Activation of STAT5 confers imatinib resistance on leukemic cells through the transcription of *TERT* and *MDR1*

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Running title: STAT5 is linked to multidrug resistance in CML cells

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Abstract

We used two imatinib resistant cell lines, K562-ADM cells, which over-expresses P-glycoprotein (a product of the *ABCB1* gene, more commonly known as *MDR1*), and K562-hTERT cells, which over-expresses the telomerase reverse transcriptase (TERT), as models to show that the acquisition of multidrug resistance in CML is associated with the enhanced phosphorylation of signal transducer and activator of transcription 5 (STAT5). The induction of P-glycoprotein expression that occurred in response to adriamycin treatment was accompanied by increased phosphorylation of BCR-ABL and STAT5, as well as increased telomerase protein expression. Intriguingly, a ChIP assay using an anti-STAT5 antibody revealed direct binding of STAT5 to the promoter regions of both the human *TERT* gene and the *MDR1* gene in K562-ADM cells. Conversely, silencing of endogenous STAT5 expression by siRNA significantly reduced both the expression of P-glycoprotein and telomerase activity and resulted in the recovery of the imatinib sensitivity of K562-ADM cells. These findings indicate a critical role for STAT5 in the induction of P-glycoprotein and in the modulation of telomerase activity in drug-resistant CML cells. Furthermore, primary leukemic cells obtained from patients in blast crisis showed increased levels of phospho-STAT5, P-glycoprotein and telomerase. In contrast, none of these proteins were detectable in the cells obtained

from patients in chronic phase. Together, these findings indicate a novel mechanism that contributes towards multidrug resistance involving STAT5 as a sensor for cytotoxic drugs in CML patients.

1. Introduction

Recently a new drug, imatinib, was developed as a specific ABL tyrosine kinase inhibitor for the treatment of chronic myelogenous leukemia (CML) [1, 2]. Imatinib is highly effective in patients with chronic phase CML, but it is less effective in patients with CML in the accelerated phase or those in blast crisis [3, 4]. Studies of imatinib-resistant CML cell lines have suggested that *BCR-ABL* amplification and overexpression might be the most common mechanism by which leukemic cells resist imatinib [5, 6]. In addition, specific mutations in the adenosine triphosphate (ATP)-binding region of the tyrosine kinase BCR-ABL are being progressively identified in a variable proportion of CML patients who become refractory to imatinib treatment [7-9]. However, it appears that additional drug resistance mechanisms must operate in a substantial proportion of patients [6], and recently telomerase has emerged as one drug resistance candidate [10]. We and others have reported that, in addition to its role preventing telomere shortening [11-13], telomerase over-expression is associated with apoptosis resistance in cancer cells. These data suggest that telomerase may be involved in drug resistance. Other drug resistance pathways have also been identified, including overexpression of the multidrug resistance gene (*MDR1*) product P-glycoprotein (P-gp) [14, 15]. P-gp is a 170 kD membrane glycoprotein responsible

for the ATP-dependent cellular efflux of a variety of compounds across the plasma membrane, thus reducing their toxicity. In the present study, we compared the similarities and differences in signaling proteins involved in drug resistance acquisition using two imatinib-resistant K562 cell lines, the P-gp over-expressing subline K562-ADM cells [16] and the telomerase over-expressing subline K562-hTERT cells [17]. Our results reveal elevated levels of p-STAT5 and telomerase in both lines. In addition, we observed direct binding of STAT5 to the promoter regions of both human telomerase reverse transcriptase *(hTERT)* and *MDR1* in K562-ADM cells, thus highlighting the importance of this transcription factor as a potential therapeutic target for drug resistant CML.

2. Materials and Methods

2.1. Cells

We used K562 cells (K562-Cont, derived from a CML patient in blast crisis) and the corresponding P-gp overexpressing multidrug resistant subline K562-ADM [16, 18]. To maintain P-gp overexpression, K562-ADM cells require the constant addition of adriamycin to the culture medium. We also used stable transformants expressing hTERT (K562-hTERT) and mock-transformed control cells (K562-Mock) established in our laboratory [17].

Primary leukemic cells were obtained from 15 CML patients, 10 of which were newly diagnosed (untreated) and in the chronic phase, and five that were newly diagnosed (untreated) and in blast crisis. Leukemic cells isolated from bone marrow samples using Ficoll-Conray density gradient, washed twice, suspended in cell banker solution (Juji Kagaku, Tokyo Japan), and stored in liquid nitrogen. Cells were thawed prior to use, and viability was determined using trypan blue dye exclusion. Cells showing >80% viability were used in experiments to exclude unreliable data derived from cell death and protein degradation; seven out of 15 samples were found to be suitable at the time of thawing. Therefore, of the initial 15 patients recruited, only cells from four chronic phase patients and three patients in blast crisis (one had

undergone transformation from the chronic phase) were studied. All samples were collected after obtaining informed consent, and the study protocol was approved by the Human Investigation Committee of our institution.

2.2. Chemicals and antibodies

Imatinib was generously provided by Novartis (Basel, Switzerland). Adriamycin was purchased from Calbiochem (La Jolla, CA, USA). Polyclonal rabbit antibodies against AKT, phospho-AKT (Ser473), phospho-STAT5 (Tyr694) and phospho-CRKL (Tyr207) were purchased from Cell Signaling (Beverly, MA, USA). Polyclonal rabbit antibodies against ABL, CRKL and STAT5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal rabbit antibodies against P-gp, HCK, phospho-HCK (Tyr209/Ser211), YB-1 and hTERT were purchased from Abcam (Cambridge, UK). The polyclonal rabbit antibody against JAK2 was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit ß-actin antiserum was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse monoclonal antibody against STAT5 was purchased from BD Pharmingen (San Diego, CA, USA). Monoclonal mouse antibodies against BCL-2 and BCL-XL were purchased from Novocastra Laboratories (Newcastle, UK) and Trevigen (Gaithersburg,

MD, USA), respectively. The monoclonal mouse antibody against phospho-tyrosine (4G10) was purchased from Upstate Biotechnology.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Isogen (Nippongene, Tokyo, Japan), and cDNA was synthesized using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA) as reported previously [19]. The cDNA (25 ng) was subjected to PCR amplification, and the relative concentrations of the PCR products were determined by comparing the ratio of the product in each lane to the levels of *β-actin*. The PCR conditions and primers used for amplification were as previously reported [19].

2.4. Telomerase assay and quantification of enzyme activity

Telomerase activity was measured using the telomere repeat amplification protocol (TRAP) as previously described [20]. PCR products were resolved by electrophoresis on a 12.5% polyacrylamide gel and visualized by staining with SYBR Green DNA stain (BMA, Rockland, ME, USA). To quantify telomerase activity in each sample, enzyme activity was expressed in arbitrary units as reported previously [21].

2.5. RNA interference

Separate aliquots of $2x10^6$ cells were transfected with a double-stranded siRNA targeting *STAT5A* mRNA or a control nonsilencing siRNA (purchased from Dharmacon, Lafayette, CO, USA) using the Amaxa nucleofection electroporation technique (Amaxa, Gaithersburg, MD, USA). Untransfected cells and control siRNA (siCont)-transfected cells were used as negative controls. After three days in culture, the cells were harvested for immunoblot analysis of STAT5, P-gp and hTERT protein levels. In some experiments, 1 μM of imatinib or solvent alone was added to the cell cultures one hour after siRNA transfection, and viable cell numbers were examined.

2.6. Telomere length analysis

Telomere length was assessed by Southern blotting using a telomere probe labeled with digoxigenin as described previously [17]. The filter was rehybridized with a triplet repeat $(CAC)_5$ probe to confirm that both K562-hTERT and K562-ADM cells and their corresponding parental cell lines were of the same genetic origin [22].

2.7. Immunoprecipitation and immunoblotting

Cells ($2x10^7$) were lysed in RIPA buffer, and precleared samples were incubated with the appropriate antibodies for 2 h or overnight. Immune complexes were separated by incubation with protein G-sepharose beads and processed for further analysis. For immunoblot analysis, samples were resolved by 5% linear or 5 to 20% gradient SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (BioRad, Hercules, CA). Blots were incubated with primary antibodies followed by incubation with goat anti-mouse or anti-rabbit horseradish peroxidase conjugated antibodies. Immune complexes were visualized using ECL (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) as previously reported [23].

2.8. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described previously with some modifications [24]. Cells were fixed in formaldehyde for 10 min at 25°C and then resuspended in lysis buffer. The lysates were subjected to sonication, and the resulting supernatants were diluted with dilution buffer, followed by preclearing with a salmon sperm DNA/protein G-Agarose slurry at 4°C. Each precleared solution was then incubated with a STAT5 antibody or normal rabbit serum overnight at 4°C. Immune complexes were isolated

by incubation with a salmon sperm DNA/protein G-Agarose slurry at 4°C. After washing the pellets, DNA elution buffer was added, and the samples were heated to 65°C, followed by treatment with 4 mg of RNase at 37°C and then 10 mg of proteinase K at 55°C. The DNA was then purified and the following PCR primers were used to amplify the promoter regions of *hTERT:* 5'-TGTCTCGAGACACTAACTGCACCCATAAT-3' and 5'-AGGAAGCTTCCTTTTTAAAGGGCTGTGTT-3', and *MDR1*: 5'-GTGTGACTGGGCAAATAATG-3' and 5'- TCATGTAGCCATTTCACCAA-3'.

2.9. Detection of point mutations in BCR-ABL and JAK2

To amplify the kinase domain of *BCR-ABL* and exon 14 of *JAK2*, the following primers were used for PCR: 5’-CGCAACAAGCCCACTGTCT-3’ and 5’-CTCCATGGCTGACGAGATCT-3’ for *BCR-ABL*, and 5’-GGGTTTCCTCAGAACGTTGA-3’ and 5’-TCATTGCTTTCCTTTTTCACAA-3’ for *JAK2*. PCR products encoding the *BCR-ABL* ATP binding site, the kinase activation loop and the *JAK2* pseudokinase domain were then cloned into the PGEM-T vector (Promega, Madison, WI) and introduced into JM109 cells. Randomly selected white colonies were screened for the appropriate insert and sequenced with forward and

reverse universal primers using the ABI377 system (Perkin-Elmer Cetus, Norwalk, CT).

2.10. Statistical Analysis

Data are expressed as the mean ± SD of three or more independent experiments. Statistical analysis was performed using the two-tailed Student's *t* test for paired data. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of imatinib on the proliferation of K562-ADM and K562-hTERT cells

As shown in Figure 1, during the first 48 h of culture, K562-hTERT cells and K562-Mock cells did not demonstrate any significant decrease in sensitivity to treatment with 1 μM imatinib. After 72 h of imatinib treatment over a broader concentration range of 5 to 10 μM, a large number of dead K562-Mock cells were observed, while K562-hTERT cells remained viable. Differences in imatinib sensitivity were more prominent between K562-ADM and K562-Cont cells. K562-ADM cells that have not been drug treated for a month are still resistant to imatinib compared with naïve K562 (K562-Cont) cells. Pretreating starved-K562-ADM cells with adriamycin somewhat restored imatinib resistance, suggesting that drug selection reactivates some preexisting pathway in these cells.

3.2. Changes in levels of apoptosis-related proteins in imatinib-resistant cells

Since telomerase has been implicated in restricting cancer cell apoptosis [25], we examined the levels of anti-apoptotic proteins in the imatinib-resistant cell lines. As shown in Figure 2A, expression of the anti-apoptotic protein BCL-2 was lower in K562-ADM and K562-hTERT cells than in the control cell lines. In contrast, BCL-XL

expression increased slightly in both K562-ADM and K562-hTERT cells, and YB-1, which is known to be involved in P-gp expression, remained stable in both cancer cell lines relative to the controls. As expected, we observed increased expression of hTERT and higher levels of telomerase activity in *hTERT*-transduced K562-hTERT cells. Surprisingly, however, we also observed increased telomerase activity in K562-ADM cells and over-expression of hTERT in addition to P-gp over-expression. Phosphorylation (Tyr694) of the transcription factor STAT5, which is associated with its activation, was also consistently observed in the imatinib-resistant these cell lines. Thus, we used K562-ADM cells and the parental K562 (K562-Cont) cell line to investigate the role of STAT5 in drug resistance related to P-gp and hTERT in the majority of the experiments (e.g. Fig.3-Fig.7).

Next, we determined whether telomerase activity correlated with telomere length in both cell lines. We found that the increase in telomerase activity observed in the K562-ADM line did not affect telomere length in these cells. In contrast, however, the telomerase over-expressing K562-hTERT cells exhibited elongated telomeric DNA. The filter was rehybridized with the minisatellite probe $(CAC)_5$ in order to confirm the initial quality and quantity of the genomic DNA (Figure 2B). The results also confirmed that both K562-hTERT and K562-ADM cells and their corresponding

parental cell lines were of the same genetic origin.

3.3. Correlation between additional genomic changes and drug resistance

To determine whether the observed imatinib resistance of the cell line results from other genomic changes, we looked for mutations in genes that are known to contribute to imatinib resistance. No single amino acid substitutions were found in the ATP-binding site and kinase activation loop (between codons 227 and 345) of the *BCR-ABL* fusion gene, which has commonly been linked to imatinib resistance. In addition, we did not observe higher levels of *BCR-ABL* mRNA in the K562-ADM cells compared to the parental cell line. Moreover, no substitutions at amino acid 617 of the JAK2 protein were detected, indicating that the V617F mutation, which is known to cause JAK2 and STAT5 activation in myeloproliferative neoplasms, was not present (data not shown).

3.4. Adriamycin transcriptionally induces hTERT and MDR1 expression

K562-ADM cells were cultured in the absence of adriamycin for one month and then challenged with the drug. We then examined the levels of *MDR1* and *hTERT* mRNA. In absence of adriamycin, weak basal expression of both genes was detectable,

but expression increased after 6 h of treatment (Figure 3A). Consistent with this increase in mRNA expression, increased levels of P-gp and hTERT protein were also detected 24 h after adriamycin addition. In addition, BCR-ABL and STAT5 were activated within 1 h of adriamycin treatment, as judged by the phosphorylation status of these proteins, and tyrosine phosphorylation of JAK2 and HCK were also enhanced after 6 h (Figure 3B).

3.5. Transcription factor STAT5 regulates hTERT and MDR1 expression in K562-ADM cells, and RNA silencing of STAT5 interferes with drug resistance

Since STAT5 is a well-known transcriptional activator, we next examined whether STAT5 could directly bind the promoter regions of *MDR1* and *hTERT* by ChIP assay. Protein-DNA complexes were cross-linked and then immunoprecipitated with STAT5 or control antibodies. The cross-linking was then reversed and DNA was extracted, followed by PCR amplification targeting the STAT5 binding sites in both of the *MDR1* and the *hTERT* promoter. STAT5 was associated with both promoters in K562-ADM cells, indicating that STAT5 is one of the transcription factors that regulates *MDR1* and *hTERT* expression in K562-ADM cells (Figure 4A). To confirm the role of STAT5 in drug resistance, we analyzed the relationship between STAT5 activation, the

functional expression of *MDR1*, and telomerase activity. K562-ADM cells were incubated with *siSTAT5* or nonsilencing siRNA for three days, and lysates were then examined by immunoblot analysis. P-gp and hTERT protein levels, together with telomerase activity, were clearly down-regulated within 72 h of transfection with *siSTAT5* (Figure 4B,C). Furthermore, knock-down of *STAT5* resulted in the recovery of K562-ADM cell sensitivity to imatinib (Figure 4D).

3.6. Changes in STAT5, P-gp and hTERT protein levels in primary leukemic blasts obtained from CML patients

We then evaluated the levels of STAT5, phospho-STAT5, P-gp and hTERT in fresh leukemic cells obtained from CML patients at different phases of the disease. As mentioned in the Methods section, only cells showing >80% viability were used, and those cells with low viability were omitted to exclude unreliable data. Using these criteria, cells from four CML patients in the chronic phase and three in blast crisis were eligible for this experiment. Primary leukemic cells obtained from the blast phase patients were positive for P-gp in two out of three cases, and cells from all three blast phase patients were positive for hTERT and p-STAT5. In contrast, cells from chronic phase leukemia patients exhibited a completely different protein profile: P-gp, hTERT

and p-STAT5 were undetectable in all cases (Figure 5).

3.7. Differences in the activation of STAT5-related signaling proteins

We next examined K562-ADM and K562-Cont cells for differences in signaling proteins that may result from STAT5 activation. Increased phosphorylation of BCR-ABL, HCK and JAK2 was observed in K562-ADM cells but not in K562-Cont cells, while no differences in the phosphorylation of CRKL, LYN and ERK in either of the two cell lines were detected (Figure 6A). A co-immunoprecipitation assay was then used to examine the association of these phosphorylated proteins with activated STAT5. The association of BCR-ABL fusion protein, HCK, and JAK2 with phospho-STAT5 was enhanced in K562-ADM cells compared to parental K562-Cont cells. HCK and JAK2 also co-immunoprecipitated with activated BCR-ABL. In addition, HCK and JAK2 co-immunoprecipitated together. These data suggest that BCR-ABL, HCK, and JAK2 form a complex with activated STAT5 (Figure 6B).

3.8. BCR-ABL activates STAT5 and induces drug resistance

To further examine how STAT5 activation affected imatinib resistance, K562-ADM cells were pre-treated with imatinib for 3 h and then stimulated with

adriamycin for up to 24 h. The optimal concentration of imatinib (5 μM) was determined by preliminary experiments (data not shown). At 5 μM, imatinib suppressed cell growth after 24 h without causing cell death, suggesting that it affects specific signaling pathways, including those involving BCR-ABL and related proteins. As is shown in Figure 7A, imatinib blocked the phosphorylation of both STAT5 and the BCR-ABL fusion protein and also suppressed the expression of hTERT and P-gp. These data indicate that BCR-ABL kinase activity up-regulates STAT5 activity in K562-ADM cells and that this pathway is negatively affected by imatinib.

4. Discussion

In the present study, we used two CML cell lines exhibiting distinct drug resistance characteristics to examine the molecular mechanisms underlying these different susceptibilities. K562-hTERT cells over-express telomerase [17], and K562-ADM cells over-produce P-gp, the product of the *MDR1* gene [16]. K562-hTERT cells, which were selected following *hTERT* gene transfer, showed resistance to imatinib with increased P-gp production. Constitutively increased telomerase activity in K562-ADM cells did not correlate with telomere elongation, suggesting that telomerase has a function other than telomere elongation. Both lines showed elevated levels of p-STAT5 and telomerase protein concomitant with increased telomerase activity.

In K562-ADM cells, P-gp expression is maintained for a prolonged period after adriamycin removal [16, 26], and the short drug exposure times needed to increase P-gp indicates that cytotoxic selection for pre-existing P-gp-expressing cells cannot be responsible for the emergence of P-gp-positive subpopulations. Rather, selective overexpression of the human *MDR1* gene, which encodes P-gp, has been shown to be related to the methylation status of the *MDR1* promoter region [27]. Thus, the emergence of *MDR1*-expressing K562-ADM cells could be due to the selection of

variants with defective methylation at the *MDR1* locus. Furthermore, recent experiments showed that re-activation of the *MDR1* promoter is more dramatic when demethylation is coupled with chromatin remodeling through inhibition of histone deacetylase activity, indicating the critical role of epigenetic regulation in *MDR1* gene expression. Such epigenetic control of P-gp expression is also supported by a recent report that focused on KU812 cells, a different CML derived cell population, [28]. Data from the cell lines used in the present study support a model in which adriamycin reactivates expression of P-gp and hTERT by stimulating interplay between these two epigenetic mechanisms. Further study will be needed to provide detailed insights into the mechanisms that regulate these genes.

STAT transcription factors play a major role in survival, proliferation, angiogenesis, and immune evasion by tumors [29-32]. STAT5, a STAT family member and transcription factor, is often persistently activated in blood malignancies by non-receptor tyrosine kinases such as BCR-ABL and SRC family kinases [29, 33-35]. We therefore speculated that STAT5 could regulate *MDR1* and *hTERT*, although such regulation had not been previously reported. Indeed, chromatin-immunoprecipitation assays revealed for the first time that STAT5 is a critical transcription factor in drug-induced expression of *MDR1* and *hTERT*. Consistent with these data,

STAT5-targeted RNA interference resulted in decreased STAT5 protein levels, abolished P-gp and hTERT protein expression, and eventually reversed the MDR phenotype such that cells once again became drug sensitive.

Given that *MDR1*-activating signaling pathways apparently culminate in the regulation of gene promoters, we first looked for differences in signaling proteins that may result in the activation of STAT5 in K562-ADM cells. We observed increased phosphorylation of BCR-ABL, HCK, and JAK2 in K562-ADM cells but not in the parental K562-Cont cell line. However, we found no differences in the phosphorylation of the BCR-ABL downstream target CRKL [36], LYN kinase, or the extracellular signal-regulated kinase (ERK) between the two cell lines. Given that these phosphorylated proteins are linked to activated STAT5, we next used a co-immunoprecipitation assay to investigate for possible associations between them. The levels of BCR-ABL fusion protein, HCK, and JAK2 that co-immunoprecipitated with phospho-STAT5 were significantly increased in K562-ADM cells relative to the parental cell line**.** HCK and JAK2 also co-immunoprecipitated with the phospho-BCR-ABL protein. In addition, HCK and JAK2 interacted with each other, suggesting the formation of a complex composed of BCR-ABL, HCK, JAK2 and STAT5. Given the concomitant early phosphorylation of both BCR-ABL and STAT5

and the late phosphorylation of HCK and JAK2, STAT5 might be activated in a biphasic manner (early and late) in K562-ADM cells following adriamycin treatment. Moreover, targeting BCR-ABL with high doses of imatinib in K562-ADM cells before adriamycin challenge suppressed STAT5 phosphorylation, supporting the link between BCR-ABL and STAT5 activation and downstream STAT5 signaling that regulates P-gp and hTERT.

One of the roles of oncogenic tyrosine kinases in cancer is to make cells resistant to genotoxic therapies [37-40]. In addition, oncogenic tyrosine kinase positive cells might repair DNA damage more rapidly and activate DNA damage-dependent checkpoints more readily, thereby allowing more time for DNA repair [41-44]. Human cells use at least six different repair mechanisms to deal with DNA lesions, and homologous recombination repair is an essential component of the drug-resistant machinery in cells transformed by BCR-ABL-related SRC family kinases [40, 44]. In fact, it has been shown that STAT5 is required for the BCR-ABL-mediated activation of RAD51 [40, 44, 45], which helps to repair breaks in double-stranded DNA. BCR-ABL-related SRC family kinases induce pronounced G2/M check-point activation in response to various chemotherapeutic agents, including cisplatin, mitomycin C, etoposide and daunorubicin. It is possible that the activation of the BCR-ABL tyrosine

kinase by genotoxic insults and the resulting prolongation of the G2/M phase might trigger STAT5 activation, leading to the overproduction of the telomere repair enzyme telomerase whose regulation is integrated with the cell cycle [46].

All of the cells collected from the patients in blast crisis showed detectable hTERT expression and STAT5 activation, and two out of three patients showed increased P-gp levels. In contrast, patients in the chronic phase of CML did not show increased levels of these proteins. Unfortunately, we could not compare clinical samples from the same patient at different disease stages, but the data from the CML patients are in line with the results obtained using the cell lines. Development of imatinib resistance is the rule rather than the exception in the blast crisis phase of CML. This may be related, at least in part, to the relative increase in the basal level of *MDR1* gene expression in blast crisis cells compared to chronic phase cells in patients, as reported here and elsewhere [47, 48]. It has been proposed that telomerase expression is essential for cell immortalization and the development of a malignant phenotype in most cancers [20]. Accumulating evidence suggests that telomerase has additional functions beyond telomere maintenance [10, 49]. Similarly, our data suggest that telomerase may contribute, in part, to drug resistance in the CML cells from the patients we analyzed.

5. Conclusions

Our results show that STAT5 tyrosine phosphorylation can occur in response to genotoxic activation of BCR-ABL, HCK, and JAK2, which are responsible for inducing the transactivation of STAT5-responsive reporter genes such as *MDR1* and *hTERT*. Thus, the STAT5 signaling pathway is an attractive target for therapeutic intervention, particularly in cases of drug resistance, and strategies designed to inhibit STAT5 activation and STAT5 mediated gene transcription may hold promise for leukemia therapy.

6. Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interest.

7. Acknowledgements

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9. Figure Legends

Figure 1. Effect of imatinib on the proliferation of K562-hTERT and K562-ADM cells.

Cells at a density of 2×10^5/ml were cultured for 72 hours with various concentrations of imatinib. Imatinib had a greater inhibitory effect on K562-Mock cells at 5 and 10 μM than on K562-hTERT cells (p=0.01, for each concentration). K562-ADM cells not exposed to drug for one month were rechallenged with adriamycin. The inhibitory effect of imatinib was most prominent in naïve K562 (K562-Cont) cells. Pretreating the starved-K562-ADM cells with adriamycin somewhat restored imatinib resistance (*P* values for the difference between adriamycin-starved and rechallenged cells at 5 μM and 10 μM were 0.13 and 0.02, respectively). K562-ADM(-) and K562-ADM(+) denote adriamycin-starved and adriamycin-rechallenged K562-ADM cells, respectively. K562 cells are the parental cells of the K562-hTERT and K562-Mock cell lines, while K562-Cont cells are the parental cells of the K562-ADM cell line. The parental K562 cell lines (labelled as K562 and K562-Cont), used to generate K562-ADM and K562-hTERT cell lines, showed some differences in their sensitivities against imatinib. There is a possibility that the characteristics of the parental K562 cell lines may have somewhat changed during the maintenance of these cells over a long period. Data are

expressed as the mean ± SD for at least three experiments.

Figure 2. Changes in apoptosis-related proteins and telomere length in imatinib-resistant cells.

(A) Differences in the expression of anti-apoptotic proteins and the correlation of the data with telomerase activity were examined in imatinib-resistant cells. Imatinib-resistant cells exhibited a high level of telomerase activity along with increased hTERT expression and activation of STAT5. The column shows the relative levels of each protein.

(B) The telomere length of the imatinib-resistant cells was examined by Southern blotting. The increased telomerase activity observed in K562-ADM cells did not affect telomere length. In contrast, telomerase over-expressing K562-hTERT cells showed marked elongation of telomeric DNA. DNA loading and integrity were verified by rehybridizing the multilocus oligonucleotide probe $(CAC)_5$ to the same filter, and no differences were detected among the samples. The results also showed K562-hTERT and K562-ADM cells and their corresponding parental cell lines were of the same genetic origin. The data show representative results from two experiments.

Figure 3. Adriamycin-induced changes in *MDR1* and *hTERT* expression and the phosphorylation of signaling proteins.

K562-ADM cells were cultured in the absence of adriamycin and then stimulated with 300 ng/ml adriamycin. Subsequently, the levels of *MDR1* and *hTERT* mRNA and protein were analyzed.

(A) The levels of *MDR1* and *hTERT* mRNA were up-regulated after 6 hours of incubation, and higher levels were detected after 24 hours.

(B) BCR-ABL and STAT5 were phosphorylated within 1 hour of treatment. In contrast, increased JAK2 and HCK phosphorylation was not observed until after more than 6 hours of treatment (relative increment of phosphorylated proteins was observed after more than 6 hours). Each experiment was performed three times, and representative results are shown.

Figure 4. Transcription factors related to *hTERT* and *MDR1* expression in K562-ADM cells, and STAT5 silencing restores drug sensitivity.

(A) After cross-linking, protein-DNA complexes were immunoprecipitated with STAT5 or control antibodies. Purified DNA was then amplified by PCR to investigate each binding site in the *MDR1* and *hTERT* promoters. The imunoprecipitate and the same

number of cells as used in the ChIP assay were also subjected to immunoblotting to confirm the efficiency of immunoprecipitation. Each experiment was performed three times and representative data are shown. (B) K562-ADM cells were incubated with si*STAT5* or nonsilencing siRNA for three days, followed by immuboblot analysis. P-gp and hTERT protein levels were markedly down-regulated within 72 hours after si*STAT5* transfection. (C) Telomerase activity was decreased in accordance with a reduction in the levels of hTERT. (D) One hour after transfection with siRNA, 1 μM of imatinib or solvent alone was added to the cells. Knock-down of *STAT5* restored the drug sensitivity of K562-ADM cells. Data represent the mean ± SD of three independent experiments.

Figure 5. Changes in STAT5, P-gp, and hTERT protein in primary leukemic cells.

Expression of STAT5, phospho-STAT5, P-gp, and hTERT was evaluated in fresh leukemic cells obtained from CML patients at different disease phases. Primary leukemic cells from patients in blast crisis were positive for P-gp in two out of three cases, while hTERT and activated STAT5 were detected in all three cases (asterisk represents positivity). In contrast, P-gp, hTERT, and p-STAT5 were undetectable in

leukemic cells collected from patients in the chronic phase.

Figure 6. Differences in the activation of STAT5-related signaling proteins and the association of these phosphorylated-proteins with STAT5.

(A) Increased phosphorylation of BCR-ABL, HCK, and JAK2 was only observed in K562-ADM cells. The levels of CRKL, LYN, and ERK phosphorylation did not differ between the two cell lines. The column shows the relative value of each phosphorylated-protein. ※: reprobed with 4G10. (B) The association of the phosphorylated-proteins depicted in (A) with activated STAT5 was examined by co-immunoprecipitation. The BCR-ABL fusion protein, HCK and JAK2 co-immunoprecipitated with phospho-STAT5 in K562-ADM cells. HCK and JAK2 also co-immunoprecipitated with activated BCR-ABL. In addition, HCK and JAK2 interacted with each other, suggesting the formation of a complex containing BCR-ABL, HCK, JAK2 and STAT5. The figure shows representative results from three experiments.

Figure 7. Effect of the ABL kinase inhibitor, imatinib, on STAT5 activation and a schematic representation of the acquisition of drug resistance by CML cells.

(A) Exposure of K562-ADM cells to adriamycin (300 ng/ml) induced rapid activation of BCR-ABL and STAT5. Preincubation with imatinib (5 μM for 3 h) blocked the phosphorylation of STAT5 as well as activation of the BCR-ABL fusion protein throughout the entire exposure period. The expression of hTERT and P-gp were also suppressed. Each experiment was performed three times and representative data are shown.

(B) Schematic representation of the acquisition of drug resistance by CML cells.

Figure 1

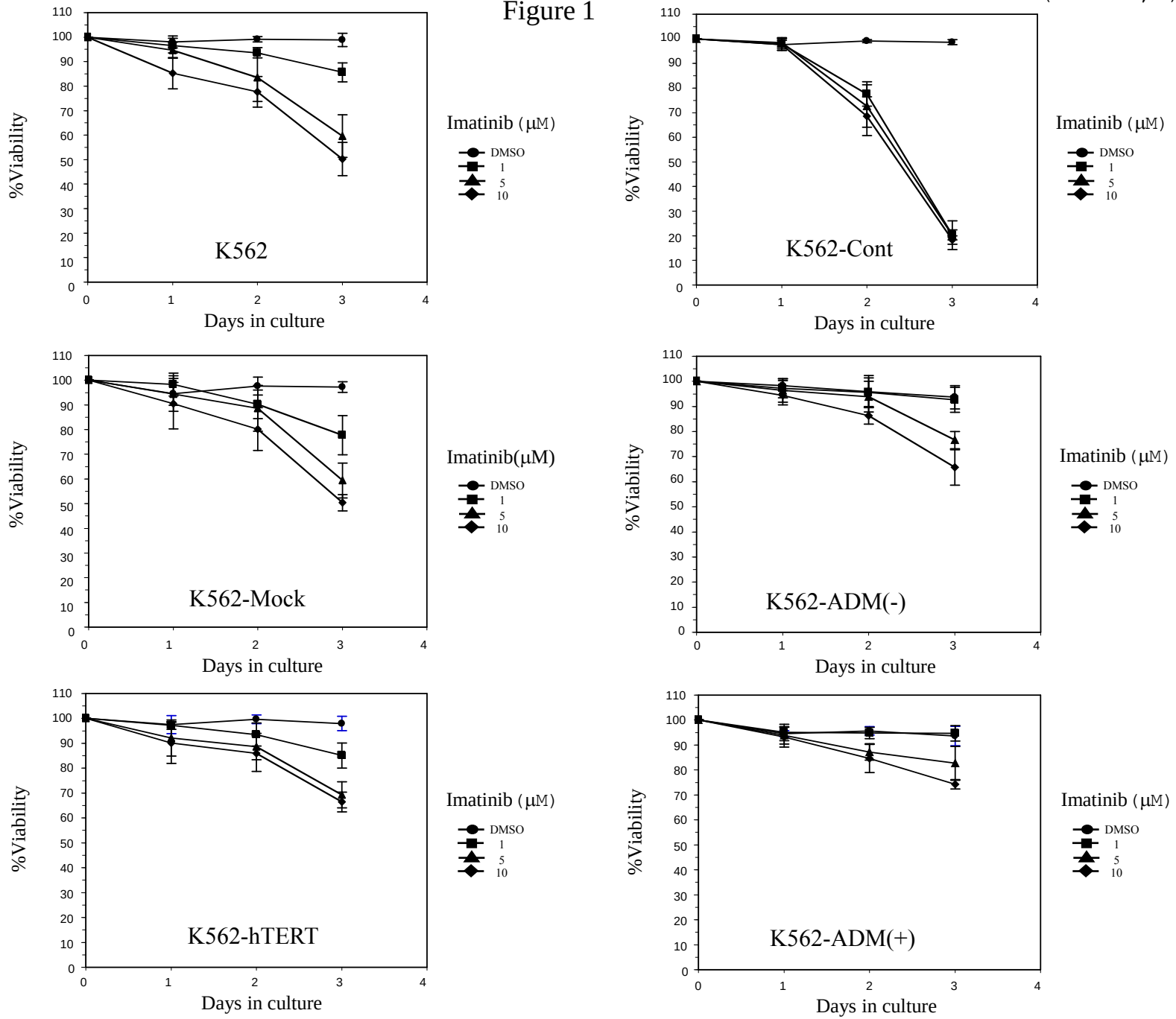

%Viability
Days in culture
Imatinib (μM)
DMSO
1
5
10
K562
K562-Cont
K562-Mock
K562-ADM(-)
K562-hTERT
K562-ADM(+)

Figure 2

(Yamada,O)

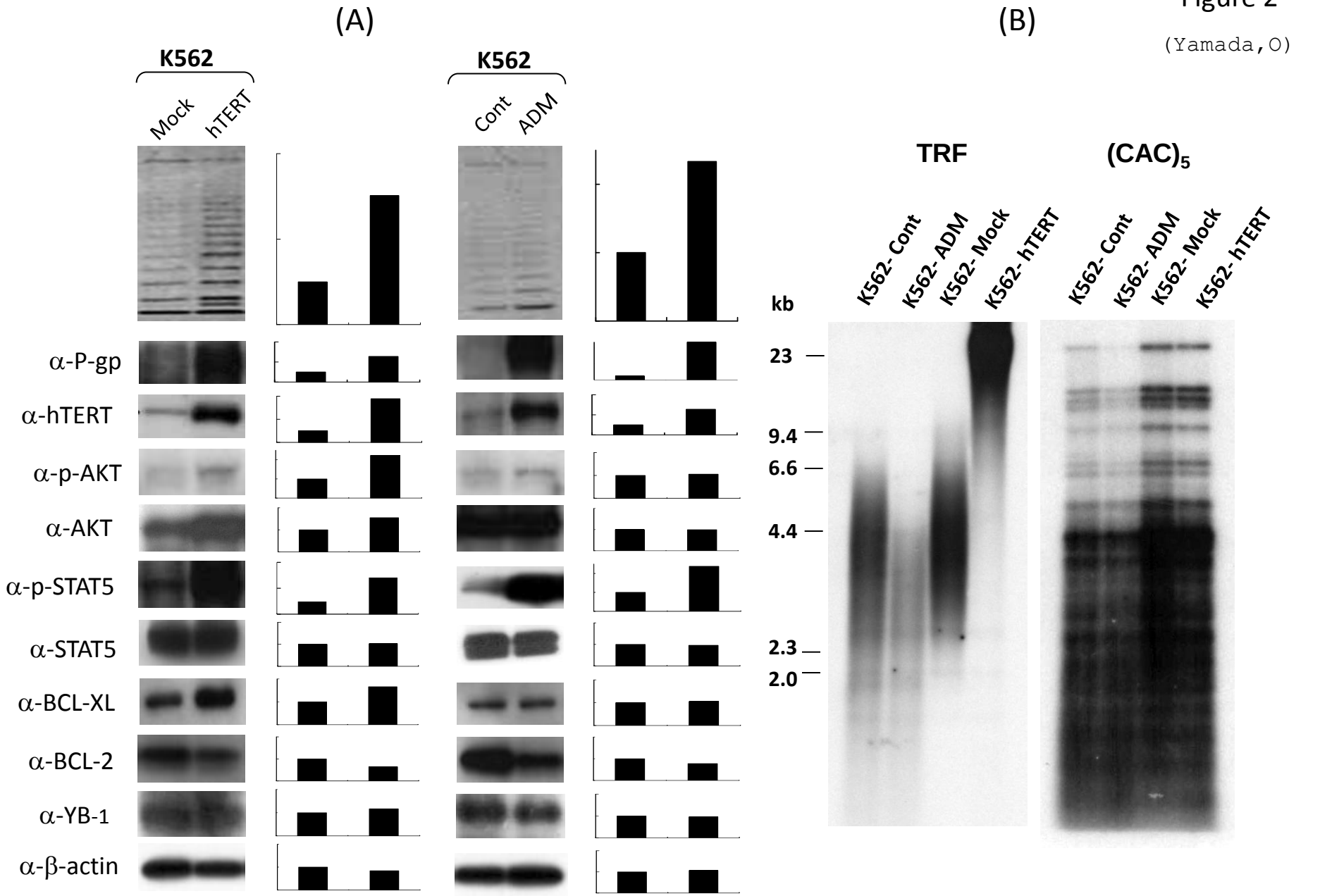
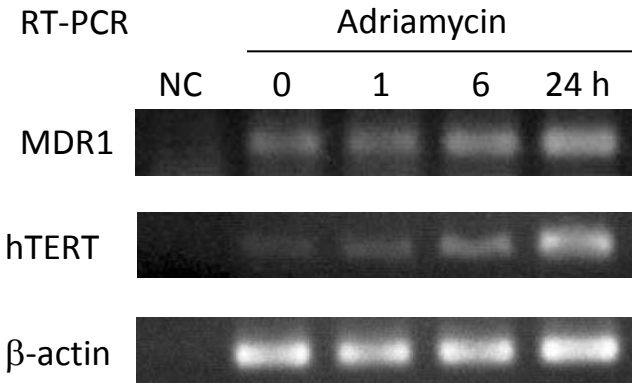


Figure 3

(Yamada,O)

(A)



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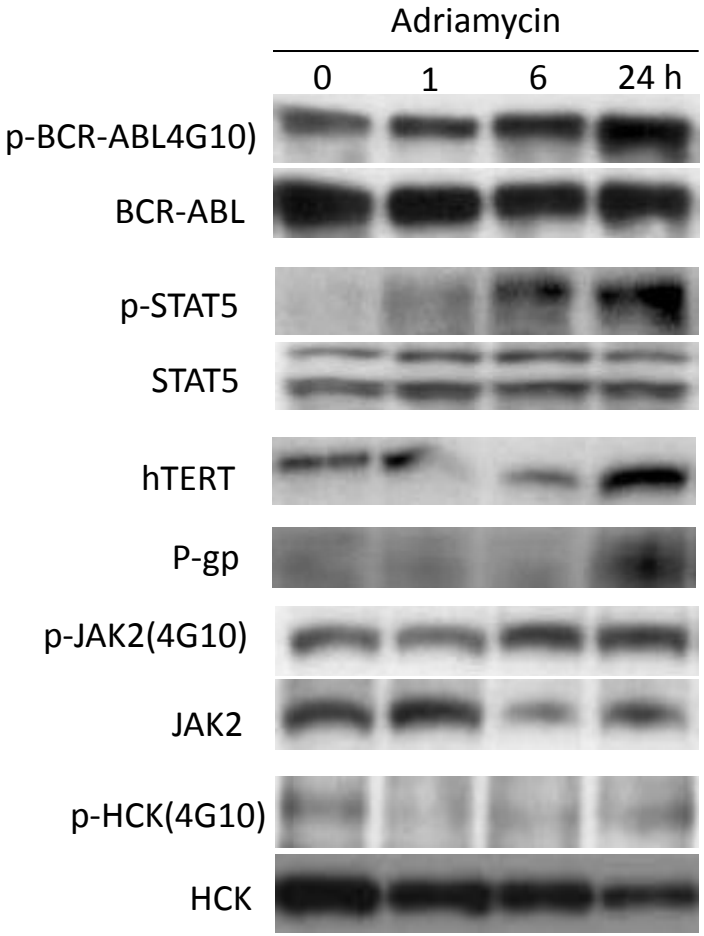


Figure 4
(Yamada,O)

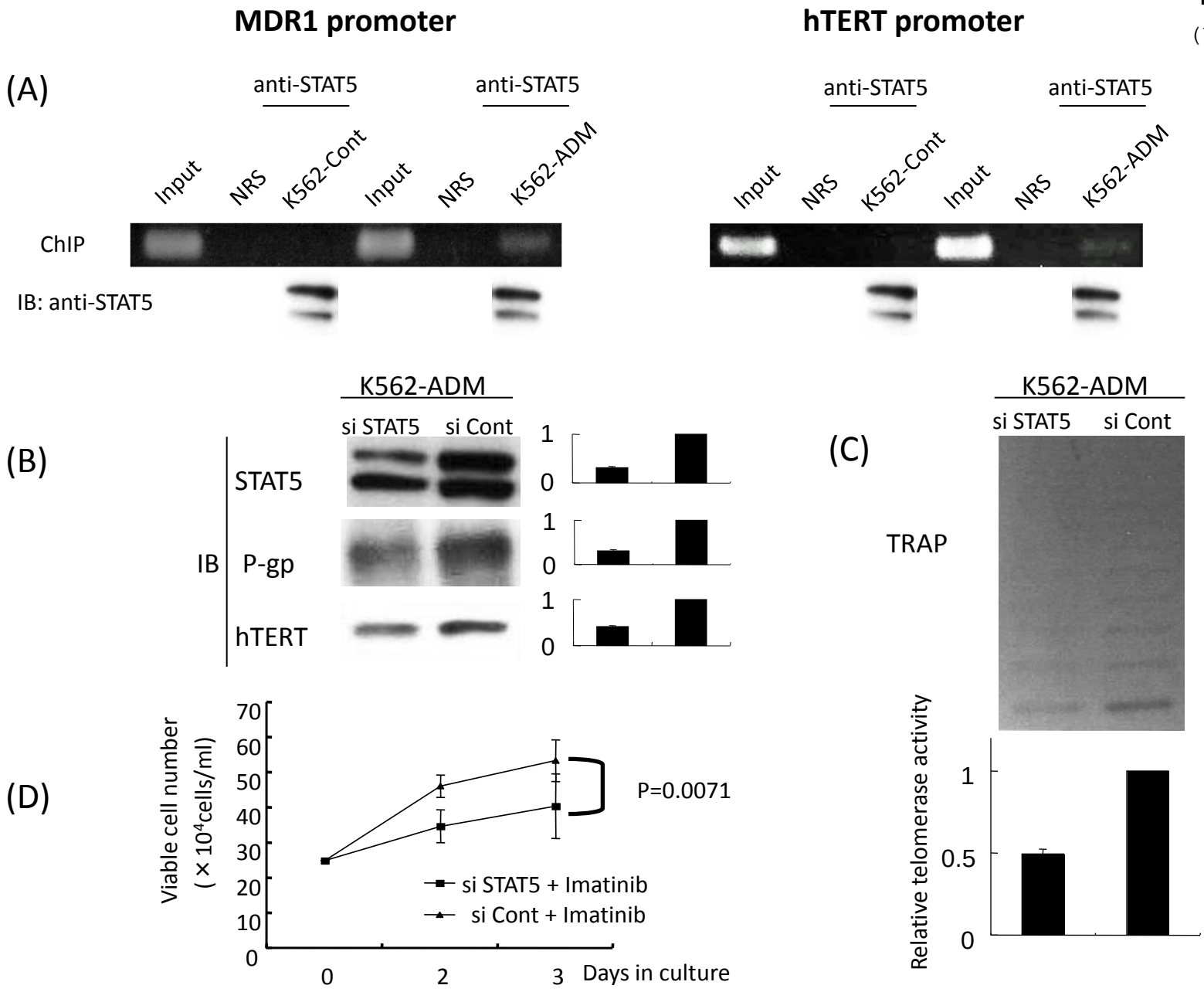


Figure 5

(Yamada,O)

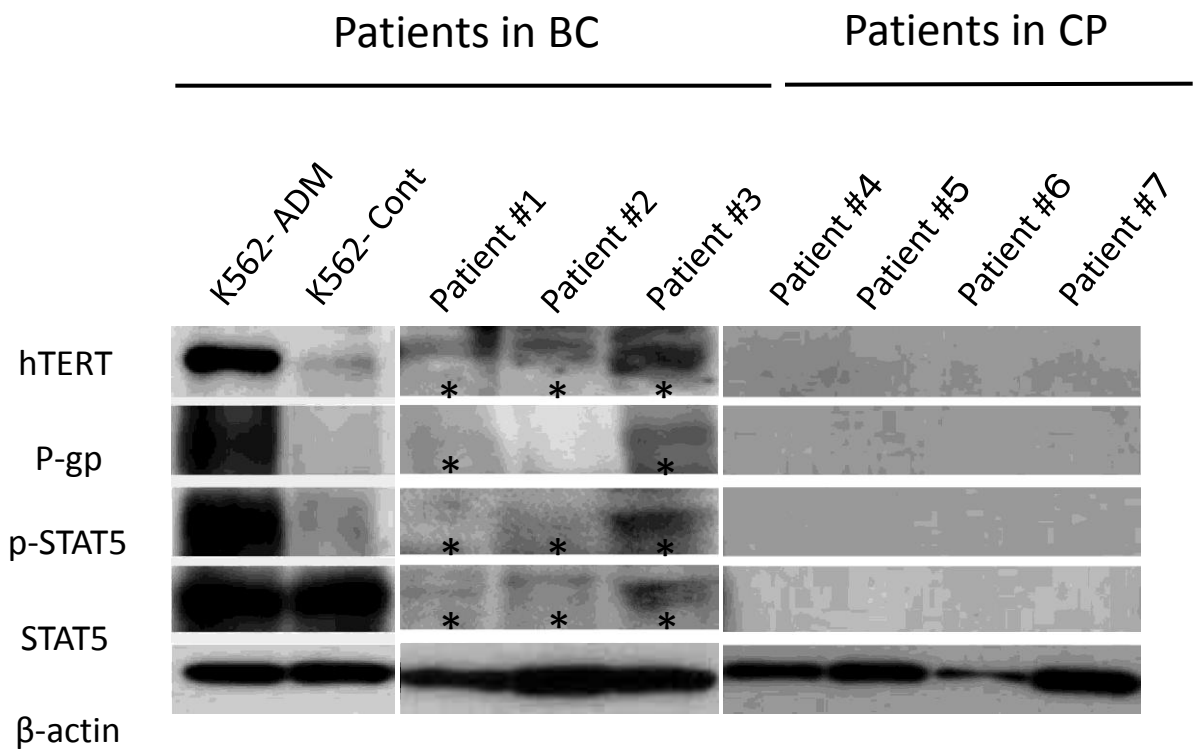


Figure 6
(Yamada,O)

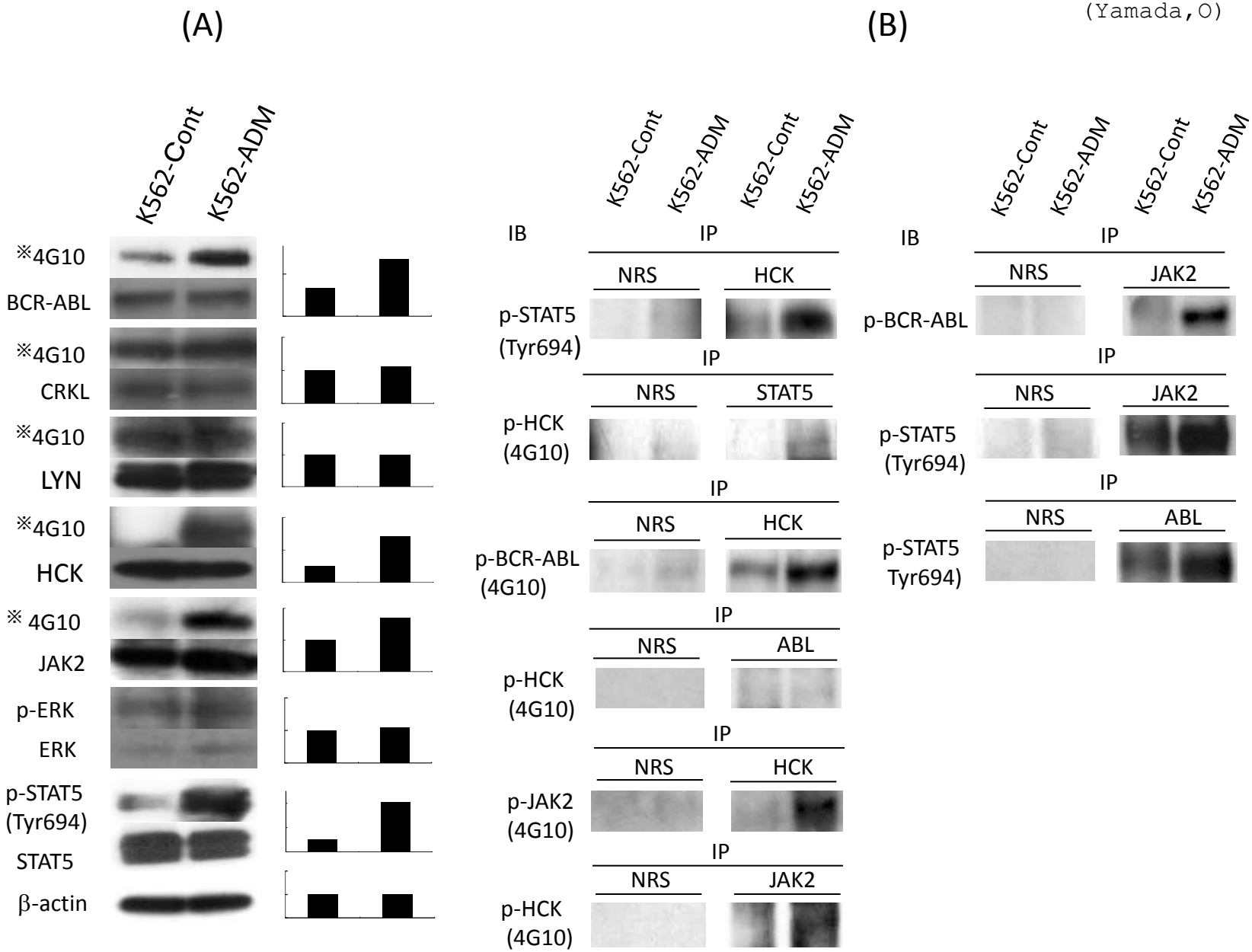
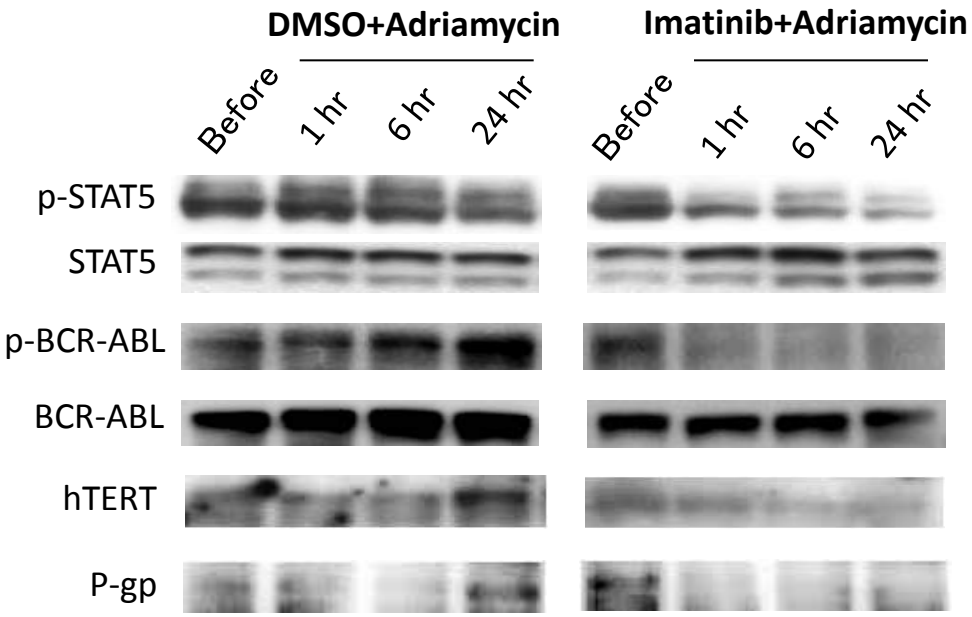


Figure 7

(Yamada,O)

(A)



(B)