Introduction of the T315I gatekeeper mutation of *BCR/ABL1* into the Philadelphia chromosome-positive lymphoid leukemia cell line using the CRISPR/Cas9 system

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Original article

Running Title: CRISPR/Cas9 to model T315I-positive Ph+ ALL

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Abstract

Imatinib and second-generation tyrosine kinase inhibitors (TKIs) dramatically improved the prognosis of Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL). However, TKI-resistance due to the T315I gatekeeper mutation of *BCR/ABL1* is crucial for further improving the prognosis. To establish a human model of Ph+ ALL with the T315I mutation, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is proper since it enables inducing specific mutation via homologous recombination (HR) repair if cellular endogenous HR pathway is intact. Here we applied the CRISPR/Cas9 system to introduce T315I mutation into the Ph+ lymphoid leukemia cell line KOPN55bi, in which the HR pathway seemed to be active due to its resistance to an inhibitor for poly (ADP-Ribose) polymerase-1. Single guide RNA targeting at codon 315 and single-strand oligodeoxynucleotide containing ACT to ATT nucleotide transition at codon 315 were electroporated with recombinant Cas9 protein. The dasatinib-resistant sublines were obtained after one-month selection with the therapeutic concentration of dasatinib, which acquired T315I mutation due to HR. T315I-acquired sublines were highly resistant to imatinib and second-generation TKIs but moderately sensitive to the therapeutic level of ponatinib. This authentic human model is helpful for developing new therapeutic strategies overcoming TKI-resistance in Ph+ ALL due to T315I mutation.

Keywords

- Philadelphia chromosome
- Acute lymphoblastic leukemia
- Tyrosine kinase inhibitors (TKIs)
- T315I mutation
- Genome editing

Highlights

- T315I-positive Ph+ lymphoid leukemia cell line was established by genome editing.
- T315I-acquired sublines were highly resistant to imatinib and second-generation TKIs.
- T315I-acquired sublines were moderately sensitive to therapeutic level of ponatinib.

1. Introduction

Philadelphia (Ph) chromosome causes chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) {Faderl, 1998 #74;Schlieben, 1996 #77}. Ph chromosome generates the *BCR/ABL1* fusion gene and plays an essential role in leukemogenesis. In Ph+ ALL patients, therapeutic outcomes were extremely poor when they were treated with conventional multiagent chemotherapy. Of note, the development of imatinib dramatically improved the prognosis of Ph+ ALL {Wassmann, 2006 #61;Abou Dalle, 2019 #27}. Imatinib inhibits the tyrosine kinase activity of BCR/ABL1 by binding to the adenosine triphosphate (ATP) pocket of the ABL1 kinase domain {Savage, 2002 #123}. Of clinical importance, mutations in the ATP pocket residues of the *BCR/ABL1* gene are frequently observed in relapsed Ph+ ALL patients treated with imatinib {Soverini, 2014 #36}. To overcome imatinib-resistance due to the mutations in the ATP pocket, second-generation tyrosine kinase inhibitors (TKIs) such as nilotinib {Weisberg, 2006 #82;Kim, 2015 #65} and dasatinib {Ottmann, 2007 #64;Shah, 2004 #83} were developed. However, the T315I gatekeeper mutation of *BCR/ABL1* shows marked resistance to imatinib as well as second-generation TKIs {Soverini, 2014 #36}. Indeed, about two-thirds of the relapsed Ph+ ALL cases acquired T315I mutation after treatment with dasatinib-combined chemotherapy {Soverini, 2014 #36;Rousselot, 2016 #28}.

Pharmaco-biological significance of the *BCR/ABL1* mutations in the TKI-sensitivity has been generally tested in murine interleukin-3 (IL-3) dependent Ba/F3 cells that were introduced *BCR/ABL1* or its mutant cDNAs by retrovirus vector {Warmuth, 2007 #56;Shah, 2004 #87}. Despite their utility, gene transduction methods have several limitations. It induces overexpression of *BCR/ABL1* gene derived from cDNA under the control of the viral promoter. Moreover, the introduced cDNA lacks introns and the 3'-untranslated region (UTR) of the target gene, which are involved in transcriptional and post-transcriptional regulation of gene expression {Sandberg, 2008 #122;Mayr, 2009 #125}. Indeed, in the *ABL1* and *BCR/ABL1* genes, microRNA is reported to be involved in post-transcriptional regulation through 3' UTR {Bueno, 2008 #126}. Similarly, alternative splicing of the *BCR/ABL1* gene is reported to be involved in TKI resistance {Lee, 2008 #124}. Furthermore, the reciprocal ABL1/BCR fusion gene had been reported to be involved in leukemogenesis [18]. Under these circumstances, direct induction of the T315I mutation into the intrinsic BCR/ABL1 gene of leukemia cells is the ideal model. In addition to the Ba/F3 system, several TKI-resistant sublines of Ph+ leukemia cell lines were established after a long-term culture in the presence of increasing concentrations of imatinib or dasatinib {Hirase, 2009 #17;Hekmatshoar, 2018 #78;Mahon, 2003 #31;Mahon, 2000 #32;Scappini, 2004 #14;Yuan, 2010 #16;Tang, 2011 #38}. Although some TKI-resistant sublines of Ph+ leukemia cell lines acquired T315I mutation, upregulation of efflux P-glycoprotein (P-gp), overexpression of BCR/ABL1 protein {Hekmatshoar, 2018 #78;Mahon, 2003 #31;Mahon, 2000 #32;Scappini, 2004 #14;Yuan, 2010 #16;Tang, 2011 #38}, and downregulation of influx organic cation transporter 1 (OCT-1) {Hekmatshoar, 2018 #78} have been noticed as additional factors for their TKI-resistance. Therefore, the development of an authentic human model of T315I-acquired Ph+lymphoid leukemia is mandatory.

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 is a new genome editing technology {Cong, 2013 #59}. In this system, guide RNA of a 20-nucleotide sequence complementary to the target DNA enables Cas9 nuclease to interact with a specific site. Subsequently, Cas9 induces a double-strand break (DSB) of DNA at the site adjacent to a protospacer adjacent motif (PAM). DSB is repaired by either non-homologous end-joining (NHEJ) or homologous recombination (HR) {Cong, 2013 #59;Maruyama, 2015 #48}. The NHEJ is a predominant pathway and disrupts target sequence by insertions or deletions. In

contrast, HR induces site-specific genome editing in the presence of single-strand oligodeoxynucleotides (ssODN) as a repair template.

Therefore, genome editing by HR with the CRISPR/Cas9 system may be helpful in introducing the T315I mutation into Ph+ leukemia cell lines. In this context, since the HR pathway is frequently disturbed in cancer cells {Nieborowska-Skorska, 2017 #49;Slupianek, 2011 #47}, verifying whether the HR pathway is intact in the target Ph+ leukemia cell lines is necessary. Based on this hypothesis, we recently tried to introduce the T315I mutation of *BCR/ABL1* into human Ph+ myeloid leukemia cell lines {Tamai, 2018 #42}. We selected three cell lines that were resistant to olaparib, one of the inhibitors for poly (ADP-Ribose) polymerase-1 (PARP-1) {Farmer, 2005 #84;Nickoloff, 2017 #60}. As a result, we successfully obtained T315I-acquired imatinib-resistant sublines of three myeloid cell lines after a short-term selection with imatinib at therapeutic concentration. However, thus far, no human model of T315I-acquired Ph+ lymphoid leukemia has been developed by HR using the CRISPR/Cas9 system.

The present study tried to develop a human model of Ph+lymphoid leukemia with the T315I mutation of *BCR/ABL1* by genome editing with the CRISPR/Cas9 system. We selected an olaparib-resistant dasatinib-sensitive Ph+ lymphoid leukemia cell line as a target. We successfully established the T315I-acquired sublines as the result of HR.

2. Materials and Methods

2.1. Cell lines and reagents

KOPN55bi was established in our ancestry laboratory from the patient with CML lymphoid blast crisis (BC) of CML, as previously reported {Uno, 2003 #76}. K562 and TCCS were established from CML-BC with the p210 BCR/ABL1 {Kano, 2001 #80}. K562 was purchased from American Type Culture Collection (ATCC). TCCS was provided from Prof. N Komatsu (Juntendo University). Each cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS).

Imatinib were purchased from Selleck Chemicals (Tokyo, Japan). Nilotinib and dasatinib were kindly provided by Dr. Tetuzo Tauchi in Tokyo Medical University. Ponatinib were purchased from Chem Scene (New Jersey, USA). Imatinib, nilotinib, dasatinib, ponatinib were dissolved in dimethylsulfoxide (DMSO) and stored at -80^{0}C, except for ponatinib stored at -20^{0}C. Daunorubicin was purchased from Sigma-Aldrich (Missouri, USA) and stored at -20^{0}C. The stock solutions were diluted to the required concentrations with serum-free medium before use.

2.2. Introduction of the T315I mutation by the CRISPR/Cas9 system

The sequence of sgRNA was 5′-aactcagtgatgatatagaacgg-3′, and the sequence of template ssODN was 5′-cgttcacctcctgccggttgcactccctcaggtagtccaggaggttcccgtaggtcatgaaTtcGAtAatgatatagaacgggggct cccgggtgcagaccc -3′ (four capital letters indicate mutated nucleotides) as we previously reported {Tamai, 2018 #42}. Both sgRNA and template ssODN were synthesized by Integrated DNA Technologies (Coralville, IA, USA). KOPN55bi cells were pre-treated with 10 nM of SCR7 (Cayman Chemical, Ann Arbor, MI, USA) for 24 hours before electroporation. Subsequently, 5×10^5 of the pre-treated cells were transfected with recombinant Cas9 (Integrated DNA Technologies) nuclease and guide RNA as a ribonucleoprotein complex with template ssODN by electroporation using the Neon electroporation transfection system (ThermoFisher Scientific, Waltham, MA, USA). The

electroporated cells were transferred to four wells of 96-well plate and cultured in the presence of 10nM of SCR7. Forty-eight hours after electroporation, the cells were mixed with untreated parental cells and plated into 12 wells of a 24-well plate. After 13-day culture in the absence of TKIs, the cells were cultured in the presence of 100nM of dasatinib. During the selection of dasatinib-resistant sublines, half the volume of the culture medium in each well was exchanged approximately every ten days with fresh medium containing 100 nM of dasatinib. When the dasatinib-resistant sublines were selectively expanded in each well of the 24-well plate, the cells were transferred to culture flasks and expanded in the absence of dasatinib for further experiments.

2.3. alamarBlue cell viability assay

Briefly, 5×10^4 cells were plated into a 96-well plate in triplicate and cultured for 72 hours in the presence or absence of six concentrations of each drug. The cells were additionally incubated for 5 hours with alamarBlue (Bio-Rad Laboratories, Hercules, CA, USA). Absorbance was monitored at 570 nm by a microplate spectrophotometer using absorbance at 600 nm as a reference. The cell survival was calculated by the percentages of optical densities of treated wells to those of untreated wells. The concentrations of drugs required to reduce the viability of treated cells to 50% of untreated cells were calculated, and the median of three independent assays was determined as 50% inhibitory concentration (IC50) for each drug.

2.4. Cell proliferation

For evaluation of cell growth, cells were seeded into 75 cm^2 culture flasks at 0.75×10^6 cells/ml in triplicate and incubated at 37 °C with 5% CO_2. Cells were harvested every day, stained with trypan blue solution (T6146, Sigma-Aldrich, St. Louis, MO, USA), and enumerated under a phase-contrast microscope with a hemocytometer.

2.5. RT-PCR analyses

RNA was extracted using PureLink RNA Mini Kit (ThermoFisher Scientific), and cDNA was synthesized using SuperScript II Reverse Transcriptase (ThermoFisher Scientific). RT-PCR analysis of the *ABL1* transcript was performed with forward primers in exons 1a (5′-gtgggctgcaaatccaagaag-3′) or 1b (5′-aggaatcatcgaggcatggg-3′) of the *ABL1* gene and a reverse primer in exon 2 of the *ABL1* gene (5′-gtccagcgagaaggttttcc-3′). K562 and TCCS were used as the positive and negative control, respectively {Tamai, 2018 #42}. RT-PCR analysis of *BCR/ABL1* transcript was also performed with forward primer in exon 6 of the *ABL1* gene (5′-ctgctgtacatggccactca-3′) and reverse primer in exon 7 of the *ABL1* gene (5′-ctctcgggtgcagtccattt-3′).

2.6. Direct sequencing

Genomic DNA was extracted using PureLink Genomic DNA Mini Kit (ThermoFisher Scientific). Direct sequencing of each PCR product was also performed using the forward primer specific for intron 5 of the *ABL1* gene (5′-ccacacgagcacagtctcag-3′).

2.7. Flow cytometric analysis of P-gp

Cells were stained with a phycoerythrin (PE)-conjugated anti-P-glycoprotein antibody (Nichirei, Tokyo, Japan) or IgG1-PE isotypic control (Beckman Coulter, Brea, CA, USA). The cells were analyzed by flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA, USA).

2.8. Western blot analyses

Cells were solubilized in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 0.05% NaN3, 1 mM phenylmethylsulfonyl fluoride, 100 μM sodium vanadate) on ice. To evaluate the phosphorylation status of signal transduction molecules, parental cells and T315I-acquired subline #2 were cultured in the presence or absence of dasatinib at 100 nM for 12 hours. The cell lysates were separated on a SDS-polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane. Each membrane was incubated with anti-ABL1 (#554148, BD Biosciences, Franklin Lakes, NJ, USA), anti-OCT-1 (SLC22A1 Antibody, Novus Biologicals, Colorado, USA), anti-phospho-c-ABL (#2861, Cell Signaling Technology, Beverly, MA, USA), anti-phosphotyrosine (4G10, Cell Signaling Technology), anti-STAT5 (#610192, BD Biosciences, Franklin Lakes, NJ, USA), anti-phospho-STAT5 (#9351S, Cell Signaling Technology), anti-p44/42 MAPK (#9102, Cell Signaling Technology), anti-phospho-p44/42 MAPK (#9101S, Cell Signaling Technology), anti-Tubulin (T5168, Sigma Aldrich, St. Louis, Missouri, USA) at 4°C overnight. Subsequently, the membranes were incubated with secondary anti-mouse or anti-rabbit horseradish peroxidase-labeled (Medical & Biological Laboratories, Nagoya, Japan) at room temperature for 1 hour. Then, the blots were developed using an enhanced chemiluminescence detection (ECL) kit (GE Healthcare, Little Chalfont, UK).

3. Results

3.1. Introduction of the T315I mutation into KOPN55bi cells using the CRISPR/Cas9 system

We analyzed olaparib sensitivities of Ph+ lymphoid leukemia cell lines that were available in our laboratory. Among 16 cell lines, 6 cell lines were highly resistant to olaparib (IC50 > 20 μM) {Tamai, 2018 #42}, suggesting that the intrinsic HR pathway may be intact in these cell lines. Among six olaparib-resistant Ph+ lymphoid leukemia cell lines, KOPN55bi was the most sensitive to dasatinib. Thus, we selected KOPN55bi as a target. KOPN55bi has the p210 type of BCR/ABL1. Digital karyotyping based on the SNP array analysis (Fig.1a) revealed that KOPN55bi has a loss of heterozygosity in chromosome 9 but not in chromosome 22. To verify whether KOPN55bi has an intact *ABL1* gene, we performed RT-PCR analyses (Fig.1b). In KOPN55bi, RT-PCR with the primers in exons 6 and 7 of the *ABL1* gene, which are present in both the *ABL1* and *BCR/ABL1* transcripts, revealed the right size of products. In contrast, no PCR products were detectable by RT-PCR with the primers in exons 1a or 1b and 2 of the *ABL1* gene, which are specific for the *ABL1* transcript. These observations indicated that KOPN55bi has a *BCR/ABL1* fusion gene but not an intact *ABL1* gene.

Before transfection, we pre-treated KOPN55bi cells with SCR7 {Maruyama, 2015 #90} to enhance HR efficiency. SCR7 is an inhibitor for DNA ligase IV, which is a crucial enzyme in the NHEJ pathway. Subsequently, we electroporated recombinant Cas9 protein with the sgRNA and the ssODN. We selected the following sgRNA and ssODN (Fig.1c) that showed the highest HR efficiency in our previous establishment of T315I-sublines in three Ph+myeloid leukemia cell lines {Tamai, 2018 #42}: the reverse sgRNA targeting at the PAM

site 12 base-pair upstream of codon 315 and the anti-sense ssODN containing a single nucleotide transition of ACT to ATT at codon 315. To avoid the re-cutting of a repaired target loci, the ssODN additionally contains three silent point mutations. After electroporation, the cells were cultured in 4 wells of a 96-well plate in the presence of SCR7 for 48 hours. Subsequently, the cells were transferred to 12 wells of a 24-well plate and cultured for 11 days in the absence of TKI to allow expansion of the cells that acquired T315I mutation as a result of HR (Fig.1d). Then, the cells were cultured in the presence of 100 nM of dasatinib. The concentration (100nM) of dasatinib for selection was equivalent to a maximum serum concentration (C_{max}) level during dasatinib therapy {Rousselot, 2010 #81;Takahashi, 2012 #79} and is high enough to eradicate dasatinib-sensitive parental cells completely. Consistently, the parental cells were entirely killed within 2-week incubation with 100 nM of dasatinib. After 4 to 5 weeks of selection, the dasatinib-resistant cells were expanded in 10 out of 12 wells of the 24-well plate and transferred to culture flasks to expand in the absence of TKIs for further experiments.

3.2. Establishment of T315I-acquired sublines of KOPN55bi

To verify that dasatinib-resistant sublines acquired the T315I mutation of *BCR/ABL1* as a result of HR, we performed direct sequencing of the PCR products (Fig.2a). We confirmed the acquisition of the T315I mutation as well as three silent point mutations in all of the dasatinib-resistant sublines. We next analyzed the significance of T315I mutation on cell growth. T315I-acquired sublines grew at a similar rate as their parental cells. There was no significant difference in cell growth between parental cells and three T315I-acquired sublines (Fig.2b). Considering that the dasatinib-resistant sublines were cultured in the presence of 100 nM of dasatinib for approximately 1 month, we tested the possibility that T315I-acquired sublines may also gain other mechanisms for TKI-resistance. We performed Western blot analysis of BCR/ABL1 (Fig.2c) and confirmed that BCR/ABL1 protein expression levels were not upregulated in the T315I-acquired sublines compared with their parental cells. We next performed a flow cytometric analysis of P-gp (Fig.2d) and confirmed that the cell surface expression levels of P-gp were not upregulated in the T315I-acquired sublines. We finally evaluated OCT-1 expression levels (Fig.2e), although its involvement in the influx of dasatinib is controversial {Yilmaz, 2015 #93}. We confirmed that OCT-1 expression levels were not downregulated in the T315I-acquired sublines. These observations demonstrated that dasatinib-resistant sublines of KOPN55bi acquired the T315I mutation as a result of HR-mediated genome editing with the CRISPR/Cas9 system.

3.3 Specific resistance to TKIs in T315I-acquired sublines of KOPN55bi

We then validated the specific resistance to imatinib and second-generation TKIs in the T315I-acquired sublines of KOPN55bi. We first tested the sensitivity to dasatinib by alamarBlue cell viability assay (Fig.3a). Parental cells of KOPN55bi were sensitive to the anti-leukemic activity of dasatinib in a dose-dependent manner. In contrast, all of the 10 T315I-acquired sublines were highly resistant to dasatinib up to 500 nM. We next evaluated the phosphorylation status of BCR/ABL1 and signal transduction molecules in response to dasatinib treatment by Western blotting (Fig.3b). After 12-hour treatment with 100 nM of dasatinib, STAT5 and p38/p40 MAPK as well as BCR/ABL1 were highly dephosphorylated in parental cells. In contrast, BCR/ABL1 and STAT5 were not dephosphorylated in the T315I-acquired subline. Partial dephosphorylation of p44/p42 MAPK was observed in T315I-acquired subline, probably due to the inactivation of the Src pathway by dasatinib. We further evaluated the sensitivities of T315I-acquired sublines to imatinib (Fig.3c) and

nilotinib (Fig.3d) using the alamarBlue cell viability assay. In parental cells treated with 1 μM of imatinib or 100 nM of nilotinib, cell viabilities were significantly decreased to approximately 70% of control. In contrast, the T315I-acquired sublines showed a complete resistance up to 10 μM of imatinib and 1 μM of nilotinib. We finally analyzed the sensitivity to daunorubicin, one of the typical chemotherapeutic agents, by alamarBlue cell viability assay (Fig.3e). Of note, parental cells and T315I-acquired sublines were almost equally sensitive to daunorubicin.

These observations indicated that the T315I-acquired sublines showed a specific resistance to imatinib and second-generation TKIs.

3.4. Ponatinib-sensitivity in T315I-acquired sublines of KOPN55bi

Ponatinib is a potent TKI that can inhibit all critical kinase domain mutations of BCR/ABL1, including T315I {O'Hare, 2009 #21}. Thus, we analyzed the sensitivity of the T315I-acquired sublines to ponatinib using alamarBlue assay (Fig.4a). Parental cells were highly sensitive to ponatinib in a dose-dependent manner. In the treatment with 40 nM of ponatinib, which is almost equivalent to a therapeutic serum concentration {Cortes, 2013 #71}, the median cell viability of parental cells in three independent analyses was 36%. The median IC50 value of ponatinib in parental cells was 0.9 nM. Of note, although significantly less sensitive than the parental cells, the T315I-acquired sublines were moderately sensitive to ponatinib in a dose-dependent manner. The median cell viability of three T315I-acquired sublines at 40 nM of ponatinib in three independent analyses was 62% (range: 50–65%). The median IC50 value of ponatinib in three T315I-acquired sublines was 310 nM (range: 32–750 nM). We next examined the effect of ponatinib on the tyrosine phosphorylation status in the T315I-acquired sublines and their parental cells. We performed Western blot analysis using an anti-phosphotyrosine antibody (Fig.4b). Treatment with 10 nM of ponatinib markedly dephosphorylated cellular proteins in parental cells, but only marginally in the T315I-acquired sublines. In contrast, treatment with 40 nM of ponatinib markedly dephosphorylated cellular proteins in the T315I-acquired sublines and their parental cells. These observations indicated that the therapeutic concentration of ponatinib showed anti-leukemic activity against the T315I-acquired sublines, but in comparison with parental cells, ponatinib at a lower concentration was less effective against the T315I-acquired sublines.

4. Discussion

In the present study, we established an authentic human model of Ph+ lymphoid leukemia cell line with T315I mutation of BCR/ABL1 as the result of HR using CRISPR/Cas9 system.

Previously, two human Ph+ lymphoid leukemia cell lines with the T315I mutation (SK9 and SU/SR) were established in different ways {Hirase, 2009 #17;Okabe, 2010 #45}. SK-9 was directly established from a Ph+ ALL patient who relapsed with the acquisition of T315I mutation of *BCR/ABL1* during imatinib-combined chemotherapy {Okabe, 2010 #45}. Thus, in the case of SK-9, no parental cell line with an imatinib-sensitive phenotype is available. SU/SR is a T315I-acquired imatinib-resistant subline of an imatinib-sensitive SU-Ph2, which was established from a Ph+ ALL patient {Hirase, 2009 #17}. SU/SR was established from SU-Ph2 after an approximately 3-month culture with increasing concentrations of imatinib from low (0.01 μM) to high (10 μM) concentration. Importantly, previous studies demonstrated that long-term exposure of Ph+ leukemia cell lines to increasing concentrations of TKIs induced TKI-resistance by the following mechanisms: overexpression of BCR/ABL1 protein due to amplification of *BCR/ABL1* gene, upregulation of efflux P-gp expression {Hekmatshoar, 2018 #78;Mahon, 2003 #31;Mahon, 2000 #32;Scappini, 2004 #14;Yuan,

2010 #16;Tang, 2011 #38}, and downregulation of influx OCT-1 expression {Hekmatshoar, 2018 #78}.

Thus, to minimize the involvement of these diverse mechanisms for TKI-resistance, we first expanded cells for 13 days in the absence of TKI. Then we selected T315I-acquired cells in the presence of dasatinib, considering that it is highly effective for Ph+ lymphoid leukemia as a dual Src and Abl inhibitor {Shah, 2004 #83}. In contrast, imatinib and nilotinib are not potent Scr inhibitor {Hantschel, 2008 #138;Mahon, 2008 #137}. In fact, parental KOPN55bi cells were sensitive to dasatinib, but not to imatinib and nilotinib. For selection, we used 100 nM of dasatinib, since 100 nM is equivalent to serum Cmax level during dasatinib therapy. Indeed, 100 nM of dasatinib was enough to kill the parental cells of KOPN55bi completely. After the approximately 1-month selection with dasatinib, we successfully obtained the T315I-acquired sublines as a result of HR. Moreover, we confirmed that protein expression levels of BCR/ABL1, P-gp, and OCT-1 in the T315I-acquired sublines were almost identical to those in parental cells. These observations demonstrated that the T315I-acquired sublines of KOPN55bi are unlikely to obtain known mechanisms for BCR/ABL1 kinase-independent TKI-resistance during dasatinib selection. In addition to KOPN55bi, we tried to introduce T315I mutation into 3 olaparib-resistant Ph+ALL cell lines (KCB1, Kasumi 8, and YAMN91), but we could not obtain their T315I-acquired subline, probably due to their highly vulnerable phenotype to electroporation.

Using our human model of T315I-acquired Ph+ lymphoid leukemia, we evaluated the efficacy of ponatinib. Ponatinib is designed to overcome TKI-resistance induced by ABL kinase domain mutations, including T315I {O'Hare, 2009 #21}. The anti-leukemic activity of ponatinib was evaluated in the murine Ba/F3 cells that were transduced *BCR/ABL1* or its mutant cDNAs by retrovirus vector {Warmuth, 2007 #56;O'Hare, 2009 #21}. In the Ba/F3 model system, the IC50 values of ponatinib in the cells expressing native *BCR/ABL1* or T315I mutant were 0.5 nM and 11 nM, respectively {O'Hare, 2009 #21}. In our human model of KOPN55bi, the IC50 values of ponatinib in the parental cells and the T315I-acquired sublines were 0.9 nM and 310 nM, respectively. The serum concentration of ponatinib was reportedly 40 nM in patients who received daily doses of ≥ 30 mg {Cortes, 2013 #71}. The median cell viabilities of the parental cells and the T315I-acquired sublines of KOPN55bi at 40 nM of ponatinib were 36% and 62%, respectively.

Thus, our human model suggested that acquisition of T315I mutation may lead to moderate resistance to ponatinib when patients are treated with a reduced dose of ponatinib. Indeed, it has been reported that the T315I clone was selectively developed in a Ph+ ALL patient who had been treated with low-dose (15 mg/day) of ponatinib as maintenance therapy after hematopoietic stem cell transplantation {Noetzli, 2017 #72}. Of clinical importance, the T315I clone was reportedly removed by increasing the dose of ponatinib to 45 mg/day in combination with a single dose of vincristine, followed by donor lymphocyte infusion {Noetzli, 2017 #72}. Taken together, this previously reported case along with our findings in the T315I-acquired sublines of KOPN55bi show that dose-reduction of ponatinib is not recommended for Ph+ ALL patients who acquired the T315I mutation of *BCR/ABL1*.

In conclusion, we successfully established a Ph+ lymphoid leukemia cell line with T315I mutation by HR using the CRISPR/Cas9 system. Our human model is a useful tool to investigate the pharmaco-biological significance of T315I mutation and to develop new therapeutic strategies to overcome the T315I mutation in Ph+ALL.

Figure legend

Fig.1 Introduction of T315I mutation into KOPN55bi cell line. (**a**) Digital karyotyping of KOPN55bi based on the SNP array analysis. In each chromosome, the top panel indicates zygosity, the middle panel indicates a copy number, and the bottom panel indicates chromosomal construction. (**b**) RT-PCR analysis of the *ABL1* and *BCR/ABL1* transcripts in KOPN55bi. At the top of the panel, schematic of *ABL1* and *BCR/ABL1* transcripts was illustrated. Arrows indicate primers for RT-PCR analyses. K562 and TCCS are positive and negative control for the *ABL1* transcript, respectively. Each number indicates the exon number of the *ABL1* gene used for primers. The molecular size marker was electrophorated in the left lane. (**c**) Schematic diagram of the sgRNA and the ssODN as a template. Top and second lines indicate wild-type amino acid and nucleotide sequences, respectively. Third and bottom lines indicate sequences of sgRNA and ssODN, respectively. Arrows indicate the directions of sgRNA and ssODN. Boxes indicate wild type and mutated codon 315. Closed arrowhead indicates the cleave site of Cas9 nuclease. In the sgRNA sequence, the PAM site is underlined. Open arrowheads indicate four mutated nucleotides. (**d**) Transfection and selection workflow

Fig.2 Establishment of T315I-acquired sublines. (**a**) Genomic sequence of the PCR products from parental cells and dasatinib-resistant sublines (#1-3) of KOPN55bi. Wild type genomic sequence and four mutations in template ssODN for HR are indicated at the top of the panel. Arrowheads indicate mutated nucleotides. (**b**) Time course analysis of cell growth in T315I-acquired sublines and their parental cells. The vertical axis indicates viable cell number. (**c**) Western blot analysis of BCR/ABL1 in KOPN55bi parental cells and T315I-acquired sublines (#1-3). The upper panel indicates the blot of the anti-ABL antibody. The lower panel indicates the blot of anti-Tubulin antibody as an internal control. (**d**) Flow cytometric analysis of cell surface expression of P-gp in KOPN55bi parental cells and T315I-acquired sublines (#1-3). Grey lines and black shades indicate fluorescence intensities of isotype control and anti-P-gp antibody, respectively. (**e**) Western blot analysis of OCT-1 in KOPN55bi parent cells and T315I-acquired sublines (#1-3). The upper panel indicates the blot of the anti-OCT-1 antibody.

Fig.3 Sensitivities of KOPN55bi parental cells and T315I-acquired sublines to TKIs and chemotherapeutic agent. (**a**) Dose-response curves of dasatinib in parental cells and 10 T315I-acquired sublines. Horizontal and vertical axes indicate the log concentration of dasatinib and % viability in the alamarBlue assay, respectively. (**b**) Western blot analysis of tyrosine phosphorylation in KOPN55bi parental cells and T315I-acquired subline #2. Cells were cultured in the absence (-) or presence (+) of 100 nM dasatinib for 12 hours. Top and middle panels indicate the blots of anti-phospho-c-ABL (p-c-ABL), anti-STAT5, anti-phospho-STAT5 (p-STAT5), anti-p44/42 MAPK, anti-phospho-p44/42 MAPK (p-MAPK). The bottom panel indicates the blot of anti-alpha Tubulin antibody as an internal control. Dose-response curves of imatinib (**c**), nilotinib (**d**), and daunorubicin (**e**) in parental cells and three T315I-acquired sublines. Horizontal and vertical axes indicate the log concentration of each drug and % viability, respectively. Error bars indicate standard errors in triplicate analyses.

Fig.4 The anti-leukemic activity of ponatinib in parental cells and T315I-acquired sublines of KOPN55bi. (**a**) Dose-response curves of ponatinib in parental cells and three T315I-acquired sublines (#1-3). Horizontal and vertical axes indicate the log concentration of ponatinib and % viability, respectively. Error bars indicate standard errors in triplicate analyses. (**b**) Western blot analysis of tyrosine phosphorylation in KOPN55bi parental cells and T315I-acquired subline (#2). Cells were incubated in the absence (-) or presence of 10 nM or 40 nM

of ponatinib for 12 hours. The top panel indicates the blots of the anti-phospho-tyrosine antibody. The bottom panel indicates the blots of anti-Tubulin antibody as an internal control

Conflict of interest disclosure

The authors declare no competing interests.

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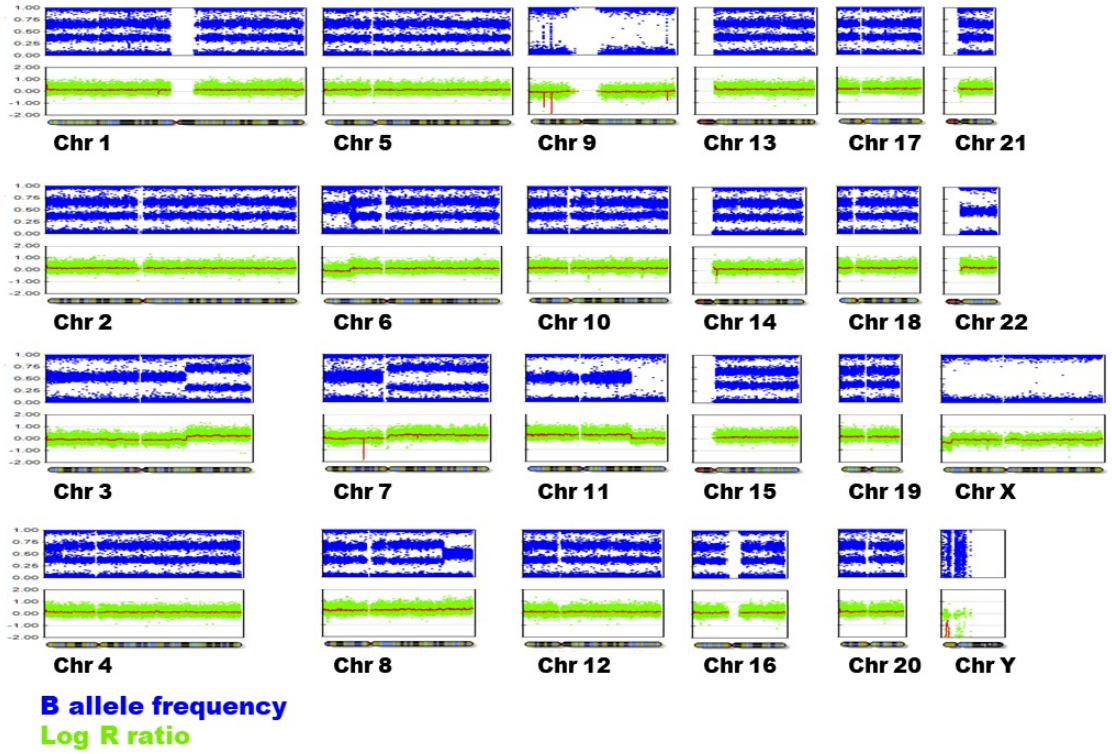
Author contributions

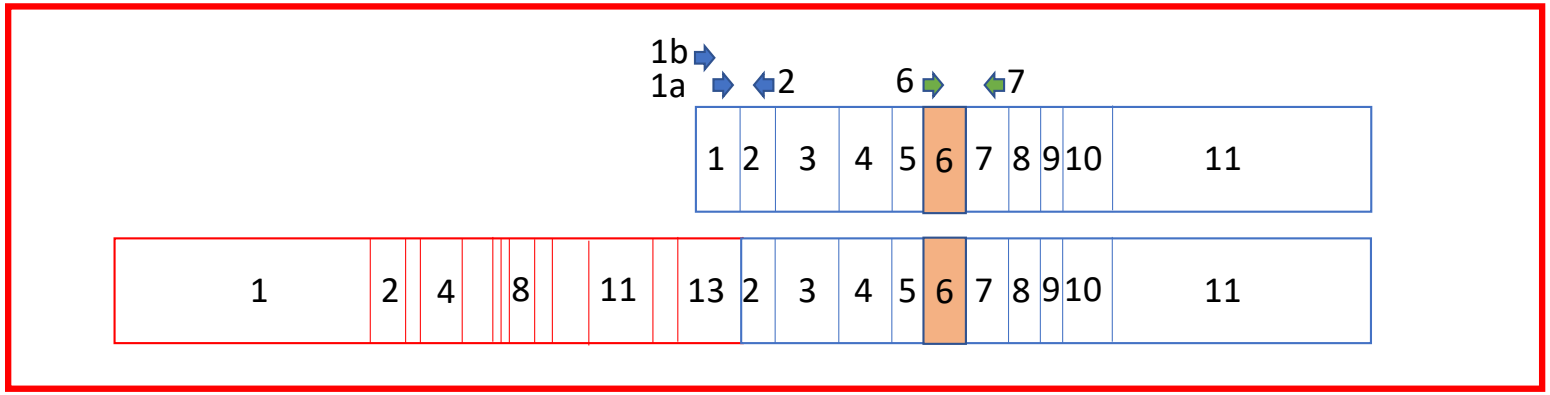
T.N. performed research, analyzed data, and wrote the manuscript; M.T., D.H., and T.I. suggested the concept of the study and designed the study; K.K., S.K., A.W., K.A., and K.G. analyzed data. T.I. revised the manuscript and supervised the overall study process.

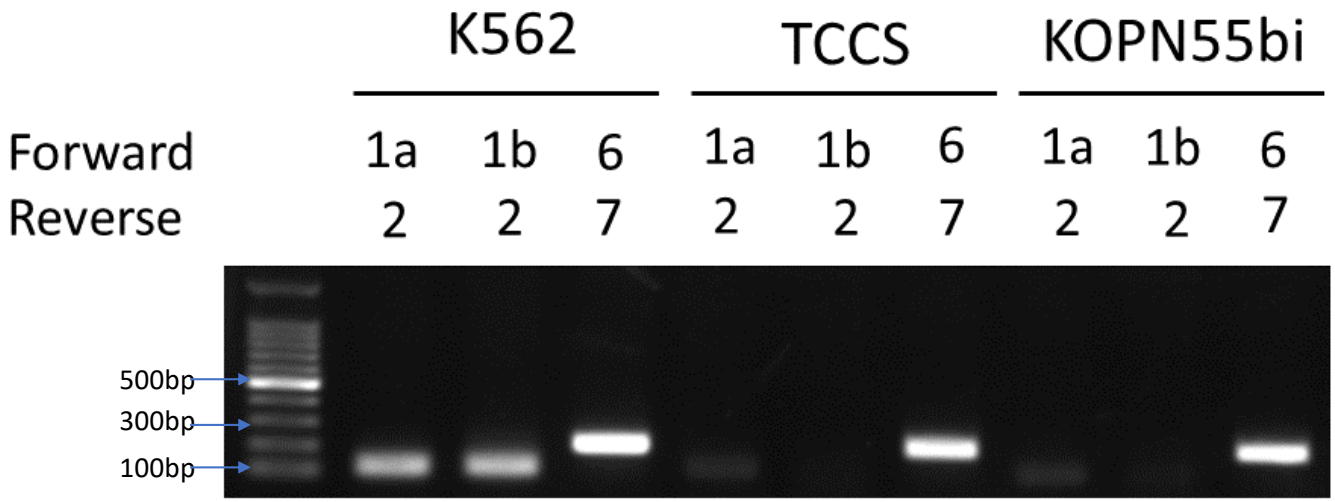
References

1a

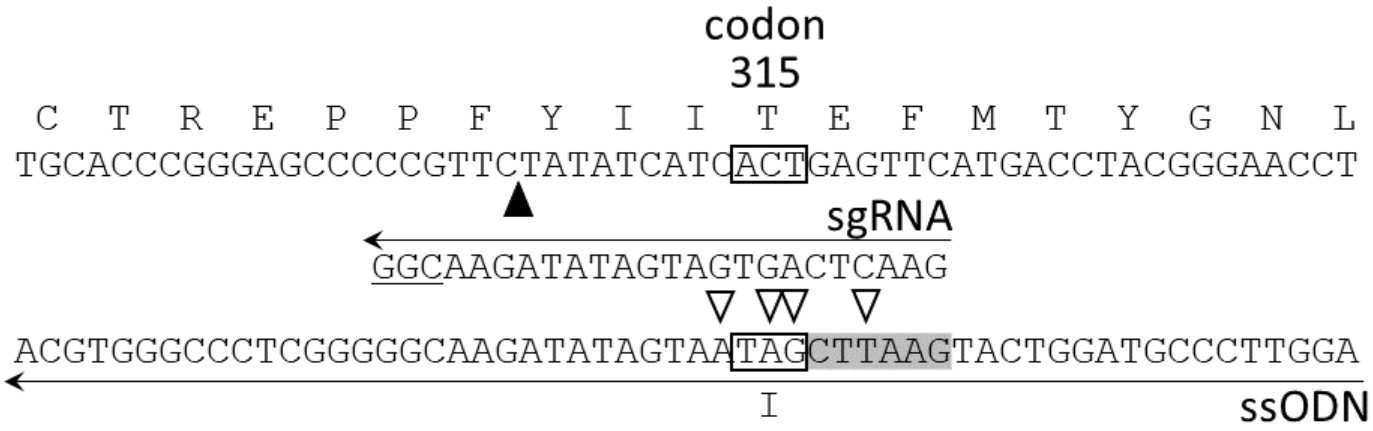

Chr 1
Chr 5
Chr 9
Chr 13
Chr 17
Chr 21
Chr 2
Chr 6
Chr 10
Chr 14
Chr 18
Chr 22
Chr 3
Chr 7
Chr 11
Chr 15
Chr 19
Chr X
Chr 4
Chr 8
Chr 12
Chr 16
Chr 20
Chr Y
B allele frequency
Log R ratio

1b

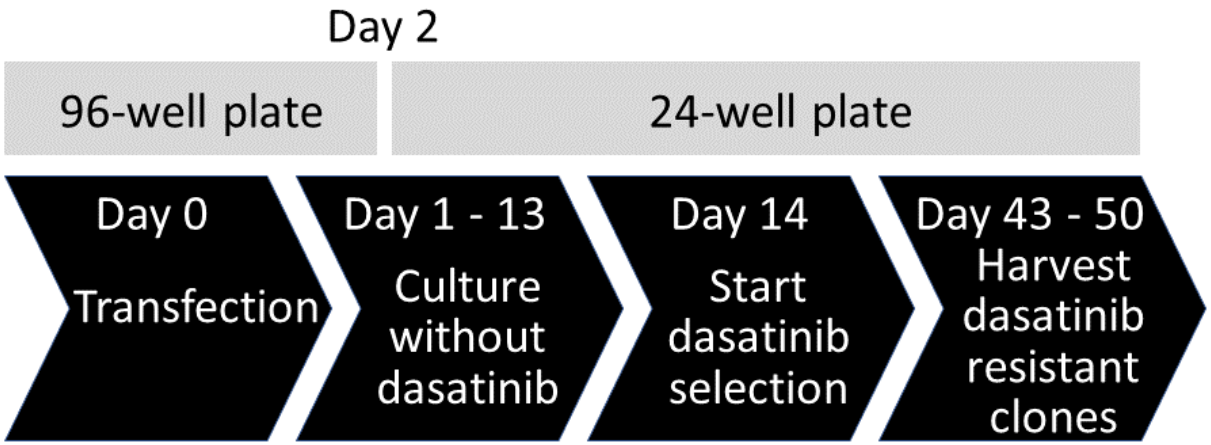

1b
1a
2
6
7
1 2 3 4 5 6 7 8 9 10 11
1 2 4 8 11 13 2 3 4 5 6 7 8 9 10 11


K562
TCCS
KOPN55bi
Forward
Reverse
1a 1b 6 1a 1b 6 1a 1b 6
2 2 7 2 2 7 2 2 7
500bp
300bp
100bp

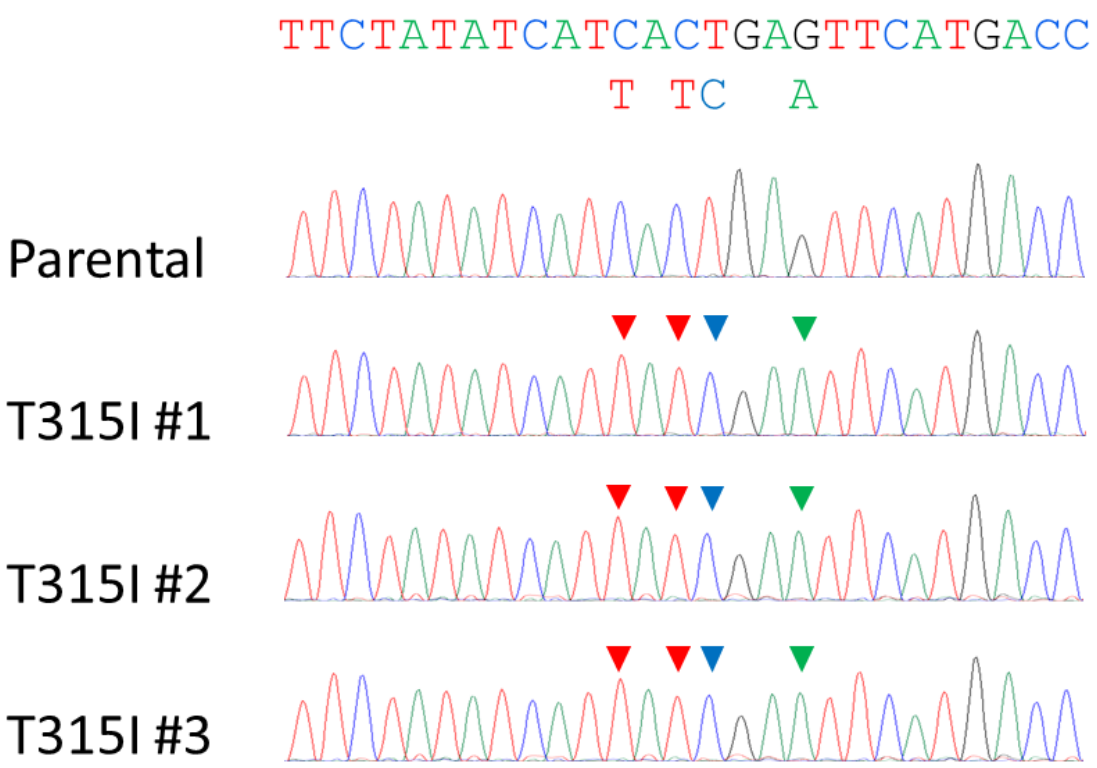
1c


codon
315
C T R E P P F Y I I T E F M T Y G N L
TGCACCCGGGAGCCCCCGTTCTATATCATCACTGAGTTCATGACCTACGGGAACCT
sgRNA
GGCAAGATATAGTAGTGACTCAAG
ACGTGGGCCCTCGGGGGCAAGATATAGTAATAGCTTAAGTACTGGATGCCCTTGGA
I
ssODN

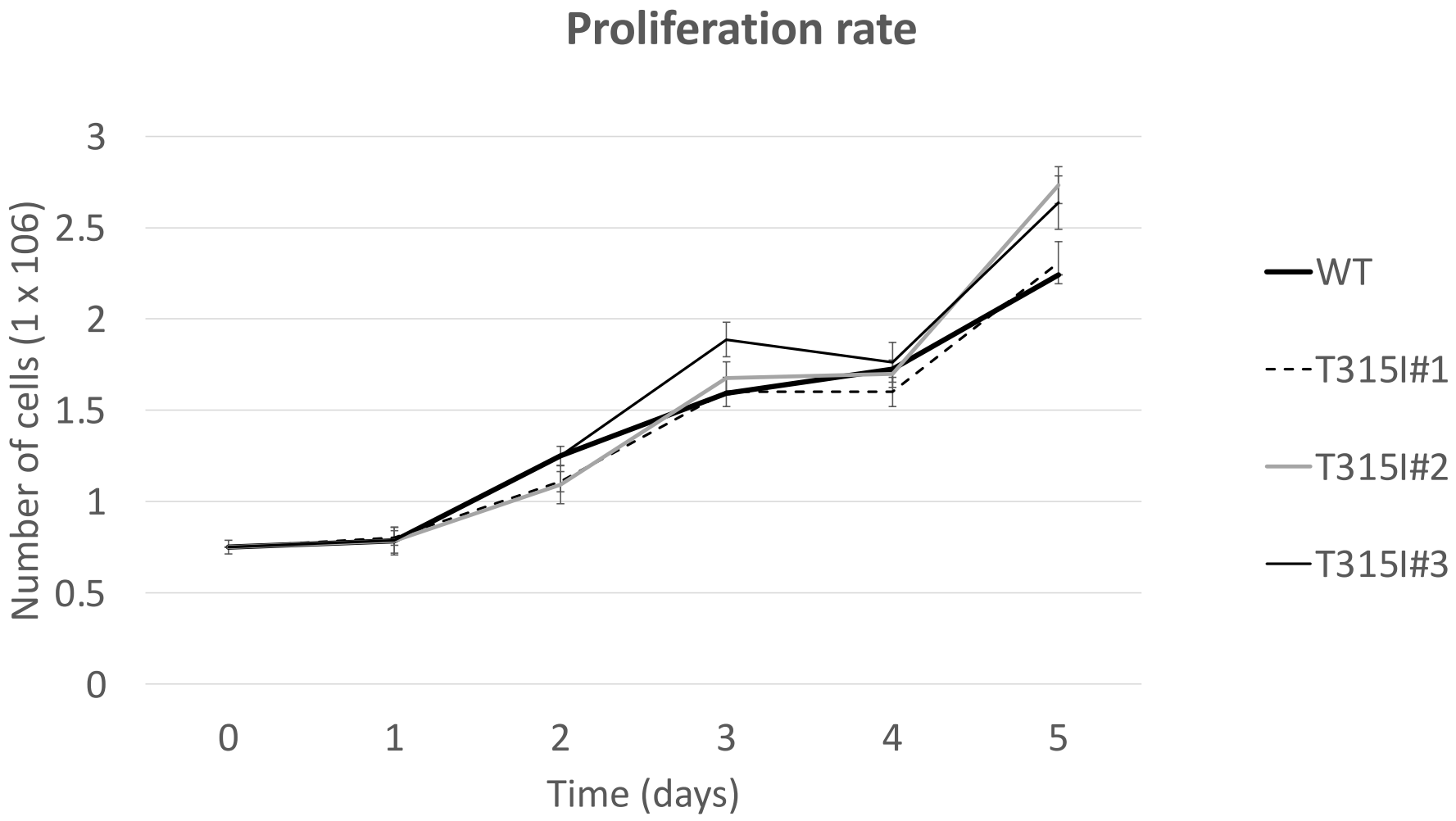
1d


Day 2
96-well plate
24-well plate
Day 0
Transfection
Day 1 - 13
Culture without dasatinib
Day 14
Start dasatinib selection
Day 43 - 50
Harvest dasatinib resistant clones

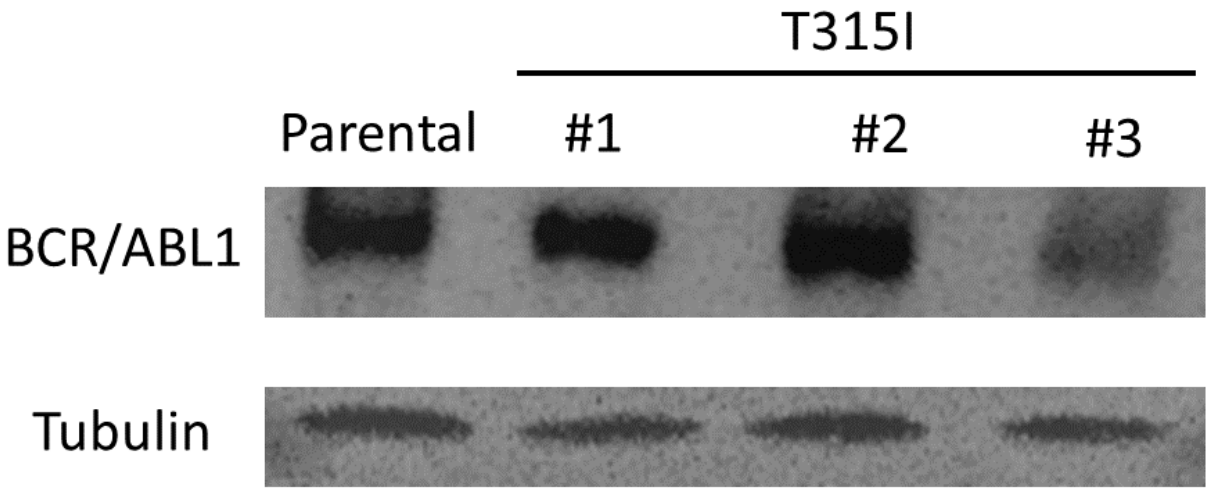
2a


TTCTATATCATCACTGAGTTCATGACC
T TC A
Parental
T315I #1
T315I #2
T315I #3

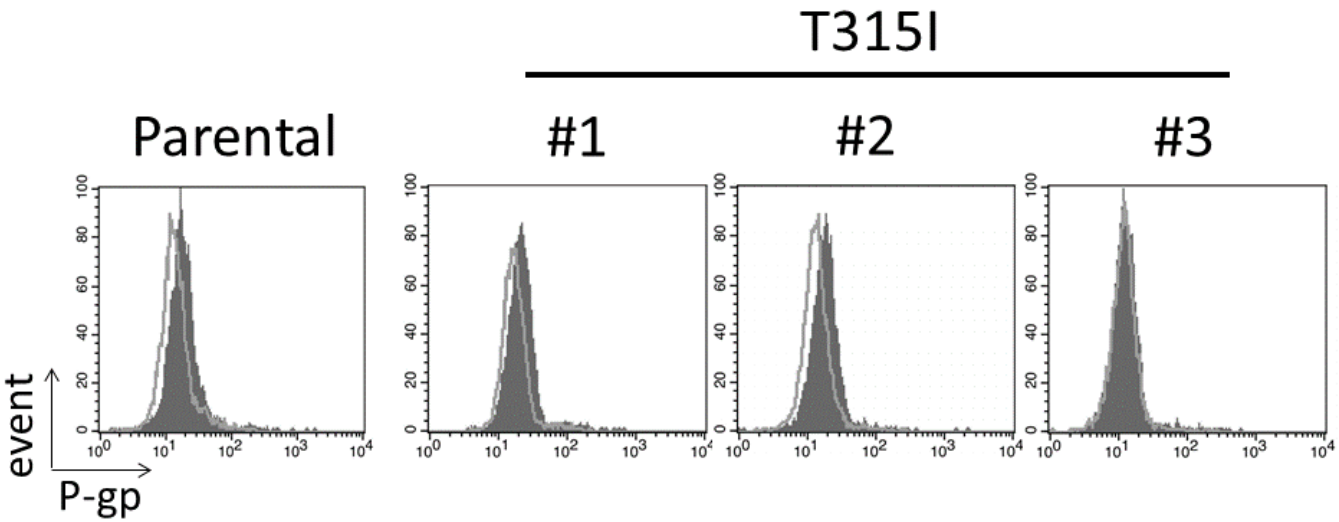
2b


Proliferation rate
Number of cells (1 x 106)
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
Time (days)
WT
T315I#1
T315I#2
T315I#3

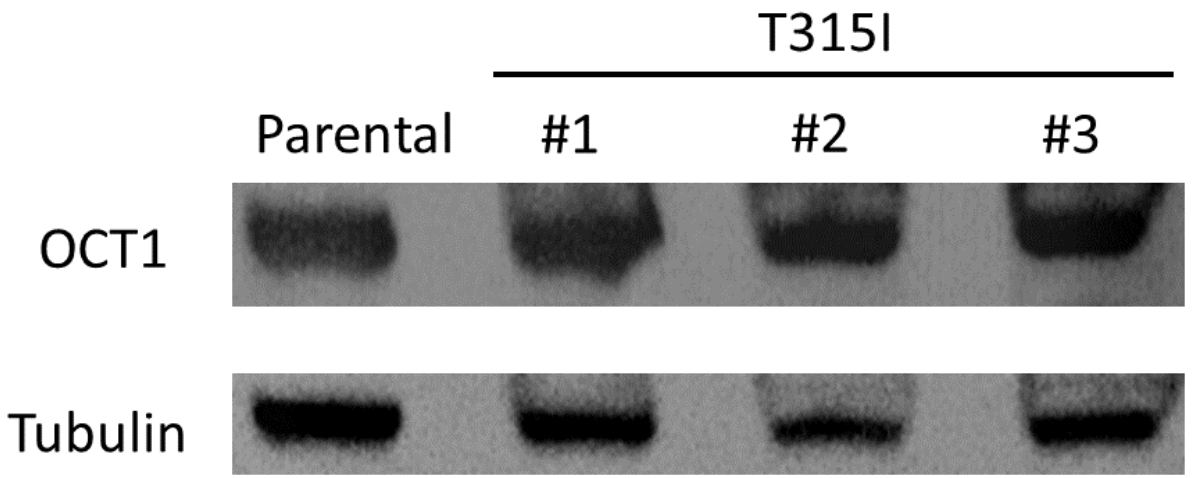
2c



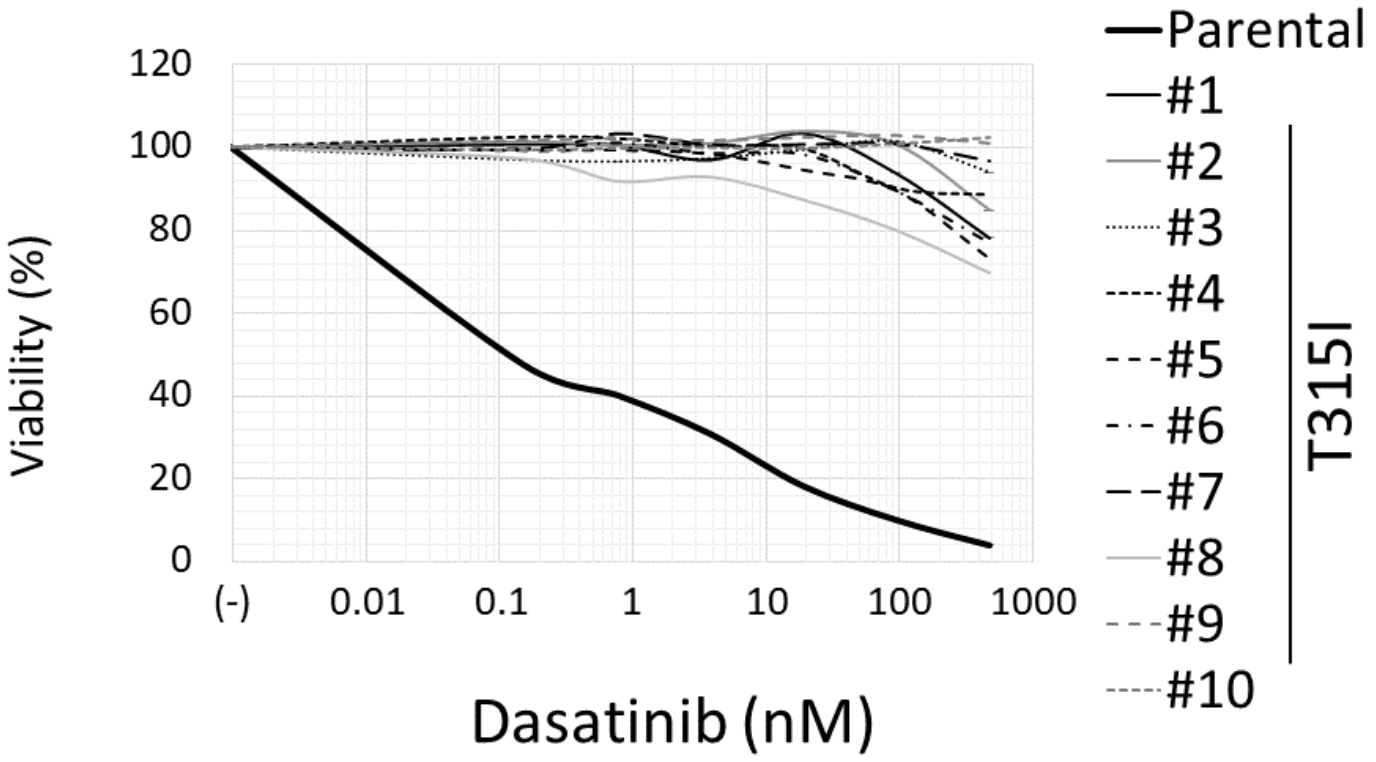
2d



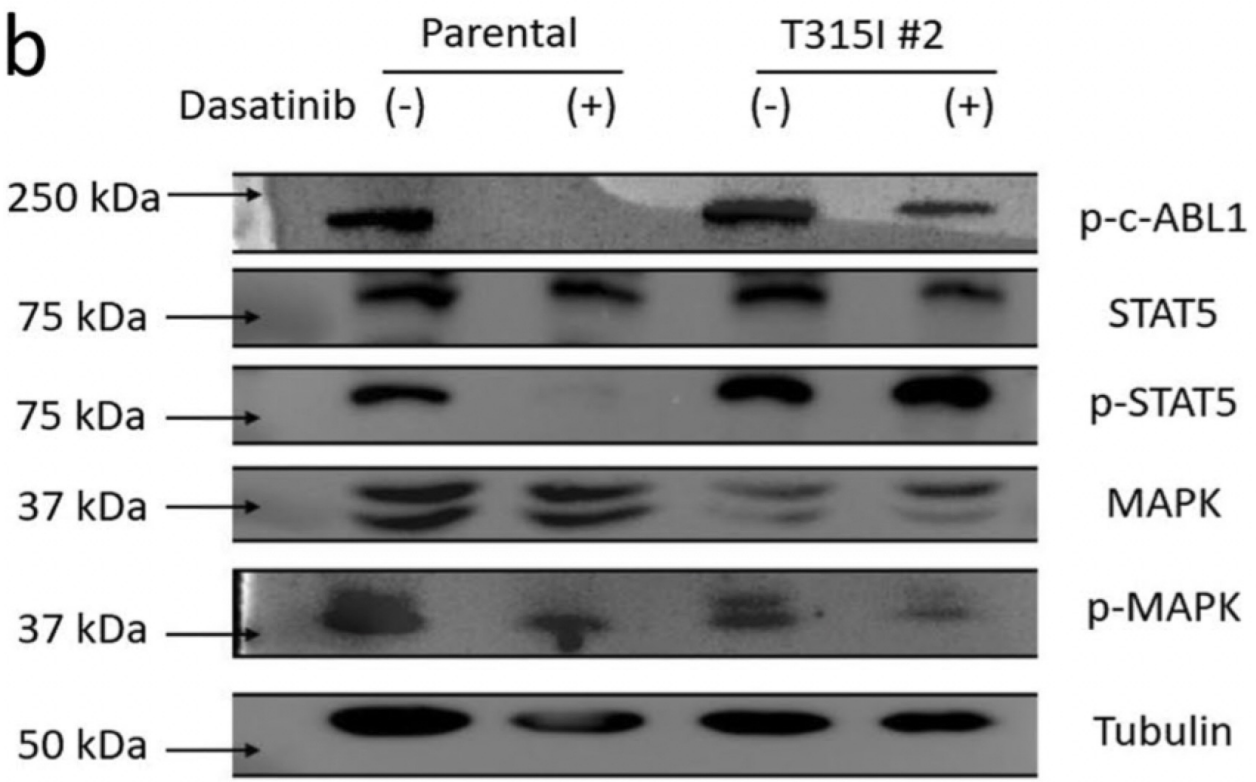
2e



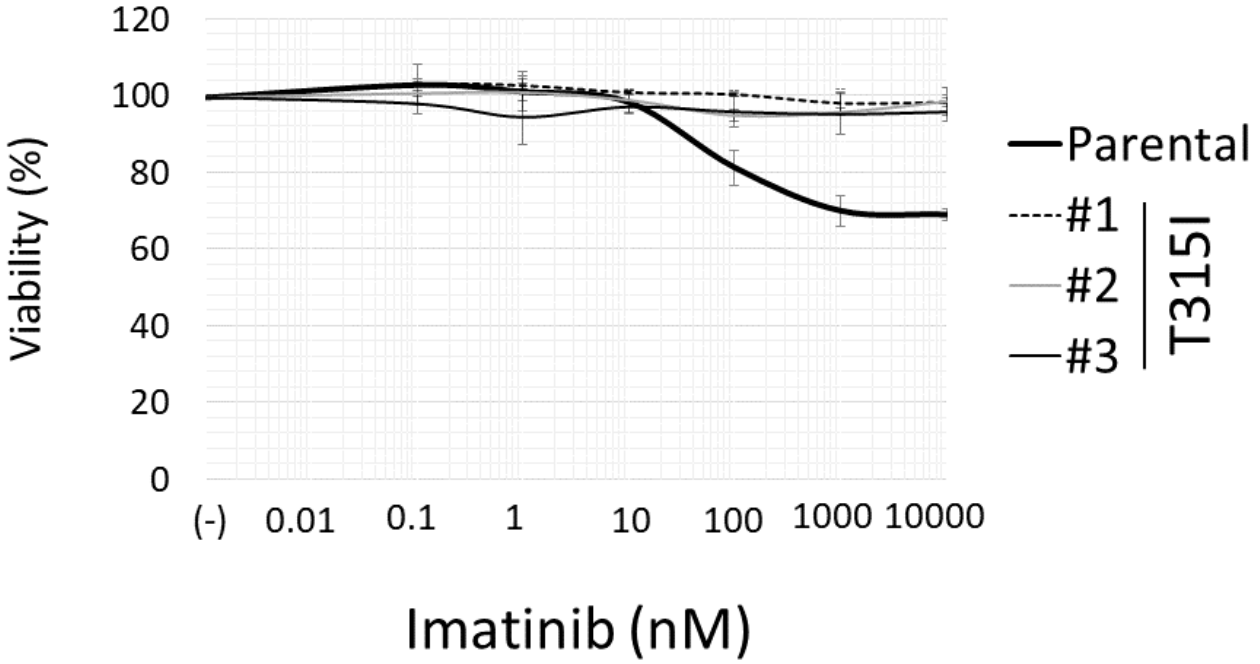
3a


120
100
80
60
40
20
0
Viability (%)
(-)
0.01
0.1
1
10
100
1000
Dasatinib (nM)
Parental
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
T315I

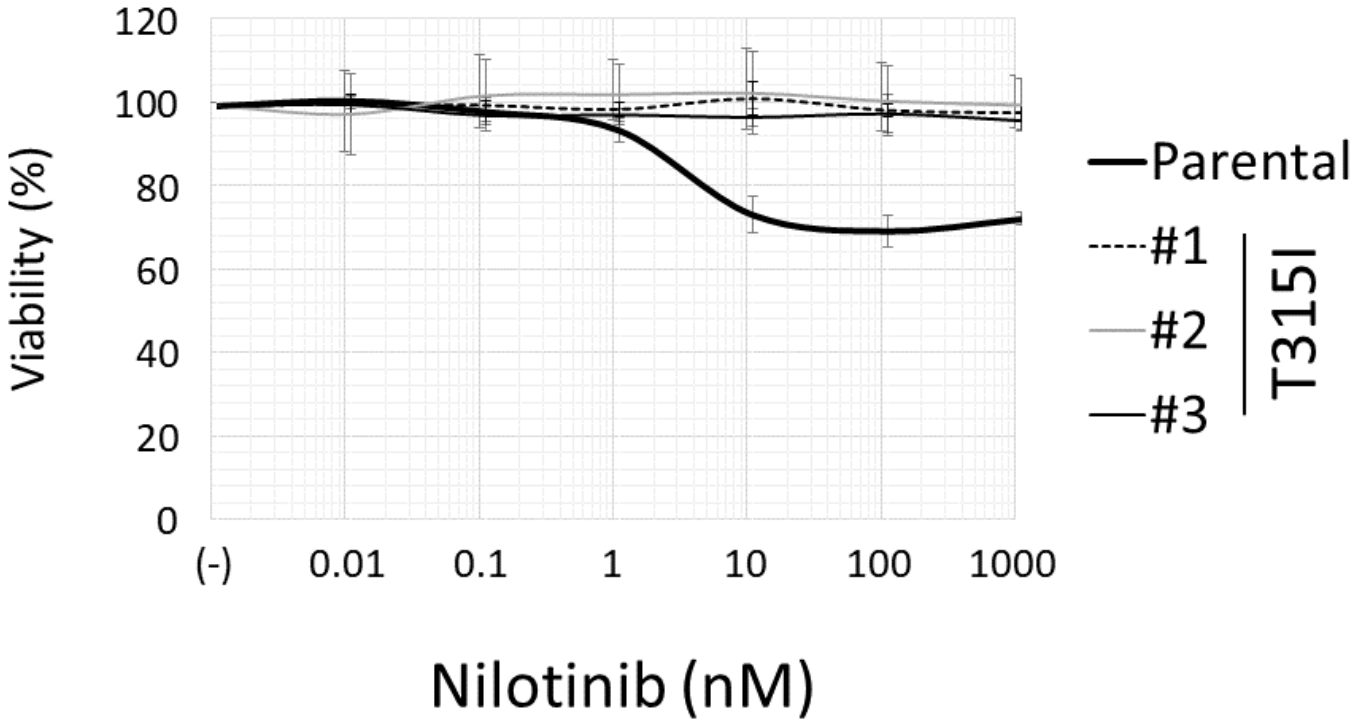
3b


Parental
T315I #2
Dasatinib (-) (+) (-) (+)
250 kDa
75 kDa
75 kDa
37 kDa
37 kDa
50 kDa
p-c-ABL1
STAT5
p-STAT5
MAPK
p-MAPK
Tubulin

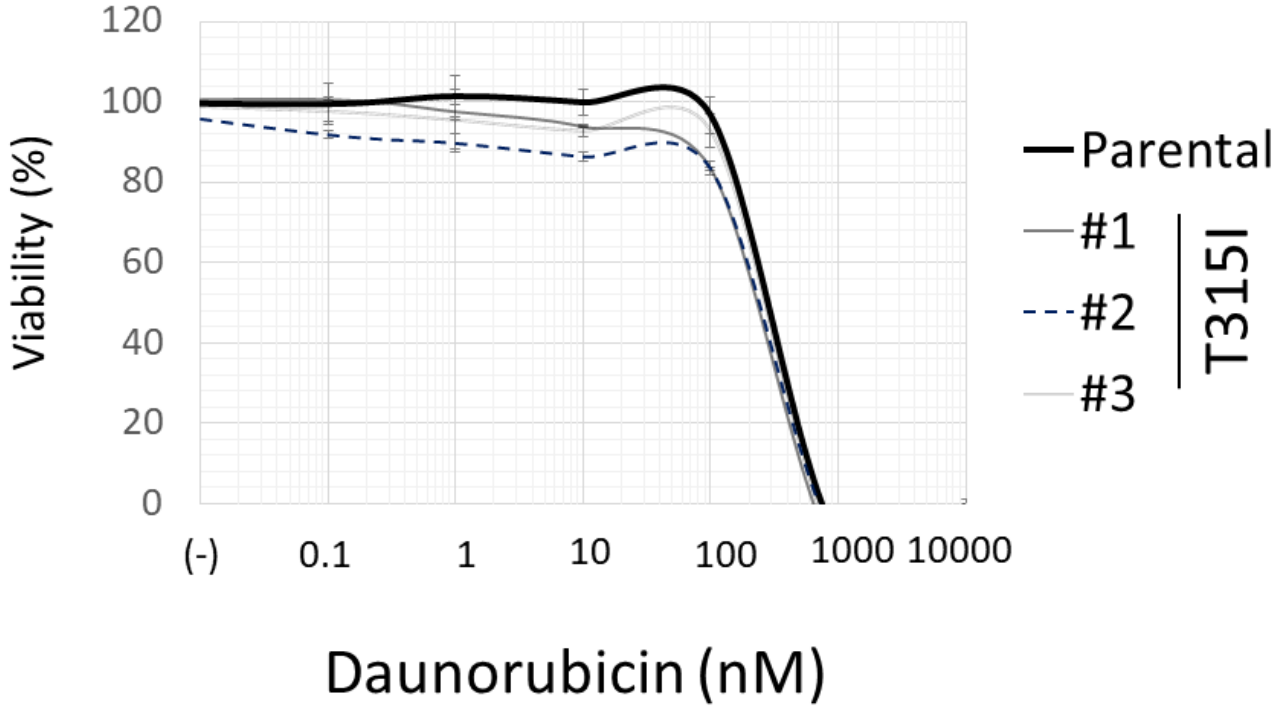
3c


120
100
80
60
40
20
0
Viability (%)
(-)
0.01
0.1
1
10
100
1000
10000
Imatinib (nM)
Parental
#1
#2
#3
T315I

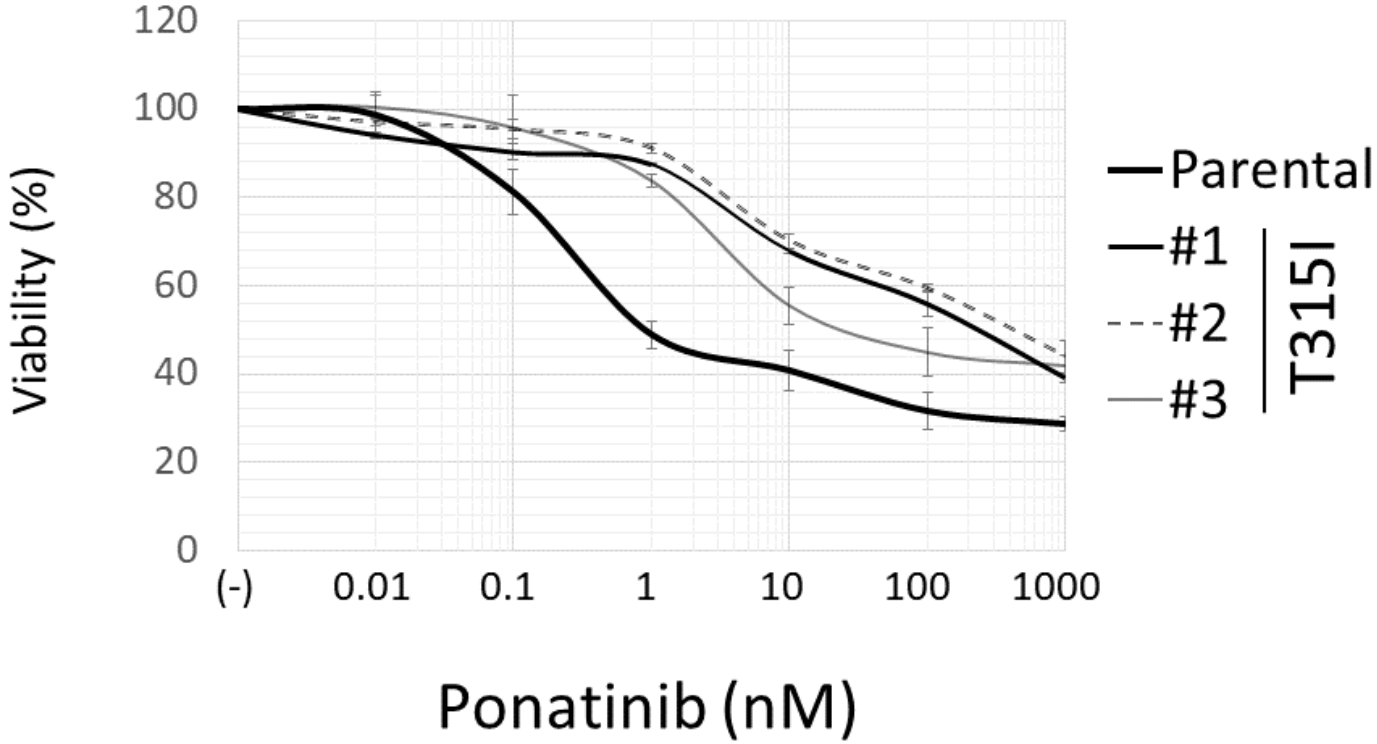
3d


120
100
80
60
40
20
0
Viability (%)
(-)
0.01
0.1
1
10
100
1000
Nilotinib (nM)
Parental
#1
#2
#3
T315I

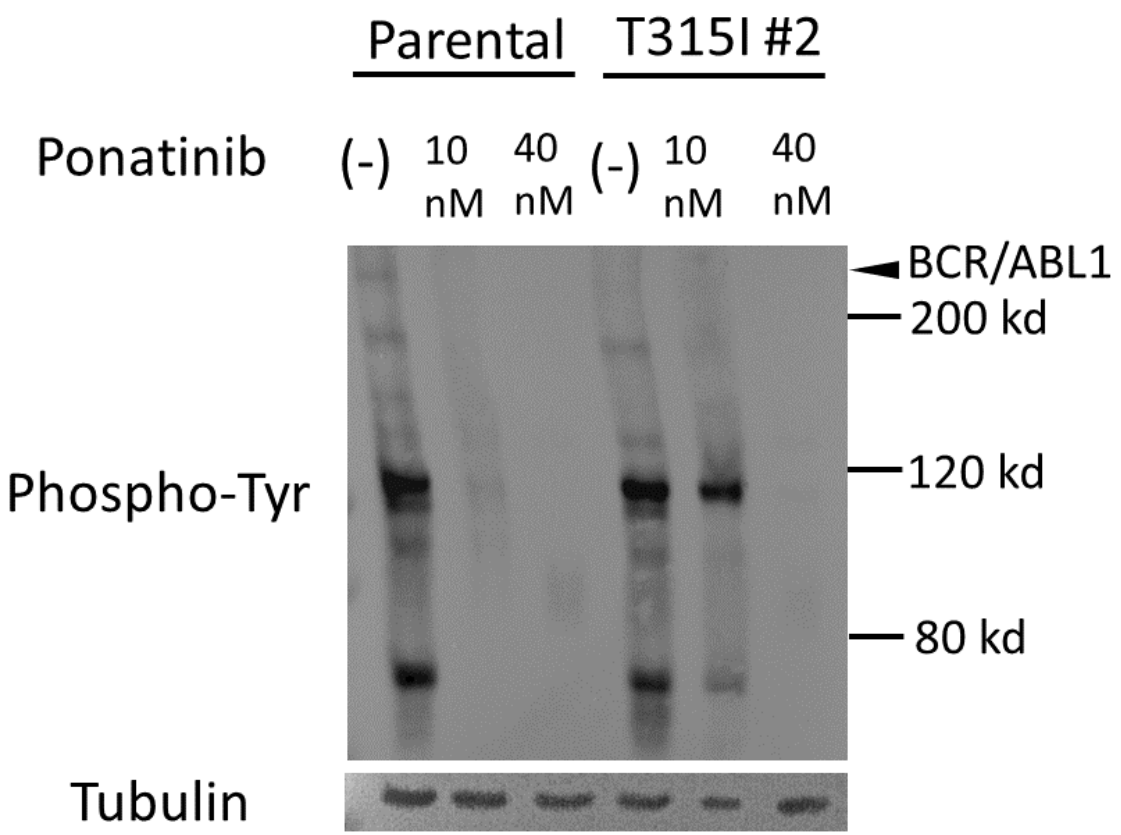
3e


120
100
80
60
40
20
0
Viability (%)
(-)
0.1
1
10
100
1000
10000
Daunorubicin (nM)
Parental
#1
#2
#3
T315I

4a


120
100
80
60
40
20
0
Viability (%)
(-)
0.01
0.1
1
10
100
1000
Ponatinib (nM)
Parental
#1
#2
#3
T315I

4b


Parental
T315I #2
Ponatinib
(-)
10 nM
40 nM
(-)
10 nM
40 nM
BCR/ABL1
200 kd
120 kd
80 kd
Phospho-Tyr
Tubulin