The intracellular negative regulator genes of the Wnt signaling in imatinib treatment

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Abstract

Aim: Tyrosine kinase inhibitors are a targeted and successful treatment modalities in the treatment of Chronic Myeloid Leukemia (CML). Imatinib mesylate is the most common of these inhibitors, but resistance reactions limit its use. It is believed that regulators in the Wnt signaling pathway play an important role in the treatment of CML. The aim of this study is to investigate possible expression changes of intracellular negative regulator genes after imatinib mesylate treatment in chronic myeloid leukemia cell line.

Materials and Methods: Total RNA isolation was performed from K562 cells treated with imatinib mesylate for 24 hours at 0.5 µM concentration and control native K562 cells. Following cDNA synthesis, expression changes of eight intracellular negative regulator genes were analyzed by Real-Time PCR.

Results: When the results were evaluated, it was determined that the expression of 5 genes increased and the expression of 3 genes decreased in K562 cells treated with imatinib.

Conclusion: When we suppressed Bcr-Abl with Imatinib in K562 cells, it was observed that there were changes in the expression levels of the intracellular negative regulator genes of the Wnt signaling pathway. These changes are important in defining new targets in CML treatment. To understand the roles of these negative regulators, it is important to conduct detailed research to develop new strategies for CML treatment.

Keywords: CML; imatinib; Wnt negative regulator genes

INTRODUCTION

Chronic myeloid leukemia is a myeloproliferative neoplasm characterized by Bcr-Abl fusion gene (1). It is known that Bcr-Abl oncoprotein causes the development of CML by showing abnormal tyrosine kinase activity through pathways such as Wnt/β-catenin signaling (2-4). While conventional treatments (hydroxyurea, busulfan, interferon alfa, etc.) were used in the treatment of CML patients, nowadays stem cell transplantation and the use of tyrosine kinase inhibitors (TKI) have enabled more successful results (5). Imatinib mesylate (IM) (Glivec, Gleevec, STI -571; Novartis, Basel, Switzerland) prevents the growth and development of hematopoietic cells expressing Bcr-Abl and induce apoptosis in these cells by preventing ATP binding to the ATP-binding site of ABL kinase. Thus, activation of signaling pathways lead to leukemic phenotype by causing functional modifications in many genes in the cell cycle, cell adhesion, cytoskeleton organization, and apoptotic pathway (6).

IM treatment results in the loss of the selection advantage that Bcr-Abl provides to cells. Therefore it is important to how the changes expression of Wnt signaling pathway genes play an significant role in CML development will change after IM treatment. It has been shown that the Bcr-Abl protein prevents the degradation of β-catenin and is necessary for the activity of Bcr-Abl in the Bcr-Abl related transformation process in β-catenin (7). The continuity of self-regenerative properties of Bcr-Abl (+) CML cells requires the presence of non-phosphorylated, active nuclear β-catenin from serine/threonine. Serine/threonine phosphorylation is accomplished by the Axin/APC/GSK3β/CKIα degradation complex and is associated with the degradation of the β-catenin. Bcr-Abl phosphorylates β-catenin and prevents the β-catenin from binding to the degradation complex. β-catenin, phosphorylated from tyrosine residues, binds to the TCF4 transcription factor, initiating transcription of target genes. The TKI IM suppresses Bcr-Abl. The suppressed Bcr-Abl can not perform the tyrosine phosphorylation of
the β-catenin. Tyrosine non-phosphorylated β-catenin binds to the degradation complex and is degraded after serine/threonine phosphorylation (8). It has been shown that the absence of β-catenin decreases the Bcr-Abl level and weakens the phosphorylation of proteins in Bcr-Abl induced CML (7). In vivo and in vitro studies have shown that cells become more sensitive to IM treatment when β-catenin is suppressed. In brief, Wnt signaling plays an important role in the CML treatment process.

It is known that deregulation of the Wnt signaling pathway is effective in the progression of many hematopoietic malignancies, including CML. As the role of the Wnt signaling pathway in the progression of leukemia is understood, the development of molecules targeting this pathway and therapies for these molecules has also gained momentum. There are two different classical Wnt signaling pathways as canonical (β-catenin dependent) and non-canonical (β-catenin independent) Non-canonical Wnt pathways are divided into two groups as Wnt/Ca2+ and Wnt/planar cell polarity (PCP) (9). Recent researches have shown that the hyperactivation of the Wnt signaling pathway may result from a different mechanism based on abnormal hypermethylation of negative regulators of the Wnt signaling (10).

Negative regulators of the Wnt signaling exhibit significant anti-cancer effects by targeting abnormalities in the Wnt signaling (11). Many cancer studies have shown that negative regulators of Wnt signaling are downregulated, and the increased expression of negative regulatory genes can significantly repress drug resistance, invasion, proliferation, and metastasis of cancer cells due to the inhibition of the Wnt signaling pathway (12-14). Negative regulators that regulate the Wnt signaling, in particular, are becoming targets for treatments. However, although there are studies in the literature related to secreted or transmembrane proteins of these negative regulators, the role of genes that intracellular negative regulators of the Wnt signaling, such as BTRC (Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase), CTBP1 (C-terminal binding protein), CTNNBP1 (Catenin, beta interacting protein 1), NDK1 (Naked cuticle homolog 1), SOX17 (SRY-box (Sox) transcription factor 17), CSNK1A1 (Casein kinase 1, alpha 1), PRICKLE1 (Prickle homolog 1), CCND1 (Cyclin D1), in the cancer treatment process is not known.

Knowing the genes that IM affects and the pathways it follows will enable both the understanding of the resistance mechanism and the development of new treatment protocols, especially in leukemias where resistance develops and resulted in treatment unresponsiveness. Therefore, in this study, it was aimed to investigate whether there is any change in the expression of intracellular negative regulators in Wnt signaling after IM was applied to the chronic myeloid leukemia cell line K562.

MATERIALS and METHODS

Cell culture
K562 cell line obtained from DSMZ-ACC10, cryopreserved (Leibniz-Institut DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Cells were grown in RPMI-1640 which contains 10% fetal bovine serum (FBS), 1% L-glutamine under 5% CO2 at 37°C. The cells were passaged when they were 70-80% confluency.

Imatinib treatment
After the cultivation of K562 cells, the cells were treated with 0.5 μM IM (15) and than the cells were passaged to the new growth medium without serum and incubated for 24 h at 37°C. After 24 h, RNA isolation was performed from the IM treated and non-treated K562 cells.

Total RNA isolation and complementary DNA synthesis
Total RNA isolation was performed by using RNAasy Mini Kit (Qiagen) from K562 cell lines. After the detection of the RNA purity by using nanodrop, samples were converted to the complementary DNA (cDNA). cDNAs were synthesized by using SuperScript First-Strand Synthesis System and RT-PCR was performed to detect the expression levels of the target genes.

Real-Time PCR Analysis
Real-time PCR was performed by using Light Cycler Fast Start DNA Master PLUS SYBR Green I Kit (Roche Diagnostics) and LightCycler 2.0 (Roche Diagnostics). The reaction mix was consisted of 0.25 pmol primers, 200 μM dNTP, 2 units of Taq polymerase, 1X reaction buffer, and 5 μl cDNA in a total volume of 20 μl. The expressions of eight intracellular negative regulator genes of the Wnt signaling were calculated and normalized. PCR primers were shown in supplementary material. β-actin gene was used for the reference gene. All of the reactions were replicated three times. PCR products were checked with agarose gel for nonspecific bands.

<table>
<thead>
<tr>
<th>Supplementary material</th>
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<tbody>
<tr>
<td>Gene Name</td>
</tr>
<tr>
<td>CTNNBIP1</td>
</tr>
<tr>
<td>CCND1</td>
</tr>
<tr>
<td>BTRC</td>
</tr>
<tr>
<td>NDK1</td>
</tr>
<tr>
<td>SOX17</td>
</tr>
<tr>
<td>CSNK1A1</td>
</tr>
<tr>
<td>PRICKLE1</td>
</tr>
<tr>
<td>BTP1</td>
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<tr>
<td>β-ACTIN</td>
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</table>

Statistical Analysis
Expression fold change was calculated by using the 2^(-Delta Delta CT) method. Fold changes higher than 1 were accepted as up-regulation while fold changes lower than 1 showed down-regulation of the expression. p values of the Ct values were calculated by Student’s t-test and p values lower than 0.05 were accepted as statistically significant. The statistical significance of
expression levels was also calculated by using Qiagen RT² Profiler PCR Array Gene Expression Analysis software.

RESULTS

Total RNA isolation was performed from K562 cells treated with Imatinib mesylate for 24 hours at 0.5 µM and control native K562 cells. Following cDNA synthesis, expression changes of eight intracellular negative regulator genes were analyzed by Real-Time PCR. BTRC, CTBP1, CTNNBIP1, NKD1 and SOX17 gene expressions were found to be increased and CSNK1A1, PRICKLE1 and CCND1 gene expressions were found to be decreased in fold change results were shown in Table 1 and 2. Fold regulation indicated the fold change results in a biologically meaningful way (Figure 1).

According to the KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis, it was determined that the majority of the genes were involved in canonical Wnt signaling pathway. Apart from this, the significantly upregulated and downregulated genes associated with the cell growth and the cell cycle were shown in Tables 1 and 2.

**Table 1. Genes with increasing expression levels and KEGG analysis**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene name</th>
<th>FC</th>
<th>p</th>
<th>NR</th>
<th>C</th>
<th>N-C</th>
<th>CD</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTRC</td>
<td>Beta-transducin repeat containing</td>
<td>3.52</td>
<td>&lt;0.05</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTBP1</td>
<td>C-terminal binding protein 1</td>
<td>10.98</td>
<td>&lt;0.05</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTNNBIP1</td>
<td>Catenin, beta interacting protein 1</td>
<td>3.92</td>
<td>&lt;0.01</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKD1</td>
<td>Naked cuticle homolog 1 (Drosophila)</td>
<td>3.49</td>
<td>&lt;0.05</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOX17</td>
<td>SRY-box 17</td>
<td>6.81</td>
<td>&lt;0.01</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FC, fold change; C, canonical; N-C, non-canonical; NR, negative regulation; CD, cell division; CC, cell cycle

**Table 2. Genes with decreasing expression levels and KEGG analysis**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene name</th>
<th>FC</th>
<th>p</th>
<th>NR</th>
<th>C</th>
<th>N-C</th>
<th>CD</th>
<th>CC</th>
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</thead>
<tbody>
<tr>
<td>CSNK1A1</td>
<td>Casein kinase 1, alpha 1</td>
<td>0.38</td>
<td>&lt;0.05</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>PRICKLE1</td>
<td>Prickle homolog 1</td>
<td>0.13</td>
<td>&lt;0.05</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
<td>0.24</td>
<td>&lt;0.05</td>
<td>+</td>
<td></td>
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</tr>
</tbody>
</table>

FC, fold change; C, canonical; N-C, non-canonical; NR, negative regulation; CD, cell division; CC, cell cycle

DISCUSSION

Aberrant regulation of the Wnt signaling pathway is known to play a role in the development of solid tumors such as breast, prostate, and colorectal cancer, as well as chronic myeloid and lymphocytic leukemia, multiple myeloma, and acute myeloid leukemia. It has been stated that mutations in components such as APC, Axin, or β-catenin cause an excessive increase in the signal cascade (16). It is known that the canonical pathway plays an important role in the development of CML with the activation of various targets such as c-MYC, ROK13A, Cadherin, MDI1, PRICKLE 1,
transcriptional repressions are thought to be 

expression is decreased in many cancers (26). Such 
determined that CTNNBIP1 is suppressed and its 
β-catenin signaling pathway. In addition, it has been 
between β-catenin and TCF4 and inhibiting the Wnt/

It has been determined that the response to IM therapy 
in CML is particularly dependent on the mechanisms 
related to Bcr-Abl oncoprotein mutations and Bcr-Abl 
amplification. However, some patients do not respond 
to this treatment. Therefore, mechanisms independent 
of Bcr-Abl may also be effective in the development or 
treatment process of the disease (19).

In this study, the change in the expression levels of eight 
genes that are intracellular negative regulators of Wnt 
signaling in IM-treated K562 cells were investigated. Five 
genes were determined to be upregulated. However, a 
significant decrease in the expression of genes such as 
PRICKLE1, CCND1 and CSNK1A1 was detected.

CCND1 (Cyclin D1) was observed in cells treated with 
IM. Cyclin D1 cooperates with TCF / LEF to organize 
a transcriptional program involving multiple targets 
(20). The decrease in CCND1 expression appears to be 
associated with inhibition of cancer cell proliferation by 
suppressing Wnt signal activation.

Casein kinase 1α (CK1α), encoded by CSNK1A1, is a 
member of the CK1 protein family with serine/threonine 
protein kinase activity (21). CSNK1A1 suppresses 
intracellular β-catenin accumulation and p53 activity. 
Järås et al. showed that when the activity of CSNK1A1 
suppressed through shRNA or small molecule inhibition, 
they observed a selective loss in the leukemia population, 
and they encountered a significant therapeutic result. 
They observed that loss of CSNK1A1 in leukemia 
cells leading to mandatory cell differentiation had 
important consequences such as the absence of Rps6 
phosphorylation and induction of p53. Additionally, 
CSNK1A1 inhibition has been shown to induce p53 
via β-catenin and MDM2 (22). Decreased CSNK1A1 
expression in K562 cells treated with IM may be associated 
with apoptosis of CML cells.

BTRC is involved in major regulatory mechanisms, 
including cell cycle progression, regulation of cell division, 
and signal transduction pathways. It is one of the 
intracellular negative regulators of the Wnt signaling (23). 
The best-characterized cancer-suppressing function of 
BTRC relates to the degradation of β-catenin, a key 
molecule in the canonical Wnt signaling pathway (24). 
Loss of function in BTRC causes β-catenin stabilization, 
activation of the Wnt signaling pathway, and proliferation 
of cancer cells through mitosis (25). In line with the data 
we obtained, the increase in BTRC expression in IM-
treated K562 cells is an expected result.

CTNNBIP1 binds to β-catenin, preventing the interaction 
between β-catenin and TCF4 and inhibiting the Wnt/ 
β-catenin signaling pathway. In addition, it has been 
determined that CTNNBIP1 is suppressed and its 
expression is decreased in many cancers (26). Such 
transcriptional level repressions are thought to be 
quite important in the inhibition of specific genes in the 
pathway. It is thought that CTNNBIP1 acts as a very 
important tumor suppressor in suppressing the Wnt 
signaling not only in hematological malignancies but 
also in solid organ cancers (27). The forced expression 
of CTNNBIP1 in solid tumor cells results in elevated levels 
of β-catenin, which has been shown to strongly inhibit 
the proliferation of these cells by inducing cell cycle G2 
arrest and cell death, reduce cell invasion and cause the 
induction of cell apoptosis (27). Likewise, CTNNBIP1 
in human glioblastoma cells has been shown to inhibit 
cell proliferation, reduce cell invasion, bring about cell 
cycle progression arrest, and cause the induction of cell 
apoptosis. According to our results, the increase in this 
gene in cells treated with IM can be considered as one of 
the effects of IM on β-catenin stabilization.

CtBP is a transcriptional corepressor documented to 
inhibit Wnt signaling by binding to TCFs or preventing TCF-
binding to β-catenin. (28). Overexpression of the CtBP1 
gene has been associated with increased cell proliferation, 
migration, invasion, and survival. For this reason, it has 
become the target of therapeutic agents in tumor types 
with high expression (29). CtBP1 has also been shown 
to interact with the Adenomatous Polyposis Coli (APC) 
tumor suppressor to suppress the expression of Wnt 
target genes (30). Thus, CtBP contributes to the inhibition 
of the interaction of β-catenin with TCF/LEF, and possibly 
paradoxically acts as a tumor suppressor. However, APC 
often mutates in many cancers and mutations in APC that 
disrupt the CtBP-APC complex result in abnormal Wnt 
signaling and cancer progression. It is known that the 
development of hematological malignancies as in CML 
may be due to the mutation of the APC genes (31). When 
we examined our IM treated cells, we considered that the 
fold increase in CTBP1 gene expression may be due to 
the regulation effect of IM on APC mutations. However, 
to verify this, it is necessary to observe whether there is 
a posttranscriptional change by studying at the protein 
level.

PRICKLE1 is known as a regulator of the noncanonical 
Wnt/PCP pathway. At the same time, PRICKLE 1 has been 
reported to suppress β-catenin activity and cell growth. 
It suggested that PRICKLE 1 is a negative regulator of 
the Wnt signaling pathway and is a putative tumor 
suppressor in human HCCs (32). However Wnt/β-catenin 
pathway is inactive in CLL cells and the expression of 
PRICKLE1 genes, involved in the PCP pathway, increases 
in CLL patients (22). Therefore, the role of PRICKLE 1 in 
hematologic malignancies is not clear. In our study, the 
PRICKLE1 gene expression level in IM-treated cells was 
significantly decreased. Therefore, it can be said that IM 
can target the PCP pathway in CML cells (33).

NKD1, another inhibitor of the Wnt signaling pathway, 
inactivates DVL, a scaffold protein. It has been reported 
that the decrease in NKD1 expression, which is thought to 
be due to promoter hypermethylation, has been observed in 
cancers such as hepatocellular carcinoma, gastric cancer, 
small lung adenocarcinoma, and breast invasive ductal
carcinoma. However, in some types of cancers such as colon cancer and hepatoblastoma, it was observed that its expression increased, so it was assumed that NKD1 plays a different role in different cancer types. Zhou et al. have shown that reduced NKD1 expression in cytotogenetically normal acute myeloid leukemia is correlated with adverse clinical outcomes. NKD1 increased significantly after induction chemotherapy reached complete remission in patients with acute myeloid leukemia. NKD1 has been shown to play a key role in both leukemogenesis and oncogenesis may be a potential biomarker and selective therapeutic target for the treatment of AML (34). In our study, the increase in NKD1 expression after IM application also showed that this gene may be effective in CML treatment.

SRY-box (Sox) transcription factors provide an HMG DNA binding domain and regulate the identity and role of stem cells in multiple tissues. SOX17 activates endodermal target genes and also plays an important role in maintaining fetal and neonatal hematopoietic stem cells. SOX17 suppresses β-catenin/TCF mediated transcription to spatially restrict gene expression domains (35). SOX17 expression has been shown to bind directly to the β-catenin promoter region, inhibiting the proliferation, tumor formation, and activity of the Wnt/β-catenin signaling pathway and downregulating target genes such as cyclin D1 (36).

**CONCLUSION**

In conclusion, IM is a tyrosine kinase inhibitor used in chronic myeloid leukemia and its mechanism of action is still being investigated due to resistance developing in some patients. In this study, the effect of IM on the expression levels of genes known as intracellular negative regulators of the Wnt signaling pathway in CML cell line K562 was investigated. Findings suggest that the response or non-response to IM may be related to more complex mechanisms that need to be investigated. Our findings provide preliminary data on studies showing that intracellular negative regulators of the Wnt signaling pathway may play a role in the treatment of CML.

_Competing Interests: The authors declare that they have no competing interest._

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**REFERENCES**


