlenmesi ve gen anlatımının düzenlenmesinde önemli roller üstlenmektedirler. Zara bağlı olan JAK proteinleri, sitokinler aracılığıyla aktif hale geçerler ve sitoplazmada bulunan STAT'ların aktivasyonunu sağlarlar. STAT proteineleri, nukleusa göç ederek, transkripsiyon faktörü olarak iş görür ve çeşitli genlerin anlatımını düzenlerler. Bir çok kanser türünde JAK/STAT genlerinin aşırı anlatımı tespit edilmiştir.

Bu çalışmada yeni tanı, tirozin kinaz inhibitörü tedavisi alan ve moleküler yanıt kaybı oluşan ve direnç gösteren 23 kronik miyeloid lösemi (KML) hastasında JAK-STAT genlerinin ekspresyon düzeylerinin belirlenmesi ve genlerinin ekspresyon düzeyleri ile hastalığın klinik seyri arasındaki ilişkinin ortaya konması amaçlanmıştır.

Elde ettiğimiz sonuçlar, *Jak3, STAT1, STAT2, STAT3 ve STAT4 ve STAT5A* genlerinin ekspresyon seviyelerinin ilaca direnç gösteren hastalarda diğer hasta gruplarına oranla daha yüksek olduğu belirlenmiştir. *Jak1, Jak2, Tyk2 ve STAT5B* genlerinin yeni tanılı hastalarda, ilaca direnç gösteren hastalara göre daha fazla anlatımı yapılırken, *STAT5A*'nın bazı yeni tanılı ve ilaca direnç gösteren hastalarda yüksek seviyede anlatımının yapıldığı belirlenmiştir.

Bu çalışma ile, KML hastalarının uygulanan ilaçlara verdiği yanıt veya gösterdiği direnç ile JAK/STAT sinyal ileti yolağı genleri arasındaki ilişki ortaya konmuştur. Söz konusu genler KML tedavisinde yeni hedef olabilecek ve aynı zamanda direncin öntahmini için de bir belirteç olabilecektir.

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CHAPTER 1

INTRODUCTION

1.1. Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm that is characterized by a specific chromosomal abnormality as called Philadelphia chromosome (Ph+). The incidence of this disease is 1-2 cases per 100.000 adults and it represents %15 of newly diagnosed cases of leukemias in adults (Jemal, et al. 2010). Philadelphia chromosome was first time described and named by Peter Nowell & David Hungerford in 1960 followed by John Bennett & Rudolf Wirchow were described CML at first time in 1845 (Nowell and Hungerford 1960, Erikson, et al. 1986). Ph chromosome (Ph+) is found in more than 90% of CML patients which results from a reciprocal translocation between the Abelson murine leukemia gene *(ABL1)* on long arm of the chromosome 9 and the breakpoint cluster region gene *(BCR)* on long arm of the chromosomes 22 (Rowley 1973, Nowell and Hungerford 1960). After this translocation, *BCR* and *ABL* genes come together at chromosome 22, which is also called Ph chromosome and start to encode BCR/ABL fusion protein. This constitutively active BCR/ABL tyrosine kinase protein has vital roles in different cellular mechanisms such as cell growth, proliferation, apoptosis, angiogenesis and initiation of leukemias (Figure 1.1) (Schiffer 2007). As a result of alternative splicing patterns of BCR/ABL, this protein may have different domains according to their molecular weight. 210- kDa BCR/ABL proteins are encoded in %90 of Ph+ CML patients while 190 kDa BCR/ABL protein is produced in %35 of acute lymphocytic leukemia (ALL) patients and 230 kDa BCR/ABL is mostly expressed in neutrophilic leukemia patients (Chan, et al. 1987, Calderon-cabrera, et al. 2013).

Chronic myeloid leukemia have been identified with 3 distinct stages which are; chronic phase (CP), accelerated phase (AP) and blast crisis (BC). Chronic phase is relatively benign and characterized by Ph positivity as the one and only genetic abnormality in differentiated leukemic cells. There are less than 10 % leukemic cells in bone marrow or blood in CP patients (Mughal and Goldman 2006). After 5-7 years

most of chronic phase patients transitions to accelerated phase, which is more malign and consists increasing numbers (10-20 %) of blasts in blood and bone marrow. These stages followed by blast crisis that is known as termination phase and resulting in intensely bleeding, organ failures and additional genetic abnormalities in Ph^+ cells such as trisomy 8, trisomy 19, an extra Ph chromosome and isochromosome 17q. Also more than 20 % of blasts exist in bone marrow and blood in BC patients (Sawyers 1999, Jabbour, et al. 2009).

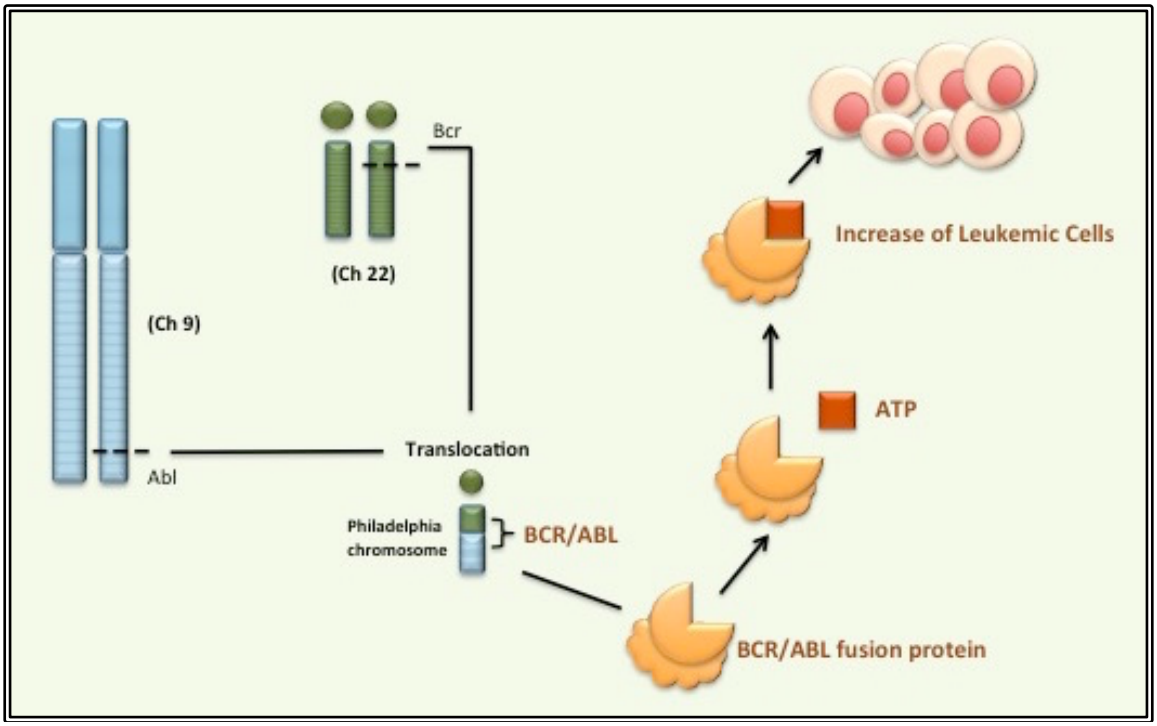

Figure 1.1 Philadelphia chromosome

1.2. Treatment of CML

Throughout history of treatment strategies of CML, blocking of tyrosine kinase activity has become the main purpose since BCR/ABL was discovered. Before, there was some limited treatment options such as busulfan or hydroxyurea, despite their cytotoxic effects and poor prognosis (Bolin, et al. 1982). Then, interferon-alpha (IFNα) had been applied in treatment of CML and Ph+ ALL patients. It basically based on inhibition of viral infections and proliferation and demonstrated significant positive hematologic and cytogenetic responses than previous applications. IFNα treatment or

IFNα combining with cytarabine, which is a DNA synthesis inhibitor, was seen as more beneficial for patients despite lack of positive responses until the invention of tyrosine kinase inhibitors (TKIs) (O'Brien, et al. 2003). After TKIs were developed; they presented the most useful option for first-line CML treatment. Previous applications were combined with TKIs to figure out previous difficulties increase the survival rates of patients (Hamad, et al. 2013). On the other hand hematopoietic stem cell transplantation is also one of the useful treatments for some patients.

1.2.1. Imatinib

Imatinib mesylate (Gleevec®; Novartis Pharmaceuticals, NJ, USA) was the first TKI to be approved by FDA for the frontline treatment of CML. It specifically binds to the ATP-binding side of inactive form of BCR/ABL protein and prevent the binding of ATP and activation of BCR/ABL kinase protein. This blockage results in the inhibition of phosphorylation of related proteins that involved in initiation of leukemias. Imatinib can also inhibit different proteins other than BCR/ABL such as platelet-derived growth factor or Kit (Druker and Lydon 2000).

After demonstration of imatinib, treatment of CML was completely changed and the new process was also called "imatinib era". The influence of imatinib was evidenced with a study in which imatinib was compared with the combination of IFNα and cytarabine. This study is known as IRIS (International Randomized Study of Interferon and STI571) and imatinib was applied at 400 mg dose daily to newly diagnosed CML patients during 19 months. As a result, imatinib was shown to be dramatically more effective than IFNα, according to comparing of complete hematologic response (CHR) and major cytogenetic response (MCyR) of differently treated CML patients. Also survival rates of imatinib treated patients were ascendantent for imatinib (O'Brien, et al. 2003).

Despite imatinib is the most advanced drug for first-line treatment of CML, all patients may not respond positively and the treatment can fail eventually by appeared drug resistance. This lead up to development of second generation TKIs such as dasatinib or nilotinib for those who fail the imatinib treatment in first-line or develop resistance to imatinib.

1.2.2. Dasatinib

Dasatinib (Sprycel®; Bristol-Myers Squibb, NY, USA) was approved by FDA in 2006, is also a BCR/ABL TKI which affects both active and inactive forms of BCR/ABL, 320 times more potent than imatinib and more sensitive to mutated catalytic domain of BCR/ABL (O'Hare, et al. 2005, Jabbour, et al. 2011). Dasatinib is also used for Ph+ ALL patients who are resistant to their first-line therapies. BCR/ABL is not the only target of dasatinib; it also inhibits Src family kinases, Kit, PDGFR which are also other important pathways in leukemia initiation (Shah, et al. 2004).

It was shown that dasatinib is against all BCR/ABL mutations that cause imatinib resistance except T315I mutation (threonine to isoleucine mutation at codon 315) (Ramchandren and Schiffer 2009). Dasatinib has higher level of endurance and efficiency then other TKIs whereas clinical phase studies showed its potential both as cytogenic and hematological responses. Dasatinib also could have ability to overcome imatinib resistance that originated from BCR/ABL overexpressions and mutations (Jabbour, et al. 2011).

1.2.3. Nilotinib

Nilotinib (Tasigna®; Novartis Pharmaceuticals, NJ, USA) as another TKI; is an analog of imatinib and 30 times more potent than imatinib at inhibiton of BCR/ABL activity. FDA approved it in 2007 for the patients who failed their imatinib treatment. More than 90 % of the failure patients that resistant or unresponsive to imatinib was shown to reached normal levels of white blood cells in their bone marrow after nilotinib treatment for approximately 5 months (Kantarjian, et al. 2006). As imatinib does, nilotinib binds to inactive forms of BCR/ABL and blocks binding of ATP and activation of kinase activity of BCR/ABL protein (Elias, et al. 2007).

It was demonstrated that nilotinib could overcome almost all BCR/ABL mutations, except T315I, likely to dasatinib. Nilotinib can be used for patients who show failure or develop resistance to imatinib during treatment in chronic phase. Like others, nilotinib also can inhibit other signaling pathways such as PDGFR and c-Kit but conversely to dasatinib, it does not affect Src kinases. (Kantarjian, et al. 2006).

1.2.4. Ponatinib

Ponatinib (Iclusig®; ARIAD Pharmaceuticals, Inc.) is another orally available TKI approved by FDA in 2012, applicaple for resistant to other TKIs in chronic myeloid leukemia or Ph+ acute myeloid leukemia patients.

In contrast to other TKIs, ponatinib is capable of inhibiting BCR/ABL with T315I mutation. Ponatinib also could target STAT or Akt pathways. It was demonstrated in many different studies that ponatinib inhibit proliferation in either imatinib resistant or sensitive CML cells (Miller, et al. 2014).

Although ponatinib has high efficiency against resistance to other TKIs, it has serious side effects seen in 12-15% of patients.

1.2.5. Stem Cell Transplantation

After the success of TKIs was presented, allogenic stem cell transplantation became disfavour for preferred first-line therapy in CML. This process constituted from transfer of healthy stem cells from a donor, who could be a relative or not, to the patient.

For the success of this process, both individual must have a tissue type that matches. Even so there are many possible risks, such as graft-versus-host disease, various types of infections, mortality or the risk of another malignancy; but stem cell transplantation could be the only proper treatment for some CML patients (Jabbour, et al. 2007).

1.3. Multidrug Resistance in CML

Multidrug resistance is a crucial problem caused by effecting of drug metabolism by a series of BCR-ABL dependent and independent factors. As a result, treatment options for CML may not be efficient on patients after a period of time. In order to overcome drug resistance; understanding the molecular mechanisms of TKIs resistance and mutations and overexpression of BCR-ABL, drug transporters, bioactive sphingolipids, microRNAs and cancer stem cells are needed to be investigated and fully illustrated.

1.3.1. BCR/ABL Mutations

Point mutations in kinase domain of BCR/ABL are the most frequently seen mechanisms of drug resistance in CML patients. These mutations may effect binding of TKIs to BCR/ABL and inhibit their actions. There are 4 different types of mutations identified in BCR/ABL; those which effect ATP binding domain, those occurs in catalytic domain, those apperars in P-loop site, those occurs in activation domain which cause a conformational difference that also effect TKI binding (Figure 1.2) (Diamond and Melo 2011).

The first mutation identified in resistant CML patients was substitution of aminoacid threonin to isoleucine at position 315 of BCR/ABL kinase protein (T315I) (Gorre, et al. 2001).

T315I, G250E, M244V, M351T, and E255K/V are frequently seen mutations in imatinib resistance, indicated with many different studies. These mutations are mostly detected in CP or AP while a few of them presented in BP as well. Other than imatinib resistance, there are many other mutations resulting in nilotinib and/or dasatinib resistance. T315I, F317L and V299L mutations have been found in dasatinib resistance while E255K/V, T315I, F359C/V and G250E mutations mostly seen in nilotinib resistance (Soverini, et al. 2013).

On the other hand, multiple mutations also could be seen in CML patients those are who generally related with poor prognosis. It was shown that multiple mutations mostly exist in patients (14 %) who resistant to both nilotinib and dasatinib as well, after imatinib failure (Parker, et al. 2011).

It was also detected that in resistant cell lines, certain myeloid differentiation genes are downregulated together with mutations in BCR/ABL. All trans retinoic acid (ATRA) was applied in order to reach myeloid differentiation and it was shown that it prevent DNA damages and decrease the level of BCR/ABL mutations. Combination of ATRA with other TKIs could be a promising application to overcome BCR/ABL mutation dependent drug resistance (Wang, et al. 2014). In another study, E35, a derivative of emodin was used to prevent drug resistance on T315I mutated cells. E35 inhibited proliferation in CML cells, which have T315I mutation by activating apoptotic pathways (Li, et al. 2014).

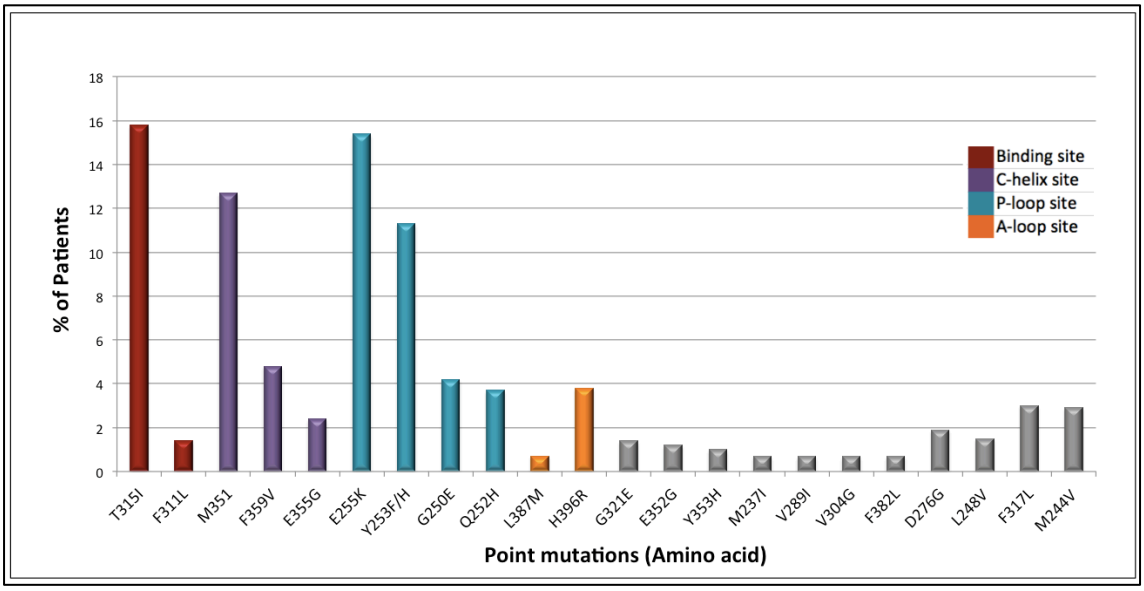

Figure 1.2. Relative incidence of BCR/ABL kinase domain mutations (Adapted from: Hughes, et al. 2006)

1.3.2. Overexpression of BCR/ABL

Abnormal level of BCR/ABL was identified as the most frequently seen cause of drug resistance in CML cell lines. Increased amount of BCR/ABL in resistant CML clones observed as the cause of imatinib resistance (Barnes, et al. 2005). In clinical studies it was confirmed that overexpression of BCR/ABL is related to failure of imatinib treatment of imatinib resistance. In a study conducted with 66 imatinib resistant patients, normal level of BCR/ABL production was detected in only 2 patients, exhibiting that drug resistance in CML is clearly related with BCR/ABL amplification (Hochhaus, et al. 2002). It was also detected in some patients BCR/ABL overexpression may arise from having multiple copies of the Ph chromosome (Campbell, et al. 2002).

In many studies, increasing amount of BCR/ABL protein and higher expression levels of BCR/ABL gene has been found in TKI resistant cell lines (Mahon, et al. 2000, Mercedes, et al. 2001). Increasing levels of BCR/ABL may reduce drug intake mediated by drug transporters (Mahon, et al. 2000). BCR/ABL related drug resistance is not just limited for imatinib, it was also shown that BCR/ABL overexpression cause nilotinib resistance via upregulation of p53/56 Lyn kinase and MDR-1 gene (Mahon, et al. 2008). BCR/ABL upregulation with GCS and SK-1 genes were detected in nilotinib resistant cell lines. Although mutation-related resistances are more frequently observed then

overexpression-related drug resistance in TKI resistance seen in clinical process. Camgoz, et al. showed there were no mutation in nilotinib-binding region of BCR/ABL but only BCR/ABL overexpression in resistant cell lines (Apperley 2007, Camgoz, et al. 2011).

1.3.3. Drug Transporters

Drug transporters are the proteins that located on the cell membrane, responsible for transport molecules through the membrane and act as one of players in BCR/ABL independent TKI resistance. ABC transporters, that are highly conserved and biggest protein family, are responsible for import/export of amino acids, inorganic compounds or drugs, through the cell membrane.

In chronic myeloid leukemia, multidrug resistance (MDR) is a cross-resistance mechanism against TKIs that is basically mediated by P-glycoprotein (P-gp). P-gb is encoded by *MDR1* also known as *ABCB1* gene. P-gp acts like a membrane-associated pump and decreases the level of drug concentration within the cells according to requirements of cells. MDR also plays role in imatinib resistance as well as other TKI resistances. It was shown that *P-gp* or *MDR1* overexpressing cells are likely to have lower levels of imatinib in the cell (Hegedus, et al. 2002, Widmer, et al. 2003, Jiang, et al. 2007). The other important gene, *ABCG2* encodes BRCP protein, which is another drug efflux pump embedded in the cell membrane. BRCP protein is overexpressed in various types of cancer cells including CML stem cells and also related to imatinib resistance. *ABCB1* and *BCR/ABL* are overexpressed in TKI resistant CML cell lines, compared to sensitive cell lines (Jordanides, et al. 2006, Hu, et al. 2008, Engler, et al. 2010). In a study, it was shown that imatinib sensitivity was significantly restored by verapamil application, which is a protein-pump inhibitor. In another study, imatinib combining with verapamil was shown to inhibit proliferation in doxorubicin-resistant K562 cells (Mahon, et al. 2007).

In contrast to this efflux pumps; human organic cation transporter 1 (OCT1) is another drug transporter and responsible for imatinib intake into the cells (Thomas, et al. 2004). It was reported that the patients who has *OCT1* gene overexpression are tend to reach complete cytogenic response more (CCyR) than the others (Crossman, et al. 2005).

ABCA transporters also play roles on TKI resistance in CML. *ABCA3* is overexpressed in resistant cell lines in compared to normal cells. Inhibition of *ABCA3* via siRNAs was shown to restored imatinib sensitivity in K562 CML cells (Chapuy, et al. 2009).

1.3.4. Bioactive Sphingolipids

Bioactive sphingolipids include ceramide, ceramide-1-phosphate, sphingosine kinases (SK) and sphingosine-1-phosphate (S1P) and play vital roles on many cellular mechanisms such as proliferation, cell growth, migration, angiogenesis, senescence and apoptosis. In many studies it was demonstrated that these sphingolipids also have roles on leukemia initiation by mostly inhibition of apoptosis with different mechanisms (Jarvis, et al. 1996, Hannun and Obeid 2008).

Ceramide is one of the major molecules in regulation of apoptosis and the key molecule of bioactive sphingolipid mechanism. Decreased level of ceramide was detected in leukemic cells while sphingosine kinase-1 was evaluated as a regulator of apoptosis and inhibition of *SK-1* restored imatinib sensitivity in resistant CML cell lines. (Bonhoure, et al. 2008). Based on their roles on apoptotic mechanism, suppression of *SK1* was also tested on imatinib resistant cell lines resulting in increasing of ceramide level and induction of cell death (Baran, et al. 2007). Overexpression of *S1P* also was shown to be important on drug resistance mechanism by inhibiton of apoptosis in CML cells (Ekiz and Baran 2010). A study showed that glucosylceramide synthase (GCS) was increased at both mRNA and protein levels, in drug-resistant K562 cells as compared to sensitive ones, providing that targeting *GCS* could restore TKI sensitivity by ceramide accumulation in the cells (Baran, et al. 2011). It was detected that nilotinib sensitivity is also related with bioactive sphingolipids. Nilotinib induces apoptosis by upregulating ceramide synthase genes and inhibit *SK-1* gene in CML cells (Camgoz, et al. 2011). In another study it was demonstrated that inhibiton of *GCS* and *SK-1* genes decrease nilotinib resistance in CML cells, suggesting that targeting this proteins, involved in drug resistance in addition to tyrosine kinase inhibitors, could enhance the effectiveness of CML treatment (Camgoz, et al. 2013).

Ceramide metabolizing genes have roles on dasatinib sensitivity as well as other TKIs. Dasatinib downregulates expression levels of antiapoptotik *GCS* and *SK-1*, while

inducing ceramide synthase (*CerS*) genes such as *CerS2, CerS5* and *CerS6* genes in CML cell lines, meaning that targeting bioactive sphingolipids that involved in CML initiation in addition to dasatinib or other TKIs treatment could be more effective in CML patients (Gencer, et al. 2011). It was also demonstrated that BCR/ABL stability is regulated by SK-1, S1P and S1P2 signaling via modulation of PP2A. In order to overcome TKI resistance, targeting SK-1/S1P2 line in addition to BCR/ABL remarks a novel approach for CML patients (Baran, et al. 2011).

1.3.5. MicroRNAs

microRNAs are small non-coding RNAs which regulate the expression of a number of genes either transcriptional or post-transcriptional level. There are various types of miRNAs involved in hematological malignancies such as miR-15a that also have roles on differentiation of hematopoietic stem cells or miR-17-19 is downregulated during treatment stage of CML patients (Hu, et al. 2010).

Overexpression of different types of miRNAs was also detected in CML, chronic lymphocytic leukemia or multiple myelomas. Downregulation of miR-217 was shown to be responsible for TKI resistance in contrast to aberrant expression of miR-17 or miR-21 was detected as inducing drug resistance in CML patients (Firatligil, et al. 2013, Seca, et al. 2013, Nishioka, et al. 2014). miR-203 is another types of miRNA which is a tumor suppressor miRNA is hypermethylated in most hematological malignancies including ALL, AML and CML (Chim, et al. 2011).

1.3.6. CML Stem Cells

Origination of cancer from stem sells (CSCs) is a model, proposing that a few cells in a huge tumor population are capable to reproduce themselves and initiate tumor occurrence. These unusual potent cells are called cancer stem cells and they were first identified in leukemias. Bonnet and Dick reported that isolation of CD34+/CD38- cells from AML patients and injection into non-obese diabetic with severe combined immunodeficiency disease mice (NOD/SCID) resulted in occurance of new tumorigenic tissue and leukemic blasts in mice (Park, et al. 1971, Bonnet and Dick 1997).

CSCs are responsible for TKI resistance as well as cancer initiation, tumor maintenance, angiogenesis and metastasis. CML originated CD34+ stem cells were shown as resistant to both imatinib and dasatinib (Graham, et al. 2002).

BMS-214662, a farnesyltransferase inhibitor was reported to initiate apoptosis in CSCs taken from BC patients when combined with all TKIs (Copland, et al. 2008). PIM kinase pathway also has role on CSCs dependent tumors to sustain. PIM kinase inhibitors cause inhibition of CD25+ AML stem cells activity via downregulation of STAT5 and degradation of Myc oncoprotein, meaning that PIM inhibitor combining with TKIs could be a new promising therapy for CSCs (Guo, et al. 2014).

Jak/STAT pathway also involve in CSCs activation in hematological malignancies. Applications of Jak2 inhibitors combining with TKIs selectively target CML stem cells and inhibit their activation resulting in overcome drug resistance (Lin, et al. 2014). On the other hand, STAT3 inhibition may raise TKI sensitivity in either CSC-dependent or BCR/ABL independent drug resistance in CML (Eiring, et al. 2014).

Lastly, a new marker for CML stem cells, Dipeptidylpeptidase IV (CD26), has been recently identified and promising that used as a target for CML treatment (Herrmann, et al. 2014).

1.3.7. Epigenetics

Epigenetic is simply defined as heritable changes in gene expression that does not include changes in DNA sequence. Epigenetic changes can be influenced by environmental factors such as age, lifestyle and nutrition and have important roles on survival, proliferation, differentiation, homeostasis and other cellular mechanism. These changes can be classified as methylations/demethylations, acetylations/deacetylations, phosphorylations/dephosphorylations and histone modifications (Bird 2007).

The roles of epigenetic changes in chronic myeloid leukemia and TKI resistance were demonstrated in many different studies (Jelinek, et al. 2011, Polakova, et al. 2013). Abnormal methylation level is related with CML maintenance, shortened survival rates and resistance to TKIs (Jelinek, et al. 2011). Hypermethylation in promoter site of *HOXA4* gene, which is a tumor suppressor gene, was detected in imatinib resistance CML patiens (Elias, et al. 2012). H3K27me3 histone modification was detected at the

promoter site of pro-apoptotic BIM and BID genes, which are demethylated in imatinib resistance CML cells, at the same time (Bozkurt, et al. 2013).

In order to overcome epigenetic mechanism dependent drug resistance, histone deacetylase inhibitors may be used in CML treatment. Based on these findings; CML patients were treated with hydralazine and valproate, which are deacetylase inhibitors, there have been dramatically restoring of TKI sensitivity in CML patients at the end of the study, suggesting that epigenetic therapy could be a promising choise to reversal of drug resistance (Cervera, et al. 2012).

1.4. Molecular Biology of CML

Chronic myeloid leukemia is characterized by BCR/ABL fusion protein, which formed as a result of the fusion of *Bcr* gene on chromosome 22 with the *Abl* gene on chromosome 9. BCR/ABL kinase protein appears as a result of this reciprocal translocation and the size of BCR/ABL protein can vary depends on the breakpoint site of the *Bcr* gene. The most important variants are 190KDa, 210 KDa and 230 KDa BCR/ABL isoforms. 190KDa is associated with in ALL, 210KDa BCR/ABL is frequently seen in CML while 230 KDa BCR/ABL isoform is found in CNL (Calderon-cabrera, et al. 2013).

BCR/ABL fusion protein has kinase activity and also mediates the expression of certain genes via other signaling pathways. These signaling pathways have vital importance in various cellular functions such as cell proliferation, growth, apoptosis, metastasis, differentiation and survival. Activation or inhibition of these pathways via BCR/ABL may cause leukemogenesis, cancer maintenance and proliferation.

1.4.1. Ras Signaling Pathway

Ras signaling pathway has many important roles on different cellular functions and cell maintenance. However its high expression may cause differentiation of hematopoietic cells to cancer cells and also uncontrolled cell proliferation. BCR/ABL initiate a Ras-dependent protein kinase cascade including MEK (MAP kinase/ ERK kinase), MAPK (mitogen-activated protein kinases) and ERKs (extracellular signal-regulated kinases) (Marais, et al. 1995). These protein cascade resulting in activation of

transcription factors such as CREB or c-Myc and activate certain genes involved cell proliferation, differentiation and also cancer initiation. BCR/ABL may have a regulatory role on protein Grb2 that can interact with phosphorylated proteins via its SH2 domain while its SH3 domain interact with mSos and transitions GDP binding form to GTP binding form that finally activates Ras protein (Puil, et al. 1994). There is an indirect relationship between the activation of Ras pathway through BCR/ABL protein.

1.4.2. Phosphatidylinositol-3 Kinase Signaling Pathway

Phosphatidylinositol-3 kinase (PI3K) is also one of the important regulatory signaling pathways on cell proliferation, differentiation, growth, motility and survival. It may cause leukemogenesis when it turns to constitutively active form. It was also shown that blocking of PI3K pathway can eliminate Ph+ cells in bone marrow that execute the interaction between BCR/ABL and PI3K pathway (Skorski, et al. 1995). BCR/ABL binds to p85 that is the regulatory subunit of PI3K resulted in activation of PI3K and initiation of turning PIP2 to PIP3 that activate Akt. Akt protein can activate many other proteins as its downstream targets that have roles on cell proliferation such as Mdm-2, mTOR and caspase-9 (Franke, et al. 1997).

Based on previous studies PI3K inhibitors and imatinib were combined and it was shown that they have synergistic effect of initiation apoptosis on Ph+ CML cells (Klejman, et al. 2002). Also PI3K inhibition increases the effectiveness of nilotinib on both CML stem cells and progenitor cells that suggesting PI3K also plays role on TKI resistance and its effectiveness (Airiau, et al. 2013).

1.4.3. c-Myc

c-Myc is a transcription factor and a nuclear oncogene that mostly has roles on cell cycle regulation as well as proliferation, cell growth and differentiation (Nieborowska-Skorska, et al. 1994). Higher level of *c-Myc* is detected in Ph+ CML cells (Sawyers, et al. 1992). Inhibition of c-Myc also dramatically showed decreasing of cell proliferation on Ph+ CML cells, suggesting that c-MYC interacts with BCR/ABL and important in CML cell growth (Nieborowska-Skorska, et al. 1994). In another

study, it was suggested that c-Myc and its ubiquitination might have roles on CML occurance by initiating leukemia stem cells (Reavie, et al. 2013).

1.4.4. Nuclear Factor Kappa B

Nuclear factor kappa B (*NF-κB*) is another transcription factor and a potent oncogene; regulates certain genes that have roles on apoptosis, proliferation, apoptosis, metastasis and angiogenesis. NF-κB is kept in the cell as its deactivated form by its inhibitor (IκB). Phosphorylation of IκB causes its degradation, which allows *NF-κB* to enter the nucleus and activate a number of antiapoptotic genes such as FLIP, cIAP, survivin, Bcl-2 and Bcl-XL (Braun, et al. 2006).

Abnormal activation of NF-κB was detected in various hematological disorders such as CML, AML or lymphoid malignancies (Braun, et al. 2006). As a result, targeting NF-κB was offered a new therapy strategy for CML. Inhibition of NF-κB combined with imatinib caused overcoming of drug resistance in CML which provide that NF-κB may have a role on drug resistance in CML patients (Cilloni, et al. 2006).

1.4.5. Protein Phosphatase 2A

The relationship between protein phosphatase 2A (PP2A) and BCR/ABL has been demonstrated in many studies that suggesting activation of PP2A could be an effective target for CML treatment. It was shown that PP2A is suppressed in both Ph+ CML cells and CD34+ CML stem cells compared to normal cells. BCR/ABL activity induces the expression of SET protein, which is inhibitor for PP2A, resulting PP2A inactivation in CML cells. BCR/ABL can induce SET protein directly by its kinase activity or indirectly via Jak2 gene. Other than SET protein, CIP2A is also an inhibitor for PP2A, was shown to be highly expressed in especially the patients in blast crisis (Neviani, et al. 2005, Samanta, et al. 2009, Lucas, et al. 2011).

Activating PP2A may ensure a potent treatment for CML. FTY720 (Fingolimod, Gilenia®, Novartis Pharmaceuticals) is a PP2A activator and S1P inhibitor either, which is approved by FDA for multiple sclerosis patients, is currently studied for clinical using in hematological malignancies. FTY720 was demonstrated to induce apoptosis in both TKI sensitive and resistant cell lines. It is already known that inhibiton

of S1P can overcome TKI resistance through RAS/MAPK signaling, providing that FTY720 could be a novel agent for CML treatment as well (Baran, et al. 2007, Bonhoure, et al. 2008, Alinari, et al. 2012).

1.4.6. Wnt/β-catenin Signaling Pathway

Wnt/ β-catenin signaling pathway has roles in many different cellular processes such as proliferation, self-renewal, differentiation and also hematological malignancies. BCR/ABL could directly activate this pathway and play roles on staging of CML. Also it was shown that Wnt pathway involved in drug resistance in CML cells via BCR/ABL interaction with β-catenin. Higher levels of β-catenin were detected in imatinib resistant CML cells comparing to normal cells (Seke Etet, et al. 2012).

Inhibition of Wnt/β-catenin pathway via AV65, a Wnt inhibitor, was demonstrated to decrease proliferation and induce apoptosis in CML cell lines, including even the cells with T315I BCR/ABL mutation. This inhibitor combining with TKIs showed synergistic effect on CML cells and may overcome drug resistance (Nagao, et al. 2011).

Wnt/β-catenin pathway has important roles in CML stem cells and their maintenance as well as Ph+ CML cells. Targeting this pathway in addition to other inhibitons also promising to overcome stem cell related TKI resistance (Graham, et al. 2002).

1.5. Jak/STAT Signaling Pathway

Jak/STAT is a major intracellular signaling pathway that involved in immune response, cancer development (mostly leukemias) and hematopoiesis by stimulating cellular growth, proliferation and differentiation. This pathway relies on transmition of outside signals into the cells through cell membrane. The Jak/STAT mechanism composed of 2 main components including Janus kinase (Jak) and signal transducers and activators of transcription (STAT) proteins. Janus kinases, have four different types (Jak1, Jak2, Jak3 and Tyk2), found as an inactive form and associated with receptor protein on the cell membrane whereas there are seven types of STAT protein (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) that are located in cytoplasm

and activated by Jak kinases. The system is started with conformational change and trans-activation of Jak proteins by a signal from a number of cytokines. This activation is followed by STAT phosphorylation at tyrosine residue in transactivation domain of STAT protein. Phosphorylated STAT proteins become dimerized by interacting phosphotyrosine of one STAT and SH2 domain of another STAT, as a result, they either affect different pathways and cause signaling cascades or move into the nucleus and directy activate their target genes involved in proliferation, differentiation, cell growth, cell death or survival. Abnormal activation of Jak/STAT signaling pathway proteins are found in many types of hematological malignancies including AML, MDS, B-cell lymphomas and CML (Valentino and Pierre 2006).

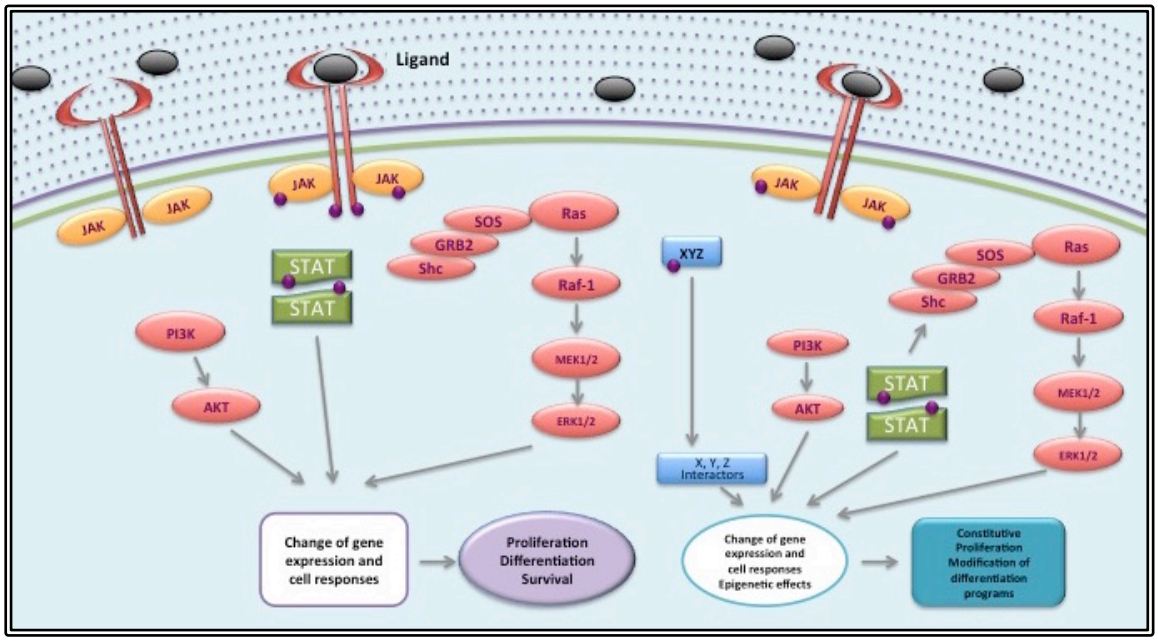

Figure 1.3. Jak/STAT Signaling Pathway

1.5.1. Structure of Jak and STAT Proteins

A typical Jak structure has seven homology regions including JH1, JH2, JH3, JH4, JH5, JH6 and JH7. JH1 domain that also known as catalytic tyrosine kinase domain, encode the kinase while JH2, a pseudokinase domain is basically responsible for catalytic activity of JH1. JH4-JH7 domains are involved in binding other proteins to Jaks (Kisseleva, et al. 2002, Valentino and Pierre 2006). JH4–JH7 region constitutes the

FERM domain domain (four-point-one, Ezrin, Radixin, Moesin) that involved in the interactions between JAKs and other kinases (Zhu, et al. 1999).

STAT proteins also have different conserved domains, which include carboxy-terminal transcriptional activation domain (TAD), SH2 domain, linker domain, DNA binding domain (DBD), coiled-coiled domain (CCD) and amino terminal domain (NH2). The phosphorylated tyrosine residue is found in transcriptional activation domain and the differences in this domain identify the types of STAT proteins as well as responsible for gene activation. DNA binding domain becomes prominent when STAT proteins move into the nucleus and activating target genes by binding the promoter site of these genes. Other domains are mostly invoved in interaction between STAT and other proteins (Strehlow and Schindler 1998, Kisseleva, et al. 2002).

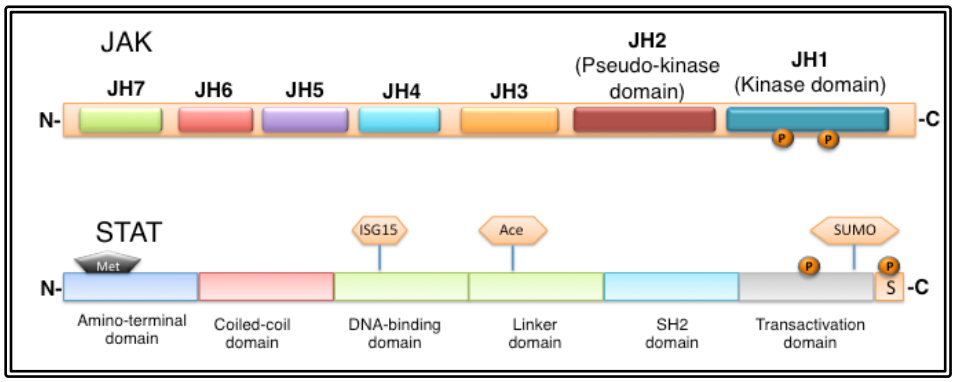

Figure 1.4. Diagram of JAK and STAT structure.
(Adapted from: Shuau and Liu 2003)

1.5.2. Jak1

Jak1 protein as a member of Janus family of kinases also play roles in cytokine signaling by IL-2, IL-4, gp130 receptors family and class II cytokine receptors (Schindler and Strehlow 2000). It was also shown that Jak1 suppression represents post-natal lethal phenothype in mice, meaning that is important for survival as well as signal transduction (Rodig, et al. 1998). Jak1 is also involved in metastasis, tumor progession and most importantly drug resistance in different cancer types such as ovarian and lung cancer (Kim, et al. 2012, Wen, et al. 2014).

Point mutations that commonly located in FERM domain and JH2 domain as V658F mutation in Jak1 protein also found in T-cell and B-cell ALL (Flex, et al. 2008). Hovever, %1 of mutations that occurred in Jak1 have been detected in AML (Tomasson, et al. 2008).

1.5.3. Jak2

Despite most of their activator cytokines are the mutual, conversely to Jak1, knockout Jak2 mice displayed embryonic lethal phenotype (Wu, et al. 1995). There have been many Jak2 somatic mutations detected including in pseudokinase domain and kinase domain in myeloproliferative disorders and also interestingly in Down syndrome (Kearney, et al. 2009).

Substitution of valine residues to phenylalanine aminoacid at 617 position (V617F) is the most frequently seen mutation in Jak2 found in many hematological disorders including Ph- and Ph+ CML as well (Gu, et al. 2013, Xu, et al. 2014). As a result of V617F mutation Jak2 dependent signaling cascade including STATs, MAPK/ERK and PI3K pathways can be activated without cytokines. Pseudokinase domain of Jak2, which normally suppress kinase domain and keep the protein inactive form in non-stimulated cells, is deactivated by V617F mutation resulted in constitutively phoshorylation and activation of Jak2 protein itself. This activation cause phosphorylation of STAT or other targets of Jak kinases and expression of abnormal levels of cell proliferation, growth and survival genes, eventually (James, et al. 2005). It was shown that approximately %30 of CML patients carry V617F mutations, therefore, detection of this mutation became an clinically important marker for the treatment options in CML (Pahore, et al. 2011). However, in contrast to myeloid disorders, V617F mutation does not exist in lymphotic leukemias (Ross, et al. 2005)

1.5.4. Jak3

Jak3 expression is also another important player in Jak/STAT signaling, although Jak3-/- mice grow normally and do not display any lethal phenotype either pre- or post-natal state (Nosaka, et al. 1995).

Jak3 mutations are mostly detected in ALL and rarely found in T-cell leukemia and lymphomas (Zhang, et al. 2012). L156P, E183G and R172Q are the most frequently found mutations that occur in FERM domain of Jak3 in hematological malignancies (Elliott, et al. 2011)

1.5.5. Tyk2

Tyk2 is the first described member of Jak family and most important one in Jak family involved in transmition of IL-6, -10 and -12 signaling (Schindler and Strehlow 2000). In contrast to other janus kinases Tyk2 mutations does not involved in hematological malignancies with a few exceptations. It was demonstrated that Tyk2-STAT1 pathway initiate upregulation of *Bcl2* gene expression resulting in ALL cell proliferation (Sanda, et al. 2013)

1.5.6. STATs

Constitutive STAT activation is described and well understood in hematological malignancies however mutations in *STAT* genes does not seem very frequent. Different group of cytokines effect different STAT protein and caused activation of different genes (Stark, et al. 1998).

STAT1 or STAT2 regulate the genes, which induce cell cycle arrest and apoptosis while STAT3 was shown as mostly regulate the genes that encode cytokines resulting in auto-activation of STAT pathway. STAT1 and STAT2 also play roles in regulation of immune response in case of viral infection or anti-tumor immunity (Leung, et al. 1995, Schindler, et al. 2007, Chen and O'Shea 2008). STAT3 is one of the important players on cancer initiation as it was demonstrated that overexpression of STAT3 found in malign tumors. STAT3 is also capable to activate various genes that responsible for inflammation response of the cells. It was also demonstrated that STAT3 have roles on epigenetic mechanism by promoting DNA methylation (Zhang, et al. 2007). On the other hand, activation of STAT3 that mediated by the action of upstream epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) was shown in different types of solid tumor cells (Stark, et al. 1998).

STAT5A, STAT5B and STAT6 are the most common STAT family members involved in hematological malignancies as well as STAT3. It was shown that BCR/ABL fusion protein could directly activate STAT5 in CML cells while STAT6 overexpression was found in leukemia and lymphomas (Lin, et al. 2000, Bruns, et al. 2006).

Table 1.1. STAT family activation and their target genes

STAT protein	Key activators	Main target genes	Example genes
STAT1	IFNγ, IFNα and IFNβ	T_H1 type immunostimulatory and pro-apoptosis	TBX21, CD80, CD40, IL-12, CDKN1A and caspases
STAT2	IFNα and IFNβ	T_H1 type immunostimulatory and pro-apoptosis	CD80 and CD40
STAT3	IL-6, IL-10, IL-23, IL-21, IL-11, LIF and OSM	T_H17 type, anti-apoptosis, pro-proliferation, angiogenic	IL-17, IL-23, BCL-X, BCL2, MCL1, CCDN1, VEGF
STAT4	IL-12	T_H1 type, IFNγ	IFNγ
STAT5A STAT5B	IL-2, GM-CSF, IL-15, IL-7, IL-3, IL-5, growth hormones	Anti-apoptosis, pro-proliferation, differentiation	BCL-XL, CCDN2, FOXP3
STAT6	IL-4 and IL-13	T_H2 type, anti-apoptosis	GATA3 and BCL-2

It was also demonstrated that Jak/STAT pathway could be activated by BCR/ABL fusion protein either. STAT5 is the main protein that constitutively activated in Ph+ cells however in some cases STAT1 was the one that replaced with STAT5 (Chai, et al. 1997). Different domains of BCR/ABL are capable to activate different STAT proteins. For instance STAT6 can be activated by just 190KDa BCR/ABL whereas both 190 KDa and 210 KDa BCR/ABL can activate STAT5 (Danial and Rothman 2000). Despite promising data obtained from different studies that illustrate the underlying mechanism of differential STAT activation, all mechanism has not been fully understood yet.

Jak/STAT pathway dependent drug resistances in CML patients have been demonstrated in many different studies. *SOCS-3* methylation by STAT3 is found to be responsible for imatinib resistance in Ph+ cells (Al-Jamal, et al. 2014). There are many downstream signaling pathways including MAPK, PI3K/AKT of Jak/STAT signaling that are activated by different receptors involved in CML and ALL. STAT pathway may also play roles in TKI resistance by its downstream signaling pathways such as STAT5 cause TKI resistance by activating AKT signaling pathway in leukemia, providing that targeting STAT pathway also could be a potential therapy in order to overcome drug resistance (Bibi, et al. 2014). Imatinib and nilotinib resistance in Ph+ CML cells may show up via Jak2/STAT5 pathway as well as other factors (Wang, et al. 2007).

1.6. Aim of the Project

Chronic myeloid leukemia is a hematological disorder, observed at a frequency of 1-2 per 100.000 and represent 15% of all leukemias (Jabbour and Kantarjian 2012). It is also one of the well explained disease that has various types of molecular interactions and targeted by molecular therapy. Despite high efficiency of tyrosine kinase inhibitors, the success of therapy is limited to multidrug resistance phenomenon. Recent developments in drug resistance mechanism in CML showed that many molecular interactions and signaling pathways are involved in mediating TKI resistance in patients. Jak/STAT signaling pathway is one of these pathways that have significant roles in various cellular mechanisms including leukemia initiation and drug resistance (Al-Jamal, et al. 2014).

The aim of this study is to examine the relationship between expression levels of Jak/STAT genes and clinical outcome of chronic myeloid leukemia (CML) patients who are newly diagnosed; treated with imatinib, nilotinib or dasatinib; responded positively to TKIs; imatinib, nilotinib or dasatinib resistant; or lost of their molecular response.

CHAPTER 2

MATERIALS AND METHODS

1.1. Chronic Miyeloid Leukemia Patients & Bone Marrow Samples

Patient samples were obtained with informed consent in accordance with Hematology Departments of Ege University, 9 Eylul University, Gulhane Medical School (GATA), Baskent University, Adana Teaching and Medical Research Center, and Erciyes University. This study including 14 newly diagnosed CML patients, 5 patients who are CML diagnosed and treated with imatinib, 1 patient who positively responded to imatinib, 1 patient who lost molecular response, 1 imatinib resistant patient and 1 patient who has both imatinib and nilotinib resistance.

Table 2.1. Characteristics of the patients enrolled

Patients	Gender	Institution	Diagnosis
1	M	9 Eylül University	Newly Diagnosed CML
2	M	9 Eylül University	Newly Diagnosed CML
3	M	9 Eylül University	Newly Diagnosed CML
4	M	9 Eylül University	Newly Diagnosed CML
5	M	9 Eylül University	Newly Diagnosed CML
6	F	Ege University	Newly Diagnosed CML
7	M	Ege University	Newly Diagnosed CML
8	M	Başkent University	Newly Diagnosed CML
9	F	9 Eylül University	Newly Diagnosed CML
10	F	GATA	Newly Diagnosed CML
11	M	GATA	Newly Diagnosed CML
12	M	GATA	Newly Diagnosed CML
13	M	GATA	Newly Diagnosed CML
14	F	GATA	Newly Diagnosed CML
15	M	Ege University	Diagnosed CML in 2008 treated with 1x400mg imatinib

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Table 2.1. (cont)

16	M	Ege University	Diagnosed CML in 2008 treated with 1x400mg imatinib
17	M	Ege University	Diagnosed CML in 2005 treated with 1x300mg imatinib
18	F	Ege University	Diagnosed CML in 2005 treated with 1x400mg imatinib
19	M	9 Eylül University	Diagnosed CML and treated with imatinib for 3 months
20	M	9 Eylül University	Positively responded to imatinib
21	F	9 Eylül University	Loss of molecular response
22	F	Ege University	Resistant to imatinib
23	F	Erciyes University	Both imatinib and nilotinib resistant, treated with dasatinib

2.2. Isolation of Mononuclear Cells from Bone Marrow Samples

Approximately 1/3 amount of bone marrow samples, Ficoll-Poque was put into 15ml falcon tubes. Bone marrow sample was placed onto the Ficoll solution gently. This mixture was homogenized for 10 min at 180 rpm in a shaker then centrifuged at 400g for 15 min. After the centrifugation plasma of bone marrow is placed at the top of the tube followed by mononuclear cells then ficoll and finally eritrocytes and granulocytes are placed at the bottom of the falcon tube. Mononuclear cells were removed with a help of a sterile pipette and transferred into another tube. The collected cells were washed with over 5-times volume of sterile serum physiologique, at least twice and purified from ficoll.

2.3. Isolation of Total RNA from Mononuclear Cells

Total RNA isolation was performed with NucleoSpin® RNA II Purification Kit, according to instructions of manufacturer. Collected mononuclear cells were centrifuged and up to $5x10^6$ cells were collected in a microcentrifuge tube (1.5 ml). In order to lyse the cells, 350 µl RA1 buffer and 3.5 µl β-mercaptoethanol were added to the cell pellet and the mixture was homogenized gently. The mixture was transferred into NucloSpin® filter (violet ring) with a collection tube (2 ml) and centrifuged at 11,000 g for 1 min to clear the lysate. Filter was discarded and 350 µl ethanol (70%) was added onto the

samples and mixed by pipetting to adjust RNA binding. Whole mixture was transferred to NucloSpin® RNA Column (blue ring) with a collection tube (2 ml) and centrifuged at 11,000 g for 30 sec in an attempt to bind RNAs to the column. 350 µl membrane desalting buffer was added onto each sample and centrifuged again at 11,000 g for 1 min. 95 µl of DNase reaction mixture, which have been prepared before in another centrifuge tube with the mixture of 10 µl reconstituted rDNase and 90 µl reaxion buffer for rDNase were added directly to the center of membrane of RNA bind column and incubated for 15 min at room temperature. Silica membrane was washed three times with 200 µl RAW2 and 600 µl RA3 that both centrifuged at 11,000 g for 30 sec and with 250 µl RA3 centrifuged at 11,000 g for 2 min, respectively. In order to elute the RNAs, 60 µl of RNase-free H_2O was applied and the samples were centrifuged at 11,000 g for 1 min. Concentrations of isolated RNAs were measured by Nanodrop ND-1000 at 260/280nm and 260/230nm ratios.

2.4. Conversion of mRNAs to cDNA via Reverse Transcriptase Reaction

cDNA synthesis examined from isolated RNAs (1 µg of each total RNA) by Thermo Scientific® First Strand cDNA Synthesis Kit. After all ingredients for cDNA synthesis were prepared they incubated at 42 ºC for 1 h and then stopped at 72 ºC for 10 min with the help of a thermal cycler.

Table 2.2. Ingredients for cDNA synthesis

Ingredients	Amount
RNase-free water	12-X µl
Total RNA (1µg)	X µl
10X Buffer	4 µl
Random Hexamer Primer (0.5 µg/l)	1 µl
dNTP (10 mM)	2 µl
RNase Inhibitor	0.5 µl
Reverse Transcriptase (200U/µl)	0.5 µl
Total Volume	**20 µl**

2.5. Detection of Expression Levels of JAK-STAT Genes by Quantitative Reverse Transcriptase Chain Reaction (qRT-PCR)

Synthesized total cDNAs were used to detect the expression levels of Jak/STAT genes (*Jak1*, *Jak2*, *Jak3*, *Tyk2*, *STAT1*, *STAT2*, *STAT3*, *STAT4*, *STAT5A*, *STAT5B*, *STAT6*) and β-actin as internal positive control by qPCR according to Thermo Scientific® DyNAmo SYBR Green qPCR Kits instructions. qPCR data analysis was performed with use of Roche LightCycler® its software.

Table 2.3. qPCR reaction setup

Components	Amount
2X Master Mix	10 µl
Primer Mix	5 µl (Final concentration: 0,5 µM each)
Template cDNA	5 µl (2-5 ng/µl in final reaction)
Total	**20 µl**

Table 2.4. Primer sequences used for qPCR

JAK1-F	5'-TCTTGGAATCCAGTGGAGGCATAAA-3'
JAK1-R	5'-CACTCTTCCCGGATCTTGTTTTTCT-3'
JAK2-F	5'-GAGCCTATCGGCATGGAATA-3'
JAK2-R	5'-ACTGCCATCCCAAGACATTC-3'
JAK3-F	5'-TATCCTTGACCTGCCAGTCC-3'
JAK3-R	5'-GTAGGCAGGCCTTGTAGCTG-3'
TYK2-F	5'- GACCAGAAGGAGATCACCCA -3'
TYK2-R	5'- CTGTCTCGTAGAAGGCCAGG -3'
STAT1-F	5'- CCGTTTTCATGACCTCCTGT -3'
STAT1-R	5'- GTGCTCTGAATATTCCCCGA -3'

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Table 2.4 (cont)

STAT2-F	5’-GCAGCACAATTTGCGGAA-3’
STAT2-R	5’-ACAGGTGTTTCGAGAACTGGC-3’
STAT3-F	5’-AACTCTTGGGACCTGGTGTG -3’
STAT3-R	5’-GGCTTAGTGCTCAAGATGGC-3’
STAT4-F	5’-TCAAGACCAACAGAAAGGGG-3’
STAT4-R	5’-ACACCGCATACACACTTGGA-3’
STAT5A-F	5'-GAAGCTGAACGTGCACATGAATC-3’
STAT5A-R	5’-GTAGGGACAGAGTCTTCACCTGG-3’
STAT5B-F	5’-CAACAGGCCCATGACCTACT-3’
STAT5B-R	5’-GTAGCAGACTCGCAGGGAAC -3’
STAT6-F	5’-TACTACCCCCACAGACCTGC-3’
STAT6-R	5’-CATGTTGGGGTGTGTCTCAG-3’
β-actin-F	5’-CAGAGCAAGAGAGGCATCCT-3’
β-actin-R	5’-TTGAAGGTCTCAAACATGAT-3’

Table 2.5. qPCR cycling protocol

Step	**Temperature**	**Time**
Initial denaturation	95°C	15 min
Denaturation	94-95°C	10 s
Annealing	* °C	20 s
Extention	72°C	30 s
Final extention	95°C	5 s
Melting curve	Annealing* + 7 °C	1 min
Number of cycles	45	

***** Annealing temperatures are optimized for each gene separately and applied as given below:

Table 2.6. Annealing temperatures for qPCR cycling protocol

51°C	*Jak1, Jak2, Jak3* and *Tyk2*
52°C	*STAT2*
53°C	*STAT6* and *β-actin*
54°C	*STAT1, STAT2* and *STAT5B*
56°C	*STAT3* and *STAT5A*

For qPCR analysis, β-actin gene was used as internal positive gene (reference gene). $2^{(-\Delta CT)}$ method, which is a comparative calculation was used for data analysis. ΔCT refers to: CT value of target gene – CT value of reference gene.

Jak1, Jak2, Jak3, Tyk2, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and *STAT6* genes were normalized to β-actin and graphics were plotted according to final calculations.

CHAPTER 3

RESULTS

3.1. Expression Levels of *Jak1* Gene in CML Patients

Jak1 gene has higher expression levels in newly diagnosed CML patients (number 3, 5 and 7) and diagnosed and having imatinib treatment patients (number 16 and 19) as compared to imatinib resistant patients (number 22) and positively responded patient (number 20) ($p<0,05$). Also the patient who lost molecular response (number 21) has lower expression of *Jak1* gene as compared to newly diagnosed and currently imatinib treated patients ($p<0,05$). The highest expression levels of *Jak1* is detected in currently imatinib treated patients (number 16 and 19) (Figure 3.1).

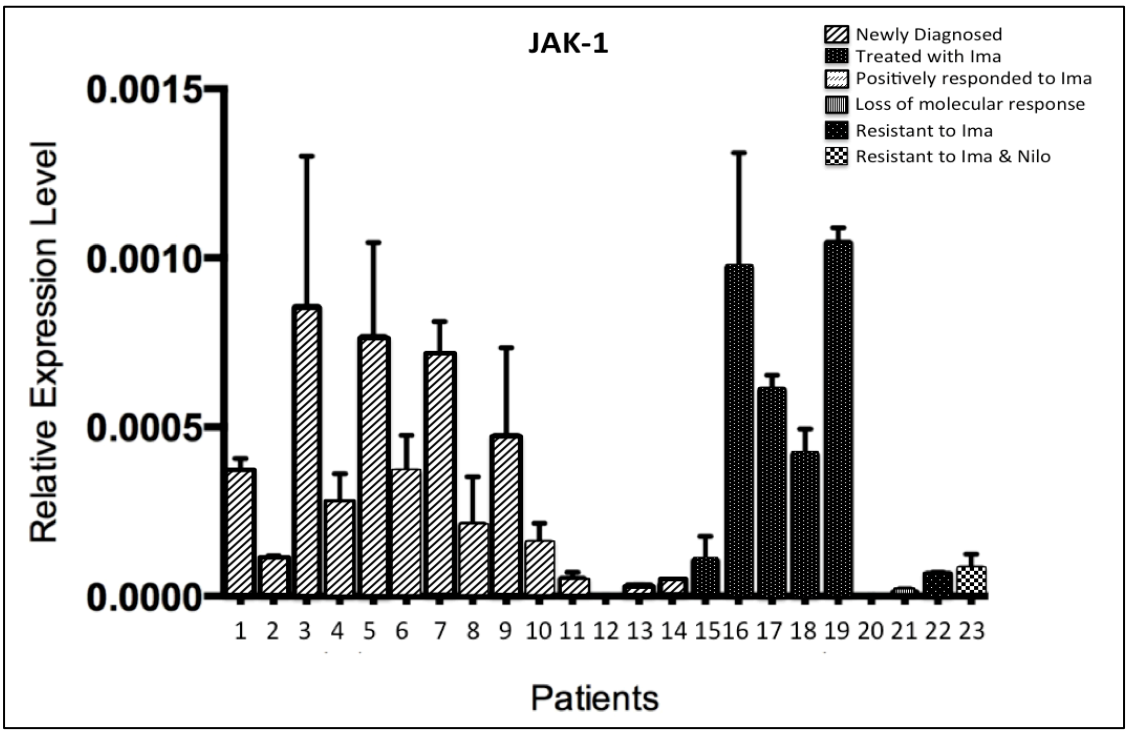

Figure 3.1. Expression levels of *Jak1* gene in CML patients (normalized to β-actin, error bars represent standart deviation (SD), and $p<0.05$ was considered significant)

3.2. Expression Levels of *Jak2* Gene in CML Patients

As similar to expression scale of *Jak1* gene, *Jak2* is also highly expressed in newly diagnosed CML patients (number 4, 5, 6, 7 and 8) than positively responded patient (number 20) ($p<0,05$). In currently imatinib treated (number 15 and 19), in imatinib resistant (number 22), and in resistant to both imatinib and nilotinib (number 23) patients, there was higher expression levels of *Jak2* as compared to positively responded patient (number 20), but it was statistically insignificant ($p>0,05$). The lowest expression levels of *Jak2* gene was detected in imatinib resistant (number 22), both imatinib and nilotinib resistant (number 23) patients, newly diagnosed (number 12) and having imatinib treatment (number 18) patients (Figure 3.2).

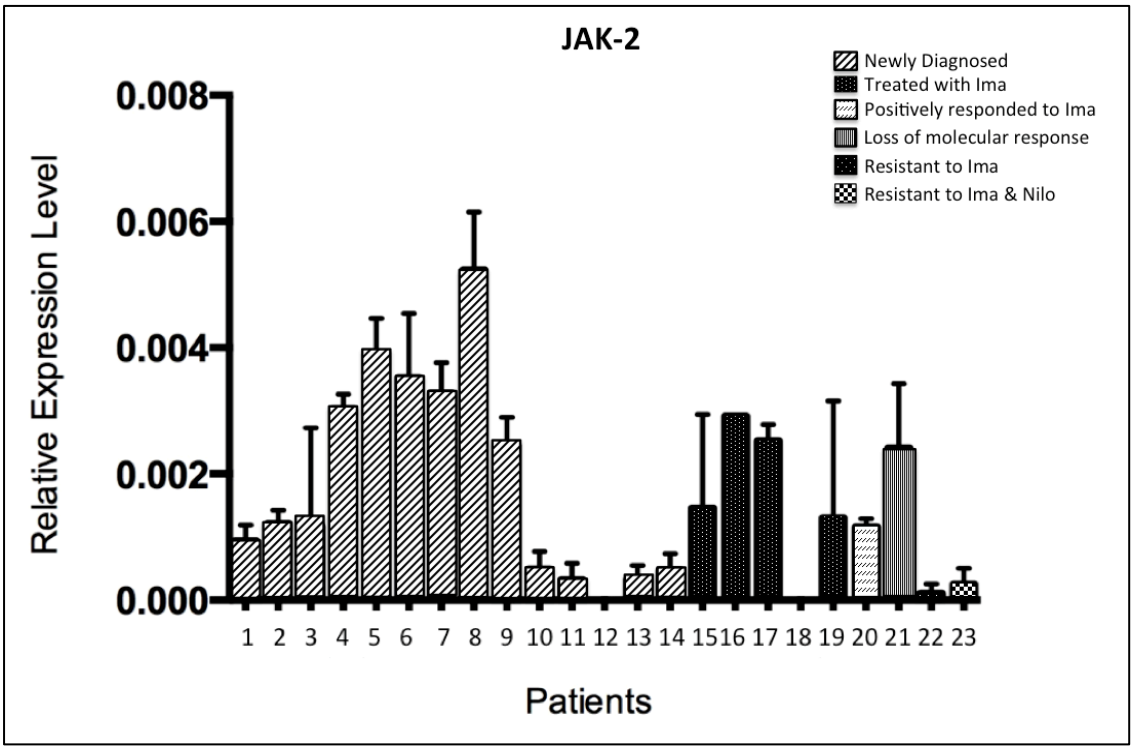

Figure 3.2. Expression levels of *Jak2* gene in CML patients (normalized to β-actin, error bars represent SD, and $p<0.05$ was considered significant)

3.3. Expression Levels of *Jak3* Gene in CML Patients

Interestingly enough, *Jak3* gene is expressed at roughly similar levels in most of the newly diagnosed CML patients. In currently imatinib treated patient (number 18), *Jak3* gene expression was in a very small amount as compared to all the other patients. On the other hand, in patient having imatinib treatment (number 15), imatinib resistant patient (number 22), and both imatinib and nilotinib resistant patient (number 23), there was higher level of *Jak3* gene expression as compared to positively responded patient (number 20) ($p<0.05$). Rest of the patients did not represent any significant differences as compared to positively responded or resistant patients ($p>0.05$) (Figure 3.3).

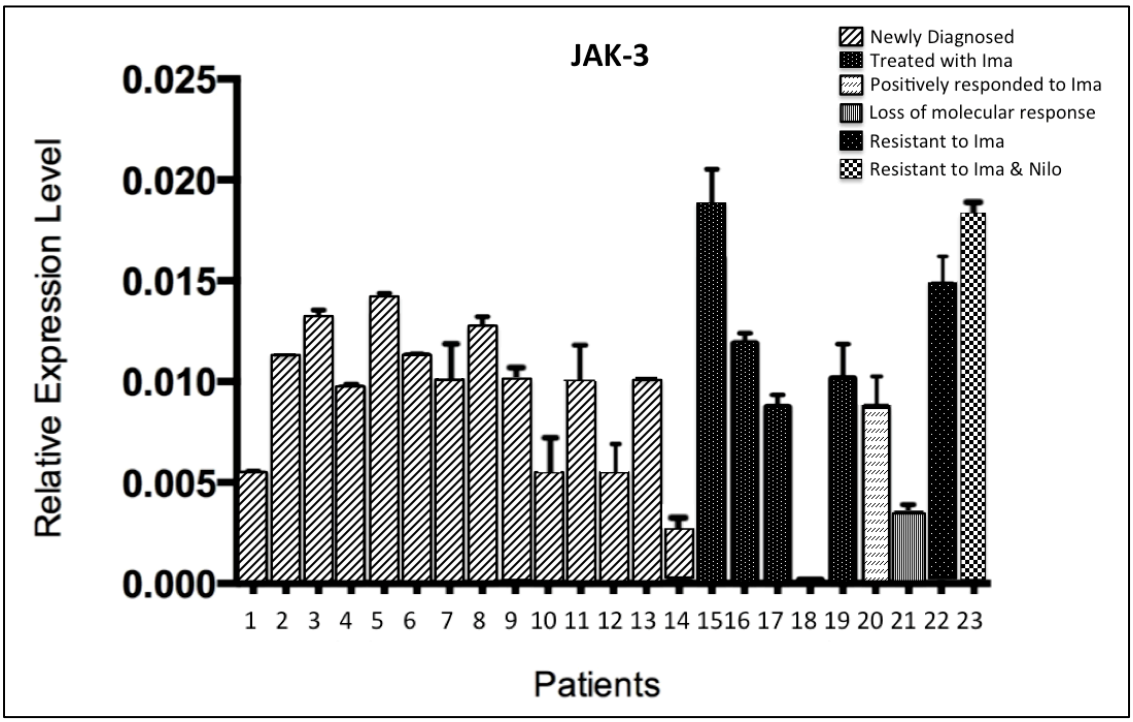

Figure 3.3. Expression levels of *Jak3* gene in CML patients (normalized to β-actin, error bars represent SD, and $p<0.05$ was considered significant)

3.4. Expression Levels of *Tyk2* Gene in CML Patients

Despite there was no significant difference between different patient groups; *Tyk2* has slightly higher expression in newly diagnosed patients than the others. The patient who positively responded to imatinib (number 20) and the newly diagnosed patient (number 8) have the highest expression levels of *Tyk2* as compared to all the other patients ($p<0.05$). Despite patient number 20 has higher expression levels of *Tyk2* than both imatinib and nilotinib resistant patient, this difference was not statistically significant ($p>0.05$). However, in one of the newly diagnosed patients (number 8), there were higher expression levels of *Tyk2* gene than both imatinib and nilotinib resistant patient (number 23) ($p<0.05$) (Figure 3.4).

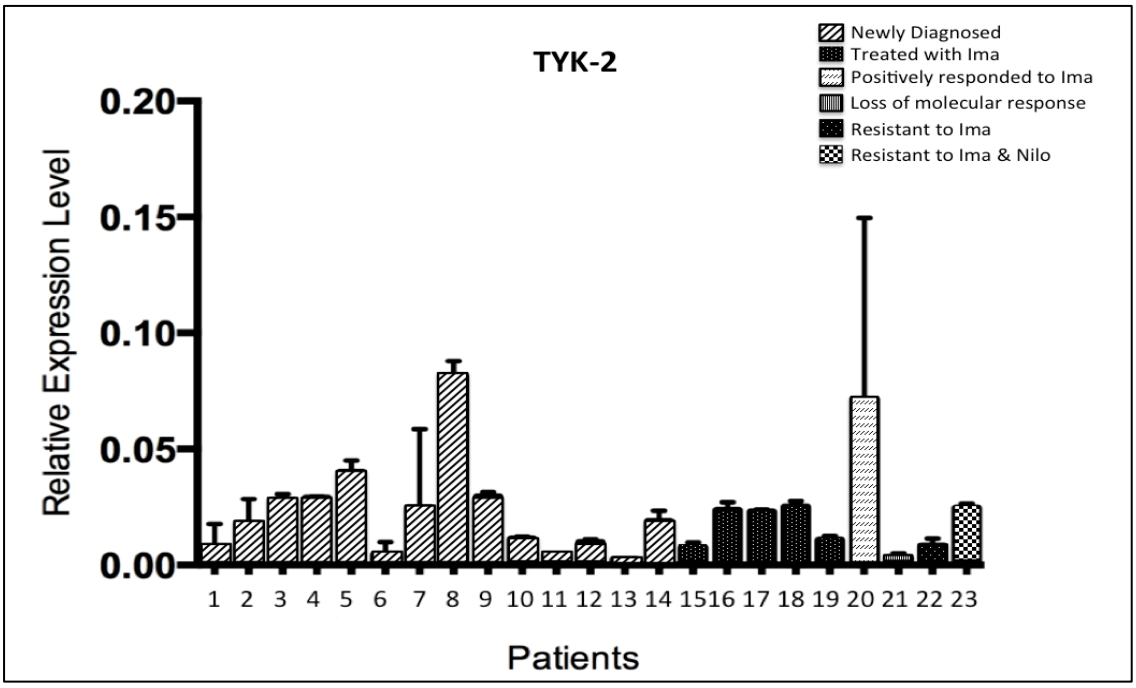

Figure 3.4. Expression levels of *Tyk2* gene in CML patients (normalized to β-actin, error bars represent SD, and $p<0.05$ was considered significant)

3.5. Expression Levels of *STAT1* Gene in CML Patients

STAT1 gene gene expression was the highest in patient resistant to both imatinib and nilotinib (number 23) ($p<0.05$). The patient having imatinib treatment (number 15) and newly diagnosed patients (number 4 and 9) have significantly higher expression levels of *STAT1* gene comparing to positively responded patient (number 20) ($p<0.05$). Overall, newly diagnosed patients (number 4-9) have partially higher expression levels as compared to the others (Figure 3.5).

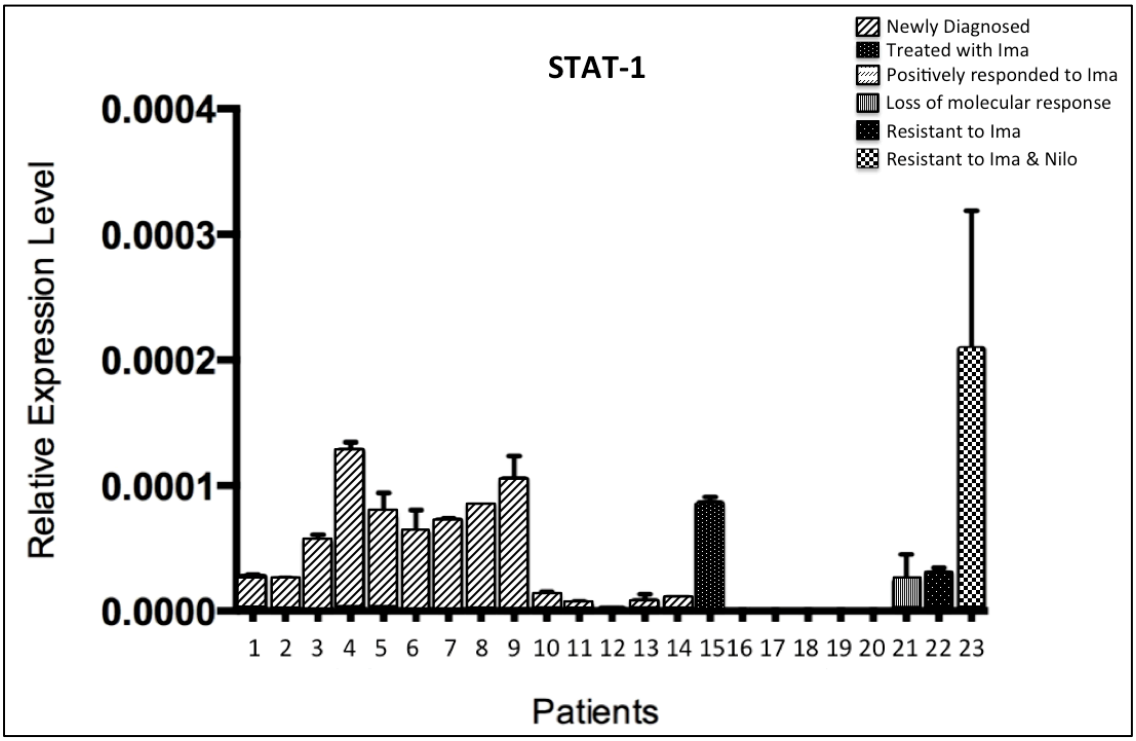

Figure 3.5. Expression levels of *STAT1* gene in CML patients (normalized to β-actin, error bars represent SD, and $p<0.05$ was considered significant)

3.6. Expression Levels of *STAT2* Gene in CML Patients

There was higher expression level of *STAT2* gene in both imatinib and nilotinib resistant patient (number 23) as compared to positively responded patient (number 20) and all patients having imatinib treatment ($p<0,05$). However, in one of the newly diagnosed patients (number 9), the highest expression levels of *STAT2* were determined. In newly diagnosed patients (number 4, 5, 6, 7, 10, 11, and 14), and patients having imatinib treatment (number 17 and 19), there were higher expression of *STAT2* gene compared to positively responded patient (number 20) ($p<0,05$). On the other hand, expression levels of *STAT2* gene in only imatinib resistant patient (number 22) was not found to be significant than positively responded one (number 20) ($p>0,05$) (Figure 3.6).

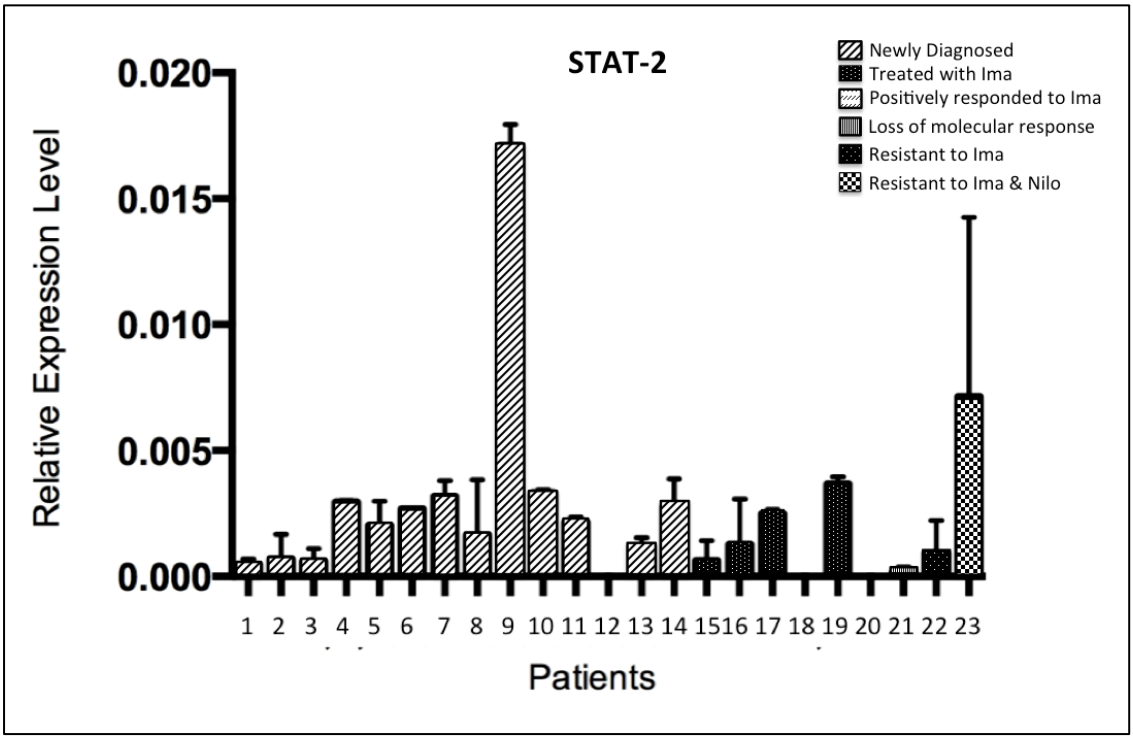

Figure 3.6. Expression levels of *STAT2* gene in CML patients (normalized to β-actin, error bars represent SD, and $p<0.05$ was considered significant)

3.7. Expression Levels of *STAT3* Gene in CML Patients

The highest expression level of *STAT3* gene was detected in both imatinib and nilotinib resistant patient (number 23) in this group. Also the newly diagnosed patients (number 3, 4, 5, 7, 8, and 9); TKI treated patients (number 15, 16, 17, and 19); patients lost the molecular response (number 21); both imatinib and nilotinib resistant patient (number 23) showed significantly higher expression level of *STAT3* gene as compared to positively responded patient (number 20) ($p<0,05$). Overexpression of *STAT3* gene stands out especially in the drug resistant patient. In contrast to other newly diagnosed patients, the patients numbered from 10 to 14 have relatively lower *STAT3* expression levels whereas one of the patients treated with imatinib (number 18) has the lowest level of *STAT3* gene expression ($p<0,05$) (Figure 3.7).

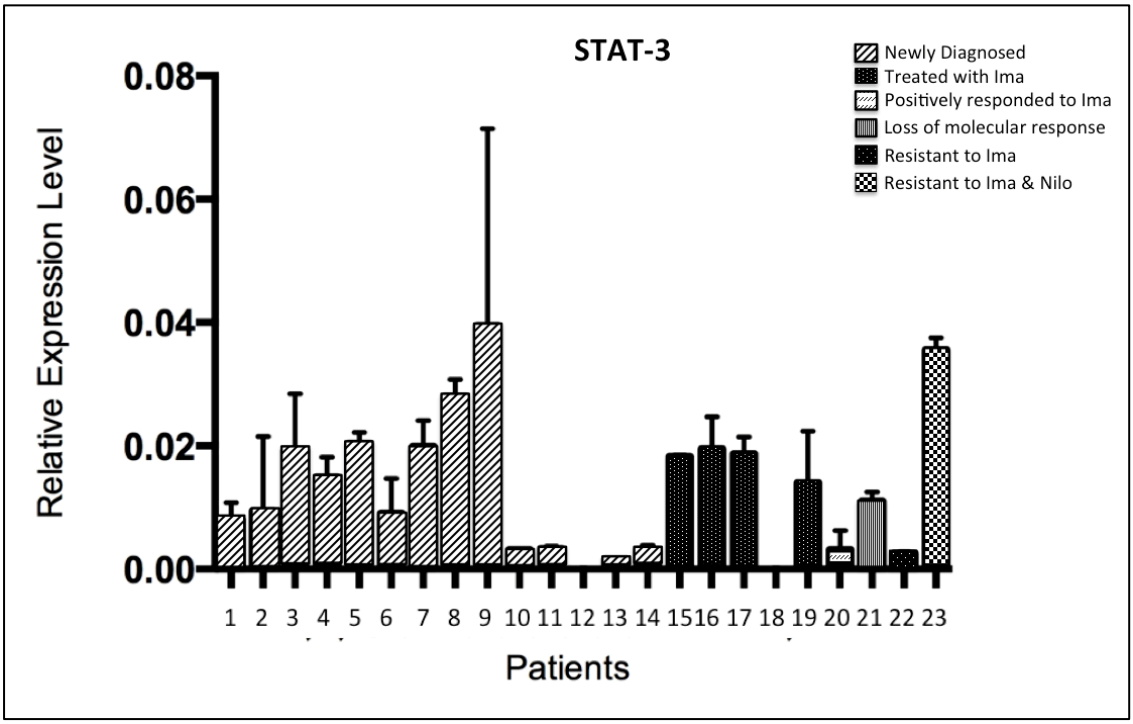

Figure 3.7. Expression levels of *STAT3* gene in CML patients (normalized to β-actin, error bars represent SD, and $p<0.05$ was considered significant)

3.8. Expression Levels of *STAT4* Gene in CML Patients

STAT4 gene has significantly higher expression in the patient who both imatinib and nilotinib resistant (number 23), compared to all other patient groups ($p<0,05$). It was also shown that, the newly diagnosed patient (number 9) and the patients having imatinib treatment (number 16 and 17) expressed *STAT4* gene at a higher level compared to positively responded patient (number 20) ($p<0,05$). Also the patient who lost molecular response (number 21) has one of the lowest level of *STAT4* expressions compared to all the others (Figure 3.8).

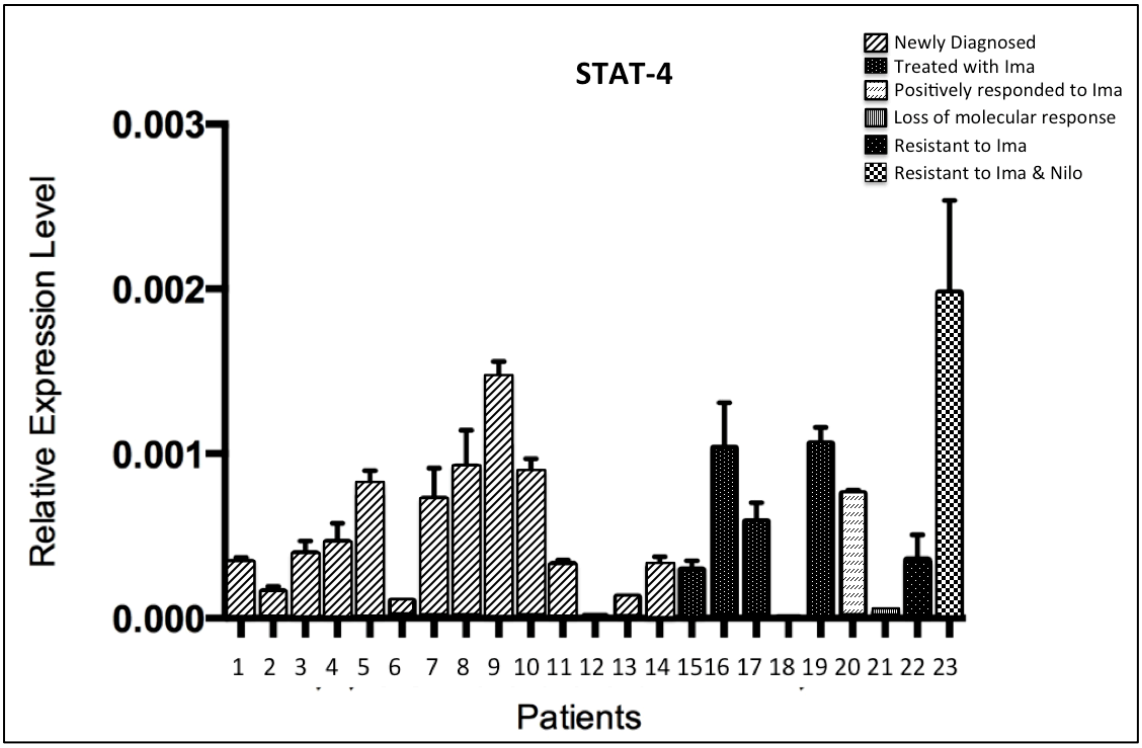

Figure 3.8. Expression levels of *STAT4* gene in CML patients (normalized to β-actin, error bars represent SD, and $p<0.05$ was considered significant)

3.9. Expression Levels of *STAT5A* Gene in CML Patients

STAT5A is highly expressed in one of the patients having imatinib treatment, (number 17) compared to all other patient groups, although not statistically significant ($p>0,05$). On the other hand, the newly diagnosed patients (number 2, 9, 10 and 14) and both imatinib and nilotinib resistant patient (number 23) have relatively higher level of *STAT5A* expression as compared to the others while the patient positively responded to imatinib (number 20) and the patient who lost molecular response (number 21) have the lowest expression levels of *STAT5A* gene in this group (Figure 3.9).

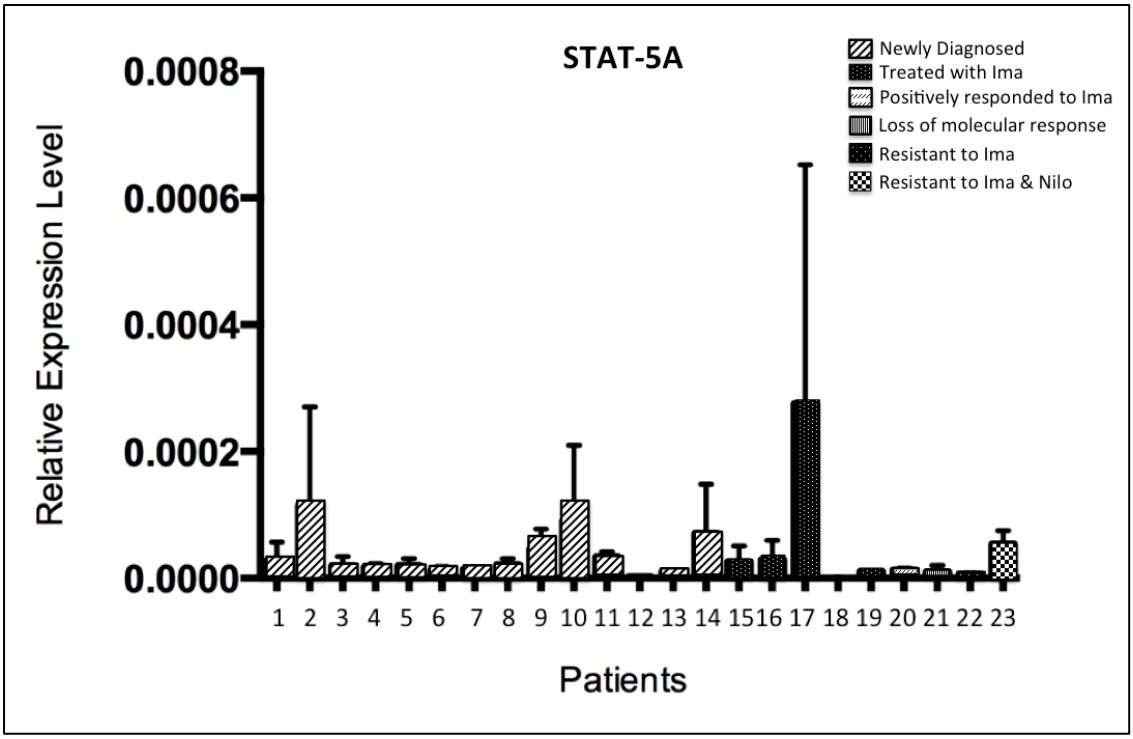

Figure 3.9. Expression levels of *STAT5A* gene in CML patients (normalized to β-actin, error bars represent SD, and $p<0.05$ was considered significant)

3.10. Expression Levels of *STAT5B* Gene in CML Patients

The highest expression levels of *STAT5B* expression was observed in newly diagnosed (number 4 and 9) compared to positively responded patient ($p<0,05$). The TKI resistant patients (number 22 and 23), the patient who lost molecular response (number 21) and the positively responded patient (number 20) have the lowest level of *STAT5B* expression in this group. Also both of the imatinib treated patients (number 15 and 19) have higher expression of *STAT5B* than positively responded one (number 20) ($p<0,05$) (Figure 3.10).

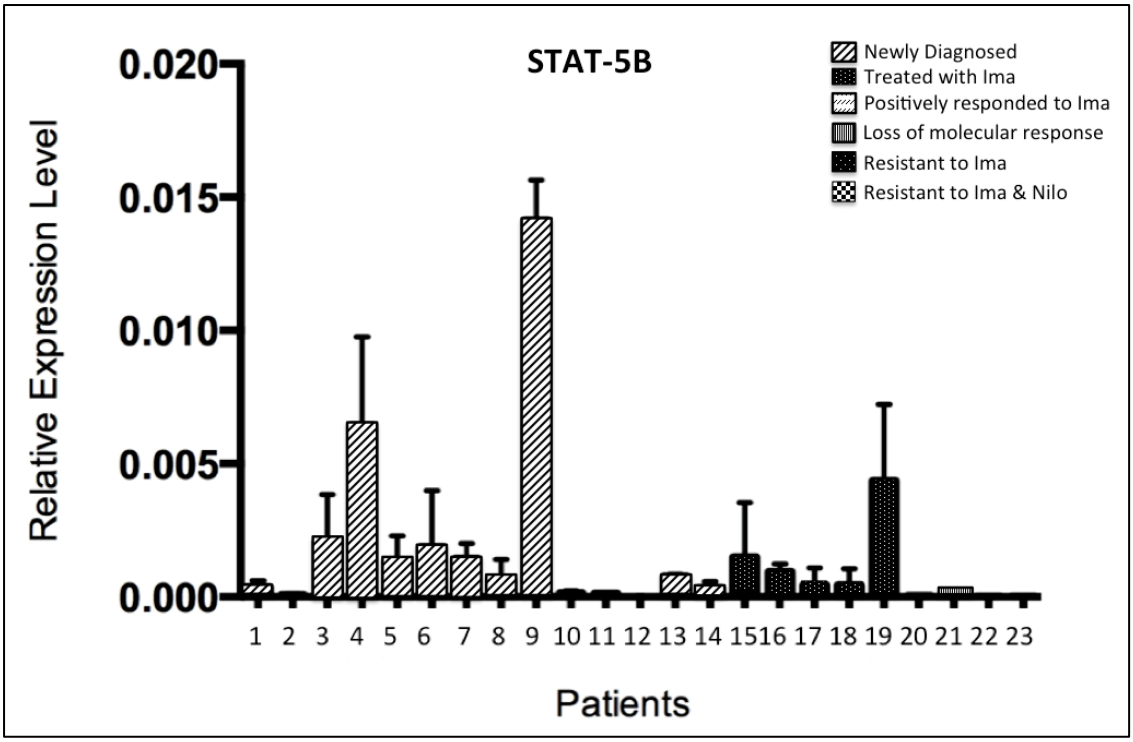

Figure 3.10. Expression levels of *STAT5B* gene in CML patients (normalized to β-actin, error bars represent SD, and $p<0.05$ was considered significant)

3.11. Expression Levels of *STAT6* Gene in CML Patients

Most of the newly diagnosed patients (number 1-8), positively responded to imatinib (number 20) and the patient who lost molecular response (number 21) have significantly higher levels of *STAT6* expression compared to all the others (p<0,05). Although newly diagnosed patients (number 9-14), imatinib treated patient (number 18) and TKI resistant patients (number 22 and 23) have dramatically lower levels of *STAT6* expression in this group (p<0,05) (Figure 3.11).

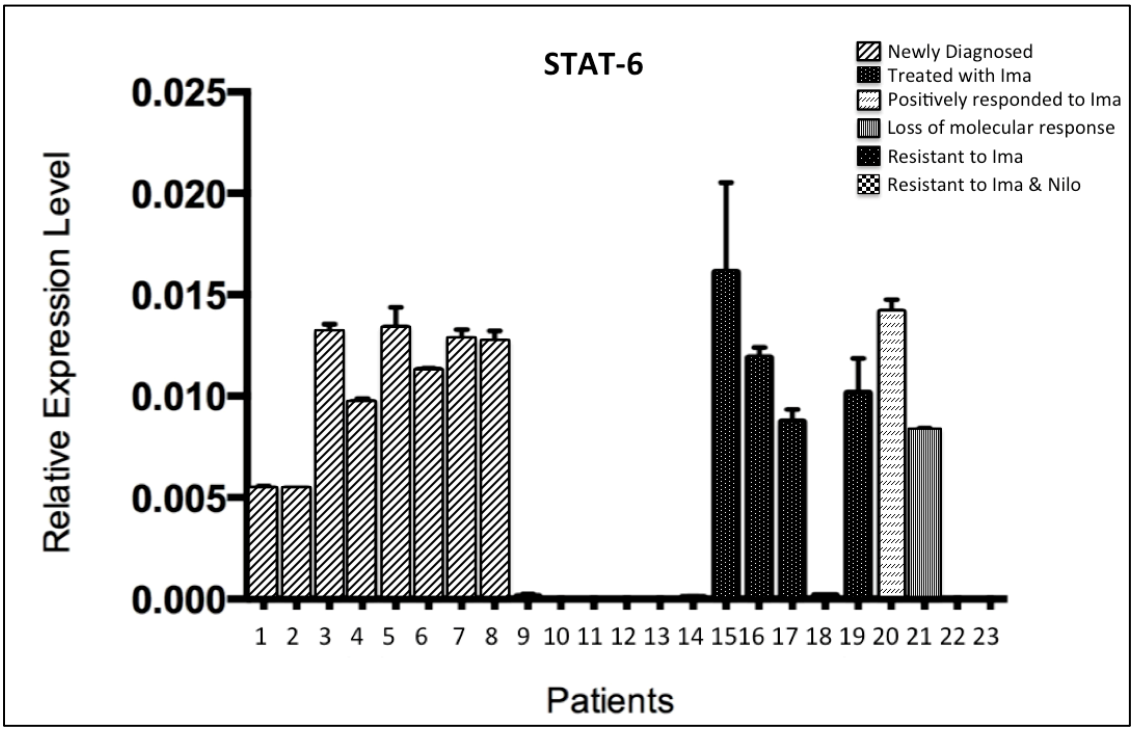

Figure 3.11. Expression levels of *STAT6* gene in CML patients (normalized to β-actin, error bars represent SD, and p<0.05 was considered significant)

CHAPTER 4

CONCLUSION

Chronic myeloid leukemia is a hematological disorder, diagnosed 1-2 cases per 100,000 people, each year and also represents 15% of all leukemias (Jabbour and Kantarjian 2012). This malignancy is characterized by BCR/ABL fusion protein that is resulted from a reciprocal translocation and has constitutive tyrosine kinase activity. Before the development of tyrosine kinase inhibitors, there were limited options for CML treatment which the most efficient one (IFN-α) induced cytogenetic response only in 20% of patients. However, after the introduction of imatinib, life expectancy and survival rates are greatly increased and CML transformed to a manageable chronic disease (Rumjanek, et al. 2013).

Although the circumstance those TKIs are highly potent for CML treatment, their therapeutic benefits may be limited by the phenomenon, called multidrug resistance. The mechanisms underlying the drug resistance can be BCR/ABL-dependent or independent. Drug transporters, microRNAs, cancer stem cells, epigenetic mechanisms and bioactive sphingolipids are involved in BCR/ABL independent drug resistance mechanisms, while BCR/ABL dependent mechanism contains, mutations of BCR/ABL or aberrant expression levels of BCR/ABL (Rumjanek, et al. 2013). In addition to them, different signaling pathways are involved in multidrug resistance status directly or indirectly by other effectors within the cell.

Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathway is one of the major signaling pathways involved in a number of cellular mechanisms such as cell growth, proliferation, differentiation, apoptosis and survival (Schindler and Plumlee 2008). After the evaluation of the vital importance of Jak2 protein in the initiation and maintenance of cancer and the discovery of Jak2V617F mutation in myeloproliferative neoplasmias; Jak/STAT pathway has gained more attention in cancer studies (Baxter, et al. 2005). Then this mutation have been identified in many other types of hematological malignancies such as ALL, CML and MDS (Steensma, et al. 2005). Jak2 was also found to have interactions with tyrosine kinase

fusion genes including BCR/ABL (Warsch, et al. 2013). It was also remarked that Jak2 might be an essential factor of BCR/ABL originated leukemogenesis (Li 2008).

In this study, we demonstrate the relationship between Jak/STAT signaling pathway genes and clinical outcome of CML patients. Bone marrow samples of 23 different patients including newly diagnosed, TKI resistant, lost molecular response and currently treated with imatinib were examined and expression levels of Jak/STAT genes (*Jak1, Jak2, Jak3, Tky2, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6*) were analyzed by qPCR.

Our results showed that *Jak1* gene is highly expressed in number 3, 5 and 7 in newly diagnosed patients and number 16 and 19 in imatinib treated patients compared to positively responded patient. Despite there is not any study represented the role of *Jak1* in CML, we detected its higher expression in some CML patients. This gene has the highest expression in imatinib-treated patients (Number 16 and 19) suggested that somehow imatinib treatment initiate the expression of *Jak1*. Number 22 and 23 who are TKI resistant patients have dramatically lower levels of *Jak1* expression than imatinib-treated patients, also confirmed that TKIs might induce *Jak1* gene, thus the resistant patients have lower expression. Conversely, the patient number 20 who positively responded to imatinib has the lowest level of *Jak1* expression. In newly diagnosed patients, number 2, 8, 10, 11, 12, 13 and 14 are likely to response negatively to TKI treatment or might develop drug resistance, if we consider they have already lower levels of *Jak1* like the patient who lost molecular response or TKI resistant ones.

Many studies indicated over activation of *Jak2* gene in CML patients. BCR/ABL was shown to phosphorylate and activate *Jak2* constitutively in CML lines (Wilson-Rawls, et al. 1996, Wilson-Rawls, et al. 1997). It was also shown that imatinib treatment decrease Jak2 phosphorylation by effecting BCR/ABL tyrosine kinase activity (Wilson-Rawls, et al. 1997). Once Jak2 become activated, it might increase c-Myc in mRNA level, effect B-catenin or inhibit PP2A pathway resulting in activation of certain transcription factors that mediate cell proliferation, leukemogenesis or apoptosis (Rubinfeld, et al. 1996, Samanta, et al. 2006, Samanta, et al. 2011). In another study, application of Jak2 inhibitor has been found to overcome drug resistance in blast crisis stage of CML patients, which TKIs are not clinically efficient (Samanta, et al. 2010). In our study, we detected most of newly diagnosed patients have higher Jak2; even number 4, 5, 6, 7 and 8 have the highest level then the other patients. According to previous studies we can say these patients may develop TKI resistance after treatment since they

have already higher level of *Jak2* gene. However in conversely to the literature, we did not detect significant high expression of *Jak2* in TKI resistant patients, suggesting that Jak2 activation may not be derived from its expression level. *Jak2* can be activated by many types of cytokines or by BCR/ABL kinase activity in chronic myeloid leukemia, but in mRNA level there might not be any change in especially TKI resistant patients. We also demonstrate that Jak2 activation may have role on loss of molecular response, by showing number 21 who has higher *Jak2* expression level compared to TKI resistant patients.

In a study, Jak3 was shown to have a physical interaction with v-Abl oncogene, which is related to initiation of leukemogenesis (Danial, et al. 1995). Its importance for drug resistance also demonstrated with a study that showed application of Jak3 inhibitor restored the sensitivity to cytotoxic agents in CLL cells (Steele, et al. 2010). We found that *Jak3*, clearly have the highest level of expression in TKI resistant patients. Number 15 who currently treated with imatinib also express *Jak3* in very high levels meaning that this patient may transform to TKI resistant in a short future. In this perspective, we can predict the clinical outcome for patient 18, as positive, who has the lowest level of *Jak3* gene. However, we could not detect any significant differences between newly diagnosed and positively responded patients.

In some studies it was suggested that Tyk2 has opposite effect on leukemia initiation, unlikely to other Jak family proteins. Tyk2 deficient mices were shown to develop Abl induced B-cell lymphoma (Stoiber, et al. 2004). The roles of Tyk2 on leukemias or cancer initiance have not cleared yet, so it is difficult to predict its role on CML as well. In our study, we have found that there are no significant expression level differences between all patients except patients number 8 and number 20. The highest level of *Tyk2* expression is detected in number 8 who is newly diagnosed and number 20 who is positively responded to imatinib. This data seems to confirm previous studies that suggest *Tyk2* has inhibitor effect on cancer initiation. Also we can predict that number 8 will positively response to TKI treatment in future, considering the condition of number 20.

The role of *STAT1* in CML and TKI resistance have not been fully illustrated even there are some studies that showed STAT1 is constitutively phosphorylated in TKI resistant cell lines, together with STAT5 (Carlesso, et al. 1996). In our study we represent the data showed the importance of *STAT1* gene in TKI resistance, clearly. Number 23, who both imatinib and nilotinib resistant patients have the highest level of

STAT1 expression while number 22 and number 21 who are imatinib resistant and who lost molecular response, respectively have slightly higher expression levels of *STAT1* compared to those who treated with imatinib, positively responded to imatinib and half of newly diagnosed patients. This result clearly evaluated that *STAT1* and drug resistance in CML patients has a positive correlation. We also could predict, number 15 who currently treated with imatinib and two of newly diagnosed patients (number 4 and 9) might develop drug resistance since they have higher level of *STAT1*, comparing to others.

As we indicated for *STAT1*, *STAT2* also has a positive correlation with drug resistance in CML patients. Number 9 who is newly diagnosed have the highest level of *STAT2*, followed by number 23 who resistant to both imatinib and nilotinib. This results evaluate that *STAT2* expression have an important role for TKI resistance in CML patients. We suggest to being watchful about patient number 9 and keeping this patient under observation since its very serious high level of *STAT2* may be correlated with TKI resistance in future. Number 20, who positively responded to imatinib, express *STAT2* at the lowest level, which also confirmed our prediction. One of the patients who currently treated with imatinib, number 18, has also the lowest level of *STAT2* expression, seems to be successful in TKI treatment considering both drug resistant patients and the patient who positively responded.

In many different studies STAT3 showed to induce drug resistance in CML patients (Bewry, et al. 2008, Nair, et al. 2012, Sayed, et al. 2014). Also it was showed that interestingly, STAT3 makes compensation for STAT5 in extraordinary situations (Bewry, et al. 2008). In another study, inhibition of Jak2 and Tyk2 was shown to blocked STAT3 activation and restore the sensitivity to nilotinib in CML cells (Nair, et al. 2012). Suppression of Jak2/STAT3 pathway or STAT3 inhibition decreases the level of imatinib resistance in both TKI resistant and sensitive cell lines (Bewry, et al. 2008, Stella, et al. 2013). Inhibition of STAT3, STAT5A and STAT5B both mRNA and protein levels showed to induced apoptosis in K562 CML cells (Kaymaz, et al. 2013). In addition to previous studies, we demonstrated tat *STAT3* and *STAT4* is highly expressed in number 23, both imatinib and nilotinib resistant patient. Interestingly, expression levels of *STAT4*, *STAT3* and *STAT2* are similar to each other in same patients. Number 12 and number 18 have the lowest expression levels of *STAT3* and *STAT4* gene, as well as *STAT2*; both probably will positively response to TKIs. Also

patient number 9, have the highest expression of *STAT3* and *STAT4*; again we strongly suggest that one needs to be followed during TKI treatment.

STAT5 is one of the most well defined modulator in TKI resistance that belongs STAT family of proteins. Higher levels of STAT5 are correlated with increased resistance to imatinib, in CML cells (Warsch, et al. 2011). In vivo studies also confirmed that if STAT5 is aberrantly expressed both mRNA and protein levels in v-ABL injected mice, imatinib resistance comes up (Warsch, et al. 2011). In another study, it is determined that the level of STAT5A is highly associated with BCR/ABL mutations (Warsch, et al. 2012). Despite Jak2 is the main regulator of STAT5; it was also showed that STAT5 might be activated by Jak2-independent way and also has vital roles on TKI resistance (Warsch, et al. 2013). In a study it was evaluated that STAT5A has roles on TKI resistance and stress protection while STAT5B does not (Casetti, et al. 2013). Our data exhibit that expression level of *STAT5A* is correlated with TKI resistance but not *STAT5B*. Both imatinib and nilotinib resistant patient has one of the highest level of *STAT5A* expression while number 17 that currently treated with imatinib has the highest, also might transform to develop resistance. Expression level of *STAT5A* gene in number 9 is slightly higher than other newly diagnosed patients in addition to number 2 and number 10, likely to other STAT expressions. Number 9 has the highest level of *STAT5B* expression, compared to other patients, meaning that following this patient during clinical process is necessary. Conversely to *STAT5A*, resistant patients have the lowest level of *STAT5B* while imatinib treated ones have higher than the others and also the patient that lost molecular response have slightly higher expression levels of *STAT5B* suggesting this gene may have role on mediating molecular response as well as TKI treatment.

Unlikely to other STAT family of proteins, *STAT6* is highly expressed in patient number 20, who responded positively to imatinib. It was the most difficult gene in Jak/STAT pathway to reach a conclusion and correlate between clinical outcome and TKI treatment. Also half of the patients of newly diagnosed have significantly high level of *STAT6* while the other half have the lowest level of *STAT6* expression. Also *STAT6* is highly expressed all the patients who currently treated with imatinib, compared to others, except number 18. The patients who resistant to TKIs have the lowest expression level of *STAT6*, suggesting that *STAT6* is down regulated in drug resistance conditions. Number 9, 10, 11, 12, 13, 14 and number 18 might be failure in TKI treatment considering their low expression levels of *STAT6*.

All in all, we demonstrated for the first time, that there is a correlation between all component of Jak/STAT signaling pathway in the regulation of tyrosine kinase inhibitors induced-apoptosis and drug resistance in chronic myeloid leukemia. Also we showed that *Jak3, STAT1, STAT2, STAT3* and *STAT4* genes are highly expressed in drug resistant patients. Clinical outcome after TKI treatment in newly diagnosed patients could be predictable and treatment options could shape according to this foresight. On the other hand, targeting Jak/STAT signaling pathway in addition to TKI application could be a new therapeutic approach to reach complete cure in CML and overcome drug resistance. But in order to reach more confident results, we are planning to increase the number of patients enrolled this study.

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