EXPRESSION LEVELS OF BIOACTIVE SPHINGOLIPID GENES IN NEWLY DIAGNOSED AND DRUG-RESISTANT CHRONIC MYELOID LEUKEMIA PATIENTS AND THEIR IMPACT ON THE CLINICAL PROGRESS

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ABSTRACT

EXPRESSION LEVELS OF BIOACTIVE SPHINGOLIPID GENES IN NEWLY DIAGNOSED AND DRUG-RESISTANT CHRONIC MYELOID LEUKEMIA PATIENTS AND THEIR IMPACT ON THE CLINICAL PROGRESS

Bioactive sphingolipids are a family of lipids including ceramide, glucosylceramide (GC), sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P) that have important functions in cellular processes including proliferation, metastasis, invasion, inflammatory response and apoptosis. Many sphingolipid-regulated functions are directly related to cancer initiation, progression, and response or resistance to anti-cancer treatments.

Ceramide, the central molecule of the sphingolipid metabolism, functions as a tumor-suppressor inhibiting cell division, and inducing cell differentiation, senescence and apoptosis. *De novo* synthesis of ceramides is regulated by ceramide synthase gene family (CERS1-6). Although ceramide is known to be a pro-apoptotic molecule, GC and S1P which are converted from ceramides by glucosylceramide synthase (GCS) and sphingosine kinase-1 (SK-1), respectively, are anti-apoptotic.

Chronic myeloid leukemia is a hematological disorder arisen from the reciprocal translocation between BCR gene on chromosome 22, and ABL gene on chromosome 9, t(9;22)(q34;q11), resulting in the formation of Philadelphia (Ph) chromosome. Ph chromosome encodes BCR/ABL fusion protein having constitutively active tyrosine kinase activity.

In this study, we examined the expression levels of CERS1-6, GCS, SK1, and BCR/ABL genes of 66 patients that are newly diagnosed, tyrosine kinase inhibitor (TKI)-resistant, or -sensitive. Q-PCR results showed that there were higher expression levels of apoptotic CERS1-6 in the patients TKI-treated and have shown minimum hematological response than that of the patients newly diagnosed and TKI-resistant. However, expression levels of antiapoptotic GCS and SK-1 genes were significantly higher in TKI-resistant and blastic phase patients than that of the other patients. Additionally, BCR/ABL expression levels were higher in newly diagnosed and TKI-resistant patients.

ÖZET

YENİ TANI VE DİRENÇLİ KRONİK MİYELOİD LÖSEMİ HASTALARINDA BİYOAKTİF SFİNGOLİPİD GENLERİNİN EKSPRESYON DÜZEYLERİ VE KLİNİK SEYİRE ETKİLERİ

Biyoaktif sfingolipidler hücrede bölünme, büyüme, yaşlanma, metastaz, invazyon, inflamatuvar yanıt ve apoptozda önemli roller oynayan ve seramid, glukozilseramid (GS), sfingozin-1-fosfat (S1F) ve seramid-1-fosfat (Ser1F) gibi önemli üyeleri olan bir lipid ailesidir. Sfingolipidlerin kontrol ettiği fonksiyonlar, kanserin başlaması, ilerlemesi ve antikanser tedavilere verilen yanıt ve dirençlilik ile doğrudan ilişkilidir.

Sfingolipid metabolizmasının temel molekülü olan seramid, tümör baskılayıcı olarak bölünmeyi durdurucu, farklılaşma, yaşlanma ve apoptozu tetikleyici fonksiyonlara sahiptir. Seramidin *de novo* sentezi, seramid sentaz gen ailesi (SerS1-6) tarafından gerçekleştirilmektedir. Seramidin pro-apoptotik bir molekül olduğu bilinmesine rağmen glukozilseramid sentaz (GSS) ve sfingozin kinaz-1 (SK-1) aracılığıyla seramidden dönüştürülen GS ve S1F, anti-apoptotik moleküllerdir.

Kronik miyeloid lösemi, 22. kromozomda bulunan BCR geni ile 9. kromozomda bulunan ABL geni arasındaki resiprokal translokasyon t(9;22)(q34;q11) sonucunda ortaya çıkan hematolojik bir hastalıktır. Bu translokasyon sonucunda oluşan Filadelfiya kromozomu, sürekli tirozin kinaz aktivitesine sahip olan BCR/ABL füzyon proteinini kodlamaktadır.

Bu çalışmada, yeni tanılı, tirozin kinaz inhibitörü (TKİ) dirençli veya duyarlı 66 hastanın SERS1-6, GSS, SK1 ve BCR/ABL genlerinin ekspresyon seviyeleri belirlenmiştir. Real-time PCR sonuçları, apoptotik SERS1-6 genlerinin ekspresyon seviyelerinin TKİ tedavisi gören ve minimum hematolojik yanıt gösteren hastalarda yeni tanılı ve TKİ dirençli olan hastalara göre daha yüksek olduğu görülmüştür. Bununla birlikte, antiapoptotik GSS ve SK1 genlerinin ekspresyon seviyelerinin ise TKİ dirençli hastalarda ve blastik fazda olan hastalarda yeni tanılı ve ilaçla tedavi edilen ve minimum hematolojik yanıt gösteren hastalara oranla çok daha yüksek olduğu görülmüştür. Ayrıca, BCR/ABL gen ekspresyonunun ise yeni tanılı ve TKİ dirençli hastalarda çok daha yüksek seviyede olduğu görülmüştür.

To indispensible people of my life...

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CHAPTER 1

INTRODUCTION

1.1. Chronic Myeloid Leukemia (CML)

Chronic myeloid leukemia is a hematological disorder characterized by a balanced reciprocal translocation between BCR (Breakpoint cluster region) gene on chromosome 22, and ABL (Abelson murine leukemia virus) gene on chromosome 9, t(9;22)(q34;q11), resulting in the formation of Philadelphia (Ph) chromosome. This Ph chromosome codes for BCR/ABL fusion protein. This protein has constitutively active tyrosine kinase activity. BCR/ABL consists of different domains. N-terminal domain of this protein is a cap structure, which has two isoforms due to alternative splicing. Tyrosine kinase domain has two subdomains called as Src-homology domains, including SH2 and SH3 (Cea, et al. 2013, Quinta´s-Cardama and Cortes 2009).

Isoforms of BCR/ABL fusion transcripts have variable molecular weights changing among 190-, 210-, or 230 kDa due to the alternative splicing patterns. The 210 kDa-oncoprotein is mainly found in CML and 5 -10% of adults with acute leukemia, whereas the 190 kDa-oncoprotein exists in acute lymphocytic leukemia, and also the 230 kDa oncoprotein is mainly found in chronic neutrophilic leukemia. Normally, the 145-kDa-ABL protein localizes in the nucleus, and has several important functions in different cellular signaling events such as cell cycle regulation, cellular response to toxic agents, and advising about the environmental conditions (Quinta´s-Cardama and Cortes 2009).

When fused with BCR, ABL cannot translocate into the nucleus, and remains in the cytoplasm. Additionally, since it is uncontrolled, ABL gains limitless tyrosine kinase function. BCR/ABL oncoprotein affects many downstream signaling pathways causing unlimited cell proliferation, decreased cell adhesion, increased cell motility, decreased apoptosis, and increased mitogenic signaling which finally cause the generation of CML phenotype (Jagani, et al. 2008). BCR/ABL also induces several signal transduction pathways including MAPK, PI3K, and RAS, which mediate cellular proliferation and cause the disruption of the genetic maintenance (Cea, et al. 2013).

In addition to Ph chromosome in 100% metaphases, another types of chromosomal abnormalities such as trisomy 8, loss of additional region in 22q, and isochromosome 17 can be also seen in 10-50% of CML patients. While 85 % of CML patients bear t(9;22) translocation, some patients can have a translocation between chromosome 22 and one another chromosome rather than chromosome 9, or translocation between other chromosomes beside of chromosomes 9 and 22. CML patients bearing variations in chromosomal translocations show similarity with the patients with typical Ph chromosome in terms of prognosis and response (Jabbour and Kantarjian 2014).

CML is characterized by three stages: chronic, accelerated and blast crisis. While chronic phase lasts few years, accelerated phase lasts 4-6 months, and blast crisis phase lasts a few months (Perrotti, et al. 2005).

Statistically, the incidence of CML is 1-2 per 100,000 population per year. The median age of presentation is 45 to 55 years, accounting for 20% of leukemia affecting adults. As in all leukemias, males are affected more than females in CML, with a ratio of 2:1 (Frazer, et al. 2007).

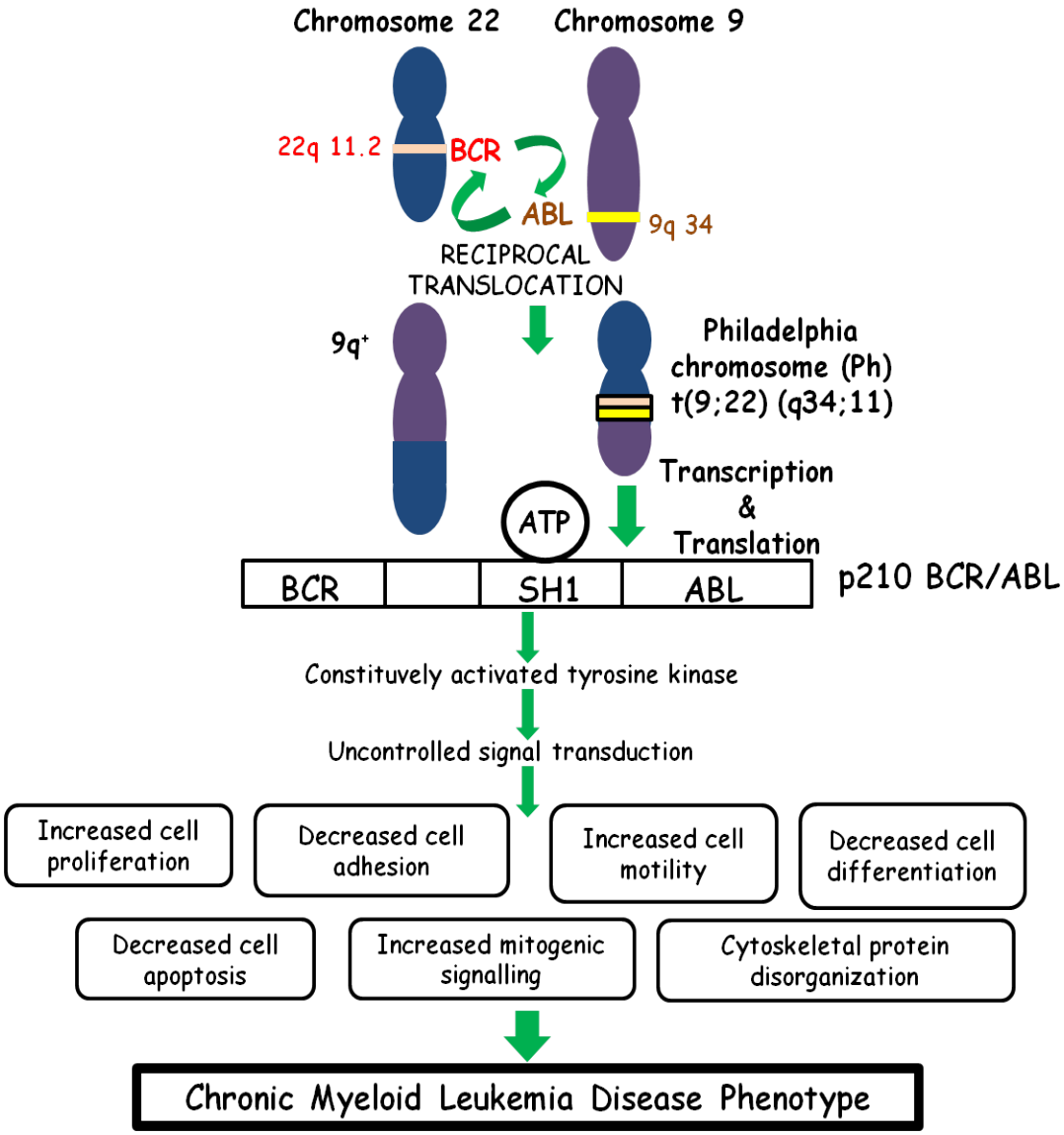

Figure 1.1. Generation of CML phenotype
(Adapted from: Frazer, et al. 2007)

1.1.1. BCR/ABL in PI3-K/Akt Signaling

Phosphoinositide-3 kinase (PI3-K) is a type of lipid kinase, and it has critical roles in many cellular processes involving survival, growth, mobility, and proliferation (Carnero 2010). Many studies have indicated that BCR/ABL expression activates majorily PI3-K (Steelman, et al. 2004, Naughton, et al. 2009). The active PI3-K converts PIP2 to PIP3 via phosphorylation, and then PIP3 in turn activates Akt. Afterwards, Akt affects the functions of many molecules such as Mdm-2, caspase-9, mTOR, Bad, and FOXO (Jagani, et al. 2008). Phosphorylation of apoptotic Bad by Akt prevents to promote its apoptotic effects. Additionally, BCR/ABL-mediated Akt activation also results in overexpression of antiapoptotic Bcl-2 (Skorski, et al. 1997). Phosphorylation of FOXO via Akt also results in the suppression of apoptosis and cell cycle arrest (Fu and Tindall 2008). A study has indicated that at chronic, acute, and blast crisis phases, CML cells treated with PI3-K inhibitor form less colonies than untreated ones. Similarly, CML cells treated with Akt inhibitor also showed decreased colony formation. These studies were also confirmed by *in vivo* studies (Jagani, et al. 2008). The other study has reported that Akt overexpression suppresses the release of cytochrome c, and by this way, it also prevents the activation of caspase-9 (Cardone, et al. 1998, Deming et al. 2004).

When BCR/ABL and Akt inhibitor-expressing marrow cells were transplanted into SCID mice, leukemia growth was significantly suppressed as compared to the control group (Skorski, et al. 1997). Furthermore, PI3-K inhibitor and imatinib have been reported to act synergistically on triggering apoptosis in CML cells at chronic and blast crisis phases. Additionally, Akt inhibitor treatment makes imatinib-resistant CML cells more susceptible to apoptosis (Klejman, et al. 2002).

1.1.2. BCR/ABL in NFκB Signaling

NFκB functions as dimers, and it can be activated by several factors including viruses, cytokines, and oxidative stress. IκB protein (inhibitor of κB) controls NFκB activity via keeping NFκB within the cytoplasm. By the phosphorylation and ubiquitinylation, IκB is degraded, and the free and active NFκB can enter the nucleus,

and by this way, it can mediate the expression of several genes related with cell survival processes (Li and Sethi 2010).

NFκB is found constitutively active in many cancer cells, and generally, it is related to the development of resistance against apoptosis. It leads to the transcription of cellular inhibitor of apoptosis proteins (C-IAPs), which suppress the activity of caspases (Danisz and Blasiak 2013). Many studies have showed that CML cells expressing BCR/ABL also overexpress the p65 (RelA), which is active subunit of NFκB (Li and Sethi 2010). In addition, constant NFκB activation was found to be necessary in order to generate tumors in nude mice by the transformation of hematopoetic cells with BCR/ABL (Jagani, et al. 2008).

1.1.3. BCR/ABL in Ras Signaling

The small GTP-binding proteins, Ras, transduce signals through tyrosine kinase receptors to the molecules found downstream (Danisz and Blasiak 2013). The connection between BCR/ABL and Ras signaling is mainly provided by the adapter protein Grb2. It binds to tyrosine residue 177 of ABL part of BCR/ABL by its SH2 domain (Ruibao 2005).

BCR/ABL expressing CML cells have also constitutively active Ras signaling. Murine bone marrow cells bearing inactive Ras also have impaired transforming potential of BCR/ ABL (Downward 2003). *In vivo* studies have been revealed that Ras and PI3-K/Akt pathways are quite significant in CML carcinogenesis (Jagani, et al. 2008).

1.1.4. BCR/ABL in JAK/STAT Signaling

Janus kinases (JAKs) are a family of protein kinases that are important in cytokine signaling (Liu, et al. 1998) The members of signal transduction and activators of transcription (STAT) family have functions in several signaling pathways such as cell growth, inflammation, differentiation, apoptosis, etc. Normally, STAT signaling is triggered with the binding of a ligand to cytokine or growth receptors, and this is followed by the phosphorylation, dimerization, and also translocation of STAT (Yu, et

al. 2009). STAT translocation into the nucleus is occurs following JAK activation by cytokine binding (Danisz and Blasiak 2013).

Since it has constitutive tyrosine kinase activity, BCR/ABL phosphorylates and activates STAT-1 and -5 constitutively. Active STAT-5 then triggers the expressions of anti-apoptotic genes such as Bcl-xL (Jagani, et al. 2008). Primarily, STAT1, -3, -5, and JAK2 are found active in CML cells bearing BCR/ABL (de Groot, et al. 2000). Majorily, STAT5 is overexpressed in CML, and when it is downregulated, imatinib-sensitive and –resistant K562 CML cell lines become more susceptible to imatinib treatment (Kosova, et al. 2010).

1.1.5. BCR/ABL and PP2A Phosphatase

PP2A is a tumor suppressor serine/threonine protein phosphatase. Studies have reported that BCR/ABL leads to the overexpression of PP2A inhibitor, SET gene in CML blast crisis. Inhibition of PP2A phosphatase by SET, in turn, leads to suppression of pro-apoptotic molecules such as Bad. PP2A inhibition also causes the overexpression of the genes such as Akt and Erk that are related with cell growth and survival (Jagani, et al. 2008).

PP2A reactivation in imatinib-resistant and –sensitive CML cell lines results in the inhibition of cell proliferation and survival molecules, the repression of BCR/ABL, and even the degradation of BCR/ABL (Perrotti and Neviani 2006).

1.2. Response Criteria in CML Therapy

1.2.1. Hematological Response

Hematological Response (HR) is a type of response that corresponds to the total count of leukocytes. HR is the most basic marker that shows the treatment goes on in a positive way. This HR could be partial or complete. In partial HR, amount of leukocytes decreases but does not reach a normal range, which changes between 4 and $11x10^9$/L. However, in complete HR (CHR) leukocyte amount decreases approximately below $12x10^9$/L (Jabbour, et al. 2008).

1.2.2. Cytogenetic Response

Cytogenetic Response (CyR) is a type of response that arises in the bone marrow rather than only in the blood. Bone marrow cells having Philadelphia chromosome (Ph+) in metaphase are counted in order to determine CyR by Fluorescent *in situ* hybridization (FISH). Cytogenetic response could be partial, major, or complete. In partial CyR, the bone marrow cells having Philadelphia chromosome (Ph+) decrease by 1-35%. In major CyR (MCyR), Ph+ cells in bone marrow decrease below ≤35%, whereas in complete CyR (CCyR), Ph+ cells could not be detected (0%) in bone marrow (Jabbour, et al. 2008).

1.2.3. Molecular Response

Molecular Response (MR) is a 3-log (0.1%) decrease in BCR-ABL transcript per a housekeeping control gene, generally ABL, (BCR-ABL/ABL) in blood or bone marrow sample. Molecular response could be determined by PCR (especially by quantitative RT-PCR), which is quite sensitive to detect BCR-ABL even as few as 1 in 100 000 cells. This MR could be also major or complete. While in major molecular response, BCR-ABL/ABL transcript reduced ≥3-log, in complete molecular response BCR-ABL transcript cannot be detected by PCR (Jabbour, et al. 2008).

Between cytogenetic and molecular response, there is a relation that 1-log (10%) reduction in BCR-ABL/ABL transcript estimates MCyR, whereas 2-log (1%) reduction estimates CCyR (Jabbour, et al. 2008).

Table 1.1. Response definitions in CML therapy

	Hematological Response (Leukocyte count)	**Cytogenetic Response (% Ph+ metaphases)**	**Molecular Response (BCR/ABL transcript level)**
Partial	$> 12x10^9/L$	1-35%	
Major		≤35%	≥3-log reduction (0,1%)
Complete	$< 12x10^9/L$	0%	BCR-ABL transcript cannot be detected

1.3. Treatment Protocols for CML

1.3.1. Allogeneic Stem Cell Transplantation

Allogeneic stem cell transplantation is carried out by engraftment of stem cells that provide blood formation of a donor that is genetically similar to the patient. Allogeneic stem cell transplantation has been used as curative therapy for CML since 1970 (Goldman and Melo 2003). Although survival chance depends on some elements such as age, disease phase, donor gender, studies have revealed that survival and disease-free life after the transplantation last approximately 10 years (Simonsson, et al. 2005). Nonetheless, the high possibility of raising graft versus host disease (GVHD) after the transplantation is a huge drawback of this treatment approach. Therefore, clinicians do not prefer this treatment protocol for the frontline therapy (Grigg and Hughes 2006).

1.3.2. Interpheron Alpha

Interpheron alpha (IFN-α), which is a biological glycoprotein, inhibits proliferation and viral infection (Frazer, et al. 2007). Many reports have revealed that interpheron alpha is the agent that is mostly used in combinatorial therapies (Baccarani, et al. 2002). A study has reported that interpheron alpha in combination with cytarabine results in higher efficacy in acquiring cytogenetic response, whereas the combination has no effect on survival rate (Guilhot, et al. 1997). However, studies have shown that use of interpheron alpha as a treatment strategy for CML results in elongated lifespan (Berger, et al. 2003).

1.3.3. Imatinib Mesylate (STI571)

Since BCR/ABL oncoprotein exists broadly in CML patients, and has constitutively active tyrosine kinase activity affecting downstream pathways related to cellular proliferation and survival, it has been considered as an open target for therapeutic applications. Therefore, tyrosine kinase inhibitors have been developed for the therapy (Pavlovsky, et al. 2009). Under normal conditions, if there is no inhibitor, ATP binds to kinase domain of BCR/ABL, and phosphorylates tyrosine residues on the substrate. Imatinib mesylate (STI571, Gleevec, Novartis, Switzerland), the widely used therapeutic agent, competitively binds to the ATP-binding pocket of BCR/ABL, and blocks ATP binding. By this way, tyrosine kinase activity of BCR/ABL is broken down (Hehlmann, et al. 2007, Fausel 2007). Since 2001, imatinib has been used as "gold standard therapy" due to its strong cytotoxic effects on CML cells (Frazer, et al. 2007). Beside of BCR/ABL kinase, imatinib can also inhibit PDGFR, c-Kit, DDR1, NQO2, and ABL (Jain, et al. 2013).

Comparative studies have revealed that imatinib has more endurable and efficient effects than IFN- α. Presently, clinicians recommend that patients recently diagnosed with CML should be treated with imatinib. Allogeneic stem cell transplantation should be referred only when imatinib therapy fails (Hehlmann, et al. 2007, Frazer, et al. 2007). A key clinical trial for TKIs and CML, International Randomized Study of Interferon and STI571 (IRIS) study involving 1106 patients treated with 400 mg/day imatinib or IFN- α in combination with cytrabine revealed that

55 % of CML patients could be treated with imatinib during 8-year-follow up, but the rest of the patients did not respond to the therapy (O'Brien, et al. 2003). Therefore, there was a need to investigate more effective second generation TKIs for CML patients who were intolerant or developed resistance to imatinib treatment (Jabbour and Kantarjian 2014).

1.3.4. Nilotinib (AMN107)

The second generation tyrosine kinase inhibitor, nilotinib (Tasigna, Novartis Pharmaceutical Corporation, NJ), inhibits the BCR/ABL oncoprotein function by binding to ATP-binding pocket of BCR/ABL. As compared to imatinib, nilotinib is more appropriate to the BCR/ABL oncoprotein, and binds more firmly. This type of binding of niolotinib resultis in more efficient and more susceptible drug action (Frazer, et al. 2007). In addition to inactive BCR/ABL, nilotinib can also bind and inhibit c-KIT, PDGFR, VEGF, ephrin receptors, and DDR1 (Jain, et al. 2013). *In vitro* studies have shown that nilotinib has 50-fold higher affinity to bind to BCR/ABL as compared to imatinib (Weisberg, et al. 2005).

Furthermore, many studies have indicated that CML patients with imatinib resistance are more susceptible to nilotinib (Larson, et al. 2012). ENESTnd study, a clinical study on the treatment of CML patients with nilotinib, has shown that nilotinib-treated patients show MMR at higher rates than the patients treated with imatinib. Additionally, transition rate into accelerated and blast phases is lower in nilotinib-treated patients as compared to imatinib-treated ones (Larson, et al. 2012).

1.3.5. Dasatinib (BMS-354825)

The other second generation tyrosine kinase inhibitor, dasatinib (Sprycel, Bristol-Myers Squibb) can bind both active and inactive conformations of BCR/ABL, contrarily to imatinib an nilotinib (Pavlovsky, et al. 2009). Since it is quite elastic in binding to several conformational structures of BCR/ABL, it has a high affinity to BCR/ABL kinase domain (Shah, et al. 2004). Despite that, due to having a potential to bind the other members of Src kinase family, dasatinib is not the most specific inhibitor of BCR/ABL kinase activity (Frazer, et al. 2007). Moreover, *in vitro* studies have indicated that dasatinib is 350-fold more effective than imatinib (Tokarski, et al. 2006).

3-year follow-up results of a phase III clinical trial, DASISION, showed that dasatinib treatment results in more effective and faster reactions than that of imatinib (Jabbour, et al. 2014).

1.3.6. Bosutinib (Bosulif)

The second generation TKI, bosutinib is a drug that inhibits Src- and BCR/ABL family kinases. Bosutinib is more effective than imatinib in CML patients who are resistant or intolerant to imatinib, and except of T315I and V299L mutations, it has an ability to inhibit most of the mutations in BCR/ABL (Cortes, et al. 2011). Clinical studies have shown that bosutinib is effective in CML patients non-responsive to imatinib, nilotinib or dasatinib treatment (Khoury, et al. 2012). An international clinical phase III study, Bosutinib Efficacy and Safety in Newly Diagnosed Chronic Myeloid Leukemia (BELA), has indicated that CCyR and MMR rates are higher in bosutinib-treated CML patients at chronic phase than that of imatinib-treated ones. Additionally, the rate of transition into accelerated and blastic phases is lower in bosutinib-treated patients (Cortes, et al. 2012).

1.3.7. Ponatinib (Iclusig)

The third generation TKI, ponatinib, is an FDA-approved drug that is effective even in CML patients with T315I mutation (O'Hare, et al. 2009). In T315I mutation, threonine at codon 315 of ABL gene switches with isoleucine. This isoleucine residue removes hydrogen bond and prevents the TKIs to bind to the ATP-binding pocket of BCR/ABL. Since ponatinib bears a particular carbon-carbon triple bond preventing the steric hindrance as a result of T315I mutation, it can bind and inhibit BCR/ABL kinase. In addition to BCR/ABL kinase, ponatinib can also inhibit Src kinase, KIT, FLT3, PDGFR, VEGFR, and FGFR (Jain, et al. 2013). In a phase I clinical trial, it has been reported that ponatinib treatment of CML patients with T315I mutation results in hematological response in all of the patients, CCyR in 75 % of the patients, and also MMR in 67 % of the patients (Cortes, et al. 2012). A phase II international clinical study, Ponatinib Ph+ALL and CML Evaluation (PACE), has reported that CCyR is seen 46 % of CML patients with T315I mutation (Jorge, et al. 2012).

1.3.8. Omacetaxine

Omacetaxine is an FDA-approved non-TKI agent derived from *Cephalotaxus harringtonia* alkaloid. It acts as a protein translation inhibitor. It binds ribosomal A subunit resulting in the prevention of the right arrangement of amino-acyl tRNA amino acid chains, and by this way, it inhibits translation (Wetzler and Segal, 2011). Omacetaxine is effective in CML patients that are resistant or intolerant to TKIs. A phase II study (Omacetaxine 202 study) has reported that more than 70 % of the patients treated with omacetaxine achieve CHR, and 16% of the patients achieve CCyR (Cortes, et al. 2013).

Current treatment protocols advise the use of imatinib, nilotinib or dasatinib for the frontline treatment of CML patients at chronic phase (O'Brien, et al. 2013). While the patients treated with second generation TKIs show higher rates of early optimal responses, long term survival effects of these TKIs require futher studies. The other treatment strategies such as allogeneic stem cell transplantation and interferon-alpha are no longer considered as frontline treatment protocols for the CML patients at chronic phase since imatinib, dasatinib or nilotinib are more effective in early optimum responses.

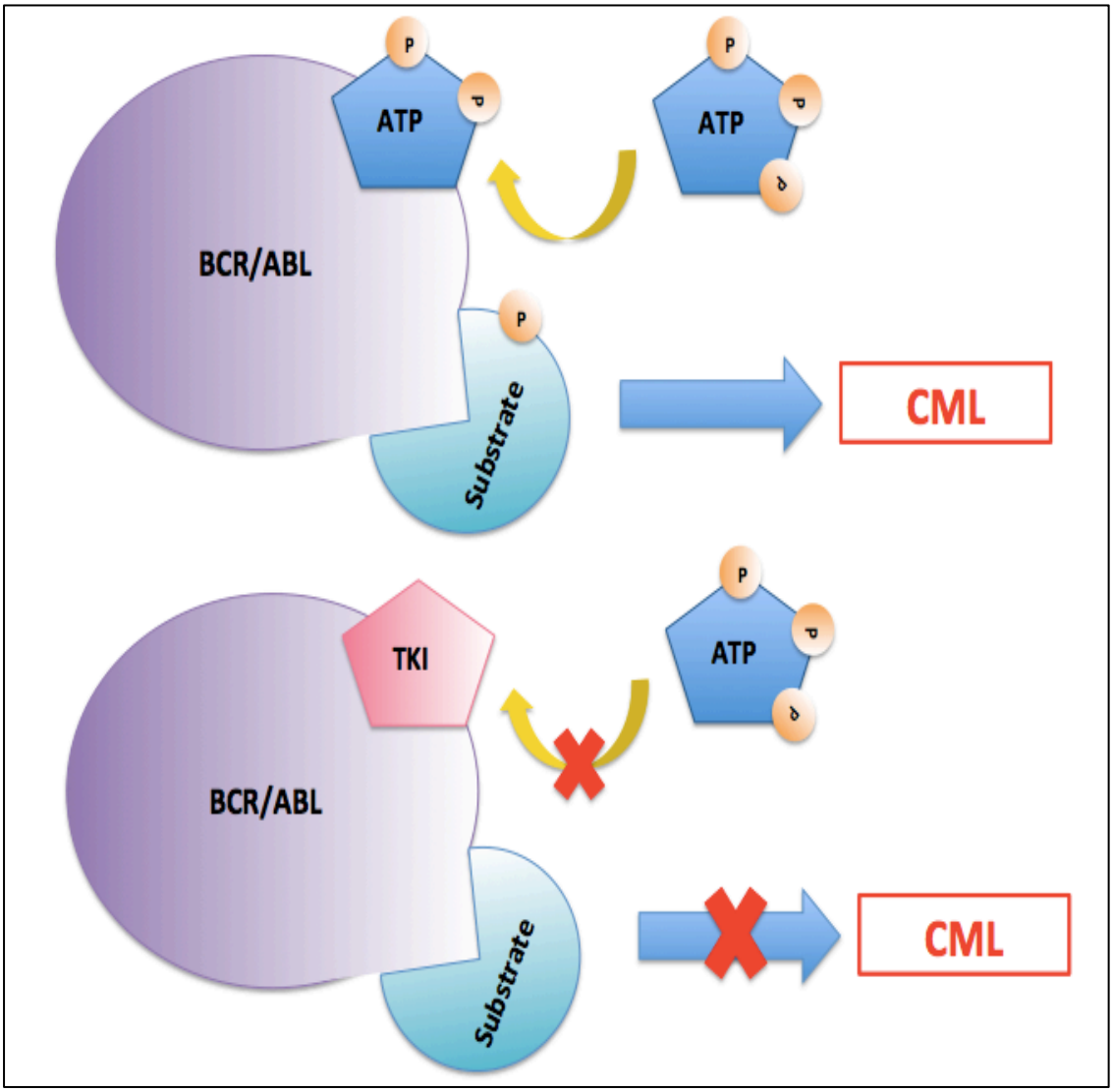

Figure 1.2. Mode of action of tyrosine kinase inhibitors

Table 1.2. Summary of therapeutic targets, recommended doses, and adverse effects of TKIs and omacetaxine used in CML therapy (Adapted from: Jain, et al. 2013)

	Therapeutic Targets	**Recommended Dose**	**Adverse Effects**
Imatinib	ABL Kit PDGFR DDR1 NQO2	400 mg 1x1/day	Myelotoxicity Periorbital edema Rash Nausea Skin pigmentation Elevated liver enzymes Diarrhea Headache
Nilotinib	ABL Kit PDGFR DDR1 NQO2 VEGF	300 mg or 400 mg 2x1/day	Elevated liver enzymes and glucose Elevated pancreatic enzymes and glucose Skin rash Myelotoxicity Diarrhea Nausea
Dasatinib	Src family PDGFR Kit	100 mg 1x1/day	Myelotoxicity Thrombocytopenia Low phosphate Diarrhea

(cont. on next page)

Table 1.2 (cont.)

Bosutinib	ABL Src family	500 mg 1x1/day	Diarrhea Elevated liver enzymes Myelotoxicity Edema Nausea Rash
Ponatinib	BCR-ABL kinase Src kinase	45 mg 1x1/day	Pancreatitis Hepatotoxicity Hypertension Rash Myelotoxicity Thrombocytopenia Edema
Omacetaxine	Protein translation inhibitor	1.25 mg/m^2 subcutaneosly 2x1/day	Myelotoxicity Thrombocytopenia Reactions at the injection site Infections Diarrhea

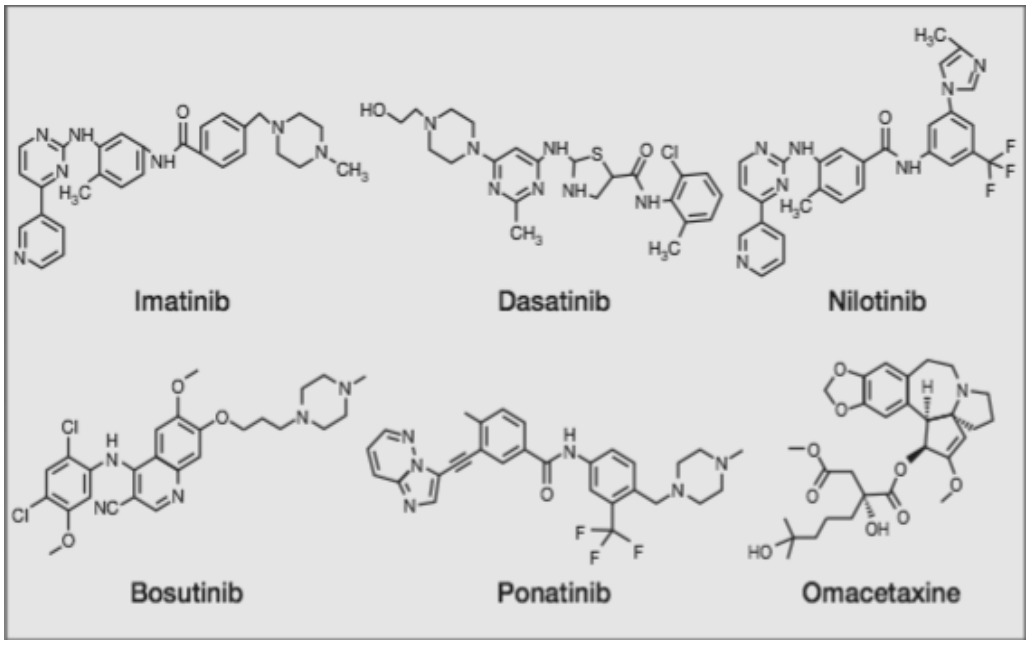

Figure 1.3. Chemical structures of TKIs and omacetaxine used in CML therapy (Source: Frankfurt and Licht 2013)

1.4. Mechanisms of Tyrosine Kinase Inhibitor Resistance in CML

In CML therapy, if the response cannot be detected enough, the disease prognosis is considered to develop resistance against the TKI used in therapy (Kimura, et al. 2014). An 8-year-follow-up study of CML patients has shown that 1/3 of CML patients at chronic phase treated with imatinib for a long time develop resistance (Deininger, et al. 2009). Resistance mechanisms developed against TKIs can be divided into two classes: BCR/ABL- dependent and –independent (Ramirez and DiPersio 2008).

1.4.1. BCR/ABL-Dependent Mechanisms of TKI Resistance

BCR/ABL-dependent mechanisms, commonly seen in CML patients, comprise point mutations generated in residues of Abl kinase domain of BCR/ABL protein, ATP-binding loop, A loop, the contact region (especially T315), and SH2 binding site (Deininger et al. 2005). Point mutations generated in P-loop account for 30-40% of point mutations (Branford, et al. 2003). Point mutation in P-loop is generally estimated with poor prognosis (Branford, et al. 2003). Generally, this type of mutation is seen in accelerated and blast crisis phases of CML.

T315I mutation, the other frequently encountered mutation, causes an important obstacle in CML therapy. This mutation results in the switch of the amino acid threonine at position 315 into isoleucine (Weisberg, et al. 2007). Patients bearing T315I mutation are resistant to imatinib, nilotinib and dasatinib (LaRosee, et al. 2002).

Moreover, another BCR/ABL-dependent resistance mechanism is BCR/ABL overexpression. Many studies have reported that the overexpression of BCR/ABL contributes to resistance in patients (Gorre, et al. 2001, Hochhaus, et al. 2002).

Alpha-1 acid glycoprotein (AGP) is the other mechanism mediating BCR/ABL-dependent TKI resistance. AGP has an ability to bind to imatinib at physiological concentrations, and results in a decrement in intracellular imatinib concentration. Subsequently, kinase activity of ABL cannot be suppressed sufficiently (Larghero, et al. 2003).

Additionally, TKIs are also substrates for P-glycoprotein (P-gp) and other efflux proteins found in cellular membrane. Many *in vitro* studies have shown that TKI resistance could be developed as a result of P-gp overexpression (Thomas, et al. 2004, Illmer, et al. 2004).

1.4.2. BCR/ABL-Independent Mechanisms of TKI Resistance

BCR/ABL-independent resistance mechanisms are also important in the generation of TKI resistance in CML therapy (Thomas, et al. 2004). This type of resistance mechanisms consists of reduction in the intracellular levels of TKIs, and the generation of multidrug resistance (MDR), which is development of cross-resistance to several anticancer agents (Gottesman, 2002). Although the mostly known mechanism of MDR is overexpression of ATP-binding-cassette (ABC) transporters, inhibition of apoptosis, detoxification of drugs, DNA repair mechanisms, and overexpression of Src family kinases are also the other mechanisms of MDR (Rumjanek, et al. 2013).

Activation of the signaling molecules such as Src family kinases, which are found downstream of BCR/ABL, is an example of BCR/ABL-independent resistance mechanism (Warmuth, et al. 1997). Src family kinases play roles in the generation of late stage CML, and BCR/ABL-independent resistance beside of cellular proliferation and survival (Dai, et al. 2004, Hu, et al. 2006). Many studies have shown that Src family kinases are responsible for oncogenic and kinase activity of BCR/ABL, and also

generation of CML disease phenotype (Meyn, et al. 2006). Overexpression of Lyn and Hck, members of Src family kinases, have been reported to be related to development of resistance against imatinib (Donato, et al. 2004).

Reduction of intracellular levels of TKIs via inhibition of drug uptake is an important obstacle in CML therapy. Imatinib uptake is carried out by organic cation transporter 1 (OCT1), encoded by SLC22 gene, whereas nilotinib and dasatinib uptake do not depend on the action of OCT1 (White, et al. 2006, Hiwase, et al. 2008). Studies comparing SLC22 gene expression levels in imatinib-sensitive and –resistant CML patients have shown that all CML patients overexpress SLC22 gene, but imatinib-resistant patients express at much higher levels than that of the sensitive ones (Corréa, et al. 2012). However, a study has demonstrated that there is no correlation between imatinib-sensitive and –resistant K562 human CML cell lines in terms of OCT1 expression (Hirayama, et al. 2008).

Reduction of intracellular levels of TKIs via activation of drug efflux is the other mechanism of TKI resistance. Multidrug resistance-associated protein 1 (MRP1 or ABCC1), breast cancer resistance protein (BCRP or ABCG2), and P-glycoprotein (P-gp or ABCB1), members of ABC transporter family, are responsible for TKI efflux (Chen and Sikic 2012). A study carried out by CML patients has reported that 55 % of patients express higher levels of P-gp although the treatment response and disease progression are not affected by the expression (Giles, et al. 1999). In another study, it has been demonstrated that CML patients at blastic phase rather than the other phases express ABCB1 at higher levels (Vasconcelos, et al. 2011). Additionally, ABCB1, ABCC1 and ABCG2 have been reported that they are expressed at higher levels in CML patients treated with imatinib for more than 6 months (Stromskaya, et al. 2008).

In spite of acquiring effective responses at early stages of the disease, CML patients treated with TKIs can become intolerant or develop resistance via several mechanisms described above. In this context, bioactive sphingolipids that have crucial functions in cancer can be considered as a potent effective strategy in CML therapy.

1.5. Bioactive Sphingolipids

Bioactive sphingolipids are a family, which is a member of membrane lipids. They have many important regulatory and structural roles in lipid bilayer. The sphingolipids ceramide, ceramide-1-phosphate, glucosylceramide, galactosylceramide, shingosine, sphingosylphospho-choline, and sphingosine-1-phosphate (S1P) are known as effector molecules of sphingolipid metabolism. They have many important roles in several cellular events including cell proliferation, senescence, apoptosis, inflammatory responses, and also migration (Ogretmen and Hannun 2004).

Ceramide is the key molecule of sphingolipid family, and it is synthesized in response to TNF-α, IL-1, Fas ligand, ionizing radiation, heat stress, oxidative stress, and chemotherapeutics (Ogretmen and Hannun 2004). Increased levels of ceramide lead to growth inhibition, apoptosis, differentiation, alteration of telomerase activity and telomere length, and senescence whereas increased S1P levels contributes to transformation, induction of cell proliferation, angiogenesis, and mobility. However, increased levels of glucosylceramide synthase (GCS), which converts apoptotic ceramide into antiapoptotic glucosylceramide, causes the development of multidrug resistance in several cancer cells (Ogretmen and Hannun 2004).

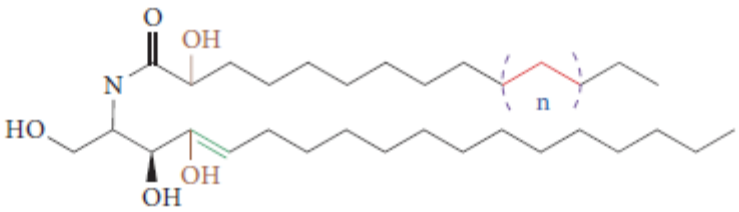

Figure 1.4. Chemical structure of ceramide
(Source: Hannun and Obeid 2008)

1.5.1. Bioactive Sphingolipid Metabolism

Ceramide, the central molecule of bioactive sphingolipid metabolism, is synthesized via two mechanisms including the *de novo* pathway, and the lipid hydrolysis, such as sphingomyelin (SM) hydrolysis. In *de novo* pathway, serine condenses with palmitoyl-CoA by serine palmitoyl transferase to generate 3-

ketodihydrosphingosine. 3-keto-dihydrosphingosine is reduced to sphinganine, which is then N-acylated by dihydroceramide (dhCeramide) synthases to produce dhCeramide or ceramide. In the hydrolytic pathway, sphingomyelin hydrolysis through sphingomyelinase generates phosphocholine and ceramide. Ceramide kinase phosphorylates ceramide, and generates ceramide-1-phosphate (Sugiura, et al. 2002).

Ceramidases convert ceramide into sphingosine (El Bawab, et al. 2002), and sphingosine kinases phosphorylate sphingosine in order to form sphingosine-1-phosphate (S1P). S1P is, in turn, converted into sphingosine by phospahatases or it is converted into ethanolamine-1-phosphate and a C16-fatty-aldehyde by the activity of lyase (Ogretmen and Hannun 2004).

1.5.2. Ceramide Synthase (CERS) Genes

De novo ceramide synthesis is conducted by CERS genes (CERS1-6) in mammals. Each of these six genes contributes to the generation of different lengths of ceramide. While CERS1 generates C18:ceramide, CERS2 and CERS4 generate C22:- and C24:ceramides, respectively. CERS5 and CERS6 generate C14:- and C16:ceramide, respectively. However, CERS3 generates C18:-, C22:-, and C24:ceramides (Teufel, et al. 2009). Generally, these ceramides varying in length have different functions in different types of cells. While C16:ceramide contributes to undergo apoptosis in LNCaP cells and hepatocytes, C18:ceramide is functional in head and neck squamous cell carcinomas (Teufel, et al. 2009).

1.5.3. Sphingolipid Compartmentalization

Enzymatic reactions are carried out in distinct cellular compartments in bioactive sphingolipid metabolism. *De novo* ceramide is carried out in the ER-associated membranes, and also on the cytosolic side of the endoplasmic reticulum (ER). Sphingomyelin and glucosylceramide synthesis are carried out in the Golgi apparatus. Once synthesized, ceramide is transferred from ER to the Golgi apparatus by two ways. Ceramide in the form of sphingomyelin is transported via CERT activity, which is the ceramide transfer protein. Ceramide in the form of glucosylceramide is transferred via vesicules. In the mitochondria, reactive oxygen species (ROS) activate

neutral sphingomyelinase (N-SMase) generating ceramide. This ceramide activates ceramide-activated protein phosphatases, PP1 and PP2A. After the activation, PP2A dephosphorylates and inactivates antiapoptotic proteins. Additionally, PP1 activates Bid, which is apoptotic. In lysosomes, ceramide is generated by acid sphingomyelinase. This ceramide activates Cathepsin-D and Bid, and then activates caspases -9 and -3, which are important in apoptosis. Additionally, ceramide generated in lysosomes is converted into sphingolipids by acid ceramidase. Ceramide is also generated in the lipid rafts of plasma membrane, and it affects crucial signaling pathways and raft function (Hannun and Obeid 2008).

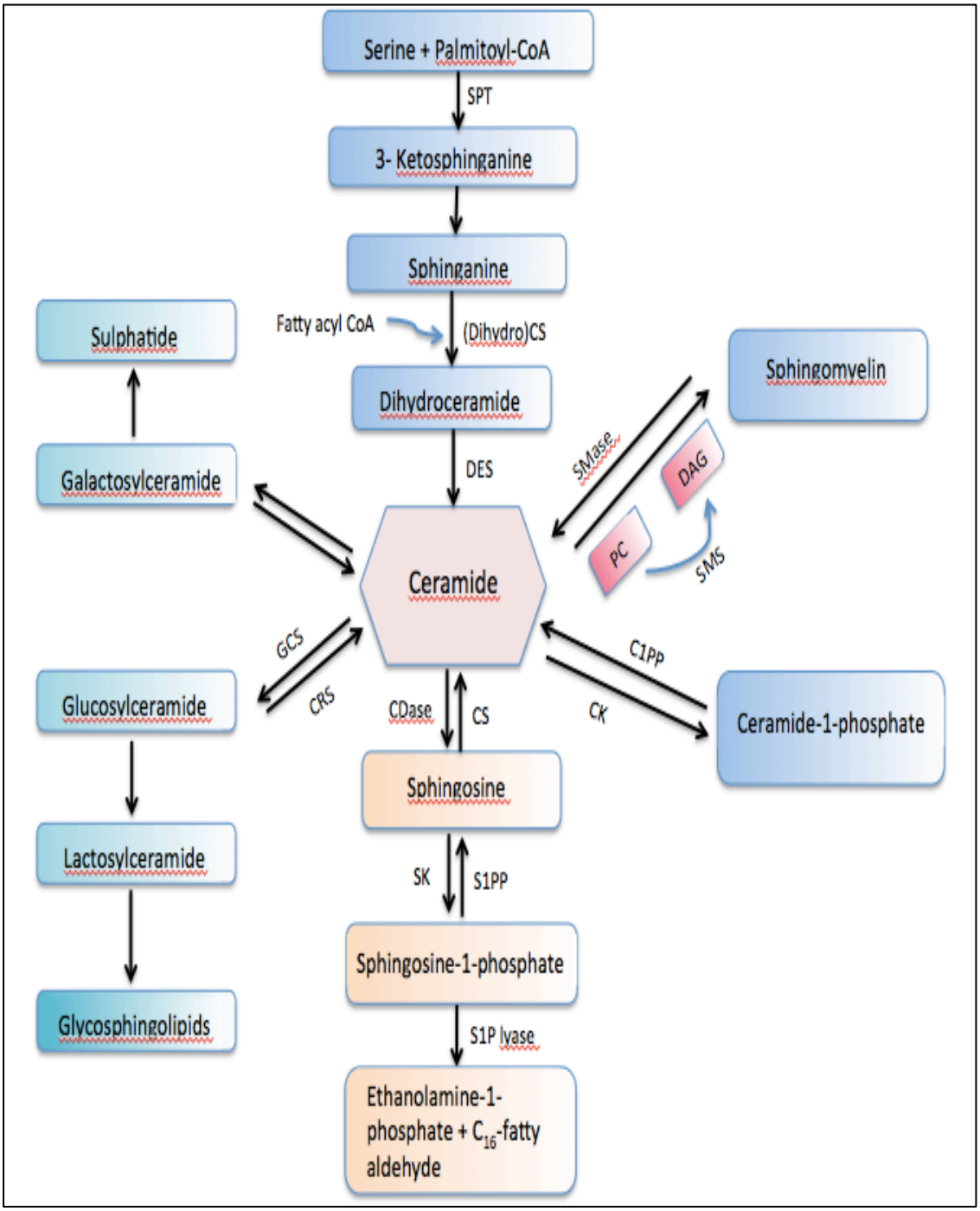

Figure 1.5. Pathways of sphingolipid metabolism
(Adapted from: Ogretmen and Hannun 2004)

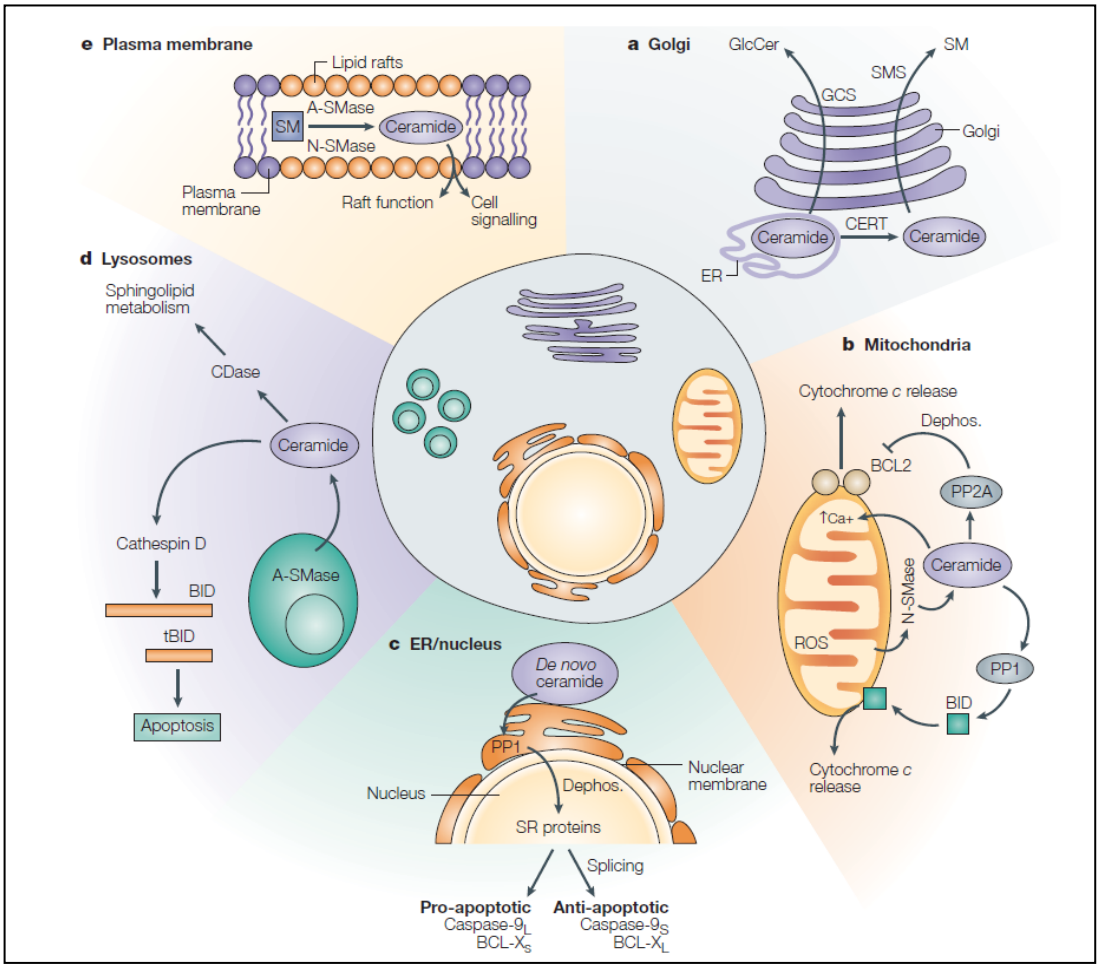

Figure 1.6. Ceramide compartmentalization
(Source: Ogretmen and Hannun 2004)

1.5.4. Ceramide and Apoptosis

In many cancer cells, ceramide is related to the apoptotic inducers including FAS/FASL, DNA damage, TNF-α, and hypoxia, which make ceramide to accumulate within cells (Pettus, et al. 2002). Chemotherapeutic agents such as daunorubicin, gemcitabine, etoposide, and FAS ligand that activate apoptotic signals trigger *de novo* generation of C16-ceramide (Ogretmen and Hannun 2004, Saddoughi, et al. 2008).

Ceramide generated by acid sphingomyelinase (A-SMase) is also important in apoptosis. Ionizing radiation and ultraviolet A (UVA) increase ceramide levels through A-SMase activity. Furthermore, TNF-α also triggers ceramide generation via A-SMase, and this ceramide, in turn, activates an endolysosomal aspartate protease, cathepsin D, which then activates caspase-9 and caspase-3 via Bid. Additionally, N-SMase is also

important in ceramide-mediated apoptosis. Generally, TNF is involved in N-SMase-mediated apoptotic pathways in many cancer cells. Moreover, ROS-mediated DNA damage triggers N-SMase-mediated ceramide accumulation (Ogretmen and Hannun 2004).

Ceramidases (CDases) are also important for the ceramide level regulation. Generally, CDases are degraded by nitric oxide (NO), and by this way, ceramide accumulates within the cell, which subsequently triggers apoptosis (Franzen, et al. 2002).

Ceramide generation can be controlled via more than one mechanism of ceramide metabolism even in the same cell type. In TNF-treated MCF-7 cells, *de novo* and N-SMase pathways are autonomously activated. Furthermore, FAS ligand binding activates the ceramide generation via A-SMase, N-SMase, and *de novo* pathways. Ceramide-induced apoptotic signaling are triggered by each pathways (Luberto, et al. 2002, Dbaibo, et al. 2001).

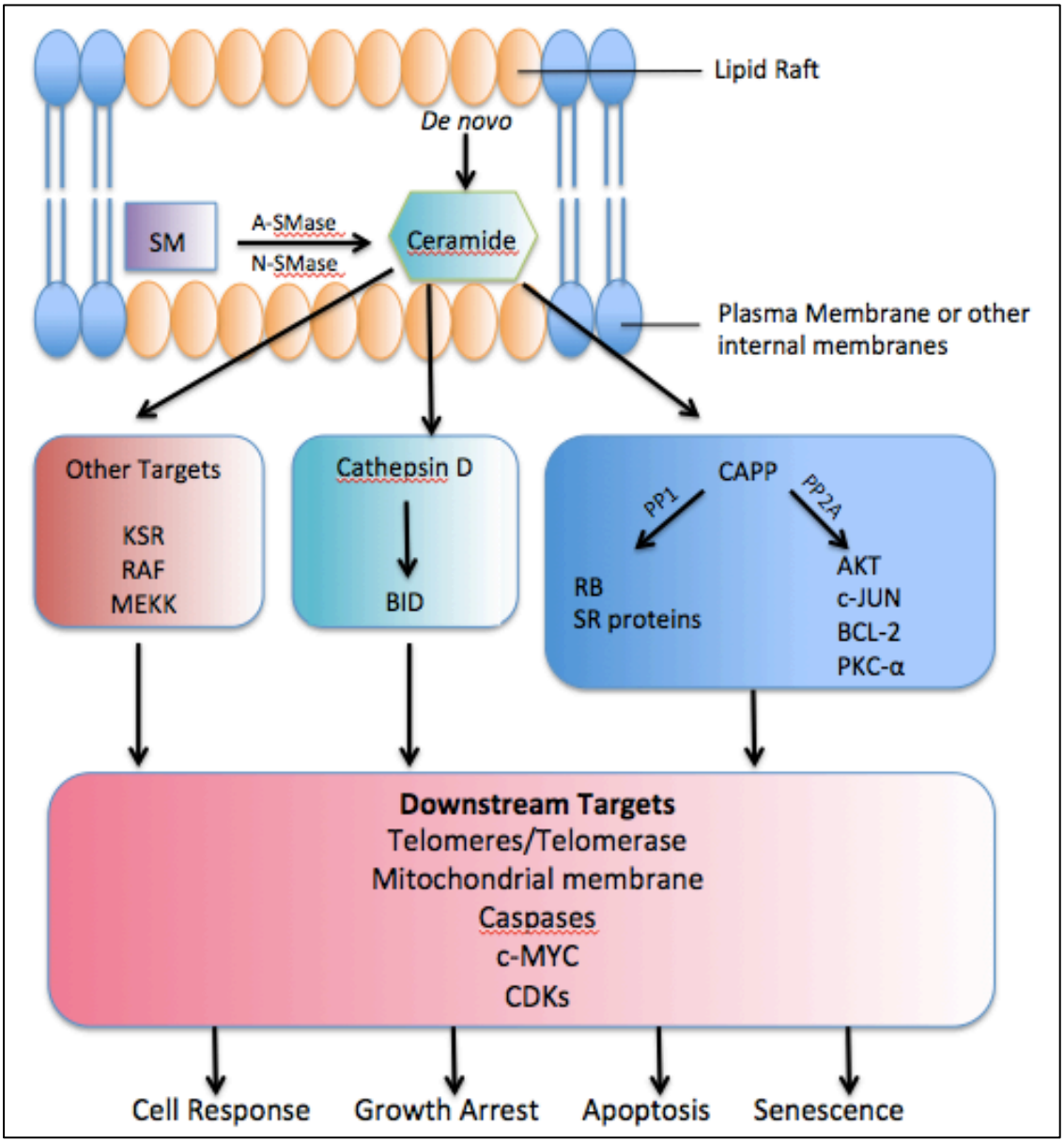

Figure 1.7. Downstream targets of ceramide
(Adapted from: Ogretmen and Hannun 2004)

1.5.5. Ceramide and Quiescence/Senescence

Generally, ceramide-mediated cell cycle arrest occurs at G0/G1 phase. TNF-mediated ceramide accumulation activates PP1, which in turn, dephosphorylates retinoblastoma (Rb) (Dbaibo, et al. 1995). Besides Rb, ceramide can dephosphorylate Cdk2, cyclin-dependent kinase, which is inactivated by dephosphorylation. p21WAF1 and p27KIP1, which are important in cell cycle regulation, are upredulated via ceramide accumulation in various cancer types. Cell cycle progression and cell growth are regulated via human N-SMase, which activates growth repression mediated by ceramide (Ogretmen and Hannun 2004, Lee, et al. 2000).

Dephosphorylation of Rb mediated by ceramide also triggers senescence via protein kinase C repression, growth factor signaling regulation, and CDK inhibition. In many cancer cells, senescence can be also triggered by ceramide via inhibition of telomerase activity (Ogretmen and Hannun 2004).

1.5.6. Sphingosine 1-Phosphate and Carcinogenesis

Contrarily to ceramide, increasing levels of S1P is related to cell proliferation, survival, and inhibition of apoptosis (Ogretmen and Hannun 2004, Cuvilier, et al. 1996). It can increase carcinogenesis potential and invasiveness of several cancer cells (Van Brocklyn, et al. 2003).

Angiogenesis can be induced by S1P via VEGF signaling in many cancer cells. VEGF signaling in turn activates RAS and MAPK signaling. S1P receptors are activated by S1P and it controls RHO GTPases, which contributes to migration of endothelial cells. These events then cause reconstruction of cytoskeleton, and apoptosis inhibition (Liu, et al. 2001, Wu, et al. 2003).

S1P-SK1 pathway can be activated by TNF-α and interleukin1 (IL-1), which result in inflammatory response development. As inflammatory reactions, SK1 activity generates S1P clusters, and then NFκB signaling is activated. Additionally, S1P triggers COX-2 and prostaglandin E2 (PGE2), which are important in inflammation (Xia, et al. 1998).

1.5.7. Sphingosine 1-Phosphate and Drug Resistance

Many studies have revealed that while increasing levels of ceramide result in apoptosis, increasing levels of S1P cause the inhibition of apoptosis (Senchenkov, et al. 2001, Radin, 2002). Antiapoptotic function of S1P in cancer cells makes it an important element for the development of drug resistance. SK1 has also been reported to decrease the apoptotic effects of the drugs used in the treatment of prostate adenocarcinoma cells (Pchejetski, 2006). PC-3 prostate cancer cells resistant to camptothecin have been reported to express higher levels of SK-1, and suppression of SK1 expression makes PC-3 cells sensitive to camptothecin (Bektas, 2005). Moreover, in kidney, breast, lung, uterus, and colon cancers, contributions of SK-1 to drug resistance have also been reported (Kawamori, et al. 2009, Visentin, et al. 2006). In these types of cancer cells, SK-1 inhibitor and S1P receptor antagonists have been reported to overcome drug resistance (Kapitonov, et al. 2009, Ponnusamy, et al. 2010). SK1 inhibition in multidrug resistant breast cancer cells leads to decrement in cell proliferation and triggers apoptosis via NFκB signaling pathway (Antoon, et al. 2012). In nilotinib-resistant chronic myeloid leukemia cells, Camgoz, et al. showed that nilotinib resistance could be reversed via inhibiting SK1 activity (Camgoz, et al. 2013). Furthermore, the mechanism of SK1 and S1P receptor-2- mediated drug resistance in chronic myeloid leukemia cells has been also reported. SK1/S1P increases BCR/ABL stability via influencing PP2A activity in imatinib-resistant K562 cells (Salas, et al. 2011).

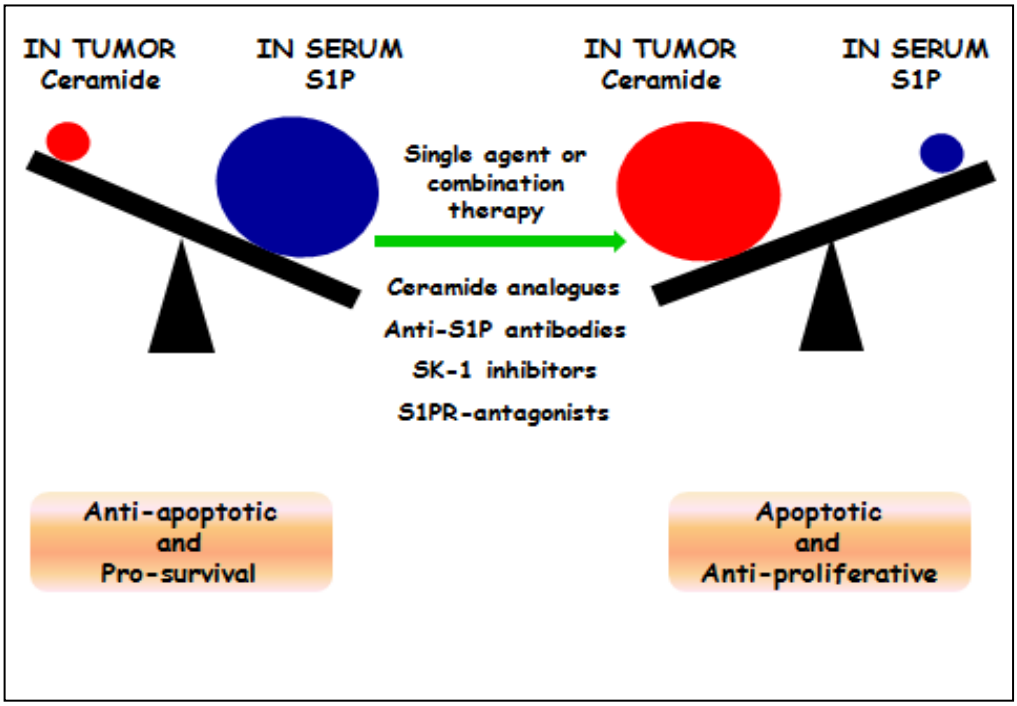

Figure 1.8. Changes in balance between ceramide and S1P in response to therapy (Adapted from: Saddoughi, et al. 2008)

1.5.8. Glucosylceramide and Drug Resistance

GCS is also found increased in drug resistant cancer cells. It has been reported that suppression of GCS activity decreases the expression levels of MDR1, and reverses drug resistance in breast cancer cells (Gouazé, et al. 2005, Baran, et al. 2011). Generation of multidrug resistant phenotype in cancer cells is related to GCS and P-gp activity (Ponnusamy, et al. 2010).

Drug resistance due to GCS overexpression in cancer cells can be reversed via GCS inhibition, and increasing intracellular levels of ceramide (Maurer, et al. 2000). Imatinib treatment results in increment in intracellular levels of ceramide in sensitive but not in resistant K562 human chronic myeloid leukemia cells (Baran, et al. 2007). Additionally, ceramide synthase activity inhibition leads to elevated apoptotic effects of imatinib in imatinib-resistant cells (Baran, et al. 2007). Moreover, GCS is overexpressed in imatinib-resistant K562 cells as compared to sensitive counterparts, and GCS inhibition by 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), which is a known GCS inhibitor, causes ceramide-induced apoptosis in both imatinib-

resistant and sensitive cells (Baran, et al. 2011). In adriamycin-resistant K-562/A02 chronic myeloid leukemia and doxorubicin-resistant MCF-7-AdrR breast cancer cells, GCS inhibition by 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) increases vinblastine uptake, and also decreases MDR1 gene and P-glycoprotein expression levels (Xie, et al. 2008, Gouazé, et al. 2005). Similar effects of PPMP have been also reported in vinblastine-resistant KB-V0.01 cervix cancer cells (Gouazé, et al. 2005, Adan- Gokbulut, et al. 2013).

1.6. The Roles of Bioactive Sphingolipids in Cancer Therapy

Understanding the molecular properties of the enzymes important in sphingolipid metabolism has provided the determination of molecular mechanisms of this metabolism. Enzyme compartmentalization suggests the complication of sphingolipid metabolism pathways. Furthermore, these enzymes have distinct functions in carcinogenesis since they found at varying levels. While carcinogenesis and ceramide levels are inversely related, it is directly related to sphingosine kinase-1 levels in various types of cancer cells (Ogretmen and Hannun 2004, Saddoughi, et al. 2008).

In brief, while ceramide is an apoptotic molecule, glucosylceramide and S1P generated from ceramide by GCS and SK1 activity, respectively, are anti-apoptotic molecules. Alterations in intracellular levels of these sphingolipids could be a novel approach for cancer therapy. Additionally, these changes might evade drug resistance, provide more effective chemotherapy, and also facilitate normal cells a defence mechanism to protect themselves from toxic materials (Ogretmen and Hannun 2004).

In vivo studies have indicated that bioactive sphingolipids have significant functions in various types of cancer. In pancreatic cancer, a cationic analogue of ceramide has been reported that it induces apoptosis via accumulating in mitochondria, and reducing resistance against gemcitabine, an anticancer agent used in pancreatic cancer therapy (Becham, et al. 2013). In addition, another group have reported that C6:ceramide treatment in combination with PDMP, GCS inhibitor, of resistant pancreatic cancer cells with nanoliposomes reduces tumor growth (Jiang, et al. 2011). In melanoma xenografts, glycosphingolipid hydrolase GBA2 has been reported that it degrades glucosylceramide, and by this way, it increases ceramide generation, which results in increased apoptosis and decreased tumor growth (Sorli, et al. 2013). In one

another study, researchers have reported that sphingomyelin supplementation in diet of rodent models with colon cancer decreases tumor growth, and importantly, no adverse effect is seen. In addition, the same group has also reported the similar results in breast cancer xenografts (Simon, et al. 2010). Moreover, lyposomal C6:ceramide treatment of mouse models with breast cancer via i.v. delivery has been reported that it suppresses tumor growth in mice (Stover, et al. 2005). SKi, which is one of the mostly used inhibitor for SK-1 and SK-2, is maintained in blood for eight hours after oral administration, and also decreases tumor size in mice (French, et al. 2006, Adan-Gokbulut, et al. 2013). A selective SK-2 inhibitor also inhibits cell proliferation, triggers apoptosis, and reduces tumor size in mice with kidney carcinoma, hepatoma, or mammary adenocarcinoma (Beljanski, et al. 2011, French, et al. 2010, Adan-Gokbulut, et al. 2013).

In clinic, Safingol, L-threo-dihydrosphingosine (DHS), is the first agent involved in clinical trials due to its sphingosine kinase inhibitor activity (Dickson, et al. 2011). When used with other chemotherapeutics, safingol exerts its effects more efficiently *in vivo* and *in vitro*. Safingol alone triggers autophagy via PI3K signaling. However, it increases the effects of some anticancer agents when used in combination (Coward, et al. 2009, Schwartz, et al. 1997). FTY-720, an FDA-approved immunosuppresant for the treatment of multiple sclerosis, has SK-1 and SK-2 inhibitory effects (Stevenson, et al. 2011, Adan-Gokbulut, et al. 2013). Additionally, FTY-720 also suppresses metastasis, reduces cell proliferation, and also triggers apoptosis via inhibiting angiogenesis in mice with melanoma or breast cancer (Pyne and Pyne 2010, Pyne, et al. 2011, Adan-Gokbulut, et al. 2013). Phase I clinical trials about sonepcizumab, a humanized S1P antibody that prevents binding of S1P to S1PRs, have revealed that sonepcizumab treatment of patients with solid tumors results in stable disease for approximately 8-12 months in most of the patients (Sabbadini, 2011, Gordon, et al. 2010, Adan-Gokbulut, et al. 2013).

1.6.1. Bioactive Sphingolipids in CML

The potential roles of bioactive sphingolipids in chronic myeloid leukemia cells are widely studied by several research groups. A group that aims to observe ABL targets in the ER has reported that ABL kinase phosphorylates the first enzyme of

sphingolipid metabolism, serine palmitoyltransferase long chain-1 (SPTLC1), which resides in the ER. BCR/ABL inhibition has been reported to trigger SPTLC1 activity and apoptosis in K562 and LAMA-84 CML cell lines (Taouji, et al. 2013). Another group reported that abnormal sphingomyelin synthase (SMS) activity, which generates sphingomyelin via depleting apoptotic ceramide and raising a mitogenic factor diacylglycerol (DAG), leads to cellular proliferation in K562 CML cells. In these cells, BCR/ABL overexpression results in increased SMS levels or vice versa. The researchers have proposed that SMS could be a downstream target for contributing to the proliferation of CML cells (Burns, et al. 2013). In nilotinib-resistant K562 CML cells, BCR/ABL, SK-1, GCS, and MRP1 genes have been found overexpressed while CERS1 and Bax genes have been found downregulated. Additionally, GCS and SK-1 inhibition leads to resensitization of CML cells against nilotinib (Camgoz, et al. 2013). The roles of GCS in CML cells have been reported in many studies. In imatinib-resistant CML cells, GCS is overexpressed as compared to sensitive counterparts. Moreover, forced overexpression of GCS in imatinib-sensitive K562 cells leads to the generation of resistance against imatinib. Inhibition of GCS by PDMP (DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), a GCS inhibitor, results in increased levels of ceramide, and also trigger of apoptosis in imatinib- treated imatinib-resistant CML cells (Baran, et al. 2011). The other studies have shown that PDMP inhibits GCS expression whereas SKi inhibits SK-1 expression, and they increase the cytotoxic and apoptotic effects of resveratrol on K-562 human chronic myeloid leukemia, and HL-60 human acute promyelocytic leukemia cells (Kartal, et al. 2011, Cakir, et al. 2011). Furthermore, PDMP and SKi together with nilotinib or dasatinib have significant synergistic apoptotic effects on K562 and Meg-01 human chronic myeloid leukemia cells (Camgoz, et al. 2011, Gencer, et al. 2011). Additionally, PDMP treatment of drug-resistant CML T315I mutant cells increases the sensitivity of these cells to imatinib, nilotinib, or GNF-2, an agent used for the inhibition of BCR/ABL *in vivo* and *in vitro*, and causes initiation of apoptosis through GSK-3 activation (Huang, et al. 2011). In a study, acid ceramidase (A-CDase) has been reported to be a downstream target for IFN regulatory factor 8 (IRF 8), a suppressor of myeloid leukemia. In chronic myeloid leukemia cells, IRF8 expression is controlled by DNA methylation, and IRF8 expression leads to inhibition of A-CDase, subsequently leads to C16:ceramide accumulation and activation of Fas-mediated apoptotic pathways. The other study has

shown that ceramide triggers apoptosis via activating p38, caspase-8, and c-Jun N-Terminal Kinase (JNK) in K562 CML cells (Nica, et al. 2008).

1.7. Aim and Originality of the Study

Chronic myeloid leukemia, a clonal myeloproliferative disease, accounts for 20% of leukemia affecting adults. The incidence of CML is 1-2 per 100,000 population per year, and unfortunately, the incidence is increasing day by day. More importantly, resistance cases are emerging against therapeutic approaches. Therefore, novel and more effective therapeutic approaches need to be investigated.

Bioactive sphingolipids, an important family of cellular membrane lipids, play critical roles in cancer. While ceramide is a powerful apoptotic sphingolipid, glucosylceramide and sphingosine 1-P are powerful antiapoptotic in cancer cells. Increased intracellular levels of ceramide result in trigger of apoptotic signaling pathways in cancer cells. However, increased levels of glucosylceramide and sphingosine 1-P lead to cell growth and proliferation, cellular transformation, angiogenesis, and also multidrug resistance.

Our previous studies showed that bioactive sphingolipids have essential roles in imatinib, dasatinib and nilotinib-induced resistance. Our data so far showed that expression levels of SK-1, GCS and BCR/ABL were significantly higher in imatinib and nilotinib-resistant CML cells when compared to their sensitive counterparts.

Under the light of these facts, the purpose of this thesis study is to determine the potential roles of bioactive sphingolipids in CML patient samples, and to carry our *in vitro* research to a more advanced level. In order to verify our *in vitro* cell culture results, expression levels of bioactive sphingolipid genes and the BCR/ABL oncogene were determined in bone marrow samples of

- newly diagnosed;
- imatinib-treated and have shown minimum hematological response;
- imatinib-resistant;
- nilotinib-treated and have shown minimum hematological response;
- nilotinib-resistant;
- dasatinib-treated and have shown minimum hematological response;
- dasatinib-resistant CML patients

So far, there has not been any report that studied bioactive sphingolipid genes in CML patient samples. Our previous *in vitro* studies have confirmed the roles of bioactive sphingolipids in drug resistance and –sensitivity of CML.

Whether the results of the studies performed in CML patient samples show a correlation between the bioactive sphingolipid genes and the progress, sensitivity and resistance of the disease, bioactive sphingolipids would be novel markers for use in the diagnosis of CML and for course of the disease. In that way, targeting bioactive sphingolipids would be in consideration with the aim of a more effective treatment of CML. As a matter of fact, by targeting bioactive sphingolipids, activity of the main cause of CML, the BCR/ABL oncogene, would be completely shut down. By this approach, two main reasons of the resistance problem, that are mutations in the target molecule or cellular amount of the target molecule, would be directly eliminated.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

Total RNA isolation kit was obtained from Macherey-Nagel (Germany). Reverse transcriptase (Moroney Murine Leukemia Virus Reverse Transcriptase), Taq DNA polymerase, dNTP set, and primers were obtained from Fermentas (USA). DyNAmo HS SYBR Green qPCR Kit was obtained from Thermo Fisher Scientific (USA). NH_4Cl and $KHCO_3$ included in lysis buffer were obtained from Sigma (USA).

2.2. Ethical Approval

This project has been approved by Ege University, Clinical Research Ethics Committee due to the use of bone marrow samples of chronic myeloid leukemia patients.

2.3. Patient Samples

We acquired bone marrow samples of 66 CML patients that were newly diagnosed; imatinib-treated and have shown minimum hematological response; imatinib-resistant; nilotinib-treated and have shown minimum hematological response; nilotinib-resistant; dasatinib-treated and have shown minimum hematological response, and dasatinib-resistant CML patients from Ege University Hospital, 9 Eylul University Hospital, Baskent University Hospital, Bayindir Hospital, Gulhane Military Medical School, and Erciyes University Hospital (Table 2.1).

All patients were informed about the study, and signed the consent form to be involved in the study. 44 males and 22 females were involved in this study. The median age of the patients was 52.

Table 2.1. Patient profiles involved in this study

PATIENT ID & NAME	AGE & GENDER	DIAGNOSIS
1. YA	58-M	Newly diagnosed CML
2. NHY	M	Shown hematological response to nilotinib treatment
3. SE	62-M	Newly diagnosed CML
4. AB	F	Loss of molecular response
5. CT	M	Newly diagnosed CML
6. EK	M	Treated with 1*400 mg imatinib
7. ZGB	58-F	Treated with 1*400 mg imatinib
8. DU	80-F	Newly diagnosed CML
9. CRG	74-M	Imatinib-resistant, treated with dasatinib
10. FM	19-F	Imatinib-resistant
11. KA	52-M	Nilotinib-resistant, treated with 800 mg nilotinib
12. ND	61-F	Newly diagnosed CML
13. MG	25-M	Treated with 1*400 mg imatinib
14. MD	36-M	Dasatinib-resistant, treated with 100 mg dasatinib
15. YA	37-M	Imatinib-resistant, treated with 1*400 mg imatinib
16. DU	81-F	Imatinib-resistant, treated with 1*400 mg imatinib
17. CRG	75-M	Dasatinib- and nilotinib-resistant, treated with nilotinib
18. SD	67-M	Treated with 1*300 mg imatinib
19. GU	66-F	Treated with 1*400 mg imatinib
20. SC	M	Newly diagnosed CML
21. AK	54-M	Treated with 1*400 mg imatinib
22. ZGB	59-F	Treated with 1*400 mg imatinib, shown MMR
23. SY	68-M	Treated with 1*400 mg imatinib
24. IC	55-M	Newly diagnosed CML
25. VT	M	Newly diagnosed CML

(cont. on next page)

Table 2.1 (cont.)

26. EO	38-M	At blastic phase, shown hematological remission
27. SB	56-M	Treated with 1*400 mg imatinib
28. YI	44-M	Newly diagnosed CML
29. SY	47-M	Treated with 50 mg 1x2 dasatinib, sensitive
30. SO	54-M	Newly diagnosed CML
31. EO	38-M	At blastic phase
32. DC	69-M	Newly diagnosed CML
33. BYO	39-F	Newly diagnosed CML
34. UA	M	Newly diagnosed CML
35. MD	36-M	Imatinib- and nilotinib-resistant, treated with dasatinib and interferon
36. MEC	52-M	Newly diagnosed CML
37. NT	65-F	Newly diagnosed CML
38. SS	54-F	Newly diagnosed CML
39. IC	55-M	Treated with 1*400 mg imatinib
40. KA	28-M	Newly diagnosed CML
41. AU	M	Imatinib- and nilotinib-resistant, treated with dasatinib
42. GT	M	Treated with 1*400 mg imatinib
43. IS	21-M	Newly diagnosed CML
44. SS	34-F	Blastic phase CML
45. AC	64-F	Newly diagnosed CML
46. TD	F	Newly diagnosed CML

(cont. on next page)

Table 2.1 (cont.)

47. AE	48-M	Treated with dasatinib
48. UT	31-M	Newly diagnosed CML
49. ES	50-M	Newly diagnosed CML
50. ZGB	60-F	Treated with 1*400 mg imatinib
51. FO	F	Newly diagnosed CML
52. DA	30-F	Newly diagnosed CML
53. SK	21-M	Newly diagnosed CML
54. IK	36-M	Newly diagnosed CML
55. IT	23-M	Newly diagnosed CML
56. NC	59-F	Newly diagnosed CML
57. NG	F	Newly diagnosed CML
58. EY	M	Newly diagnosed CML
59. AK	78-M	Treated with 1*400 mg imatinib
60. MM	82-M	Newly diagnosed CML
61. BY	45-M	Treated with 1*400 mg imatinib
62. SY	48-M	Treated with dasatinib
63. EA	52-K	Newly diagnosed CML
64. NB	48-F	Treated with nilotinib
65. AG	46-F	Newly diagnosed CML
66. AM	56-M	Newly diagnosed CML

2.4. 1X Lysis Buffer Preparation

155 mM NH_4Cl and 10 mM $KHCO_3$ are involved in the lysis buffer used for isolating bone marrow mononuclear cells.

2.5. Mononuclear Cell Isolation From Bone Marrow

Mononuclear cells from bone marrow samples of patients were isolated by lysis buffer including 155 mM NH_4Cl and 10 mM $KHCO_3$. 10 ml lysis buffer was added onto bone marrow sample in a steril falcon tube, and shaken by orbital shaker for 10 min. Then, bone marrow sample in the falcon tube was centrifuged at 8000 g for 5 min, and supernatant was removed. The pellet was homogenized with 2 ml lysis buffer, and then centrifuged again at 8000 g for 5 min, and then this step was repeated. Afterwards, the pellet was washed by 2 ml PBS, and centrifuged at 8000 g for 5 min. After the centrifugation, supernatant was removed, and the pellet was homogenized, and isolated bone marrow mononuclear cells (BMMNCs) were counted by hemocytometer.

2.6. Measurement of Cell Viability

In order to measure the viabilities of the cells, 50 µl of cells were mixed with 50 µl of trypan blue dye which is an important dye using in distinguishing dead cells from alive cells under the microscopy. After treating the cells with trypan blue dye, viable cells do not permeate this dye whereas dead cells permeate due to their broken cell membranes. Therefore, under the microscope, while dead cells were stained blue, viable cells were not. By this way, the cells were counted under the microscope by using hemocytometer and then the percentage of unstained cells were calculated. Before each RNA isolation, cell viability assay was conducted.

2.7. Total RNA Isolation and cDNA Synthesis

Total RNAs of BMMNCs isolated from the bone marrow samples of the patients were extracted using a Ribolock RNA isolation kit (Macherey-Nagel, Germany) as described by the manufacturer. Briefly, $4x10^6$ cells were centrifuged at 8000 g for 5

min, and the pellet was lysed with 350 µl RA1 buffer and 3,5 µl β-mercaptoethanol. Then the lysate was passed through insulin syringe for five times, and the cells were put on Nucleospin filter, and then cells were centrifuged at 11000 g for 1 min. After the centrifuge, filter was removed, and 350 µl ethanol was added onto the cell lysate, and then the mixture was put onto Nucleospin RNA column, and centrifuged at 11000 g for 30 s. Afterwards, 350 µl membrane desalting buffer was added onto the fitler, and centrifuged at 11000 g for 1 min. After this step, 95 µl DNase reaction mixture was added onto the column, and incubated at room temperature for 15 min. After the incubation, 200 µl RA2 buffer was added, and the column was centrifuged at 11000 g for 30 s. After the centrifugation, the column was put into a clean sterile collection tube, and 600 µl RA3 buffer was added, and then the column was centrifuged at 11000 g for 30 s. Then, 250 µl RA3 buffer was added onto the column, and again the column was centrifuged at 11000 g for 2 min. After these washing steps, the RNA was eluted from the column by 50 µl sterile RNase-free water, and centrifuged at 11000 g for 2 min. Concentrations of isolated RNAs were measured by Nanodrop ND-1000 (260/280 and 260/230 ratios). 1 µg of each total RNA was reverse transcribed using reverse transcriptase (RevertAid Reverse Transcriptase, Thermo Fisher Scientific, USA). After 10 min of incubation at 25 °C, and 60 min at 42 °C, the reactions were stopped at 70 °C for 10 min.

2.8. Quantitative Real Time Polymerase Chain Reaction (Q RT-PCR)

cDNAs were used in quantitative RT-PCR to measure the mRNA expression levels of CERS1, CERS2, CERS3, CERS4, CERS5, CERS6, SK1, GCS, BCR/ABL and β-actin. The expression levels of β-actin were used as internal positive control. All of the experiments were conducted as triplicate in two independent sets.

Expression levels of BCR/ABL were quantified and analyzed based on International Scale by using Ipsogen BCR/ABL Mbcr IS MMR Kit (Qiagen, USA). This kit provides a sensitive detection and quantification of BCR/ABL transcripts in bone marrow or peripheral blood samples of CML patients. This kit is also intended to assess molecular response level. Quantification of the expression based on IS provides accreditation to acquire reliable results. The expression levels of ABL were used as internal positive control for analyzing BCR/ABL expression levels.

Table 2.2. Primer sequences used in this study

β-actin-F	(5'-CAGAGCAAGAGAGGCATCCT-3')
β-actin-R	(5'-TTGAAGGTCTCAAACATGAT-3')
CERS1-F	(5'-CACTGCGCGCCTCTTTCG-3')
CERS1-R	(5'-ATTGTGGTACCGGAAGGCG-3')
CERS2-F	(5'- GCTGGAGATTCACATTTTAC-3')
CERS2-R	(5'-GAAGACGATGAAGATGTTGT-3')
CERS3-F	(5'-CATGATCTTGCAGGTCCTTCACC-3')
CERS3-R	(5'-CTCGTCATCACTCCTCACATCC-3')
CERS4-F	(5'-GACCTTCTCCTACAGTGCCAAC-3')
CERS4-R	(5'-GTCGCACACTTGCTGATACTGC-3')
CERS5-F	(5'-ATCTTCTTCGTGAGGCTG-3')
CERS5-R	(5'- ATGTCCCAGAACCAAGGT-3')
CERS6-F	(5'- ATCAGGAGAAGCCAAGCACG-3')
CERS6-R	(5'- AGTAGTGAAGGTCAGTTGTG-3')
GCS-F	(5'- TCCAGATACGCTTACTGACATGG-3')
GCS-R	(5'-TTGAAACCAGTTACATTGGCAGA-3')
SK1-F	(5'- TCCTGGCACTGCTGCACTC-3')
SK1-R	(5'-TAACCATCAATTCCCCATCCAC -3')

Table 2.3. Q RT-PCR conditions for β-actin

Steps	**Temperature (^{o}C)**	**Duration (min:sec)**	
Initial Denaturation	95	15:00	
Denaturation	94	00:10	**40 cycles**
Annealing	53	00:20	
Extension	72	00:30	
Melting Curve	60	1:00	

Table 2.4. Q RT-PCR conditions for CERS1

Steps	Temperature (^{o}C)	Duration (min:sec)	
Initial Denaturation	95	10:00	
Denaturation	95	00:15	**40 cycles**
Annealing	66	00:30	
Extension	72	00:30	
Melting Curve	71	2:00	

Table 2.5. Q RT-PCR conditions for CERS2

Steps	Temperature (^{o}C)	Duration (min:sec)	
Initial Denaturation	95	10:00	
Denaturation	95	00:15	**40 cycles**
Annealing	50	00:30	
Extension	68	00:30	
Melting Curve	55	2:00	

Table 2.6. Q RT-PCR conditions for CERS3

Steps	Temperature (^{o}C)	Duration (min:sec)	
Initial Denaturation	95	10:00	
Denaturation	95	00:15	**40 cycles**
Annealing	51	00:30	
Extension	72	00:30	
Melting Curve	56	2:00	

Table 2.7. Q RT-PCR conditions for CERS4

Steps	Temperature (^{o}C)	Duration (min:sec)	
Initial Denaturation	95	10:00	**40 cycles**
Denaturation	95	00:15	
Annealing	62	00:30	
Extension	72	00:30	
Melting Curve	67	2:00	

Table 2.8. Q RT-PCR conditions for CERS5

Steps	Temperature (^{o}C)	Duration (min:sec)	
Initial Denaturation	95	10:00	**40 cycles**
Denaturation	95	00:15	
Annealing	63	00:30	
Extension	72	00:30	
Melting Curve	68	2:00	

Table 2.9. Q RT-PCR conditions for CERS6

Steps	Temperature (^{o}C)	Duration (min:sec)	
Initial Denaturation	95	10:00	**40 cycles**
Denaturation	95	00:15	
Annealing	55	00:30	
Extension	72	00:30	
Melting Curve	60	2:00	

Table 2.10. Q RT-PCR conditions for GCS

Steps	Temperature (^{o}C)	Duration (min:sec)	
Initial Denaturation	95	10:00	
Denaturation	95	00:15	**40 cycles**
Annealing	62	00:30	
Extension	72	00:30	
Melting Curve	67	2:00	

Table 2.11. Q RT-PCR conditions for SK1

Steps	Temperature (^{o}C)	Duration (min:sec)	
Initial Denaturation	95	15:00	
Denaturation	95	00:10	**40 cycles**
Annealing	64	00:20	
Extension	72	00:10	
Melting Curve	69	2:00	

Table 2.12. Q RT-PCR conditions for BCR/ABL

Steps	Temperature (^{o}C)	Duration (min:sec)	
Initial Denaturation	95	00:15	
Denaturation	95	00:05	**50 cycles**
Annealing	60	00:30	
Extension	36	01:00	
Melting Curve	65	2:00	

2.9. Statistical Analysis

Statistical significance was determined using one-way analysis of variance for quantitative RT-PCR. $P<0.05$ was considered to be significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Determination of CERS1 Gene Expression Levels

Real-time PCR results were analyzed according to the expression levels of beta actin gene of each patient as reference. The results showed that patients with 12, 25, 30, 32, 36, and 38 ID numbers (#) newly diagnosed; # 2 treated with nilotinib; #7, #23, #39, #50, and #62 treated with imatinib; #15 imatinib-resistant; #17 nilotinib- and dasatinib-resistant; and also #47 treated with dasatinib showed higher levels of CERS1 expression. However, CERS1 expression levels were significantly lower in patients # 4, #9, and #10 imatinib-resistant; #11 nilotinib-resistant; #35 and #41 both imatinib- and nilotinib-resistant; # 31 at blastic phase; and also # 33 newly diagnosed (Figure 3.1 A and B).

3.2. Determination of CERS2 Gene Expression Levels

Our data demonstrated that CERS2 gene expression levels were significantly higher in patients #1, #3, #12, #25, #30, #32, #34, #36, #37, #38, #40, #44, #46, #48, #49, #52, #53, #54, #55, and #64 that have been newly diagnosed; patients #23 and #62 that have been treated with imatinib and shown major molecular response; and #26 at blastic phase and shown hematological remission; and also patients #2 treated with nilotinib, #7, #39, and #50 treated with imatinib, #15 imatinib-resistant, #17 both nilotinib- and dasatinib-resistant, and also #47 treated with dasatinib. On the other hand, CERS2 gene expression levels were found significantly lower in patients #4, #10, and #11 that have been treated with imatinib and shown loss in molecular response, resistance to IMA, and resistance to NIL, respectively, and also #35 that have shown resistance to both IMA and NIL; and #59, #63, #65 that have been treated with imatinib, dasatinib, and nilotinib, respectively (Figure 3.2 A and B).

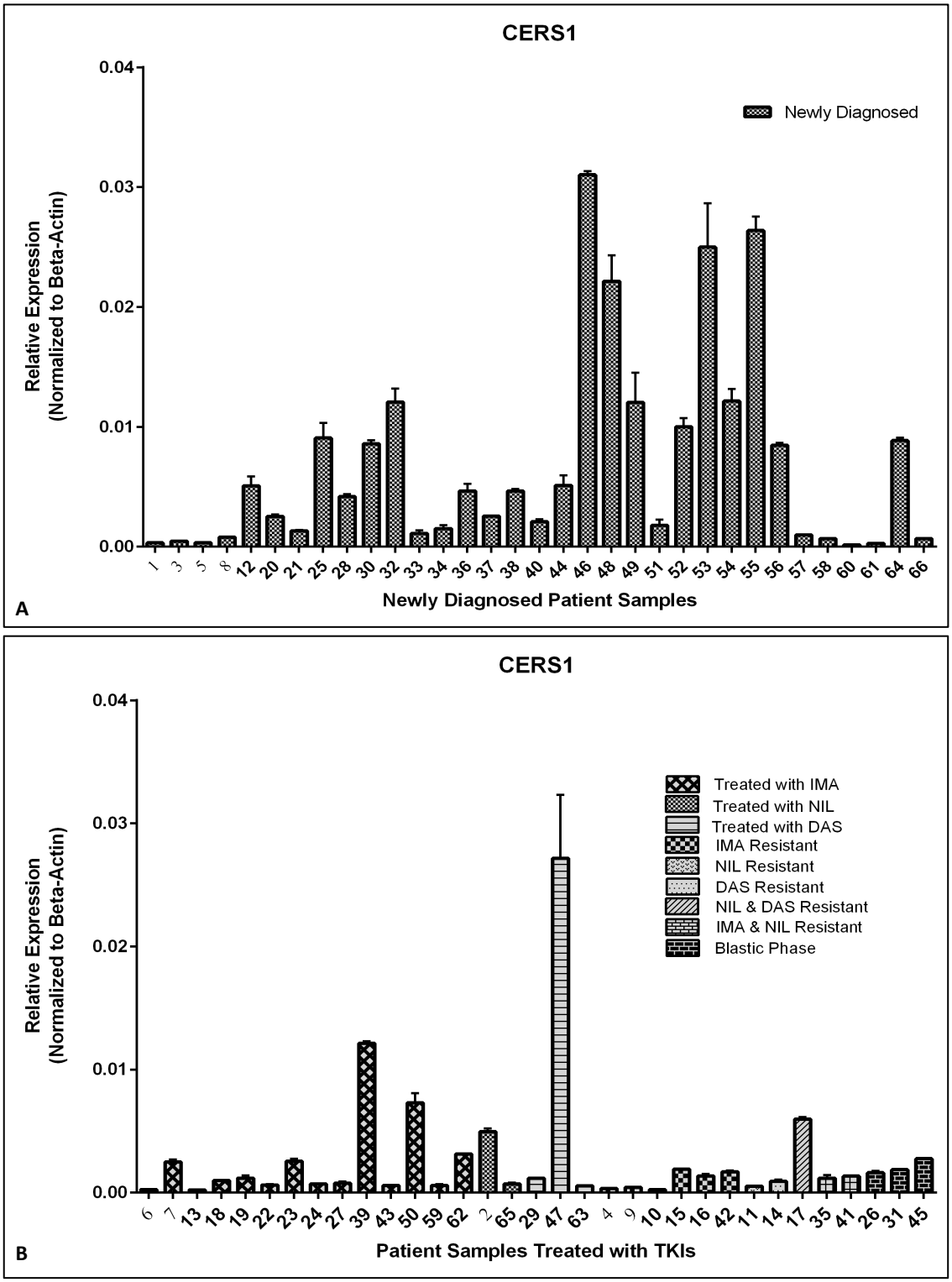

Figure 3.1. CERS1 gene expression levels of CML patients (A) CERS1 gene expression levels in newly diagnosed CML patients (B) CERS1 gene expression levels in TKI-treated CML patients. *P<0.05* was considered to be significant. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

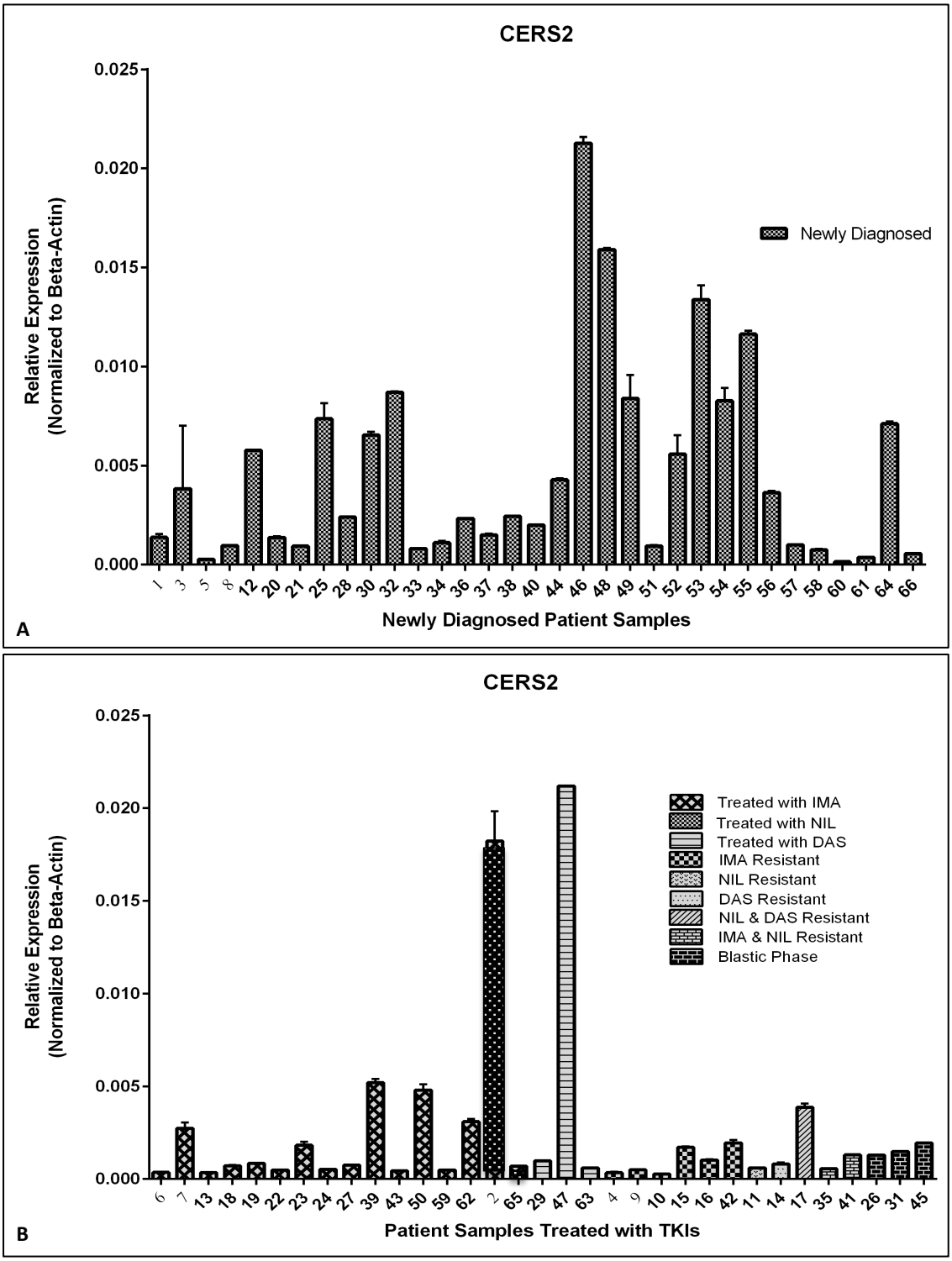

Figure 3.2. CERS2 gene expression levels of CML patients (A) CERS2 gene expression levels in newly diagnosed CML patients (B) CERS2 gene expression levels in TKI-treated CML patients. *P<0.05* was considered to be significant. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

3.3. Determination of CERS3 Gene Expression Levels

Our data demonstrated that CERS3 gene expression levels were found to be significantly higher in patients #3, #12, #25, #30, #32, #46, #48, #49, #52, #53, #54, #55, #56, and #64 that have been newly diagnosed; patients #7, #23, #39, and #50 that have been treated with imatinib; and also patients #2 treated with nilotinib; #17 resistant to both nilotinib and dasatinib; and also #47 treated with dasatinib. On the other hand, CERS3 gene expression levels were found to be significantly low in patients #9 and #10 imatinib-resistant; #11 nilotinib-resistant; #35 and #41 both imatinib- and nilotinib-resistant; and #59 treated with imatinib; #63 treated with dasatinib; and #65 treated with nilotinib (Figure 3.3 A and B).

3.4. Determination of CERS4 Gene Expression Levels

Our data demonstrated that CERS4 gene was highly expressed in CML patients #5, #8, #12, #20, #25, #30, #31, #33, #34, #36, #37, #38, #42, #46, #48, #49, #52, #53, #54, #55, #61, and #64 that have been newly diagnosed; #2 treated with nilotinib; #11 that have shown resistance to NIL; #26 that have been at blastic phase and shown hematological remission. On the other hand, CERS4 gene expression levels were found to be lower in patients #14 that have shown resistance to DAS; #1 and #3 that have been newly diagnosed; and #63 that has been treated with dasatinib (Figure 3.4 A and B).

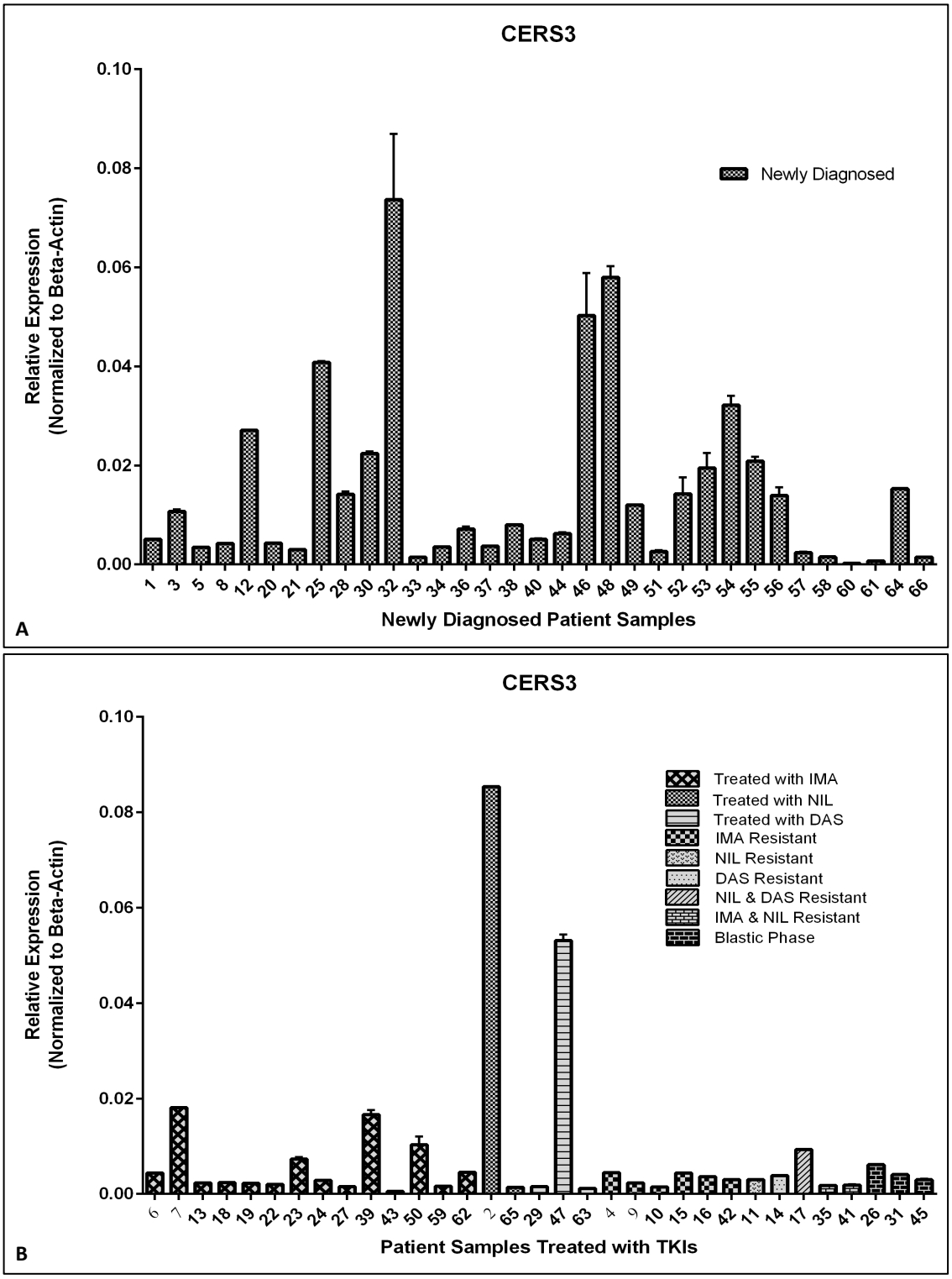

Figure 3.3. CERS3 gene expression levels of CML patients (A) CERS3 gene expression levels in newly diagnosed CML patients (B) CERS3 gene expression levels in TKI-treated CML patients. *P<0.05* was considered to be significant. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

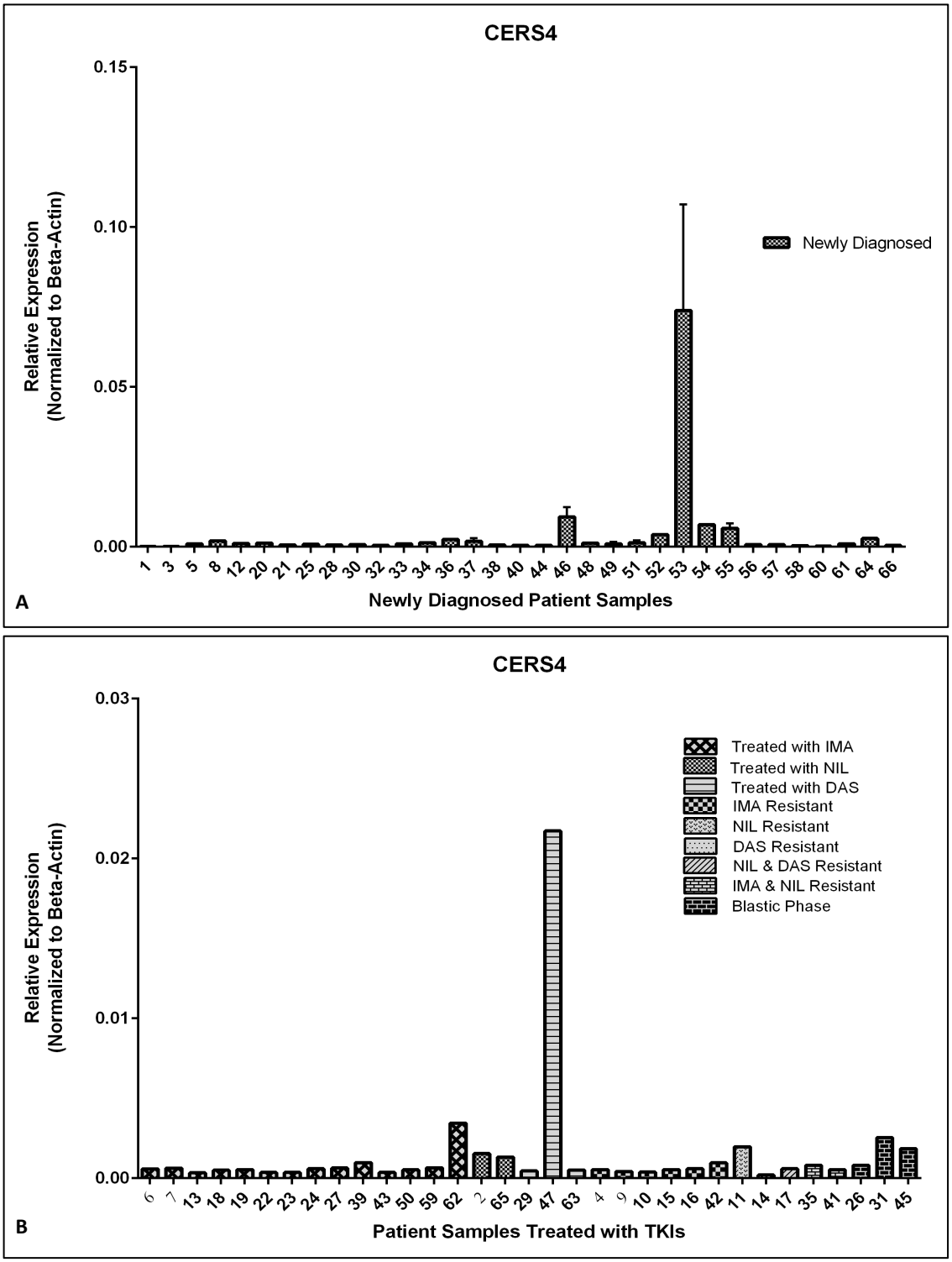

Figure 3.4. CERS4 gene expression levels of CML patients (A) CERS4 gene expression levels in newly diagnosed CML patients (B) CERS4 gene expression levels in TKI-treated CML patients. $P<0.05$ was considered to be significant. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

3.5. Determination of CERS5 Gene Expression Levels

Our data demonstrated that CERS5 gene was highly expressed in CML patients #12, #25, #28, #30, #32, #36, #44, #46, #48, #49, #53, #55, and #64 that have been newly diagnosed; #17 that have shown resistance to both dasatinib and nilotinib; and #2 treated with nilotinib; #7, #39, #50, #59, and #62 treated with imatinib; #45 at blastic phase; and #47 treated with dasatinib. On the other hand, CERS5 gene expression levels were found lower in patients #1, #3, #5, #35, #40, #51, #52, #56, #57, #58, #60, #61, and #66 that have been newly diagnosed; #6, #13, and #43 that have been treated with imatinib; #4, #9 and #10 that have shown resistance to IMA; and also #35 and #41 that have shown resistance to both imatinib and nilotinib; and also #63 and #65 that have been treated with dasatinib and nilotinib, respectively (Figure 3.5 A and B).

3.6. Determination of CERS6 Gene Expression Levels

Our data demonstrated that CERS6 gene was highly expressed in CML patients #12, #20, #21, #25, #28, #30, #32, #37, #44, #46, #48, #49, #52, #53, #54, #55, #56, and #64 that have been newly diagnosed; #17 that have shown resistance to both dasatinib and nilotinib; and #26 that have shown hematological remission; and also #2 treated with nilotinib; #7, #23, #39, #47, and #50 that have been treated with imatinib. On the other hand, CERS6 gene expression levels were found lower in patients #1, #3, and #5 that have been newly diagnosed; #6, #13, #43, #59, and #63 that have been treated with imatinib; #4 and #10 that have shown resistance to imatinib; and also #35 and #41 that have shown resistance to both imatinib and nilotinib (Figure 3.6 A and B).

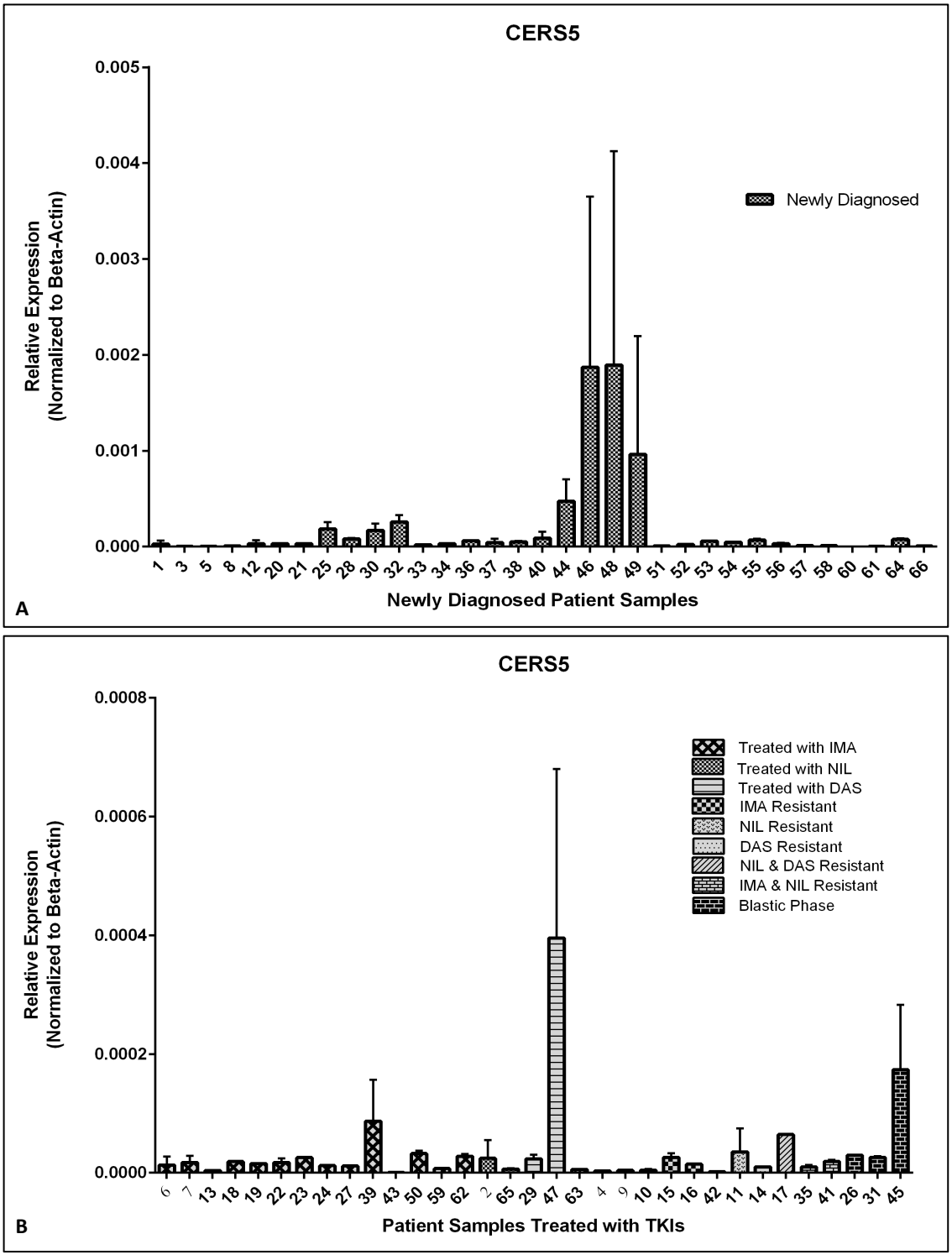

Figure 3.5. CERS5 gene expression levels of CML patients (A) CERS5 gene expression levels in newly diagnosed CML patients (B) CERS5 gene expression levels in TKI-treated CML patients. $P<0.05$ was considered to be significant. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

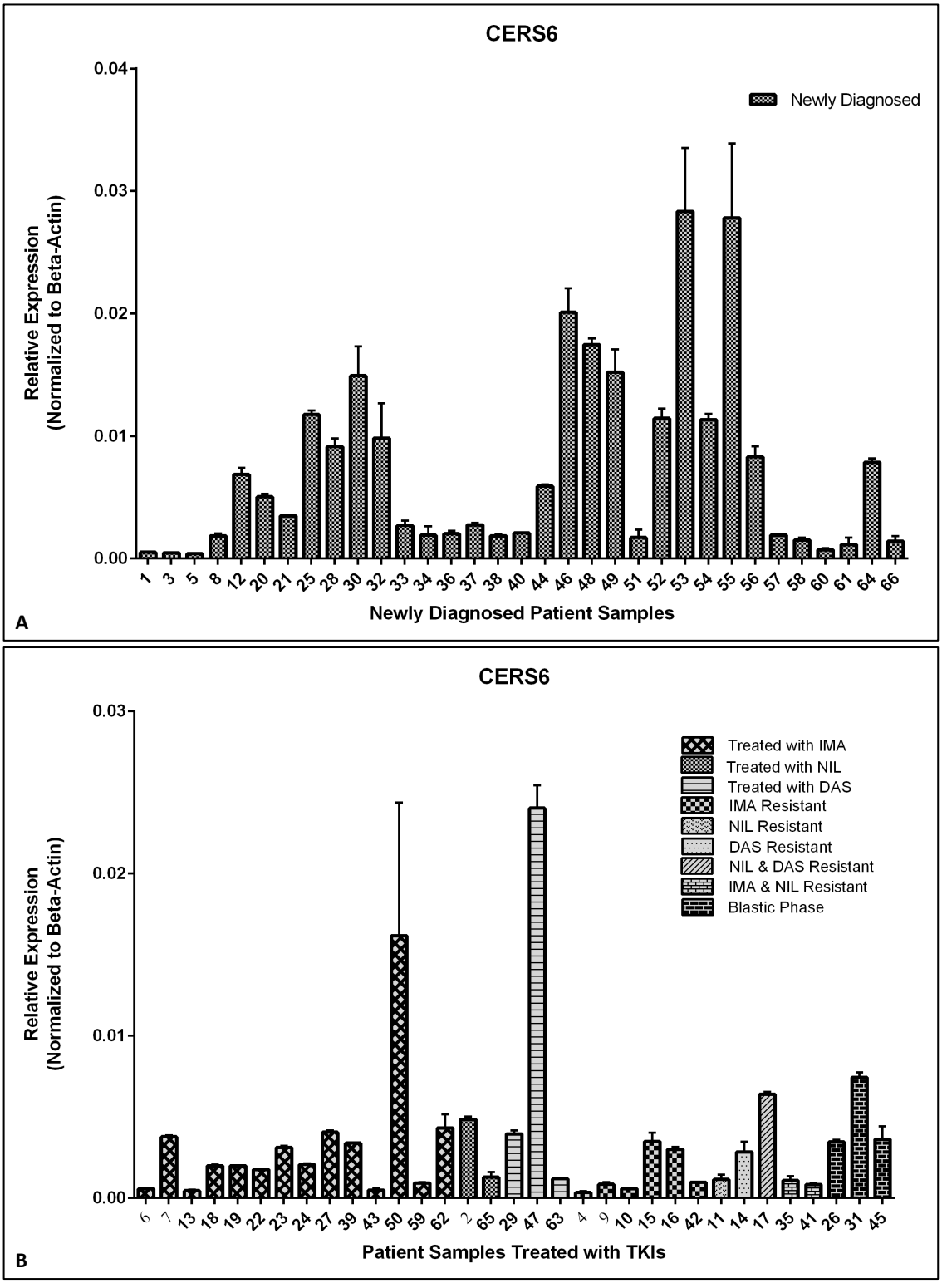

Figure 3.6. CERS6 gene expression levels of CML patients (A) CERS6 gene expression levels in newly diagnosed CML patients (B) CERS6 gene expression levels in TKI-treated CML patients. *P<0.05* was considered to be significant. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

3.7. Determination of GCS Gene Expression Levels

Our data demonstrated that GCS gene was highly expressed in CML patients #1, #3, #5, #20, #30, and #55 that have been newly diagnosed; #4 that have shown resistance to imatinib; #18 that have been treated with imatinib. On the other hand, GCS gene expression levels were found lower in patients #2 that have been treated with nilotinib and shown positive response; #17 that have shown resistance to both dasatinib and nilotinib, but have been still treated with nilotinib; #11 that have shown resistance to nilotinib; #35 and #41 that have shown resistance to both imatinib and nilotinib; #43, #47, and #59 that have been treated with imatinib; and also #32, #44, #46, #48, #52, #54, #56, #57, #58, #60, #61, #64, and #66 that have been newly diagnosed (Figure 3.7 A and B).

3.8. Determination of SK1 Gene Expression Levels

Our data demonstrated that SK-1 gene was highly expressed in CML patients #2 that have been treated with nilotinib, and shown positive response; #14 dasatinib-resistant; and also #12 and #25 that have been newly diagnosed. On the other hand, SK-1 gene expression levels were found lower in patients #6, #13, #18, #19, #22, #24, #27, #39, #43, #50, #59, and #62 that have been treated with imatinib; #10 resistant to imatinib; #29 and #47 and that have been treated with dasatinib; #35 and #41 that have shown resistance to both imatinib and nilotinib; and #26 at blastic phase; #63 and #63 treated with dasatinib and nilotinib, respectively; and also #28, #30, #32, #33, #34, #36, #37, #38, #40, #44, #46, #48, #49, #51, #52, #53, #54, #55, #56, #57, #58, #60, #61, #64, and #66 that have been newly diagnosed (Figure 3.8 A and B).

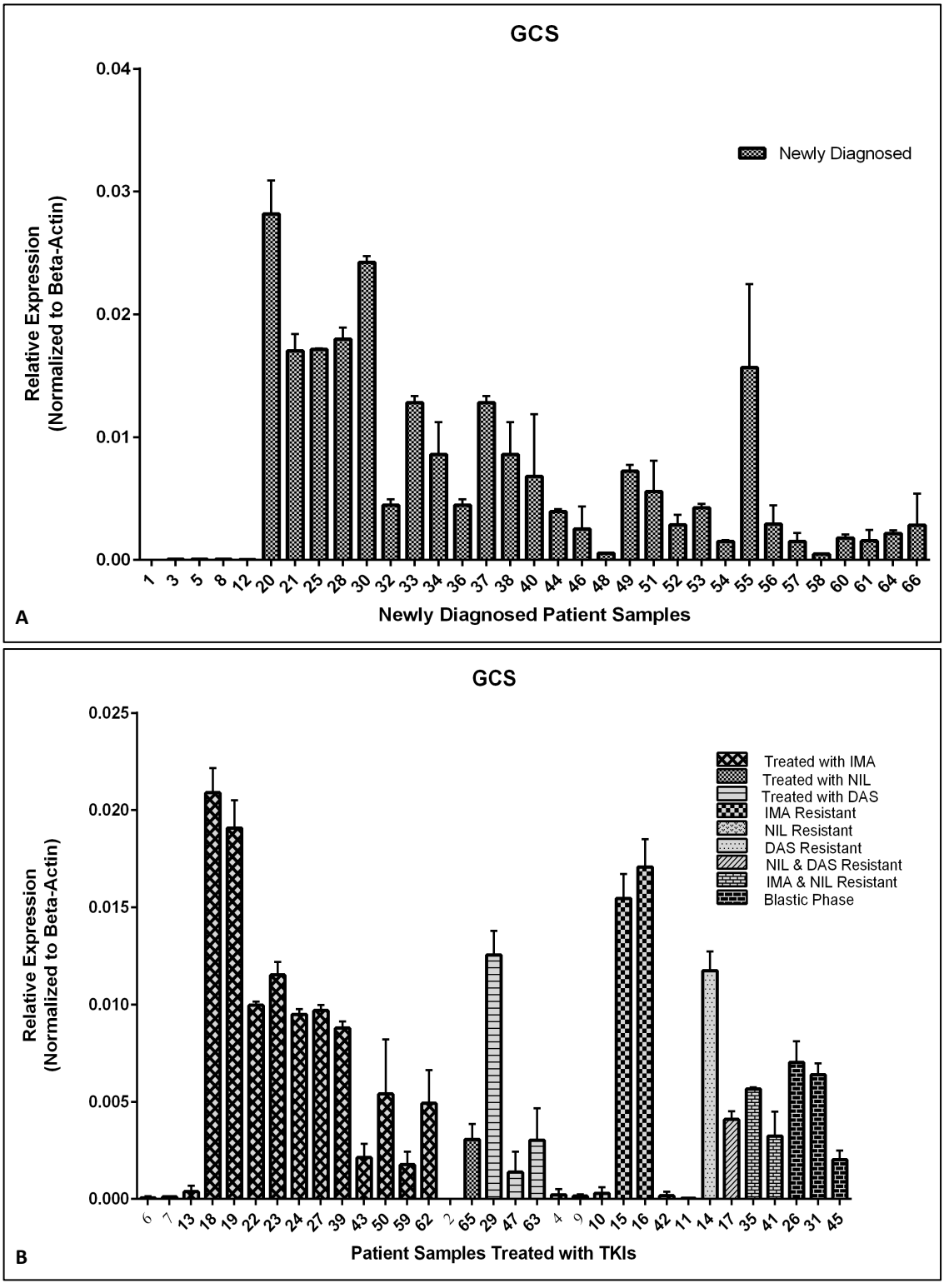

Figure 3.7. GCS gene expression levels of CML patients (A) GCS gene expression levels in newly diagnosed CML patients (B) GCS gene expression levels in TKI-treated CML patients. $P<0.05$ was considered to be significant. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

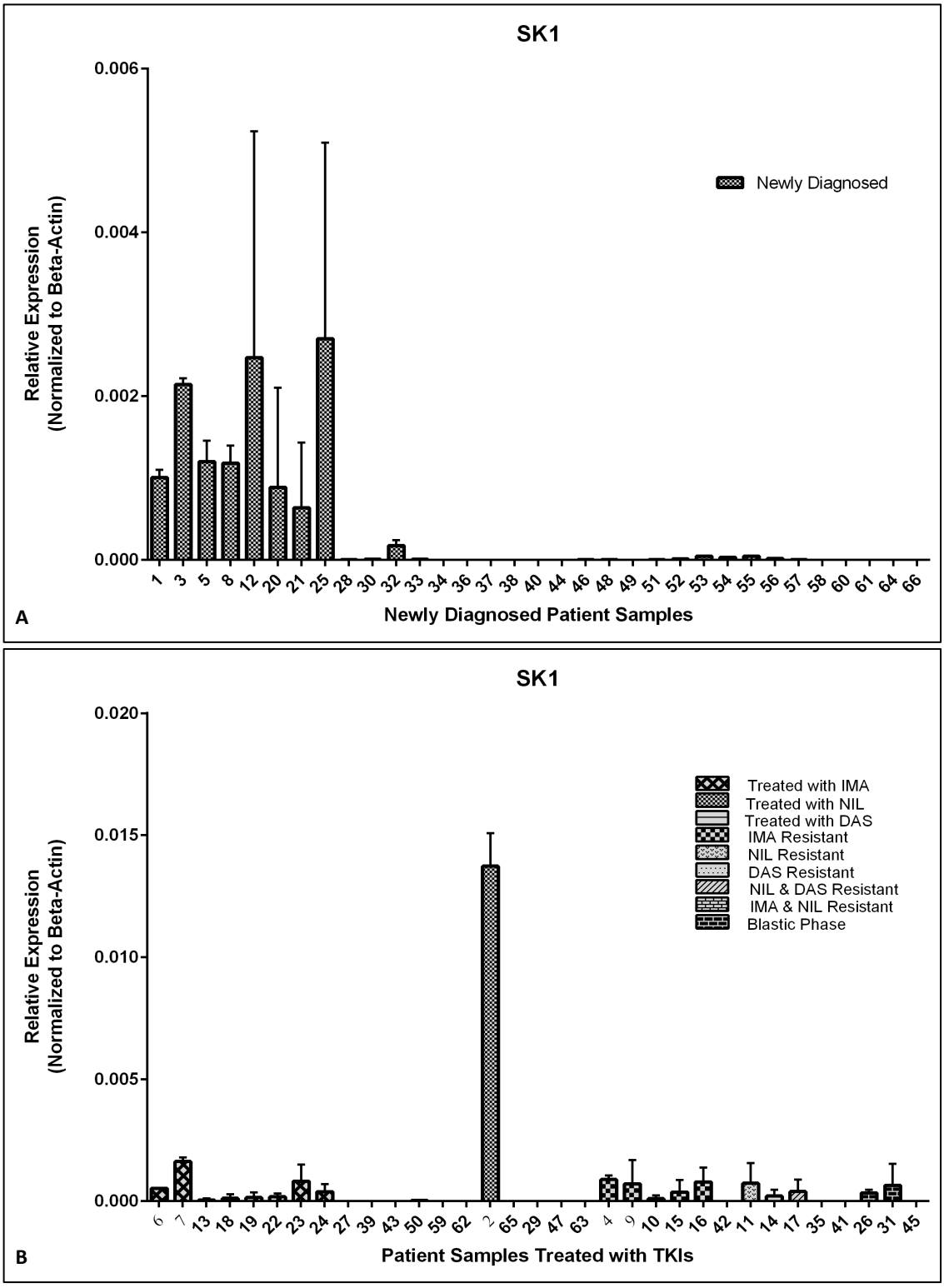

Figure 3.8. SK1 gene expression levels of CML patients (A) SK1 gene expression levels in newly diagnosed CML patients (B) SK1 gene expression levels in TKI-treated CML patients. *P<0.05* was considered to be significant. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

3.9. Determination of BCR/ABL Gene Expression Levels

Higher BCR/ABL expression levels were analyzed in patients #1, #3, #8, #25, #28, #30, #32, #33, #34, #36, #37, #38, #40, #46, #51, #53, #54, #55, #56, #64, and #66 that have been newly diagnosed; and #47 and #59 that have been treated with dasatinib and imatinib, respectively; #9, #15, and #16 imatinib-resistant; #11 nilotinib-resistant; and #26 at blastic phase. BCR/ABL expression was lower in patients #39 and #43 that have been treated with imatinib. However, BCR/ABL expression was not determined in patients #2 that have been treated with nilotinib, and shown positive response; and #19, #22, and #50 that have been treated with imatinib (Figure 3.9 A and B).

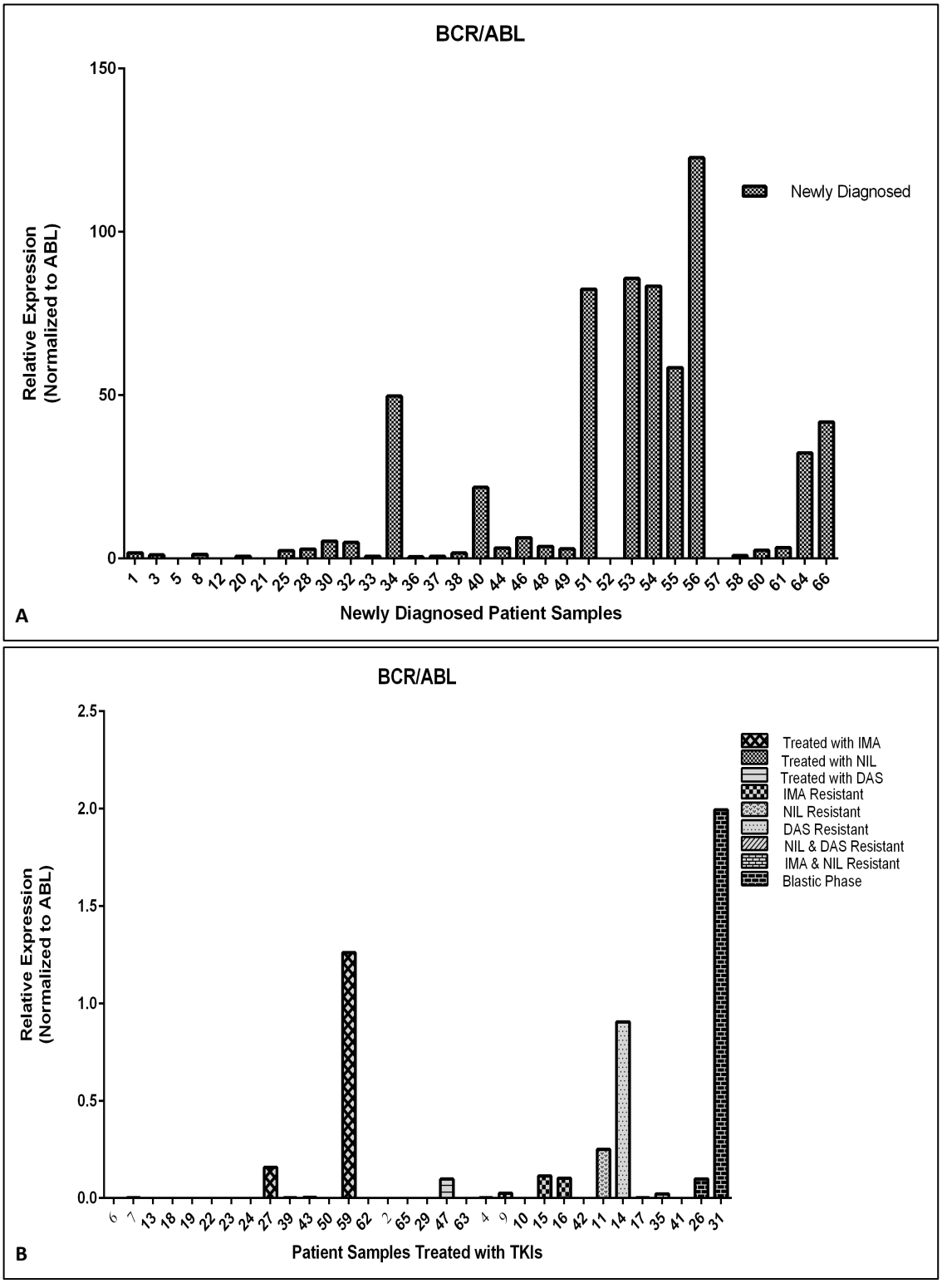

Figure 3.9. BCR/ABL gene expression levels of CML patients (A) BCR/ABL gene expression levels in newly diagnosed CML patients (B) BCR/ABL gene expression levels in TKI-treated CML patients. $P<0.05$ was considered to be significant. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs.

3.10. Comparison of the Expression Levels of Bioactive Sphingolipid Genes in the Patients' Bone Marrow Samples Acquired in Different Time Periods

We acquired bone marrow samples of some of the patients (#7, #8, #9, #14, #25, and #26) more than one time, once in six months; and compared the bioactive sphingolipid expression levels in each samples. In order to compare the expression levels, we analyzed the fold changes according to the first samples harvested from the patients.

3.10.1. Comparison of the Expression Levels of Bioactive Sphingolipid and BCR/ABL Genes in Bone Marrow Samples of Patient #7

In order to compare the changes in expression levels of bioactive sphingolipids in response to the therapy by the time, we acquired bone marrow samples of patient #7 three times when the patient was treated with imatinib, treated with imatinib and showed MMR, and treated with imatinib in different time periods. When we compared the expression levels according to the first sample, our results showed that CERS1 expression did not change when the patient was treated with imatinib and showed MMR, whereas its expression decreased approximately 4,5-fold by the time. While the expression levels of CERS2, -3, -4, 5, and -6 decreased approximately 1,7-, 2,6-, 1,8-, 1,2-, and 1,3- fold, respectively, as compared to the first sample, their expression increased when we compared the second and the third samples. Additionally, expression levels of GCS, SK-1, and BCR/ABL decreased in response to imatinib therapy by the time as compared to the first sample (Figure 3.10, Table 3.1).

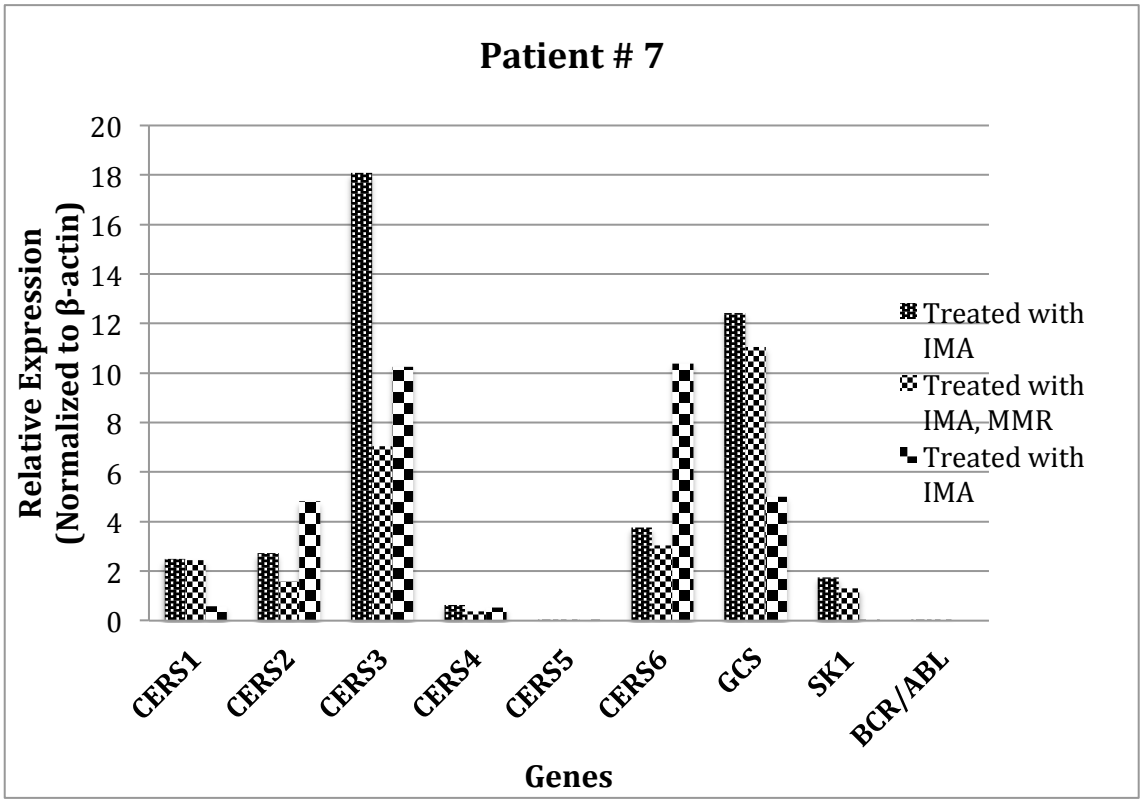

Figure 3.10. Expression levels of CERS1-6, GCS, SK1, and BCR/ABL genes in patient #7 in response to the therapy

Table 3.1. Fold changes in expression levels in response to therapy in bone marrow samples of patient #7 (Expression levels in the first bone marrow sample was assigned as reference, and fold changes were calculated based on the first sample. (-) represents decrease in expression fold change.)

	Treated with IMA, MMR (Fold change)	**Treated with IMA (Fold change)**
CERS1	1	4,5 (-)
CERS2	1,7 (-)	1,8
CERS3	2,6 (-)	1,8 (-)
CERS4	1,8 (-)	1,2 (-)
CERS5	1,2 (-)	1,2
CERS6	1,3 (-)	2,8
GCS	1,2 (-)	2,5 (-)
SK1	1,4 (-)	50 (-)
BCR/ABL	5 (-)	0

3.10.2. Comparison of the Expression Levels of Bioactive Sphingolipid and BCR/ABL Genes in Bone Marrow Samples of Patient #8

In order to compare the changes in expression levels of bioactive sphingolipids in response to the therapy by the time, we acquired bone marrow samples of patient #8 two times when the patient was newly diagnosed, and shown resistance to imatinib, but still treated with imatinib in different time periods. When we compared the expression levels according to the first sample, our results showed that expression levels of CERS1, -5, and -6 increased approximately 1,7-, 1,4-, and 1,6-fold, respectively, in response to imatinib therapy, whereas CERS4 expression levels decreased 4-fold, and CERS2 expression levels almost did not change. However, GCS expression levels increased 1,2-fold, SK1 expression almost did not change, and BCR/ABL expression decreased 12,5-fold in response to imatinib treatment (Figure 3.11, Table 3.2).

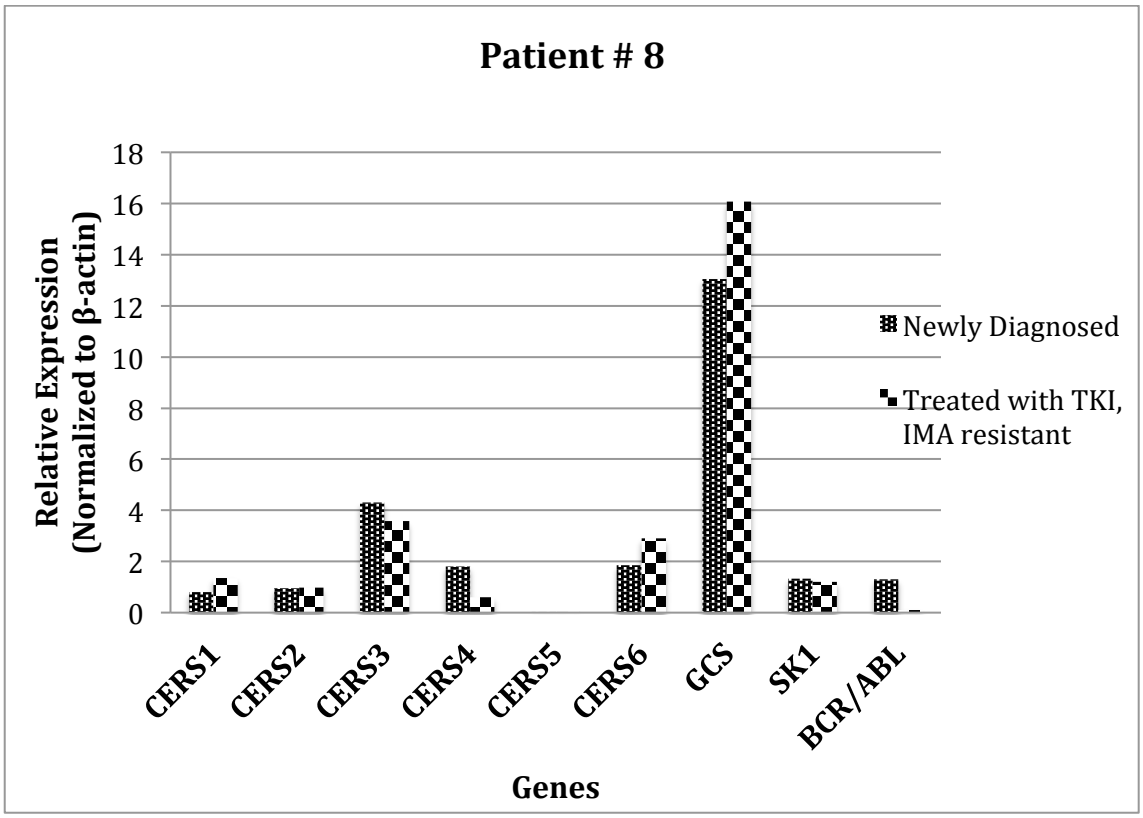

Figure 3.11. Expression levels of CERS1-6, GCS, SK1, and BCR/ABL genes in patient #8 in response to the therapy

Table 3.2. Fold changes in expression levels in response to therapy in bone marrow samples of patient #8 (Expression levels in the first bone marrow sample was assigned as reference, and fold changes were calculated based on the first sample. (-) represents decrease in expression fold change.)

	Treated with IMA, IMA resistant (Fold change)
CERS1	1,7
CERS2	1,04
CERS3	1,2 (-)
CERS4	3 (-)
CERS5	1,41
CERS6	1,57
GCS	1,23
SK1	1,1 (-)
BCR/ABL	12,5 (-)

3.10.3. Comparison of the Expression Levels of Bioactive Sphingolipid and BCR/ABL Genes in Bone Marrow Samples of Patient #9

In order to compare the changes in expression levels of bioactive sphingolipids in response to the therapy by the time, we acquired bone marrow samples of patient # 9 two times when the patient was imatinib resistant and treated with dasatinib, and also dasatinib resistant and treated with nilotinib in different time periods. When we compared the expression levels according to the first sample, our results showed that there were approximately 19-, 9-, 4-, 1,4-, 13-, and 7-fold increases in expression levels of CERS1, -2, -3, -4, -5, and -6 genes, respectively, in response to nilotinib treatment. However, there were approximately 3-, 2-, and 11-fold decreases in expression levels of GCS, SK-1, and BCR/ABL genes, respectively, in response to nilotinib treatment (Figure 3.12, Table 3.3).

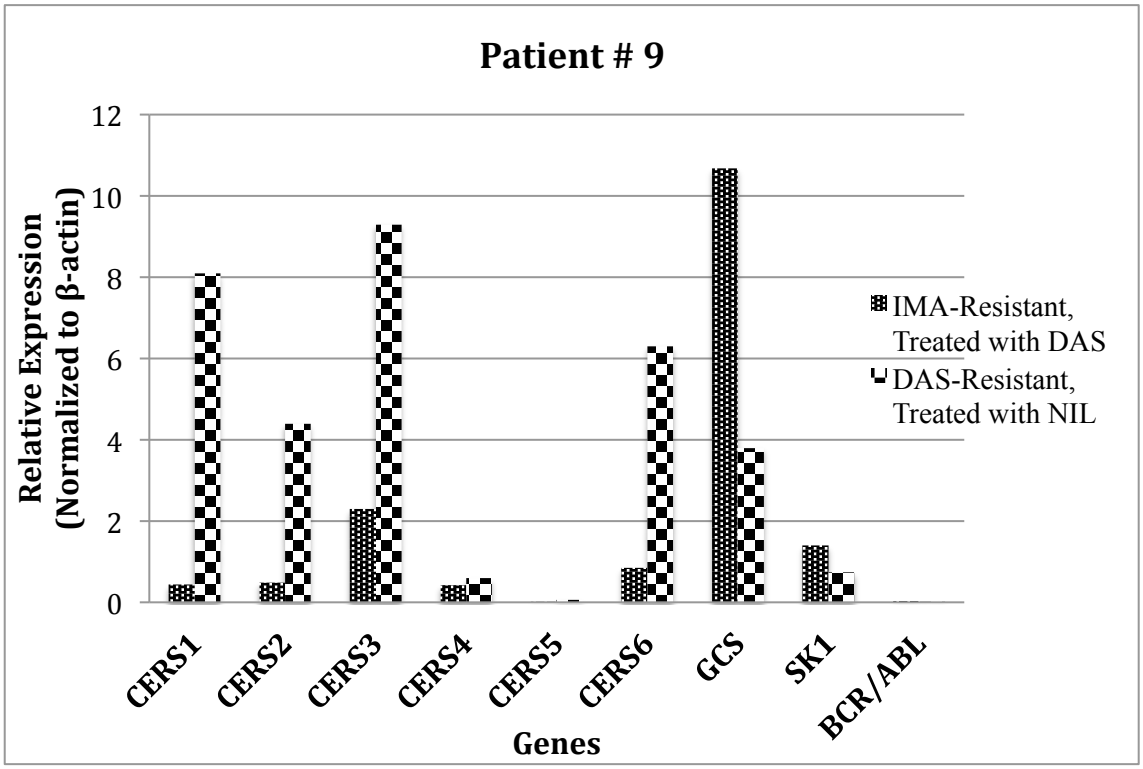

Figure 3.12. Expression levels of CERS1-6, GCS, SK1, and BCR/ABL genes in patient #9 in response to the therapy

Table 3.3. Fold changes in expression levels in response to therapy in bone marrow samples of patient #9 (Expression levels in the first bone marrow sample was assigned as reference, and fold changes were calculated based on the first sample. (-) represents decrease in expression fold change.)

	DAS-Resistant, Treated with NIL (Fold change)
CERS1	19
CERS2	9
CERS3	4
CERS4	1,4
CERS5	13
CERS6	7
GCS	3 (-)
SK1	2 (-)
BCR/ABL	11 (-)

3.10.4. Comparison of the Expression Levels of Bioactive Sphingolipid and BCR/ABL Genes in Bone Marrow Samples of Patient #14

In order to compare the changes in expression levels of bioactive sphingolipids in response to the therapy by the time, we acquired bone marrow samples of patient # 14 two times when the patient was dasatinib resistant, and also imatinib, nilotinib, and dasatinib resistant and treated with nilotinib in different time periods. When we compared the expression levels according to the first sample, our results showed that there were approximately 1,3- and 4-fold increases in expression levels of CERS1 and -4, respectively, in response to dasatinib and interferon combination treatment. However, there were approximately 1,4-, 2-, and 2,2-fold decreases in expression levels of CERS2, -3 and -6 genes, respectively, whereas CERS5 gene expression did not change in response to the therapy. Additionally, expression levels of GCS, SK-1, and BCR/ABL genes decreased approximately 2-, 5000-, and 50-fold, respectively, in response to dasatinib and interferon combination (Figure 3.13, Table 3.4).

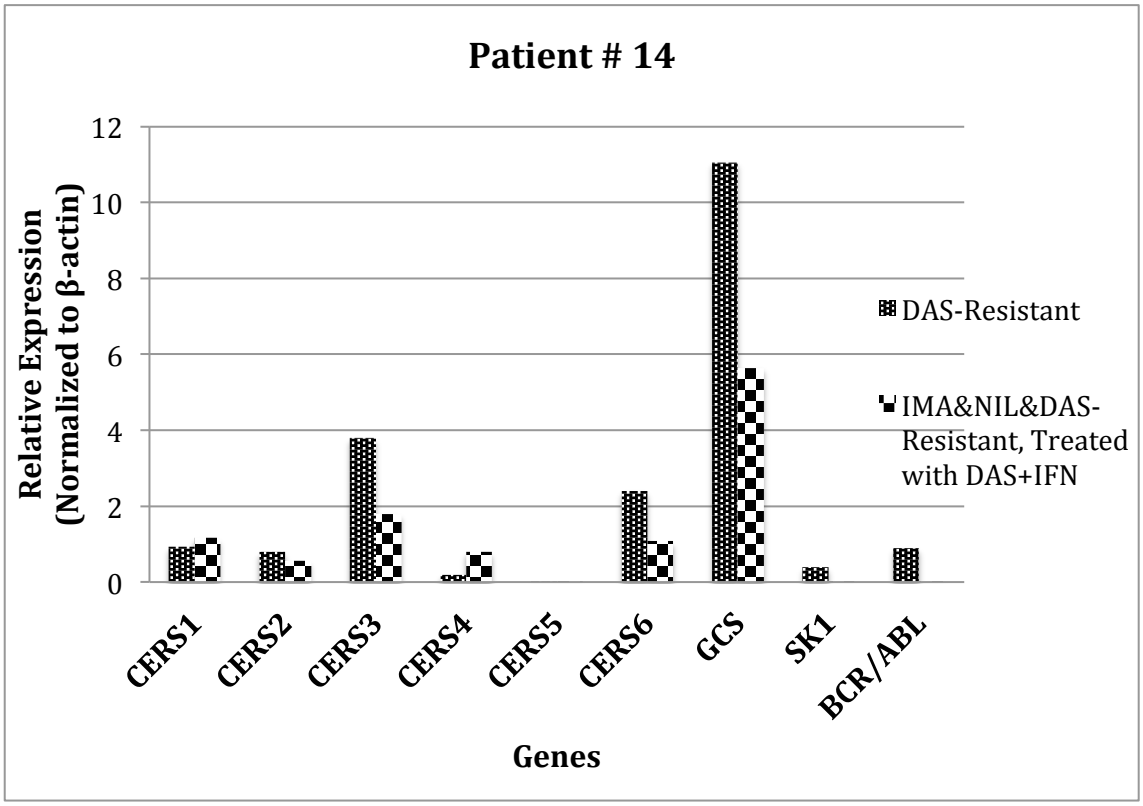

Figure 3.13. Expression levels of CERS1-6, GCS, SK1, and BCR/ABL genes in patient #14 in response to the therapy

Table 3.4. Fold changes in expression levels in response to therapy in bone marrow samples of patient #14 (Expression levels in the first bone marrow sample was assigned as reference, and fold changes were calculated based on the first sample. (-) represents decrease in expression fold change.)

	IMA&NIL&DAS-Resistant, Treated with DAS+IFN (Fold change)
CERS1	1,3
CERS2	1,4 (-)
CERS3	2 (-)
CERS4	4
CERS5	1
CERS6	2,2 (-)
GCS	2 (-)
SK1	5000 (-)
BCR/ABL	50 (-)

3.10.5. Comparison of the Expression Levels of Bioactive Sphingolipid and BCR/ABL Genes in Bone Marrow Samples of Patient #25

In order to compare the changes in expression levels of bioactive sphingolipids in response to the therapy by the time, we acquired bone marrow samples of patient # 25 two times when the patient was newly diagnosed, and also treated with imatinib in different time periods. When we compared the expression levels according to the first sample, our results showed that there were approximately 1,1- and 1,3- fold increases in expression levels of CERS1 and -4, respectively, in response to imatinib treatment. However, there were approximately 1,6-, 2,5-, 2-, and 3-fold decreases in expression levels of CERS2, -3, -5, and -6 genes, respectively, in response to the therapy. Additionally, expression levels of GCS, SK-1, and BCR/ABL genes decreased approximately 2-, 3000-, and 1250-fold, respectively, in response to imatinib treatment (Figure 3.14, Table 3.5).

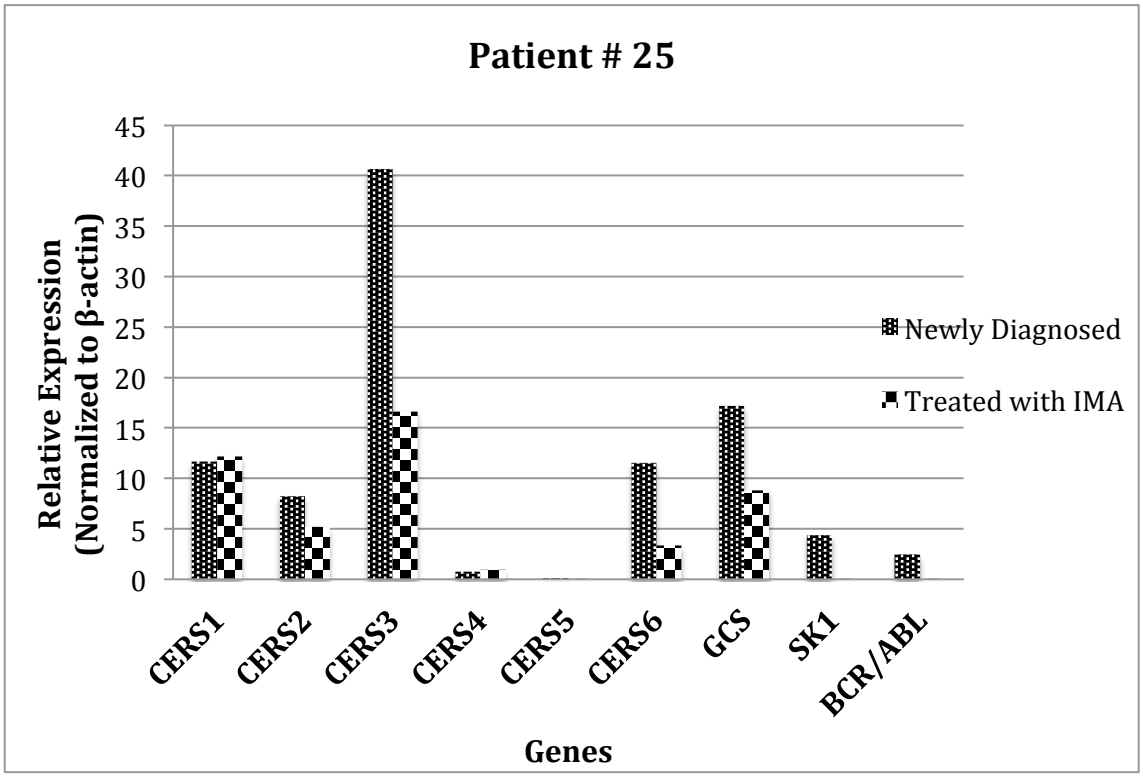

Figure 3.14. Expression levels of CERS1-6, GCS, SK1, and BCR/ABL genes in patient #25 in response to the therapy

Table 3.5. Fold changes in expression levels in response to therapy in bone marrow samples of patient #25 (Expression levels in the first bone marrow sample was assigned as reference, and fold changes were calculated based on the first sample. (-) represents decrease in expression fold change.)

	Treated with IMA (Fold change)
CERS1	1,1
CERS2	1,6 (-)
CERS3	2,5 (-)
CERS4	1,3
CERS5	2 (-)
CERS6	3 (-)
GCS	2 (-)
SK1	3000 (-)
BCR/ABL	1250 (-)

3.10.6. Comparison of the Expression Levels of Bioactive Sphingolipid and BCR/ABL Genes in Bone Marrow Samples of Patient #26

In order to compare the changes in expression levels of bioactive sphingolipids in response to the therapy by the time, we acquired bone marrow samples of patient #26 two times when the patient was at blastic phase and showed hematological remission, and also at hematological remission in different time periods. When we compared the expression levels according to the first sample, our results showed that there were approximately 1,1-, 3,1-, and 2,2- fold increases in expression levels of CERS2, -4, and -6 respectively, by the time. However, there were approximately 1,1-, 1,5-, and 1,2-fold decreases in expression levels of CERS1, -3, and -5 genes, respectively, by the time. Additionally, expression levels of GCS and SK-1 genes decreased approximately 1,1- and 2,3-fold, respectively, whereas BCR/ABL gene expression increased by 20,2-fold by the time (Figure 3.15, Table 3.6).

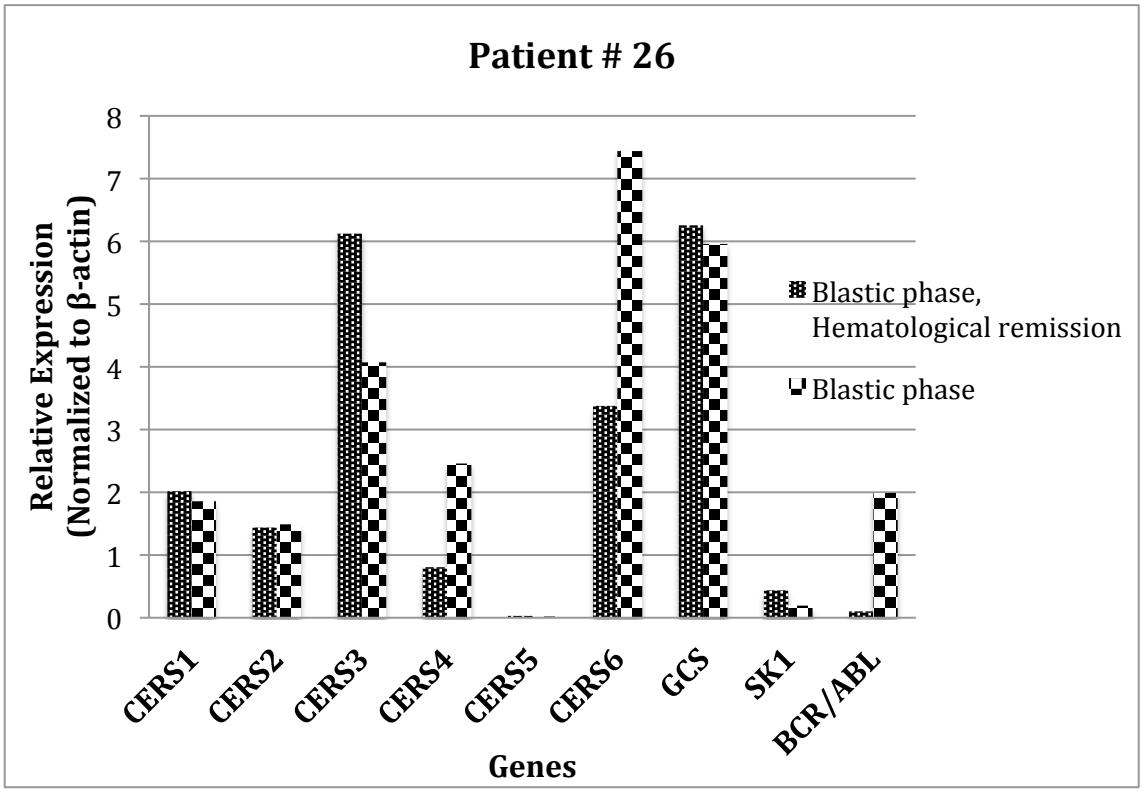

Figure 3.15. Expression levels of CERS1-6, GCS, SK1, and BCR/ABL genes in patient #26 in response to the therapy

Table 3.6. Fold changes in expression levels in response to therapy in bone marrow samples of patient #26 (Expression levels in the first bone marrow sample was assigned as reference, and fold changes were calculated based on the first sample. (-) represents decrease in expression fold change.)

	Blastic phase (Fold change)
CERS1	1,1 (-)
CERS2	1,1
CERS3	1,5 (-)
CERS4	3,1
CERS5	1,2 (-)
CERS6	2,2
GCS	1,1 (-)
SK1	2,3 (-)
BCR/ABL	20,2

3.10.7. Comparison of the Expression Levels of Bioactive Sphingolipid and BCR/ABL Genes in Bone Marrow Samples of Patient #29

In order to compare the changes in expression levels of bioactive sphingolipids in response to the therapy by the time, we acquired bone marrow samples of patient #29 two times when the patient was treated with dasatinib in different time periods. When we compared the expression levels according to the first sample, our results showed that there was approximately 1,2- fold increase in expression levels of CERS4 by the time. However, there were approximately 2,1-, 1,6-, 1,4-, 3- and 3-fold decreases in expression levels of CERS1, -2, -3, -5 and -6 genes, respectively, by the time. Additionally, expression levels of GCS and SK-1 genes decreased approximately 4,2- and 14,3-fold, respectively, by the time (Figure 3.16, Table 3.7).

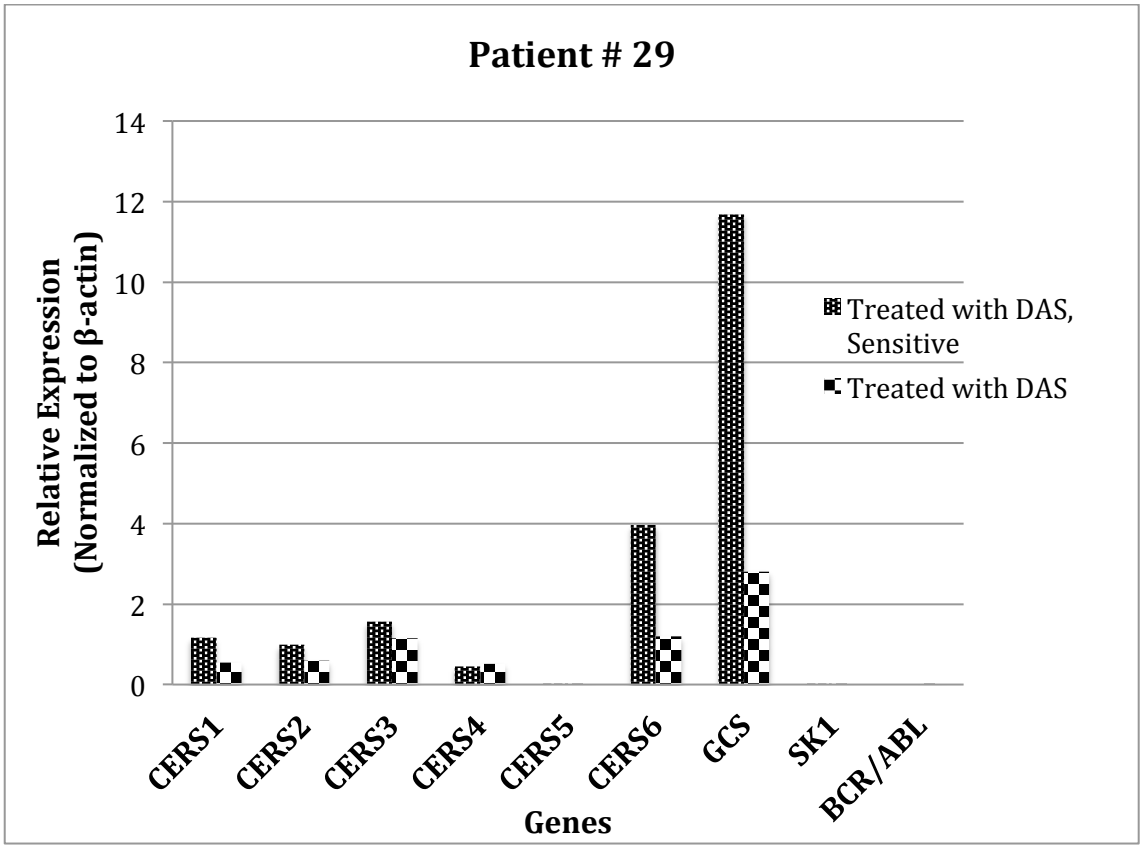

Figure 3.16. Expression levels of CERS1-6, GCS, SK1, and BCR/ABL genes in patient #29 in response to the therapy

Table 3.7. Fold changes in expression levels in response to therapy in bone marrow samples of patient #29 (Expression levels in the first bone marrow sample was assigned as reference, and fold changes were calculated based on the first sample. (-) represents decrease in expression fold change.)

	Treated with DAS (Fold change)
CERS1	2,1 (-)
CERS2	1,6 (-)
CERS3	1,4 (-)
CERS4	1,2
CERS5	3 (-)
CERS6	3 (-)
GCS	4,2 (-)
SK1	14,3 (-)
BCR/ABL	

CHAPTER 4

CONCLUSIONS

Chronic myeloid leukemia, arisen from the reciprocal translocation between BCR gene on chromosome 22, and ABL gene on chromosome 9, t(9;22)(q34;q11), resulting in the formation of Philadelphia (Ph) chromosome is a type of hematological malignancies (Goldman, et al. 2003). This Ph chromosome encodes BCR/ABL fusion protein, which has constitutively active tyrosine kinase activity. Presence of BCR/ABL in cells is considered as a determinant of CML (Quintas-Cardama and Cortes 2009). If the disease is left untreated, it can progress into blastic phase, which is eventually mortal (Melo and Barnes 2007). Frontline therapy of CML is conducted by TKIs, imatinib, nilotinib, and dasatinib. Resistance cases against these drugs can be developed by the time. This resistance can be either BCR/ABL-dependent or independent (Ramirez and DiPersio 2008). When the patients become unresponsive for the therapy, the second-line alternatives including second and third generation TKIs are used. Bosutinib, the second generation TKI, is more effective in CML patients resistant or intolerant to imatinib, nilotinib, and dasatinib. Like nilotinib and dasatinib, bosutinib is not effective in T315I mutation, which is also called as “gatekeeper mutation” (Cortes, et al. 2011, Gorre, et al. 2001). More importantly, the third generation TKI, ponatinib is significantly effective in CML patients even with T315I mutation (O'Hare, et al. 2009). Despite of the generation of these kinds of effective drugs, there is still a need for much more effective agents that cause minimal adverse effects in the patients.

Bioactive sphingolipids are important secondary messengers that have vital roles in cellular processes such as cell proliferation, differentiation, migration, invasion and metastasis, senescence, apoptosis, and also multidrug resistance. Ceramide, the center molecule of bioactive sphingolipid metabolism, is synthesized *de novo* by ceramide synthases (CERS1-6), and also it is converted into glucosylceramide by the activity of glucosylceramide synthase, and into sphingosine-1-P by sphingosine kinase-1 activity. While increased intracellular levels of ceramide result in apoptosis, increased glucosylceramide and sphingosine-1-P levels lead to cell growth and proliferation (Adan-Gokbulut, et al. 2013). Our previous *in vitro* studies have indicated that there is a

balance between ceramide and S1P in CML cells, and when this balance is broken down and equilibrium is changed to the S1P direction, CML cells start to develop resistance against imatinib (Baran, et al. 2007). Furthermore, inhibition of GCS and SK-1 by PDMP and SK-1 inhibitor, respectively, result in ceramide accumulation, and this, in turn, sensitizes the cells to the treatment, and triggers apoptosis in drug-resistant and –sensitive CML cell lines (Camgoz, et al. 2011, Gencer, et al. 2011, Camgoz, et al. 2013). Additionally, combinations of PDMP, SK-1 inhibitor, or ceramide with anticancer agents show synergistic cytotoxic and apoptotic effects in CML cell lines (Kartal, et al. 2011, Camgoz, et al. 2011, Gencer, et al. 2011, Can, et al. 2012). Moreover, BCR/ABL stability is maintained by SK-1 and S1P Receptor-2 via changing protein phosphatase 2A activity (Salas, et al. 2011).

There are some clinical studies about the potential effects of bioactive sphingolipids in cancer patients with several types of cancer such as breast, head and neck, and non-small cell lung cancers. In breast cancer patients treated with C2 and C6 ceramides, it has been reported that ceramides do not cause high grades of toxicity, and they are well tolerated (Jatoi, et al. 2003). In another study, comparisons of normal tissues with tumor tissues of 45 patients with head and neck squamous cell carcinoma have shown that C16:ceramide and C24:ceramide levels are found at higher levels, whereas C18:ceramide levels are significantly lower in tumor tissues. Additionally, lower C18:ceramide levels have been reported to be related to invasive and metastatic phenotype in these tumor tissues (Karahatay, et al. 2007). Ceramide has been also used in cancer immunotherapy. In phase I trials in head and neck cancer, antigen-presenting cells triggered with α-galactosylceramide were applied into the nasal submucosa of nine patients with head and neck cancer and the results showed that peripheral blood levels of natural killer cells significantly increased in 4 patients, and also natural killer function increased significantly in 8 patients. Additionally, the researchers did not observe any adverse effects (Uchida, et al. 2008). The same cancer immunotherapy approach has been also reported in non-small cell lung cancer. In phase I and II studies with 17 non-small cell lung cancer patients, PBMCs treated with α-galactosylceramide were co-cultured with IL-2 and GM-CSF, and then these cells were injected intravenously to the patients. The results showed that 10 of these 17 patients showed high peripheral blood levels of IFN-gamma producing cells. Additionally, median survival time of these 10 patients were longer than that of the other patients. In all of the patients, α-galactosylceramide was tolerated well, and any adverse effects were not be

observed (Motohashi, et al. 2009). In another phase I clinical study, 43 cancer patients with solid tumors were treated with safingol (SK1 inhibitor) alone and in combination with cisplatin. The results showed that 37 patients responded to the therapy, 6 patients showed stability in disease, and reversible liver toxicity was observed in a dose-dependent manner. In addition, plasma levels of S1P decreased in response to safingol treatment in a dose-dependent manner (Dickson, et al. 2011).

So far, there has not been any publication that studied the expression levels of bioactive sphingolipid genes in CML patient samples, and their effects in the clinical progress.

In this study, we analyzed the expression levels of bioactive sphingolipid metabolism genes (CERS1-6, GCS, SK-1) and BCR/ABL gene in bone marrow samples of 66 CML patients with different disease profiles, such as newly diagnosed, TKI-treated, and TKI-resistant. Our results showed that CERS1, -2, -3, and -6 expression levels were higher in most of the patients newly diagnosed and some of the patients treated with TKIs (Figure 3.1, 3.2, 3.3, and 3.6) Nonetheless, expression levels of CERS1, -2, -3, and -6 genes were significantly lower in all of the patients imatinib, nilotinib, or dasatinib resistant, and also the patients # 35 and 41 resistant to both imatinib and nilotinib (Figure 3.1, 3.2, 3.3, and 3.6). CERS1, -2, -3, and -6 expression levels were also lower in some of the patients treated with imatinib, nilotinib, or dasatinib, and all of the patients at blastic phase (Figure 3.1, 3.2, 3.3, and 3.6). However, CERS4 gene expression levels were higher in 4 out of 33 patients newly diagnosed, and the patients #11 resistant to nilotinib, #31 at blastic phase, and #62 treated with imatinib (Figure 3.4). Additionally, CERS4 expression levels were lower in 29 out of 33 patients newly diagnosed; 13 out of 14 patients treated with imatinib; and patient #14 resistant to dasatinib (Figure 3.4). On the other hand, CERS5 levels were found higher in 4 out of 33 patients newly diagnosed, and also the patients #17 both nilotinib and dasatinib resistant; #39 treated with imatinib; #45 at blastic phase; and #47 treated with dasatinib (Figure 3.5). CERS5 expression levels were lower in 29 out of 33 patients newly diagnosed; 13 out of 14 treated with imatinib; patients #2 and #65 treated with nilotinib; and #29 and #63 treated with dasatinib (Figure 3.5). CERS5 gene expression levels were also lower in all of the patients imatinib-resistant; patient #11 nilotinib-resistant; #14 dasatinib-resistant; and also #35 and #41 resistant to both imatinib and nilotinib (Figure 3.5). Furthermore, antiapoptotic GCS gene expression levels were higher in 10 out of 33 patients newly diagnosed, and 2 out of 14 patients

treated with imatinib; 1 out of 3 patients treated with dasatinib; 2 out of 6 patients resistant to imatinib; and patient #14 resistant to dasatinib (Figure 3.7). The highest GCS expression was observed in the patient #4 resistant to imatinib (Figure 3.7). Lower GCS expression levels were observed in patients #2 and #65 treated with nilotinib; #11 nilotinib-resistant, but still treated with nilotinib; #17 resistant to both dasatinib and nilotinib, but still treated with nilotinib; #41 resistant to both imatinib and nilotinib, and treated with dasatinib; #43 and #59 treated with imatinib; and also #47 and #63 treated with dasatinib (Figure 3.7). Moreover, antiapoptotic SK-1 expression levels were higher in 8 out of 33 patients newly diagnosed; and the patients #2 treated with nilotinib; #4, #9, #10, #15, and #16 imatinib-resistant; #6, # 7, #18, #23 treated with imatinib; #11 nilotinib-resistant, #14 dasatinib-resistant, #17 both nilotinib and dasatinib resistant (Figure 3.8). Nonetheless, expression levels of SK-1 were lower in 25 out of 33 patients newly diagnosed; and 8 out of 14 patients treated with imatinib; patients #29, #47, and #63 treated with dasatinib; #65 treated with nilotinib; and also #35 and #41 resistant to both imatinib and nilotinib, but treated with dasatinib (Figure 3.8). Expression levels of BCR/ABL gene were found higher in 9 out of 33 newly diagnosed patients; and the patients #11 resistant to nilotinib; #14 dasatinib-resistant; #15 and #16 imatinib-resistant; #27 and #59 treated with imatinib; #47 treated with dasatinib; and all of the patients at blastic phase (Figure 3.9). Additionally, BCR/ABL expression levels were lower in 24 out of 33 patients newly diagnosed; 12 out of 14 patients treated with imatinib; the patients #29 and #63 treated with dasatinib; and #2 and #65 treated with nilotinib; and also #35 and #41 resistant to both imatinib and nilotinib, but treated with dasatinib (Figure 3.9). Moreover, we acquired bone marrow sample of patients #7, #8, #9, #14, #25, #26, and #29 more than one time in different time periods, approximately once in 6 months. Imatinib treatment of patient #7 increased expression levels of CERS2, -5, and -6 whereas decreased GCS, SK-1, and BCR/ABL expression by the time (Figure 3.10). In the patient #8, CERS1, -2, -5, and -6 expression levels increased in response to imatinib treatment (Figure 3.11). When the patient #8 gained resistance to imatinib, GCS expression was found increased whereas SK-1 expression was almost unchanged (Figure 3.11). We acquired two samples of patient #9 when the patient was imatinib-resistant and treated with dasatinib, and also dasatinib-resistant and treated with nilotinib (Figure 3.12). Q RT-PCR results showed that although the patient #14 was resistant to imatinib and dasatinib, nilotinib treatment significantly increased the expression levels of CERS1, -2, -3, -5, and -6 genes while decreased GCS, SK-1, and

BCR/ABL expression (Figure 3.13). Combination of dasatinib with interferon increased the expression levels of CERS1 and -4 whereas decreased the expression levels of GCS, SK-1, and BCR/ABL in patient #14 (Figure 3.13). In the patient #25, imatinib treatment slightly increased the expression levels of CERS1 and -4 whereas expression levels of the other bioactive sphingolipid genes and BCR/ABL decreased (Figure 3.14). In the patient #26 at blastic phase, expression levels of CERS1, -2, and -5, and also GCS were almost unchanged whereas BCR/ABL expression significantly increased by the time (Figure 3.15).

Under the light of these results, we concluded that CERS1, -2, -3, -5, and -6 expressions are important in achievement of positive responses to TKI treatment. Decreases in expression levels of CERS1, -2, -3, -5, and -6 genes lead to development of resistance against TKIs in CML patients. However, CERS4 gene expression is not related to the positive responses to TKI treatment in CML since in most of CML patients resistant to TKIs or at blastic phase, CERS4 was expressed at higher levels. Moreover, GCS is considerably related to the development of resistance against TKIs, and also nilotinib and dasatinib rather than imatinib treatment are more effective in decreasing GCS expression in CML patients. Furthermore, in most of the cases, there is an inverse relation between gene expression levels of CERS1 and GCS, SK-1, and also BCR/ABL. We also concluded that, SK-1 expression increases especially in imatinib-resistant CML patients. Additionally, dasatinib is more effective in decreasing the expression levels of SK-1. On the other hand, although some of the patients expressing high levels of SK-1 also expressed higher levels of BCR/ABL, our results did not show a strong correlation between SK-1 and BCR/ABL expression in CML patients. This correlation might be seen as comparing protein levels. More importantly, acquirement of bone marrow samples of CML patients more than one time in different time periods showed that we could estimate the disease progression through considering the expression levels of bioactive sphingolipid genes.

Consequently, we confirmed our previous studies that showed increasing intracellular levels of CERS genes trigger TKI-induced cell death, and also GCS and SK-1 contribute to development of MDR in CML cell lines. In this study, we showed for the first time that expression levels of bioactive sphingolipids are important players in achievement of positive responses, or development of MDR to TKI therapy in CML patients. Therefore, expression levels of bioactive sphingolipid genes might be predictive for estimating disease progression. More importantly, targeting bioactive

sphingolipid genes, for instance overexpression of CERS genes or downregulation of GCS and SK-1, might be more effective in CML therapy. For this reason, bioactive sphingolipids might be novel targets as well as prognostic markers for more effective CML therapy.

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1) FOXM1 Transcription Factor in T-Cell Acute Lymphoblastic Leukemia Cell Line.
2) Molecular Mechanisms of Drug Resistance and Its Reversal in Cancer.
3) Molecular Biology of Stem Cells. (Book Chapter).
4) New Indication for an Old Well-Known Drug, Therapeutic Potential of Propranolol for Multiple Myeloma. Journal of Cancer Research and Clinical Oncology.
5) Therapeutic Potential of Targeting Ceramide/Glucosylceramide Pathway in Cancer.
6) Novel Agents Targeting Bioactive Sphingolipids for the Treatment of Cancer.
7) STAT Pathway in the Regulation of Zoledronic Acid-Induced Apoptosis in Chronic Myeloid Leukemia Cells.
8) Apoptotic Effects of Resveratrol, a Grape Polyphenol, on Imatinib Sensitive and Resistant K562 Chronic Myeloid Leukemia Cells.
9) Resveratrol Triggers Apoptosis by Increasing Intracellular Concentrations of Ceramides in Chronic Myeloid Leukemia Cells.
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