TOPK is regulated by PP2A and BCR/ABL in leukemia and enhances cell proliferation

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Abstract. Although treatment of chronic myeloid leukemia (CML) has improved with the development of tyrosine kinase inhibitors (TKIs), patients develop fatal blast crisis (BC) whilst receiving TKI treatment. Alternative treatments for cases resistant to TKIs are required. A serine/threonine protein kinase, T-lymphokine-activated killer cell-originated protein kinase (TOPK), is highly expressed in various malignant tumors. Binding of peptides to human leukocyte antigen was assessed via mass spectrometry in K562 CML cells. TOPK expression was assessed in various CML cell lines and in clinical samples obtained from patients with CML using reverse transcription-quantitative polymerase chain reaction and western blot assays. It was observed that TOPK was expressed abundantly in BCR/ABL-positive cell lines and at significantly higher levels in CML clinical samples compared with healthy donor samples. Overexpression of BCR/ABL or the presence of its inhibitor imatinib upregulated and downregulated TOPK expression, respectively, indicating that TOPK may be a target of BCR/ABL. TOPK inhibitor OTS514 suppressed proliferation of BCR/ABL-positive cell lines and colony formation of CD34-positive cells from patients with CML compared with lymphoma patients without bone marrow involvement. Furthermore, phosphorylation of TOPK was increased by protein phosphatase 2A (PP2A) inhibitor okadaic acid and was decreased in the presence of PP2A activator FTY720 compared with untreated samples. As constitutive BCR/ABL activity and inhibition of PP2A are key mechanisms of CML development, TOPK may be a crucial signaling molecule for this disease. Inhibition of TOPK may control disease status of CML, even in cases resistant to TKIs.

Introduction

Chronic myelogenous leukemia (CML) is caused by the constitutively active BCR/ABL kinase, which derives from chromosome translocation t(9;22) (q34;q11.2) in hematopoietic stem cells (1). The majority of patients with CML in the chronic phase (CP) have no symptoms and without treatment the disease develops into fatal blast crisis (BC) (2). Starting with imatinib, the development of tyrosine kinase inhibitors (TKIs) has markedly improved treatment of patients with CML; however, some patients progress to BC even with TKIs treatment (2). Survival following transition to BC is ~0.5-1 year (2) and novel treatments for these patients are urgently required.

In general, in the transformation to malignant cells, the activation of kinase signaling and inactivation of phosphatases is required (3). Inhibition of protein phosphatase 2A (PP2A) has been reported essential in CML progression (4). The PP2A complex consists of subunits A (structural subunit), B (regulatory subunit), and C (catalytic subunit) (5). PP2A suppresses cell survival and proliferation by dephosphorylating regulatory molecules, such as protein kinase B (Akt) and extracellular signal-regulated kinase (4,5). In CML, BCR/ABL presence increases the expression of SET nuclear proto-oncoprotein (SET), an inhibitor of PP2A, causing PP2A suppression and promoting cell proliferation (4). Activation of PP2A causes dephosphorylation of BCR/ABL by binding and activating tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1 and suppressing leukemogenesis (4).

Cytotoxic T cells recognize peptides bound to human leukocyte antigen (HLA) class I molecules on the malignant cells (6). These peptides are generally derived from intrinsic proteins, such as tyrosinase and melanoma-associated antigen recognized by T cells, through degradation in proteasomes (6). In the current study, the *HLA-A*24:02* gene was introduced into K562, a cell line established from CML-BC lacking HLA expression. It was determined by mass spectrometry that peptides from T-lymphokine-activated killer cell-originated protein

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kinase/PDZ-binding-kinase (TOPK/PBK) were interacting with the introduced HLA. TOPK is a serine/threonine protein kinase belonging to the mitogen-activated protein kinase kinase family (7). It consists of 322 amino acids and is known as cancer/testis antigen (8). TOPK is scarcely expressed in human normal tissues except for the testis and high TOPK expression is reported in various cancers, including lung, oral, esophageal and gastric cancers, and in hematopoietic tumor cell lines, particularly with high-grade malignancies (9-13). TOPK is phosphorylated by cyclin-dependent kinase 1 (CDK1)/cyclin B1 during the mitotic phase of the cell cycle (7). Phosphorylated TOPK activates certain proteins associated with cytokinesis of tumor cells, including histone H3, phosphatase and tensin homolog and p97 (14-16). The current study focused on the association between CML oncogenesis and TOPK. Findings of the current study suggest that TOPK was induced by BCR/ABL and regulated by PP2A. TOPK may be a therapeutic target for patients with BCR/ABL leukemia.

Materials and methods

Plasmids. *HLA*A24* in pcDNA3.1 was obtained from the RIKEN BioResource Research Center (Ibaraki, Japan). The coding region for *HLA*A24* was inserted into the *Bam*HI and *Eco*RI sites of retroviral vector pQCXIN (Clontech Laboratories, Inc., Mountainview, CA, USA) yielding HLA-A*24-pQCXIN. The HLA-A*24-FLAG sequence was generated from HLA-A*24-pQCXIN using the pQC (forward, 5'-ACGCCA TCCACGCTGTTTTGACCT-3') and FLAG (reverse, 5'-AAG AATTCTACTTATCGTCGTCATCCTTGTAATCCACTTTA CAAGCTGT-3') primers. All polymerase chain reaction (PCR) procedures were performed using LA TaqDNA polymerase (Takara Biotechnology Co., Ltd., Dalian, China) at 94°C for 5 min, followed by 30 cycles with 94°C for 30 sec, 30 sec at 55°C and 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were cloned into pGEM-T easy vector (Promega Corporation, Madison, WI, USA) by insertion into the *Bam*HI and *Eco*RI cloning sites of pQCXIN (HLA-A*24-FLAG-pQCXIN).

Total RNA was isolated from cultured K562 using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific, Inc.) at 42°C for 50 min. The TOPK sequence from K562 cDNA was amplified using TOPK v2 F (forward, 5'-GGACTGAGAGTGGCTTTCAC-3') and TOPK v2 R (reverse, 5'-CAAGCCACACTTCAGCTGAG-3') primers as described above. PCR products were subcloned into pGEM-T easy and the fragment was subsequently inserted into the *Eco*RI site of pcDNA3 (Thermo Fisher Scientific, Inc.) yielding TOPK-pcDNA3. Generation of the TOPK-FLAG sequence was achieved using TOPK-pcDNA3 with TOPK v2 F and FLAG primers. PCR products were subcloned into the *Eco*RI site of pcDNA3 or pQCXIN generating TOPK-FLAG pcDNA3 and TOPK-FLAG pQCXIN, respectively.

V245 pCEP4-HA B56α (#14532), V246 pCEP4-HA B56β (#14533), V247 pCEP4-HA B56γ1 (#14534), V248 pCEP4-HA B56γ3 (#14535), V249 pCEP4-HA B56δ (#14536) and V250 pCEP4-HA B56ε (#14536; Addgene, Inc., Cambridge, MA, USA), plasmids containing various PP2A subunits were gifts from Dr David M. Virshup (Duke-NUS Medical School, Singapore, Republic of Singapore) (17).

Patients with CML. A total of 16 patients diagnosed with CML according to clinical criteria and laboratory features were recruited between November 2002 and December 2015 at the Tokyo Medical and Dental University Hospital (Tokyo, Japan) (18). Patients were >20 years, with a median age of 63 years (range, 22-89 years) and comprised 11 males and 5 females. Only CML patients with *BCR/ABL* were included. Peripheral blood mononuclear cells (PBMCs) or bone marrow mononuclear cells (BMMCs) of patients were obtained at the time of diagnosis of CML as CP (2 PBMCs and 9 BMMCs) or BC (5 PBMCs and 3 BMMCs) according to European LeukemiaNet criteria (18). Three additional samples were obtained during TKIs therapy, two for patients with CML-CP treated with imatinib and dasatinib and one for a patient with CML-BC treated with nilotinib. These patients were resistant to TKI treatment and samples were obtained prior to switching TKIs. The clinical course of the patient with CML-BC treated with nilotinib is further described. All other samples were obtained from patients not receiving TKI treatment at time of collection. PBMCs form 4 healthy volunteers (2 males and 2 females; age, 21-51 years) and BMMCs from 3 patients with stage I-IIA malignant lymphoma without BM involvement (2 males and 1 female; age, 42-81 years) were used as control. PBMCs and BMMCs were stored as detailed below.

Cells and reagents. PBMCs and BMMCs of patients or healthy donors were isolated from fresh samples (peripheral blood, 10-20 ml or bone marrow fluid, 2-5 ml) by density gradient centrifugation (760 x g; 15 min; 20°C) using Separate-L (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). Mononuclear cells were preserved using the Cellbanker-1 (Nippon Zenyaku Kogyo Co., Tokyo, Japan) at -80°C.

MD901 lymphoma cells (19), and TMD2 (20) and TMD5 (21) leukemic cells were established and provided by Tokyo Medical and Dental University (Tokyo, Japan). TL-Oml (22) and ILT-M1 (23) T cell leukemic cells were established and provided by Dr Mari Kannagi (Tokyo Medical and Dental University). TonB210, a clone of murine interleukin (mIL) 3-dependent BaF3 pro-B cells transfected with a BCR/ABL cDNA under the control of a tetracycline-inducible promoter, was provided by Dr George Q. Daley (24). 32D, 32D/p210 myeloblasts (32D transfected with *BCR/ABL*) and TonB210/T315I pro-B cells (inducible transfectant of Ba/F3 with *BCR/ABL* T315I mutant) were established in our laboratory as previously described (25,26). PLAT-A, an amphotropic virus packaging embryonic kidney cell line (27) was kindly provided by Dr Toshio Kitamura (The Institute of Medical Sciences, Tokyo University, Tokyo, Japan). K562 leukemic cells were obtained from RIKEN BioResource Center (Tsukuba, Japan). RPMI-8226 myeloma cells, EW36 and Raji B cell lymphoma cells, MOLM1 and MV4-11 leukemic cells, and 293T cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured as follows: RPMI-1640 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for K562, MD901, Raji, TMD2, RPMI-8226, EW36 and MOLM1; Iscove's modified Dulbecco's medium (IMDM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for MV4-11; α modified Eagle's minimum essential medium (α-MEM; Sigma-Aldrich; Merck KGaA) for TMD5; RPMI-1640 with 3 U/ml

recombinant mIL-3 (PeproTech, Inc., Rocky Hill, NJ, USA) for 32D, 32D/p210, TonB210 and TonB210/T315I; Dulbecco's modified Eagle's medium (DMEM) for 293T and PLAT-A; and RPMI-1640 with 30 U/ml of IL-2 (PeproTech, Inc.) for TL-Oml and ILT-M1. Each medium contained 10% fetal calf serum (FCS; Nichirei Biosciences Inc., Tokyo, Japan). All cells were grown and treated in a humidified atmosphere at 37˚C with 5% CO_2.

TOPK inhibitor OTS514 (purity, 99.97%) was provided by OncoTherapy Science, Inc. (Kanagawa, Japan). Okadaic acid was purchased from Merck KGaA (#459620). FTY720 was purchased from Cayman Chemical Co., Ltd. (#10006292; Ann Arbor, MI, USA). Doxycycline (DOX) was purchased from Sigma-Aldrich (#D9891; Merck KGaA). Nocodazole was purchased from Merck KGaA (#487928). Imatinib was provided by Novartis Pharma K.K. (Tokyo, Japan).

K562 cells were treated with 300 ng/ml nocodazole for 0, 6, 12, 21 and 24 h to check the time-dependent effects of nocodazole. K562 cells were further treated with nocodazole for 21 h followed by treatment with 20 nM okadaic acid or 0, 5, 10 and 15 μM FTY720 for 4 h at 37˚C prior to harvesting. K562 cells expressing FLAG-tagged TOPK (K562 TOPK-F pQCXIN) were treated with or without 300 ng/ml nocodazole for 21 h to check the interaction between TOPK and PP2A. Stock solutions were prepared using dimethyl sulfoxide and solutions were diluted ≥1/1000 for treatment.

TonB210 cells were treated with 1 μg/ml of DOX for 5 h at 37˚C to induce BCR/ABL. Cells were then incubated with imatinib (10 μM) for 24 h at 37˚C. To check effects of OTS514 on survival, TonB210 and TonB210/T315I were cultured in the presence of 1 μg/ml of DOX in the absence of IL-3 for 48 h prior to treatment with 20 nM OTS514 for 48 h at 37˚C.

RT-quantitative (q) PCR. PBMCs or BMMCs from patients with CML and PBMCs from healthy donors were used in these experiments. Total RNA was extracted using from these samples using TRIzol reagent (Gibco; Thermo Fisher Scientific, Inc.).

RT was performed using oligo(dT) primers and SuperScript™ II reverse transcriptase (Thermo Fisher Scientific, Inc.) to synthesize cDNA at 42˚C for 50 min. cDNA amplification was performed using the following primers (Sigma-Aldrich; Merck KGaA): *PBK/TOPK* v2, forward, 5'-GGACTGAGA GTGGCTTTCAC-3' and reverse, 5'-CAAGCCACACTTCA GCTGAG-3'; and *GAPDH*, forward, 5'-CTGACTTCAACAGC GACACC-3' and reverse, 5'-TCCTCTTGTGCTCTTGCTGG-3'. qPCR was performed using LightCycler 480 Probes Master kit and LightCycler 480 system software version 1.5.1 (Roche Diagnostics, Indianapolis, IN, USA) using TaqMan Gene Expression assays (#4331182; PBK/TOPK, Hs00902990_m1 and GAPDH, Hs0286624_g1; Thermo Fisher Scientific, Inc.). Relative fold changes of gene expression were assessed using the $2^{-\Delta\Delta Cq}$ method (28).

Transfection and infection. For transient expression, 293T cells (3×10^5) were transfected with indicated plasmids using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A total of 2 μg DNA was used for the transfections, comprising 1 μg V245 pCEP4-HA B56α, V246 pCEP4-HA B56β, V247 pCEP4-HA

B56γ1, V248 pCEP4-HA B56γ3, V249 pCEP4-HA B56δ or V250 pCEP4-HA B56ε and 1 μg TOPK-FLAG pcDNA3. Additionally, a TOPK-FLAG (1 μg) plus pcDNA3 (1 μg) sample was established and a control using 2 μg pcDNA3. Transfection efficiency was determined by western blot analysis. As endogenous TOPK expression in 293T was very low (16), no exogenous TOPK expression was observed for the control sample at the applied exposure time.

To obtain K562 cells stably expressing HLA-A*24, PLAT-A cells (1×10^6) were transfected with 2 μg of HLA-A*24-FLAG-pQCXIN using Lipofectamine PLUS reagent (Thermo Fisher Scientific, Inc.). At 72 h of transfection, 2 ml of the culture supernatant containing recombinant retrovirus was harvested. K562 cells (1.2×10^6) were infected with the obtained recombinant retrovirus Following 48 h of infection, K562 cells were cultured in the presence of G418 (1 mg/ml; Wako Pure Chemical Industries, Ltd.) for one week and high expressing cells were selected by fluorescence-activated cell sorting (FACS) using flow cytometer (FACSAria II; Becton-Dickinson and Co., Franklin Lakes, NJ, USA). Cells (3×10^5) were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-HLA-ABC antibody (W6/32; 100 ng; #11-9983-41; Thermo Fisher Scientific, Inc.) at 4˚C for 30 min. Data obtained from flow cytometry were analyzed using FlowJo 6.3.3 (FlowJo LLC, Ashland, OR, USA). Two independent K562 clones stably expressing HLA-A*24 were selected for the immunoprecipitation experiment to identify the peptides binding to HLA.

K562 cells expressing FLAG-tagged TOPK (K562 TOPK-F pQCXIN) were obtained by the same procedure. Following one week of selection with G418, cells of interest were isolated by limiting dilution cloning. The clone that exhibited the highest exogenous expression level by western blot analysis was selected for the further analysis.

Clonogenic assay. CD34-positive cells were isolated from BMMCs obtained from 3 patients with CML and 3 patients with stage I-IIA malignant lymphoma without BM involvement as control using a Dynabeads CD34 Positive Isolation kit (Thermo Fisher Scientific, Inc.). BMMCs (4×10^7) were incubated with 100 μl of washed Dynabeads CD34 at 4˚C for 30 min with gentle rotation. Following isolation with a magnet, CD34-positive cells were released using 100 μl DETACHaBEAD solution at room temperature for 45 min. CD34-positive cells (1×10^3) were cultured in MethoCult medium (Stemcell Technologies, Inc., Vancouver, BC, Canada) for 14 days at 37˚C in the presence or absence of OTS514 (20 nM). Each treatment was assessed in triplicate and colony numbers were counted by inverted microscopy (magnification, x100). The number of visible colonies was determined as the ratio of OTS514-treated to untreated samples.

Western blot analysis. Cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 50 mM NaF, 5 mM ethylenediaminetetraacetate, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin and 10 mg/ml leupeptin (Sigma-Aldrich; Merck KGaA) and incubated on ice for 30 min. Lysates were centrifuged (17,400 x g; 4˚C; 15 min),

proteins were obtained in the supernatant and quantified using the DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (20 μg) were boiled with an equal amount of 2X SDS buffer (0.1 M Tris-HCl pH 6.8, 4% SDS, 12% 2-mercaptoethanol, 20% glycerol, 1% bromophenol blue) at 100°C for 5 min, separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with blocking buffer (5% non-fat milk or 5% BSA in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) and probed with primary antibodies overnight at 4°C followed by secondary antibodies at room temperature for 1 h. The following antibodies were used: Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (1:5,000; cat. no. NA9310), HRP-conjugated donkey anti-rabbit IgG (1:5,000; cat. no. NA934; GE Healthcare, Chicago, IL, USA), HRP-conjugated mouse anti-goat IgG (1:5,000; cat. no. sc-2354; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-PBK/TOPK (1:5,000; cat. no. #4942), rabbit anti-phosphorylated (p)-PBK/TOPK (Thr9; 1:5,000; cat. no. #4941), rabbit anti-HA-tag (C29F4; 1:5,000; cat. no. #3724), rabbit anti-p-c-ABL (Tyr245; 1:5,000; cat. no. #2861) (all from Cell Signaling Technology, Inc., Danvers, MA, USA), mouse anti-PP2A-Aα (C-20; 1:1,000; cat. no. sc-6112), rabbit anti-c-ABL (K-12; 1:1,000; cat. no. sc-131) (both from Santa Cruz Biotechnology, Inc.) and mouse anti-β-actin (1:10,000; cat. no. A1978; Sigma-Aldrich; Merck KGaA). Blots were visualized using Pierce electrochemiluminescence western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.). Densitometric analyses were performed using ImageJ 1.48 (National Institutes of Health, Bethesda, MD, USA).

Immunoprecipitation. K562 cells expressing FLAG-tagged TOPK (K562 TOPK-F pQCXIN) or 293T cells transiently transfected with TOPK-FLAG and PP2A subunits-HA were lysed in lysis buffer as described above and obtained supernatants were cleared with Protein A/G PLUS-Agarose Immunoprecipitation reagent (Santa Cruz Biotechnology, Inc.) at 4°C for 1 h. The obtained supernatant was treated with 30 μl anti-FLAG M2 Affinity gel (#A2220; Sigma-Aldrich; Merck KGaA) at 4°C overnight. An equal amount of supernatant was incubated with 10 μg mouse IgG (#0107-01; SouthernBiotech, Birmingham, AL, USA) on ice for 1 h and subsequently treated with 30 μl Protein A/G PLUS-Agarose Immunoprecipitation reagent at 4°C overnight as negative controls. Proteins were then separated by SDS-PAGE as described and subjected to western bot analysis. Total cell lysates (TCL) were analyzed prior to immunoprecipitation.

Cell-surface FLAG-tagged HLA molecules from K562 cells stably expressing HLA-A*24 (1x10^8 cells; two independent clones) were solubilized in buffered detergent solution (150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1% CHAPS) at 4°C for 1 h (29,30), reacted with anti-FLAG M2 antibody (2 μl/ml; #F3165; Sigma-Aldrich, Merck KGaA) at 4°C for 1 h and then incubated with Protein A/G PLUS-Agarose Immunoprecipitation reagent according to the manufacturer's protocols. Bound HLA molecules were dissociated from the beads through the addition of FLAG peptides (150 μg; DYKDDDDK peptide; #044-30953; Wako Pure Chemical Industries, Ltd.). Samples were further immunoprecipitated using anti-pan HLA-class I antibody (2 μl; #14-9983-80;

Thermo Fisher Scientific, Inc.) at 4°C for 1 h and treated with Protein A/G PLUS-Agarose Immunoprecipitation reagent as described above.

Measurement of living cells. Cell viability was determined using flow cytometer. Following treatment with or without 20 nM OTS514 for 48 h in K562, MOLM1, TMD5, MV4-11, TonB210, TonB210/T315I and K562 cells treated with or without 10 nM OTS514 and/or 0.5 μM imatinib for 48 h were stained with 3,3'-dehexyloxacarbocyamine iodine (40 nM; DiOC6), which is transported into the mitochondria of living cells, and propidium iodide (10 μg/ml; PI; both Molecular Probes; Thermo Fisher Scientific, Inc.), which permeates into dead cells through damaged cell membranes at 37°C for 15 min. In living cells, DiOC6 is positive and PI is negative. The total number of cells was counted using a flow cytometer. Data were analyzed using FlowJo 6.3.3.

Mass spectrometry. Peptides bound to HLA molecules were eluted in 0.2 M acetic acid solution for 10 min at 4°C (30-32) and dried in a vacuum evaporator (Centrifugal Concentration CC-101; Tomy Seiko Co., Ltd., Tokyo, Japan) for 3 h at room temperature. Subsequently, peptide samples were dissolved in 0.5% trifluoroacetic acid (TFA) in 5% aqueous acetonitrile (ACN), purified and concentrated using Pierce C18 Spin Columns (#89870; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The final peptide elution was performed using 50% ACN.

Aqueous peptide samples (2 μl) were separated by nano-flow liquid chromatography (nLC) using the Easy-nLC II (Thermo Fisher Scientific, Inc.) at room temperature. Separation was performed using a NTCC-360 column (75 μm x 150 mm; 3 μm; Nikkyo Technology Co., Ltd., Hong Kong, China) with 0.1% TFA in water as solvent A and 0.1% TFA in 70% ACN as solvent B at a flow rate of 300 nl/min. Peptide samples that eluted from the column were fragmented for amino acid sequencing using collision-induced dissociation (CID) in tandem mass spectrometer (LTQ Orbitrap velos; Thermo Fisher Scientific, Inc.) equipped with a nano electrospray ion source (positive ion mode). Acquired CID spectra were analyzed against a protein database (Swiss-Prot; https://www.uniprot.org/downloads) using proteomics software PEAKS Studio 6.0 (Bioinformatics Solutions, Inc., Waterloo, ON, Canada). The PEAKS peptide score (-10lgP) was calculated for every peptide-spectrum match. The score was derived from the P-value that indicates the statistical significance of the peptide-spectrum match (33).

Statistical analysis. For statistical analysis, Student's t-tests were performed in pairwise comparisons using EZR 3.0.2 (Saitama Medical Center, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) and Kruskal-Wallis followed by Dunn's multiple comparisons test or one-way analysis followed by Turkey's post-hoc test were performed for multiple group comparisons using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard error representative of three repeats. P<0.05 was considered to indicate a statistically significant difference.

Table I. Peptides interacting with HLA in HLA-A*24:02 transduced in K562 cells were analyzed by mass spectrometry.

| Amino acid sequence	-10lgP	Predicted protein of origin (gene symbol)
RYFDPANGKF	79.14	Elongation factor 2 (EEF2)
KFIDTTSKF	64.75	60S ribosomal protein L3 (RPL3), L3-like (RPL3L)
EYPDRIMNTF	64.03	Tubulin β-2A, 2B, 3, 4B chain (TUBB2A, 2B, 3, 4B)
SYQKVIELF	58.93	PDZ-binding kinase/lymphokine-activated killer T-cell-originated protein kinase (PBK/TOPK)
KYIHSANVL	54.66	Mitogen-activated protein kinase 1, 3, 4, 6 (MAPK1, 3, 4, 6)
NYARGHYTI	52.05	Tubulin α-1A, 1B, 1C, 3C/D, 3E, 4A chain (TUBA1A, 1B, 1C, 3C/D, 3E, 4A)
RYTDVSTRY	41.7	Thioredoxin-related transmembrane protein 2 (TMX2)
RYQKSTELL	36.58	Histone H3.3 (H3F3A)
LDKLRFGFKK	28.85	Dynein heavy chain 17, axonemal (DNAH17)
AVLGRGHF	20.34	Serine/threonine-protein kinase N3 (PKN3)

-10lgP, PEAKS peptide score.
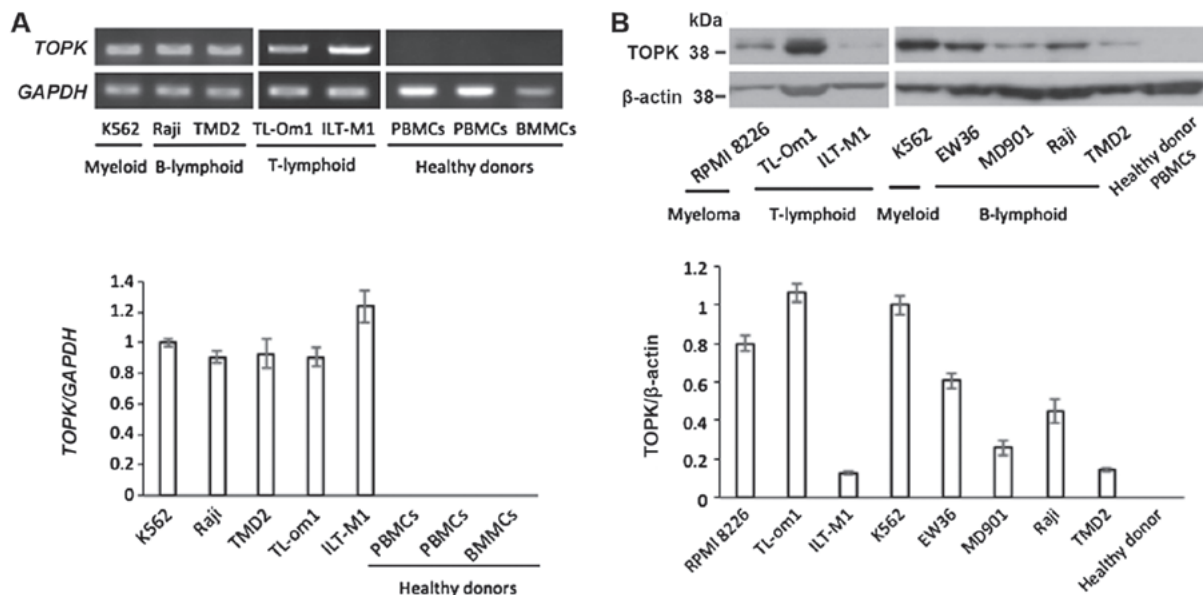
Figure 1. TOPK expression in hematopoietic tumor cell lines. TOPK (A) mRNA and (B) protein expression was assessed in human myeloid, B-cell, T-cell and myeloma cell lines, and in cells collected from healthy donors. PBMC, peripheral blood mononuclear cell; BMMC, bone marrow mononuclear cell; TOPK, T-lymphokine-activated killer cell-originated protein kinase.

Results

*Peptides derived from TOPK are binding to HLA-A*24 in HLA-A*24-overexpressing K562 cells.* To reveal novel molecular targets for hematopoietic malignancies, FLAG-tagged HLA-A*24 cDNA was introduced into HLA-deficient K562 cells. The introduced HLA molecule was isolated through two cycles of immunoprecipitation using anti-FLAG and anti-pan HLA class I antibodies. Peptides bound to HLA molecules were eluted and analyzed by mass spectrometry. A total of >200 sequences ranging from 9-11 amino acids were identified. These sequences exhibited an expected reduced amino acid complexity at anchor residue positions for HLA-A*24 (data not shown) (34).

The most promising ten peptides with the highest PEAKS peptide score (-10lgP) were selected based on assays using two independent clones (Table I). Peptides derived from TOPK were selected in further analysis, as TOPK has previously been reported as a cancer/testis antigen that serves a role in regulation of survival and proliferation of malignant cells (14).

The nature of the peptides binding to HLA dependents on active signaling pathways, as these define the sources of the metabolized proteins (29,35). It is speculated that TOPK is actively metabolized in the CML derived cell line assessed in the current study and TOPK expression in hematopoietic tumor cell lines, including K562 and clinical samples from patients with CML was further assessed.
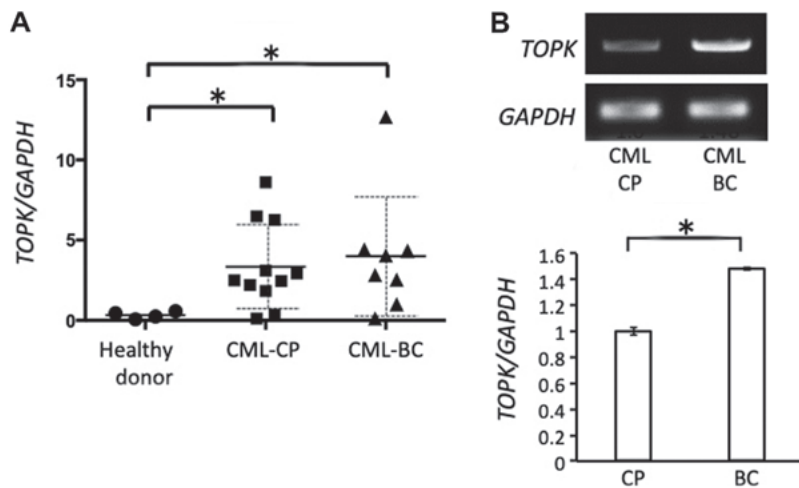
Figure 2. TOPK expression in samples from patients with CML. (A) TOPK mRNA expression determined in 20 CML clinical samples; classified into CML-CP (n=11), CML-BC (n=8) and healthy donors (PBMCs; n=4). (B) TOPK mRNA expression in PBMC samples at CML-CP and -BC from one patient. *P<0.05. CML, chronic myeloid leukemia; CP, chronic phase; BC blast crisis; PBMC, peripheral blood mononuclear cell; TOPK, T-lymphokine-activated killer cell-originated protein kinase.
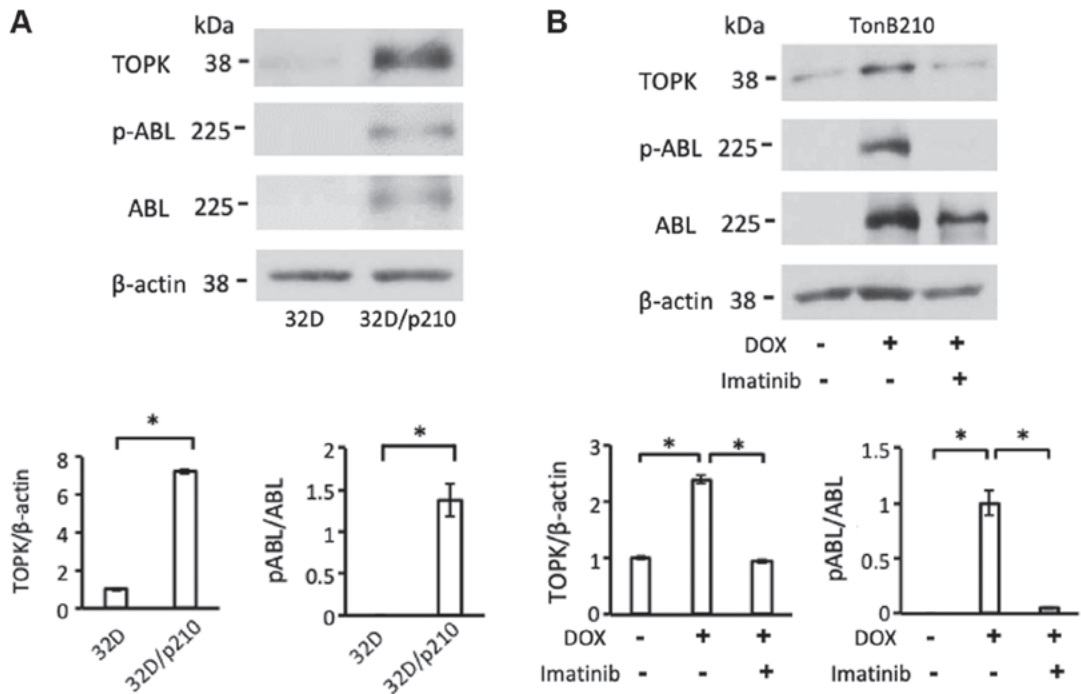

Figure 3. TOPK expression in cells expressing BCR/ABL. (A) 32D murine interleukin 3-dependent myeloblast-like cells and 32D expressing BCR/ABL (32D/p210) were examined for TOPK expression and ABL phosphorylation levels by western blot analysis. (B) DOX-induced BCR/ABL expression was monitored in TonB210 cells in the presence or absence of imatinib, and TOPK expression and ABL phosphorylation levels were determined by western blot analysis. *P<0.0001. TOPK, T-lymphokine-activated killer cell-originated protein kinase; DOX, doxycycline; p, phosphorylated.

TOPK is highly expressed in hematopoietic tumor cell lines. TOPK mRNA expression was determined by RT-qPCR in various hematopoietic tumor cell lines, in addition to PBMCs or BMMCs isolated from healthy donors. TOPK was expressed in all analyzed cell lines, including K562, Raji, TMD2, TL-om1 and ILT-M1, and expression was not detected in samples collected from healthy donors (Fig. 1A). Consistently, TOPK protein expression detected by western blotting was only observed in hematopoietic tumor cell lines, including RPMI-8226, TL-om1, ILT-M1, K562, EW36, MD901, Raji and TMD2, and not in samples from healthy donors (Fig. 1B).

TOPK expression is increased in primary CML cells. TOPK expression was analyzed in clinical samples from patients with CML-CP or CML-BC and compared with samples from healthy donors. TOPK expression was not significantly different in PBMCs and BMMCs isolated from one patient with CML-CP (data not shown). TOPK expression was significantly increased in CML-CP (2 PBMCs and 9 BMMCs) and CML-BC (5 PBMCs and 3 BMMCs) samples compared with healthy donor samples (4 PBMCs; P<0.05 and P<0.01, respectively; Fig. 2A). No significant difference in TOPK mRNA expression was observed between CML-CP and CML-BC samples
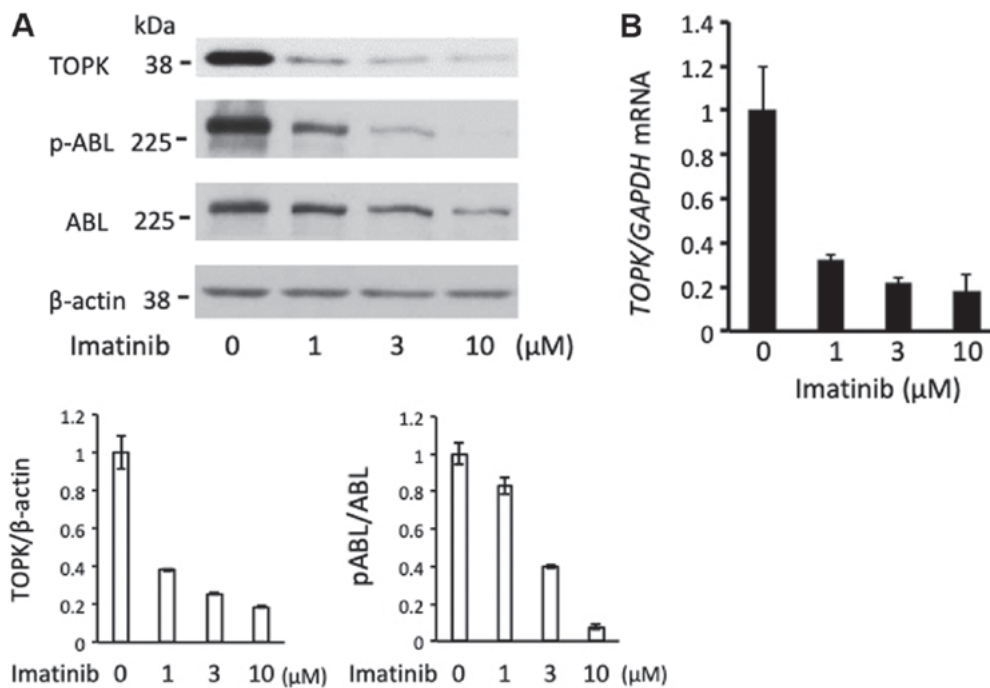
Figure 4. Imatinib suppresses TOPK expression. K562 cells were treated with imatinib (0, 1, 3 and 10 μM) overnight. (A) TOPK expression and ABL phosphorylation levels were determined by western blot analysis; results are representative of four independent experiments. (B) TOPK mRNA expression. TOPK, T-lymphokine-activated killer cell-originated protein kinase; p, phosphorylated.

(P>0.05). Expression of TOPK mRNA was further compared in CML-CP and CML-BC samples obtained from the same patients. In two out of three patients who provided clinical samples at CML-CP and CML-BC, TOPK expression was upregulated in CML-BC compared with CML-CP (Fig. 2B).

TOPK expression induces the kinase activity of BCR/ABL. 32D/p210 cells expressing BCR/ABL and the parental cell line 32D not expressing BCR/ABL (26), were compared to evaluate an association between TOPK expression and BCR/ABL. TOPK protein expression was significantly higher in 32D/p210 compared with the parental 32D (P>0.05; Fig. 3A). An association between BCR/ABL and TOPK expression was further analyzed using TonB210 cells, in which the addition of DOX induced BCR/ABL expression and BCR/ABL autophosphorylation. TOPK protein expression was significantly increased in TonB210 cells in the presence of DOX compared with the untreated cells (P<0.01; Fig. 3B). In addition, induction of TOPK in TonB210 using DOX was significantly reversed when cells were treated with imatinib (P<0.01; Fig. 3B) inhibiting BCR/ABL kinase activity and autophosphorylation of BCR/ABL. The data suggested that BCR/ABL may induce TOPK expression.

To further elaborate on this hypothesis and to clarify the association between imatinib and ABL activation and TOPK expression, imatinib was added to K652 cells at different doses. As presented in Fig. 4A, with increasing imatinib concentration, levels of TOPK and phosphorylated BCR/ABL decreased in a potential dose-dependent manner. Additionally, imatinib decreased expression levels of TOPK mRNA in a potential dose-dependent manner (Fig. 4B). Similar results regarding protein expression and phosphorylation were obtained using MOLM1 and TMD5 cells (data not shown).

The results indicate an association between TOPK expression and autophosphorylaiton of BCR/ABL.

Mitotic arrest induced phosphorylation of TOPK. Next, TOPK phosphorylation was assessed. TOPK is phosphorylated at Thr9 by CDK1/cyclin B1 in the mitotic phase (7,15). A crucial role of TOPK-Thr9 in cell cycle progression of solid tumors has been reported previously (14,36). Nocodazole induces mitotic arrest of the cell cycle by microtubule depolymerization (37). In the current study, treatment with nocodazole (300 ng/ml) enhanced the phosphorylation of TOPK in K562 cells a time-dependent manner (Fig. 5). Cell cycle analysis prior to and following 24 h treatment with nocodazole suggested an accumulation of cells in the mitotic phase (Fig. 5). At 24 h, the TOPK band appeared with an increased molecular weight on the western blot analysis, potentially due to mobility retardation caused by phosphorylation, as reported previously (14).

PP2A is associated with and dephosphorylates TOPK. PP2A is a serine/threonine phosphatase that acts as a tumor suppressor in BCR/ABL-positive cells (4). Okadaic acid is an inhibitor of protein phosphatase 1 and PP2A, and FTY 720 is an activator of PP2A. In K562 cells treated with nocodazole (300 ng/ml), phosphorylation of TOPK was enhanced in the presence of okadaic acid (200 nM; Fig. 6A) and phosphorylation was reversed by FTY 720 (5, 10 and 15 μM) in a dose-dependent manner (Fig. 6B). Treatment with okadaic acid in the absence of nocodazole revealed a TOPK band with a changed migration pattern that not detected using the p-TOPK-Thr9 antibody (Fig. 6A). This data suggested a potential phosphorylation of TOPK at a different amino acid.
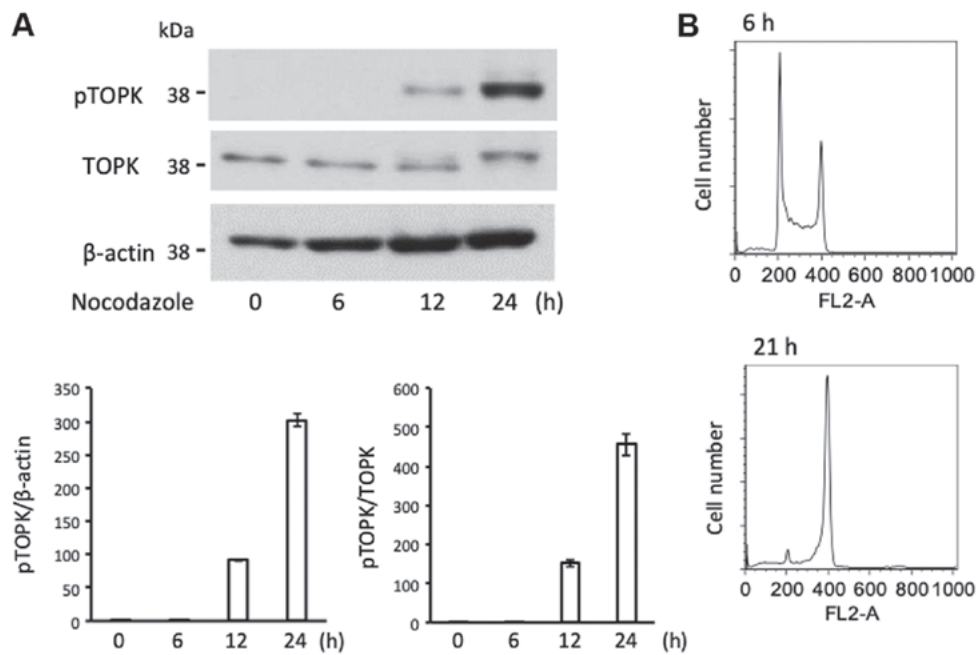
Figure 5. Phosphorylation of TOPK is induced by nocodazole. K562 cells were treated with nocodazole (300 ng/ml) for 0, 6, 12 or 24 h. (A) Western blot of TOPK phosphorylation levels and (B) cell cycle analyses at 6 and 21 h; results of the cell cycle analysis are representative of ten repeats. TOPK, T-lymphokine-activated killer cell-originated protein kinase; p, phosphorylated.
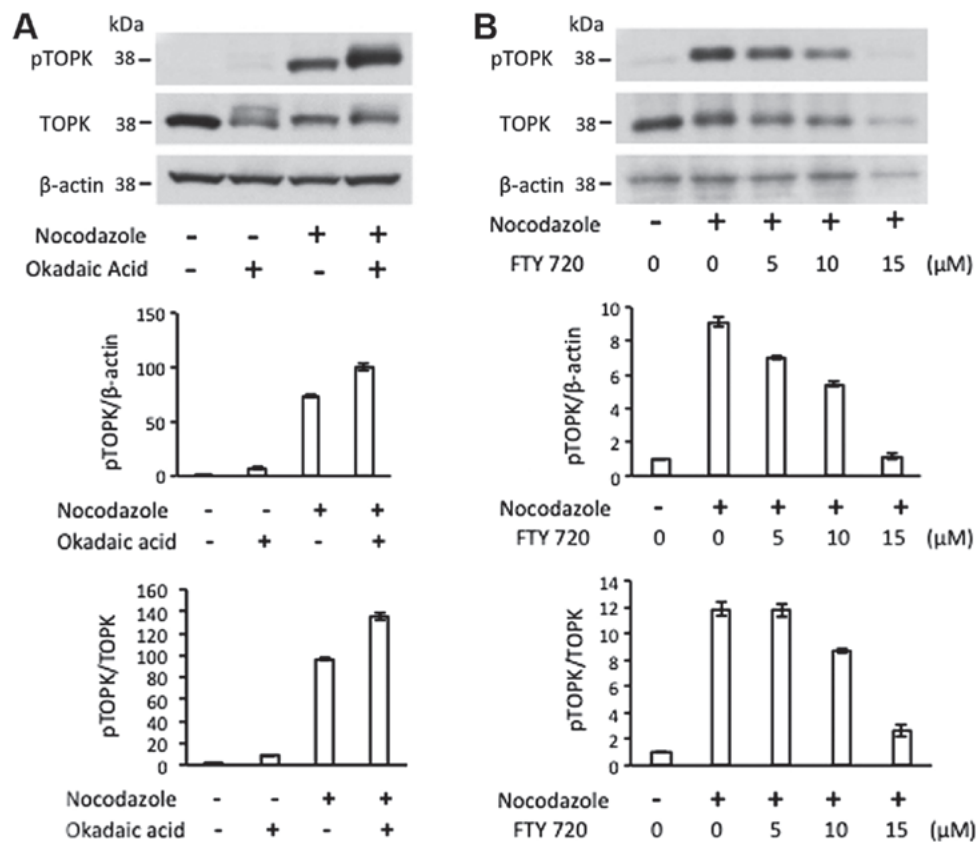

Figure 6. PP2A regulates TOPK phosphorylation. (A) K562 cells were treated with nocodazole (300 ng/ml) and PP2A inhibitor okadaic acid (200 nM). TOPK phosphorylation was examined by western blot analysis; results are representative of four repeats. (B) K562 cells were treated with nocodazole (300 ng/ml) and PP2A activator FTY720 (0, 5, 10 and 15 μM). TOPK phosphorylation was examined by western blot analysis; results are representative of four repeats. TOPK, T-lymphokine-activated killer cell-originated protein kinase; p, phosphorylated; PP2A, protein phosphatase 2A.

To further evaluate this potential connection, the association between TOPK and PP2A was studied. K562 cells were transfected with a FLAG-tagged TOPK overexpression vector. Western blot analysis of TCL suggested the presence of
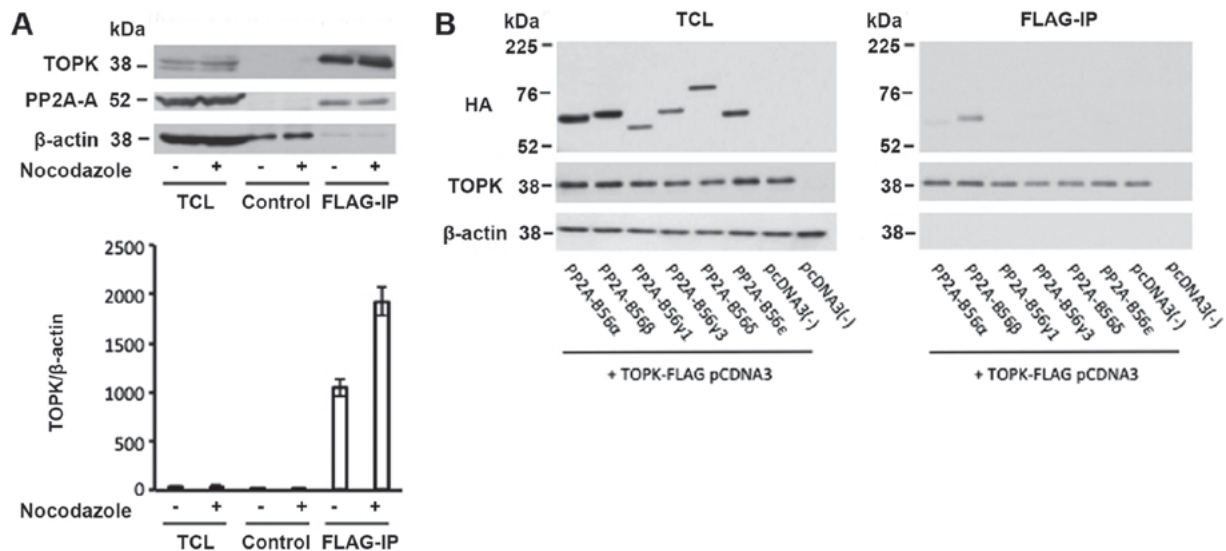
Figure 7. TOPK interacts with PP2A. (A) K562 cells transfected with FLAG-tagged TOPK were treated with nocodazole (300 ng/ml) overnight. Anti-FLAG beads were used in the IP of TOPK from TCL and anti-mouse IgG2a antibody served as negative control. TOPK or PP2A-A levels were examined by western blot analysis; results are representative of three repeats. (B) 293T cells were transfected with FLAG-tagged TOPK and various PP2A-B subunits. Anti-FLAG beads were used in the IP of TOPK from TCL, and TOPK and HA levels in the TCL and IP fraction were examined by western blot analysis; results are representative of eight repeats. TOPK, T-lymphokine-activated killer cell-originated protein kinase; TCL, total cell lysate; IP, immunoprecipitation; PP2A, protein phosphatase 2A; HA, human influenza hemagglutinin.

PP2A-A and TOPK in the cells. Although control mouse IgG was unable to precipitate TOPK and PP2A-A, immunoprecipitation of TOPK with anti-FLAG beads caused co-precipitation of PP2A-A in the presence and absence of nocodazole (Fig. 7A). This data suggested potential intermolecular interactions between TOPK and the PP2A complex. PP2A-B is a regulatory subunit and in combination with the PP2A-A and C dimer, it functions as PP2A phosphatase (5). To elucidate which PP2A-B subunit was involved in the interaction between PP2A and TOPK, 293T cells were transfected with overexpression vectors for FLAG-tagged TOPK and various PP2A-B56 subunits (α, β, γ1, γ3, δ and ε). Expression was determined in the culture supernatant to confirm transfection efficiency. Immunoprecipitation of TOPK using anti-FLAG beads suggested that PP2A-HA-B56β and, to a lesser extent, PP2A-HA-B56α interacted with TOPK (Fig. 7B). As β-actin was not detected in the immunoprecipitated samples, interference from non-specific precipitates was regarded as negligible. This result indicated that TOPK and specific PP2A-B subunits may interact.

TOPK inhibitor suppresses proliferation of BCR/ABL-positive cells. To elaborate on the effect of TOPK in BCR/ABL-positive cells, experiments were performed using TOPK inhibitor OTS514. OTS514 inhibits cell growth in several cell lines in which TOPK is expressed at high levels (38). Suppression of proliferation by OTS514 was assessed in various BCR/ABL-positive hematopoietic cell lines (K562, MOLM1, TMD5, TonB210 and TonB210/T315I). Cells were treated with 20 nM OTS514 for 48 h and evaluated by FACS analysis. In each cell line, proliferation was suppressed significantly in the inhibitor treated cells compared with the untreated controls (P<0.001; Fig. 8A).

Additionally, it was examined whether OTS514 affects CD34-positive primary cells. CD34-positive BMMCs from three patients with CML-CP and three patients with

stage I-IIA malignant lymphoma without BM involvement were used in clonogenic assays. Colony numbers were assessed at 14 days of culturing. As presented in Fig. 8B, OTS514 significantly decreased the number of colonies of the three samples from patients with CML-CP (P<0.05) compared with the untreated control. No significant differences were observed when treating the primary cells isolated the control patients. The data suggested that an inhibitory effect of OTS514 on the clonogenic capacity may be specific for CML-CP cells.

OTS514 and imatinib exhibit combinatory effects. To examine effects of the combination of OTS514 and imatinib, K562 cells were treated with OTS514 (10 nM), imatinib (0.5 μM) and a combination of both, and viability was assessed by FACS analysis. OTS514 and imatinib single treatments reduced the number of viable cells to a similar extent. Combined treatment further significantly reduced the number of viable cells compared with the single treatments (P<0.0001; Fig. 9). These results suggested that inhibition of TOPK and BCR/ABL may affect the viability of CML cells in an additive manner.

Discussion

In the present study, it was observed that peptides describing TOPK-breakdown products were interacting with HLA-A in K562 cells. Future experiments may focus on anti-TOPK CTL using by this peptide in *in vitro* analyses. Difficulties may arise from TOPK expression being associated with T cell activation and anti-TOPK T cells may be hard to detect.

It was described that TOPK was induced by BCR-ABL as a downstream target. Data suggested that expression of TOPK was induced in BCR/ABL overexpressing cells and reduced when BCR-ABL was inhibited using imatinib. Future studies evaluating the knockdown or knockout of BCR/ABL
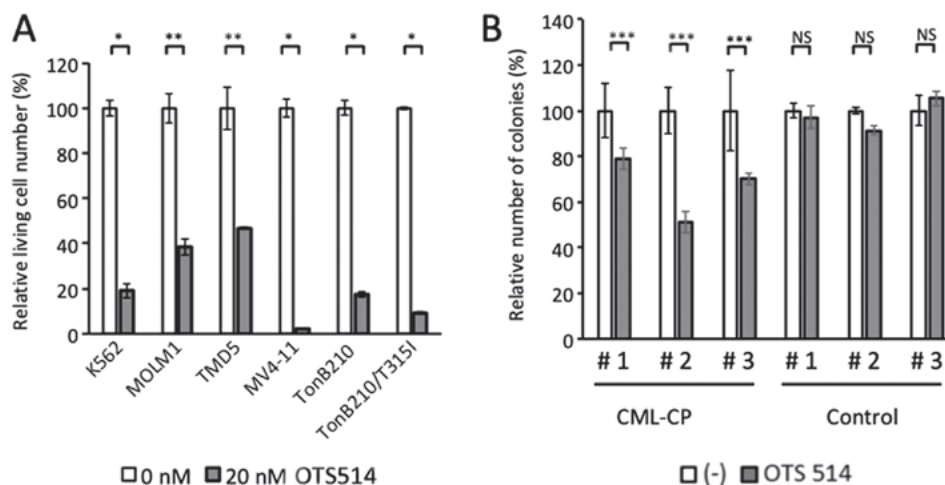
Figure 8. TOPK inhibitor OTS514 affects hematopoietic tumor cells. (A) Various hematopoietic cell lines were treated with TOPL inhibitor OTS514 (20 nM) for 48 h. Living cells were counted by fluorescence-activated cell sorting; experiments were performed in triplicate. (B) CD34-positive cells were isolated from the bone marrow cells of patients with CML-CP (n=3) and lymphoma in limited stages as control (n=3) and cultured for 14 days in the presence or absence of OTS514 (20 nM); experiments were performed in triplicate. *P<0.0001, **P<0.001 and ***P<0.05. TOPK, T-lymphokine-activated killer cell-originated protein kinase; CML-CP, chronic myeloid leukemia-chronic phase; NS, not significant.
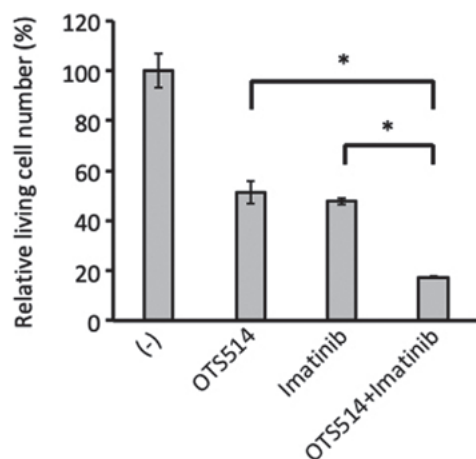

Figure 9. TOPK inhibitor OTS514 and imatinib exhibit additive effects. K562 cells were treated with OTS514 (10 nM) and/or imatinib (0.5 μM) for 48 h. Living cells were counted by fluorescence-activated cell sorting; experiments were performed in triplicate. *P<0.0001. TOPK, T-lymphokine-activated killer cell-originated protein kinase.

are required to confirm this mechanism and additional CML cell lines may be assessed. The present data indicated that BCR/ABL induced TOPK transcription, although certain cell lines, including TMD2, exhibited low TOPK protein levels and abundant TOPK mRNA, implying a potential presence of post-transcriptional regulators. The mechanism of this transcriptional induction of TOPK by BCR/ABL requires further elucidation. Previously, it was reported that transcription factor forkhead box protein M1 (FoxM1) regulates TOPK expression (39). As FoxM1 is activated by BCR/ABL (39,40), it may further be associated with TOPK upregulation by BCR-ABL.

TOPK expression was significantly increased in samples from patients with CML compared with healthy donors, suggesting that TOPK may be associated with the development of CML. Mechanisms leading to BC may vary on a case to case basis; explaining for the results reported here, where no

significant differences were observed in patients with BC and CP. In two of three patients that provided clinical samples in CP and BC, TOPK expression was increased in BC compared with CP. The case presented in more detail, was the patient exhibiting the highest overall recorded expression of TOPK. The patient initially presented with major molecular response to nilotinib (BCR/ABL international scale [IS] <0.1%), prior to occurrence of sudden myeloid BC with E255K mutation (IS=136.55%) at 11 months following diagnosis. Disease progressed rapidly and the patient succumbed 3 weeks following BC diagnosis. High TOPK expression may have contributed to the rapid progression, as TOPK expression is associated with aggressive development in other malignancies (9-13). The samples size used in the current study was small and samples for PBMCs and BMMCs were combined for the analysis. Further analysis using an increased number of clinical samples is required to elucidate CML development further.

In addition, TOPK is associated with PP2A, a key tumor inhibitor of BCR/ABL-positive cells (4). PP2A suppresses cell survival and proliferation by dephosphorylation regulatory markers (4,5). It was suggested here that PP2A may interact with TOPK. PP2A is able to accept substrates following binding of the B subunit to the dimer formed by subunits A and C (41). The B subunit defines the potential target molecule and is classified into four subfamilies (B/B55, B'/B56, B''/B72 and B'''/PR93) (42). The present study revealed that PP2A-B56β interacted most strongly with TOPK. It has been reported that PP2A-B56β mediates the interaction of the PP2A core enzyme complex with Akt (43). A downstream target of Akt, CDC-like kinase 2, was reported to phosphorylate B56β, which led to dephosphorylation of Akt by PP2A. Akt is serine/threonine kinase regulating proliferation and survival of cells and interacts with B56β (44). Based on the results presented in the current study it was hypothesized that TOPK may utilize a similar mechanism as Akt to regulate dephosphorylation by PP2A. However, further studies are required to evaluate these claims.

Suppression of cell proliferation using a TOPK inhibitor has been reported for various cell lines expressing TOPK at high levels (38). The current study confirmed that TOPK inhibitor OTS514 suppressed proliferation in hematopoietic cell lines expressing BCR/ABL with or without the T315I mutation, which confers resistance to most TKIs currently in clinical use (45). Additionally, OTS514 significantly enhanced the effect of imatinib on cell viability. TOPK inhibitors may describe novel therapeutic agents for ABL-mutated and -unmutated cases, to be used with or without TKIs. Further studies are required to examine effects of the TOPK inhibitor in various imatinib-resistant cell lines exhibiting BCR/ABL-independent and -dependent molecular mechanisms.

Treatment of patients with CML has dramatically improved since the introduction of TKIs, and the current goal is to eradicate CML stem cells and to prevent relapse following TKI treatment completion. The TOPK inhibitor OTS514 suppressed colony formation in CD34-positive cells from patients with CML patients but not in cells isolated from controls. Further studies are required to examine whether the TOPK inhibitor may promote elimination of CML stem cells.

The current study suggested that TOPK was induced by BCR/ABL and was dephosphorylated by PP2A. Further studies are warranted to evaluate the mechanisms of action and to elucidate the significance of TOPK in BCR/ABL-positive and its potential as a novel therapeutic.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EU, SS, OM and TF contributed to project conception and the design of experiments. EU, SS, RY, KW and TK performed the experiments and analyzed the results. EU, SS, OM and TF wrote the paper with contributions from all of the other coauthors. All authors read and approved the final version of the manuscript.

Ethical approval and consent to participate

Consent was obtained in compliance with the Declaration of Helsinki and all patients provided written informed consent.

Approval was obtained from the Ethics Committee of the Tokyo Medical and Dental University (Tokyo, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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