Receptor-type tyrosine-protein phosphatase kappa directly targets STAT3 activation for tumor suppression in nasal NK/T-cell lymphoma

Supplemental data for: Chen *et al*.

Files included in this data supplement: Detailed Materials and Methods, Tables, and Figures

Supplemental Materials and Methods

NKTCL cell lines and culture conditions

The SNK6[1] and NKYS[2] cell lines have been previously described. The NK92[3] and YT[4-5] cell lines were obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany). The KHYG[6] cell line was obtained from the Health Science Research Resource (Osaka, Japan). The SNK6, NKYS, KHYG, and NK92 cell lines were maintained in RPMI 1640 medium (HyClone, Thermo Scientific HyClone, Logan, UT, USA) supplemented with antibiotics, 10% heat-inactivated fetal bovine serum (FBS, HyClone), and 100 units/mL recombinant human interleukin-2 (rIL2, PeproTech, NJ, USA). The YT cell line was maintained in Iscove's modified Dulbecco medium (IMDM) supplemented with antibiotics and 20% heat-inactivated FBS, without rIL2.

Isolation of normal NK cells

Normal NK lymphocytes were isolated from the peripheral blood of 2 healthy donors. The lymphocytes were first isolated from anticoagulant-treated peripheral blood using Lymphoprep™ (Axis-Shield, Oslo, Norway), and the NK cells were then isolated from peripheral blood mononuclear cells (PBMCs) using the Dynal® NK Cell Negative Isolation Kit (Dynal Biotech, Oslo, Norway). The purity of NK cells was assessed by flow cytometry analyses using a FACSCanto II analyzer (BD Biosciences, San Jose, CA), and CD3- CD56+ NK cells were found to account for >90% of cells from each isolation.

Semiquantitative RT-PCR analysis

Total RNA and genomic DNA were extracted from fresh cell pellets of untreated and 5-aza-dC-treated NKTCL cell lines and frozen sections of 27 NKTCL tumors using TRIzol Reagent (Invitrogen, Life Technologies Corporation, Carlsbad, CA). All RNA samples were pre-treated with DNase I (DNA-*free*™ kit, Ambion Inc., Life Technologies Corporation, Carlsbad, CA) prior to RT-PCR analysis. Semi-quantitative RT-PCR was performed using total RNA, a GeneAmp RNA PCR system (Applied Biosystems, Life Technologies

Corporation, Carlsbad, CA), and *PTPRK*-specific primers, with β-*actin* as the normalization control (supplemental Table 1).

Immunohistochemical staining

Paraffin sections from NKTCL tumor specimens were studied for PTPRK and phospho-STAT3^{Tyr705} protein expression in tumor cells by immunohistochemistry using standard protocols. For PTPRK immunostaining, antigen retrieval was achieved by microwaving the dewaxed and rehydrated tissue section slides in 10 mM sodium citrate buffer (pH 5.8) for 15 minutes, followed by incubation with a rabbit polyclonal PTPRK (H75) antibody raised against amino acids 27-101 mapping within an N-terminal extracellular domain of human PTPRK (dilution 1:20, sc-28906, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and analysis using the LSAB^{TM}+ visualization kit (DakoCytomation, Glostrup, Denmark). For phospho-STAT3^{Tyr705} immunostaining, antigen retrieval was achieved by microwaving the tissue section slides in 10 mM sodium citrate buffer (pH 6.0) for 15 minutes, followed by incubation with a phospho-STAT3^{Tyr705} (D3A7) XP® rabbit monoclonal antibody (dilution 1:50, Cell Signaling Technology) and analysis using the EnVision^{TM}+ Dual link visualization kit (DakoCytomation). The expression levels of membranous and cytoplasmic PTPRK and nuclear phospho-STAT3^{Tyr705} were assessed based on the proportion of positive tumor cells: ≤50% was scored as low, and >50% was scored as high.

Western blot analyses

Western blot analyses were performed on protein extracts from cell pellets using standard protocols. After boiling, cellular proteins were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Amersham Pharmacia, GE Healthcare, Chalfont St Giles, BUCKS, UK). The blots were then blocked with 5% non-fat milk and incubated with an anti-PTPRK–rabbit IgG N-terminal antibody (H75; dilution 1:2000, Santa Cruz, CA), washed, and incubated for 1 hour with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Inc., Danvers, MA). Each step was followed by 3 washes with TBS-Tween 20 solution (Bio-Rad Laboratories, Hercules, CA). The protein bands were visualized using an enhanced chemiluminescence reagent (Amersham Pharmacia). The same membrane was then stripped and reprobed with anti-DDK tag M2 antibody (dilution 1:2000). Primary antibodies against phospho-STAT3^{Tyr705} (D3A7, Cell Signaling Technology, 1:500) and STAT3 (dilution 1:1,000, Cell Signaling Technology) were used for STAT3 phosphorylation analysis. A mouse anti-actin (1:1,000 dilution, Sigma-Aldrich) antibody was used as a loading control.

Retrovirus packaging cell line

Phoenix-Ampho cells, a 293T-derived Moloney murine leukemia virus (MoMLV) packaging

cell line (Orbigen Inc., San Diego, CA), were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L D-glucose (Gibco/BRL, Gaithersburg, MD) supplemented with antibiotics and 10% heat-inactivated FBS.

Restoration of PTPRK expression by retroviral transduction of the NKYS and KHYG cell lines

PTPRK and mock retroviral vector transductions were performed in non-*PTPRK*-expressing NKYS and KHYG cells. Full-length wild-type *PTPRK* cDNA (NM_001135648, 4341bp) with a Myc-DDK tag sequence at the C-terminus was purchased from OriGene (RC227943, OriGene Technologies, MD) and cloned into the pCMV6-Entry vector (OriGene). The insert was then subcloned into the MoMLV-based vector pBMN-GFP (Orbigen Inc.) with a downstream internal ribosomal entry site (IRES) for green fluorescent protein (GFP) expression. The construct sequence was confirmed by DNA sequencing. A pBMN retroviral vector with a Myc-DDK tag sequence was used as a mock control for indirect immunofluorescence experiments, while an empty pBMN retroviral vector was used as a mock control for various functional assays.

For retroviral transduction, 24 µg of each construct or the mock vector was transfected into Phoenix-Ampho cells (Orbigen Inc.) with 1 µg of the pHCMV-G plasmid expressing the G glycoprotein of vesicular stomatitis virus using the Fugene®6 transfection reagent (Roche Applied Science, Mannheim, Germany). Briefly, 24 hours before transduction, NKYS and KHYG cells ($5x10^6$/mL) were pre-activated with 900 U/mL rIL2 (PeproTech), while the packaging cells transfected with *PTPRK*/mock retroviral vector 2 days earlier were given fresh medium and incubated in a 5% CO_2 incubator at 32°C. On the day of transduction, the viral supernatant was preloaded onto a RetroNectin-coated 6-well plate (recombinant CH296 fibronectin, Takara Shuzo, Otsu, Japan) for 4 hours, followed by the addition of target cells and then centrifugation at 2,000 rpm for 1.5 hours at 32°. After centrifugation, the plate was incubated in a 5% CO_2 incubator at 32°C for 18 hours. Transductions were performed for three successive days for NKYS and KHYG cells. Six hours after the third transduction, the transduced cells were pelleted by centrifugation and resuspended in growth medium. As the pBMN vector used in this study is a bicistronic retroviral vector that also expresses GFP, the expression of GFP 48 hours after the last round of cell transduction indicates a successful transduction. Restoration of PTPRK expression was validated by Western blot analysis and immunofluorescence staining. The reconstituted PTPRK expression levels in NKYS and KHYG cells were approximately similar to the levels of PTPRK expression in normal NK cells. The subsequent *in vitro* functional analyses were performed on GFP-sorted cells 3 days after selection by the cell sorter (FACSAria I, Becton Dickinson, BD, Franklin Lakes, NJ).

***PTPRK* knockdown in SNK6 cells with lentiviral short hairpin RNA (shRNA)**

PTPRK knockdown was initially performed with five *PTPRK*-specific shRNAs (supplemental Table 1) using the CMV-puro (pLKO.1puro-CMV-TagRFP) lentiviral plasmid (Sigma-Aldrich) in *PTPRK*-expressing SNK6 cells. The sequences of these shRNAs were chosen carefully to ensure that they are *PTPRK* specific and will not affect the other receptor-type tyrosine-protein phosphatases. MISSION® pLKO.1-puro-CMV-TagRFP™ Positive Control Plasmid DNA was used as a mock control. The vector used in this study expressed red florescence protein (RFP) as the transduction marker.

For PTPRK knockdown, five *PTPRK* lentiviral shRNA plasmids and a control non-targeting lentiviral shRNA plasmid (Sigma-Aldrich) were used. The *PTPRK* shRNAs sequences are listed in supplemental Table 1. The sequences of these shRNAs were chosen carefully to ensure that they are *PTPRK* specific and will not knockdown other R-PTPases. HEK 293T cells were plated at $1x10^7$ cells/well on a 100-mm dish in DMEM containing 10% FBS. The next day, cells were transfected with shRNA plasmids in a reagent mixture [0.5 mg plasmid DNA, 5 mL of lentiviral packaging mix (Sigma-Aldrich), and 3 mL FuGENE6 transfection reagent (Roche Applied Science, USA)]. Culture medium containing lentiviral particles was collected 2 days after transfection and added to 6-well plates, followed by the addition of SNK6 cells ($5x10^6$, preactivated with 900 U/mL of rIL2 for 24 hours) and centrifugation at 2,000 rpm for 1.5 hours at 37°C. DMEM was replaced with RPMI for SNK6 cells the day after transduction. SNK6 cells successfully transduced with shRNA were identified by the expression of RFP 48 hours after cell transduction. Partial knockdown of PTPRK expression was validated by Western blot analysis.

Two of the five *PTPRK*-specific shRNAs (shRNA #1 and #2) that were found to be the most efficient when tested independently were chosen for further study. shRNA #1 and #2 independently knocked down approximately 70% of PTPRK expression in RFP-sorted SNK6 cells acquired using a cell sorter (FACSAria SORP, Becton Dickinson, BD, Franklin Lakes, NJ). The *in vitro* functional assays were performed on RFP-sorted SNK6 cells.

Flow cytometry analysis of phospho-STAT3 Tyr705

To determine the expression of STAT3 phosphorylation at Tyr705, a phospho-$STAT3^{Tyr705}$ antibody (D3A7, Cell Signaling Technology) was used for intracellular flow cytometry using a Cytofix/Cytoperm Fixation/Permeabilization kit (Becton, Dickinson and Company, BD Biosciences, San Jose, CA). Background staining was determined using a rabbit isotype control monoclonal IgG antibody (Cell Signaling Technology). Briefly, the cells were first fixed with 1% paraformaldehyde for 30 minutes at 4°C and permeabilized by incubation in absolute methanol for at least 20 minutes at 4° C. The cells were then stained for 2 hours in the dark at room temperature using antibody against phospho-$STAT3^{Tyr705}$ (dilution 1:200). Finally, the cells were incubated with Pacific Blue-conjugated goat anti-rabbit IgG (Life Technologies, Carlsbad, CA). The cells were washed for three times between each step, and

pSTAT3^{Tyr705} expression was analyzed by flow cytometry (BD LSR Fortessa Analyzer, Becton Dickinson, BD, Franklin Lakes, NJ) after gating on GFP- or RFP-positive populations. The flow cytometric data were analyzed using FlowJo software, version 9.3.1 (Treestar, San Carlos, CA).

Indirect immunofluorescence

For NKYS cells re-expressing PTPRK by retroviral transduction, immunofluorescence staining was performed by using an anti-DDK tag M2 antibody (dilution 1:500, Sigma-Aldrich) and an anti-phospho-STAT3^{Tyr705} antibody (dilution 1:100, Cell Signaling Technology). The signal of each primary antibody was amplified by applying a biotinylated secondary antibody (dilution 1:1,000, Vector Laboratories, Inc., Burlingame, CA) followed by fluorescein streptavidin (for the detection of the anti-DDK tag M2 antibody) and Texas Red streptavidin (for detection of anti-phospho-STAT3^{Tyr705} antibody). For SNK6 cells with partial knockdown of PTPRK by lentiviral shRNA transduction, the slides were stained with a phospho-STAT3^{Tyr705} antibody (dilution 1:100, Cell Signaling Technology) followed by a biotinylated secondary antibody (Vector Laboratories) for signal amplification and then fluorescein streptavidin (Vector Laboratories). Fluorescein streptavidin and Texas Red streptavidin both target the biotinylated secondary antibody, and they emit green and red fluorescence, respectively.

For indirect immunofluorescence, two days after viral transduction, the cells were collected and cytospun onto slides. The cells were then fixed with cold methanol, permeabilized with 0.2% Triton X-100, and blocked with 10% bovine serum albumin (BSA, Sigma-Aldrich). The first primary antibody was then applied to the slide and incubated at 4^0C overnight. Biotinylated secondary antibody was added and incubated at room temperature for 1 hour, followed by fluorescent streptavidin. The slide was washed with PBS 3 times before proceeding to the next step. If two protein molecules were to be simultaneously detected, the second antibody was applied using the same procedure as the first primary antibody after blocking the slide again with 10% BSA. Finally, the slides were treated with 2 μL/mL of Hoechst 33342 dye (Molecular Probes, Life Technologies Corporation, Carlsbad, CA) for nucleic acid staining. The mounting medium Vectashield (Vector Laboratories) was applied to each slide with a coverslip. Images were captured using a fluorescence microscope equipped with a CCD camera (Leica Microsystem Wetzler GmbH, Germany) and a Carl Zeiss LSM 510 confocal microscope, or the images were captured using the Leica Q550CW fluorescence microscope. The pBMN retroviral vector used in this study contains a downstream IRES for GFP expression as a marker for transduced cells. However, the fixation of GFP-expressed transduced cells with cold methanol prior to immunostaining denatured the GFP and destroyed its fluorescence, therefore, the GFP signal did not interfere with the subsequent imaging of indirect immunostaining using green fluorescein streptavidin.

Construction, expression, and purification of glutathione S-transferase (GST) recombinant proteins

The *PTPRK-GST* fusion protein construct was made by cloning the first phosphatase domain of *PTPRK* into the pGEX-4T-1 GST expression vector (Amersham Pharmacia) using the primers described in supplemental Table 2. A cDNA construct (RC227943, OriGene) was used as a template for the PCR amplification of the phosphatase domain. Substrate trapping mutants, D1057A (mutant 1) and C1089A (mutant 2), were made by site-directed mutagenesis using the GENEART site-directed mutagenesis system (Life Technology) and the primers described in supplemental Table 2. The wild-type *PTPRK-GST* fusion protein construct was used as a template for PCR amplification of the mutant phosphatase domains. The wild-type and substrate trap mutant proteins of PTPRK were expressed in the *E.coli* strain BL21 by induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside and then purified using glutathione beads (Thermo Fisher Scientific Inc., Waltham, MA).

Phosphatase substrate-trap assays

Substrate-trap mutant assays were performed, as previously described, for the phosphatase PTP1B.[7] In brief, 10 million NKYS/KHYG cells were treated with 100 □ l pervanadate for 30 minutes and collected by centrifugation. The cell pellet was lysed with 1 mL of lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1x complete protease inhibitor mixture, 1 mM EDTA, 1 mM benzamidine), treated with 5 mM iodoacetic acid on ice for 5 minutes, and then subjected to centrifugation at 16000 x g for 30 minutes to remove cell debris. GST only and the 3 GST-PTPRK fusions bound beads were incubated with NKYS/KHYG lysate at 4°C for 8 hours, and then the beads were pelleted and washed 3 times with lysis buffer supplemented with 1 mM DTT. The beads were then boiled, and aliquots were analyzed by SDS/PAGE and Western blotting.

***In vitro* phosphatase assay**

The STAT3-Myc-DDK fusion protein construct was created by PCR subcloning of STAT3 cDNA (SC100105, OriGene, Rockville, MD) into the pCMV6-Entry (C-terminal Myc and DDK tagged) vector (OriGene) using the primers described in supplemental Table 2. For the *in vitro* phosphatase assays, HEK293T cells overexpressing STAT3-Myc-DDK were stimulated with 10 ng/mL of IL6 for 3 hours, treated with 50 μM pervanadate for 30 minutes, and then lysed with RIPA buffer. STAT3 proteins were immunoprecipitated with anti-Myc antibody-conjugated agarose beads (Thermo Scientific, Waltham, MA). The immune complexes were washed twice in a wash buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% Nonidet P-40) containing phosphatase inhibitors (10 mM NaF and 2 mM Na_3VO_4), followed by 2 washes in the same buffer without the phosphatase inhibitors, 1 wash

in ST buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4), and 1 wash in phosphatase assay buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM DTT). STAT3 immune complexes were then incubated together with either purified GST alone (control) or a PTPRK-GST fusion protein, wild-type or mutant 2, in the absence or presence of 10 mM Na_3VO_4 at 37°C for 45 minutes. Western blot analysis was performed to quantify phospho-STAT3^{Tyr705}levels.

Cell growth assay

The cell proliferation rates were measured with the AQueous One Solution MTS Assay (Promega). The GFP/RFP-sorted transduced cells were analyzed in a 96-well plate (at densities of $1x10^4$ cells per well) in 100 µL of culture medium with 20 µL of MTS reagent. After a 3-hour incubation period at 37°C, the absorbance at 490 nm was measured with a Tecan 96-well plate reader (Molecular Devices, LLC, Sunnyvale, CA). The measurements were performed on three consecutive days.

Anchorage-independent colony formation assay

PTPRK retroviral-transduced NKYS and KHYG cells or *PTPRK*-specific lentiviral shRNA-transduced SNK6 cells ($3x10^3$ cells/well in a 24-well plate) were grown in methylcellulose (final concentration of 1.2% for NKYS and KHYG, and 0.4% for SNK6) containing the cells' respective culture media. The number of colonies formed was counted in five randomly selected microscopic fields after five days for NKYS and KHYG cells and after one week for SNK6 cells.

Cell cycle distribution and apoptosis induction assays

For cell cycle analyses, GFP-sorted *PTPRK* retroviral transduced NKYS and KHYG cells ($1x10^5$) were fixed in 70% ethanol at -20°C overnight and resuspended in PBS containing 200 µg/mL RNase A and 20 µg/mL PI (Sigma-Aldrich). Cell cycle distributions were determined by flow cytometry analyses using a FACSCanto II analyzer (BD Bioscience). For apoptosis analysis, cells (unsorted $1x10^5$) were stained with 7-amino-actinomycin D (7-AAD) (BioLegend, CA) and Pacific blue-Annexin V (BD Bioscience) to detect cells undergoing apoptosis. After 10 to 15 minutes of staining, the cell death parameters were determined by flow cytometry analyses using a FACSAria Sorp analyzer (BD Bioscience). The distributions of cells in the G_0/G_1, S, and G_2/M phases and the proportion of cells in different stages of apoptosis were analyzed using FlowJo software.

To study caspase-mediated apoptosis by Western blot analysis, a cleaved caspase-3 rabbit monoclonal antibody (Asp175; 5A1E), a cleaved caspase-9 antibody (human specific) (Asp330), and a cleaved PARP rabbit monoclonal antibody (Asp214; D64E10 XP® ; dilution 1:1,000, Cell Signaling Technology) were used.[8] Mouse anti-actin (dilution 1:1,000; Sigma-Aldrich, St. Louis, MO) was used as a loading controls.

Cell-cell interactions

PTPRK retroviral-transduced NKYS cells or *PTPRK*-specific lentiviral shRNA-transduced SNK6 cells were sorted based on GFP/RFP fluorescent protein expression. Two days after cell sorting, the cells were centrifuged and re-suspended in fresh medium. Six hours after the re-suspension, the formation of any clumps of 5 to 10 cells consisting of transduced NKTCL cells were imaged under ultraviolet light.

Pharmacological concentrations of 5-aza-dC for the NKTCL cell lines

The pharmacological concentrations of 5-aza-dC for the individual NKTCL cell lines were determined by treating the five cell lines with different concentrations of 5-aza-dC (0 to 50 μM) for three days and measuring the percentages of viable cells with the CellTiter 96®Aqueous One Solution MTS assay (Promega). The pharmacological concentrations of 5-aza-dC were determined to be 10 μM for the SNK6 and NKYS cell lines and 0.5 μM for the NK92, KHYG1, and YT cell lines.

For indirect immunofluorescence analysis, following treatment with 5-aza-dC for three days, NKYS cells (a non-PTPRK-expressing NKTCL cell line) were collected and cytospun onto slides. After fixation with cold methanol, permeabilization with 0.2% Triton X-100, and blocking with 10% BSA (Sigma-Aldrich), the slides were incubated with an anti-PTPRK–rabbit IgG N-terminal antibody (H75; Santa Cruz; dilution 1:20) at 4°C overnight. The slides were then washed with PBS three times. Next, the biotinylated secondary antibody (Vector Laboratories, Inc.) was applied, followed by fluorescein streptavidin (Vector Laboratories). The cells were then labeled with 2 μL/mL of Hoechst 33342 dye (Molecular Probes, Eugene, OR) for nucleic acid staining. The mounting medium Vectashield (Vector Laboratories) was applied to each slide with a coverslip to prevent the fluorescence signal from fading. The images were captured using a Leica DM RXA (Leica) florescence microscope (100_/0.7 NA oil objective). The florescence images were imported and analyzed using Leica CW 4000 FISH software (Applied Imaging, UK).

Bisulfite treatment and methylation analysis

Bisulfite treatment of genomic DNA was performed with the CpGenome Universal DNA Modification Kit (Chemicon, EMD Millipore Corporation, MA). The primers used in the BGS and MSP assays were designed using an online CpG island finder (http://dbcat.cgm.ntu.edu.tw) (supplemental Table 1). For BGS, the bisulfite-treated DNA was amplified using the BGS primers, the PCR products were cloned, and 8 to 10 clones were sequenced for BGS analysis. A pair of BGS primers (supplemental Table 1) that amplified both the methylated and unmethylated *PTPRK* alleles of the bisulfite-modified DNA was designed to flank the CpG island region that contained 51 CpG sites. For MSP

analysis, bisulfite-treated DNA was amplified with the *PTPRK* unmethylated- or methylated-specific primer sets (supplemental Table 1). Universal methylated DNA and unmethylated DNA (Intergen® Co., NY) were used as positive controls, while non-bisulfite-treated DNA was used as the negative control for MSP.

PTPRK gene allelic loss analysis

Multiplex PCR was performed on genomic DNA from primary NKTCL tumors using *PTPRK* and β-*globin* (as the normal allele control) gene-specific primers (supplemental Table 1). After 30 PCR cycles, the products were separated on a 2% agarose gel. Quantification was achieved via a densitometry scan of the ethidium bromide-stained gels using Molecular Imaging Software (Kodak, New Haven, CT), and samples with a *PTPRK*/β-*globin* allelic ratio of less than 0.75 were considered to have a monoallelic *PTPRK* gene deletion.

Mutational analysis of _PTPRK_

Mutations within the *PTPRK* coding region were examined using mRNA extracted from primary NKTCL tumors. The cDNA sequences covering the entire coding region were amplified with a combination of seven forward and seven reverse primers (supplementary Table 1), followed by the direct bidirectional sequencing of the PCR products after ExoSAP-IT (USB, Affymetrix Inc., Santa Clara, CA) treatment.

Statistical analyses

SPSS 19.0 (IBM Corporation, Armonk, NY) was used for data processing, univariate analysis, and graphical representation. The associations between the levels of PTPRK protein expression in tumor cells versus *PTPRK* mRNA expression in tumor specimens and the levels of PTPRK protein expression versus the levels of phospho-STAT3^{Tyr705} in tumor cells were analyzed using the two-sided Fisher's exact test. The correlations between PTPRK protein expression levels in tumor cells and various clinical features, Eastern Cooperative Oncology Group (ECOG) score,[9] and the IPI[10] were analyzed in NKTCL patients using Pearson's χ^2-test.

Similarly, the association between *PTPRK* promoter methylation status and *PTPRK* mRNA expression in tumors was analyzed using the two-sided Fisher's exact test. The correlations between *PTPRK* promoter methylation status in tumors and various clinical features, ECOG score[9] and IPI[10] were analyzed in NKTCL patients using a χ^2-test. To study the clinical relevance of *PTPRK* promoter methylation in NKTCL, survival curves were plotted using the Kaplan-Meier method and were compared using the log-rank, Breslow, and Tarone-Ware tests.

The OS of patients was calculated as the time between diagnosis and death or the last follow-up. Disease free survival (DFS) was used to estimate the response to treatment. A two-tailed probability (*p*) value <0.05 was considered significant for each analysis.

Supplemental References

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Supplemental Tables

Supplemental Table 1. The three genes encoding for PTPases located at chromosome 6q in the human genome.[11-12]

Chromosomal location	Gene	Protein, Synonyms	STAT3-specifying YXXQ motif Yes/No
6q12	*PTP4A1*	Protein Tyrosine Phosphatase Type IVA, Member 1 PRL-1	No
6q22.2-23.1	*PTPRK*	Protein Tyrosine Phosphatase, Receptor Type, K RPTPκ	Yes
6q23	*EYA1*	Eyes Absent Homolog 4 (Drosophila) Eya4	No

*RNA Guanylyltransferase and 5'-Phosphatase mRNA capping enzyme (*RNGTT*) and Epilepsy, Progressive Myoclonus Type 2A, Lafora Disease Laforin (*EPM2A)* which are also located at chromosome 6q in the human genome are not known to have PTPases activities.

Supplemental Table 2. The primer sequences used in this study.

Primer name	Forward primer	Reverse primer
For *PTPRK* RT-PCR	5'-actcggcatggatacgactg-3'	5'-ctcgggtggtagataatgag-3'
For β-*actin* RT-PCR as a control	5'-gttgctatccaggctgtgct-3'	5'-agcactgtgttggcgtacag-3'
For PCR amplification of the first phosphatase domain of *PTPRK*: 1. Wild-type 2. Mutant 1 (D1057A) 3. Mutant 2 (C1089A)	 5'-cgtggatccgtaaaaaagagcaaacttgctgctaaaaaa-3' 5'-tcacgggctggcctgcccatggagtgcccta-3' 5'-cccatcgttgtacatgccagtgctggtgctgg-3'	 5'-gaattcaaattcacagacaggtatggca-3' 5'-tagggcactccatgggcaggccagcccgtga-3' 5'-ccagcaccagcactggcatgtacaacgatggg-3'
For PCR subcloning of the coding sequence of STAT3	5'-actaagcttctctgccggagaaacagttg-3'	5'-gagcggccgcgtcatgggggaggtagcgcact -'3
shRNA sequences against *PTPRK*	shRNA#1 5'-gcgatgagcagtgtggagaaggaaactaa-3' shRNA#2 5'-gcccagactaagaacatcaat-3' shRNA#3 5'-acctgactatgaaggagttgatgcctctc-3' shRNA#4 5'-aatgtcagcctcaagatgatcctaaccaa-3' shRNA#5 5'-tgtatgtcttgttcaatggactgtgatgt-3'	
For *PTPRK* MSP-M	5'-ggcgtcgcgttcgagttc-3'	5'-ttactcgaaaaaaaacgaaaacg-3'
For *PTPRK* MSP-U	5'-ggtggtgttgtgtttgagttt-3'	5'-ctttactcaaaaaaaaacaaaaaca-3'
For *PTPRK* BGS	5'-tttgagttttggaatagggta-3'	5'-atca/gccaaactacctcaaaaac-3'
For *PTPRK* gene allelic loss by multiplex DNA PCR	5'-agtcgacttgtcccaggttaatg-3'	5'-ccatatcgtagcatcccttcct-3'
For β-*globin* gene as a normal allele control for multiplex DNA PCR	5'-caacttcatccacgttcaacc-3'	5'-gaagagccaaggacaggtac-3'
For mutational analysis of *PTPRK* mRNA	F1: 5'-cttgaaacttctcccaaactcg-3' F2: 5'-aatctcctcatttcctccgtcta-3' F3: 5'-cccaaagacattaaagattgctg-3' F4: 5'-aagccaaaggtgctcctatcagt-3' F5: 5'-ccttctagacgtacctcgctacc-3' F6: 5'-tttatccggcgagtcaagttat-3' F7: 5'-ctgctttcatcgtcacacaatac-3'	R1: 5'-attcacctctacatcccctagacg-3' R2: 5'-ttgcctgtatttcagcaatcttt-3' R3: 5'-tctgataagcactgataggagca-3' R4: 5'-ccctcacagaggtagcgaggta-3' R5: 5'-agcactgggagggtttgataact-3' R6: 5'-tccagaagtcttttacagtgttg-3' R7: 5'-aggtttcttcatggatgcacttta-3'

Supplemental Table 3. *PTPRK* mRNA expression is highly correlated with PTPRK protein expression in NKTCL cell lines and in tumor cells of primary NKTCLs.

		PTPRK mRNA		Fischer's exact test *p*-value
		High	Low	
NKTCL cell lines (n=5)				
	PTPRK protein – Low	0	4	0.025
	PTPRK protein – High	1	0	
NKTCL tumor biopsy specimens (n=27)*				
	PTPRK protein – Low	4	13	0.034
	PTPRK protein – High	8	2	

**PTPRK* mRNA from tumor-infiltrating non-neoplastic cells may account for the minor discrepancy between *PTPRK* mRNA and PTPRK protein expression in some cases of NKTCL tumor biopsy specimens.

Supplemental Table 4. Correlation of the protein expression levels of PTPRK with phospho-STAT3^{Tyr705} expression in the NKTCL cell lines and in tumor cells of primary NKTCLs.

		phospho-STAT3^{Tyr705} expression		Fischer's exact test *p*-value
		Low	High	
NKTCL cell lines (n=5)				
	PTPRK expression- negative	0	4	0.025
	PTPRK expression- positive	1	0	
NKTCL tumor biopsy specimens (n=27)				
	PTPRK expression- negative	5	12	0.040
	PTPRK expression- positive	7	3	

Supplemental Table 5. Correlation between the methylation status of the *PTPRK* promoter and *PTPRK* mRNA expression in NKTCL cell lines.

		PTPRK mRNA		Fisher's exact test *p*-value
		High	Medium/Low	
NKTCL cell lines (n=5)				
	PTPRK promoter - methylated	0	4	0.025
	PTPRK promoter - unmethylated	1	0	

Supplemental Table 6A. Summary of *PTPRK* promoter methylation, *PTPRK* gene allelic loss, and *PTPRK* mRNA expression results in individual cases of NKTCL.

	PTPRK promoter Methylated (M) /Unmethylated (U)	Monoallelic *PTPRK* gene loss Yes/No	*PTPRK* mRNA High/Medium/Low
NL1	M	No	Medium
NL2	M	No	Low
NL3	M	Yes	Low
NL4	U	No	High
NL5	U	No	High
NL6	U	No	High
NL7	U	No	High
NL8	M	Yes	Low
NL9	U	No	High
NL10	M	No	High
NL11	U	No	High
NL12	M	Yes	Low
NL13	M	Yes	Low
NL14	M	Yes	Low
NL15	U	Yes	Medium
NL16	U	Yes	Medium
NL17	M	No	Medium
NL18	M	No	Low
NL19	U	No	High
NL20	U	No	High
NL21	M	No	High
NL22	M	No	Medium
NL23	M	No	Medium
NL24	M	No	Medium
NL25	M	No	Medium
NL26	U	No	High
NL27	M	Yes	Low

Supplemental Table 6B. Correlation between the methylation status of the *PTPRK* promoter and *PTPRK* mRNA expression in NKTCL primary tumors.

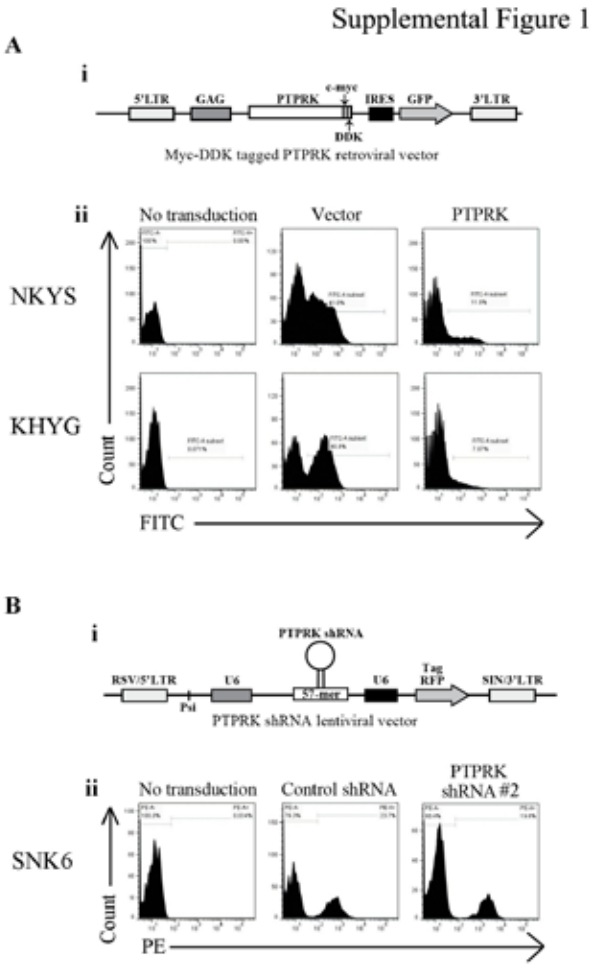
	PTPRK mRNA		Fisher's exact test
	High	Medium/Low	*p*-value
NKTCL primary tumors (n=27)			
PTPRK promoter - methylated	2	14	0.001
PTPRK promoter - unmethylated	9	2	

Supplemental Table 7. Correlation between the protein expression levels of PTPRK in tumor cells and clinical features in NKTCL patients.

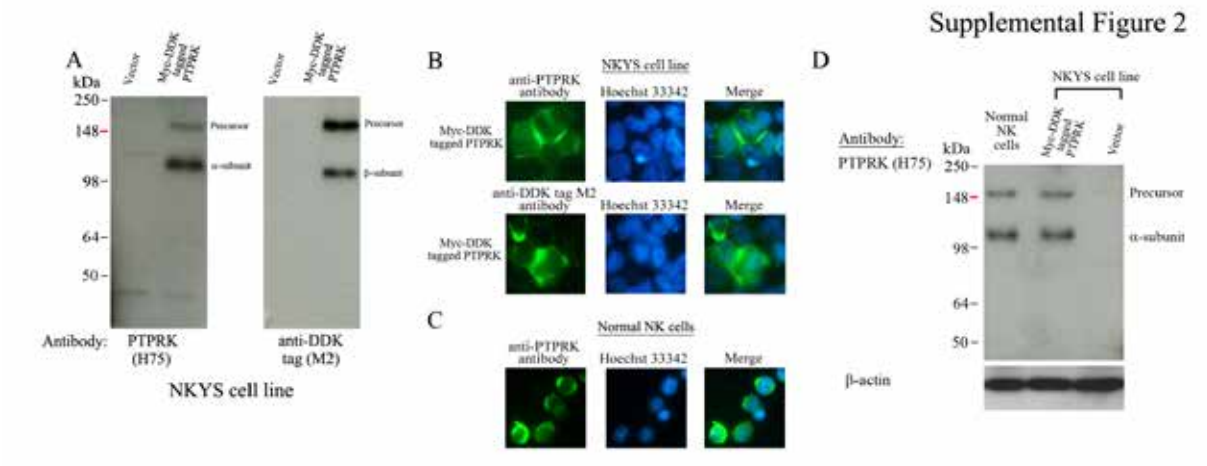
		Low PTPRK	High PTPRK	Pearson's χ^2-test *p*-value
Gender				
	Male	9	7	0.384
	Female	8	3	
Age				
	≤ 60 years	13	6	0.365
	> 60 years	4	4	
Stage				
	1, 2	5	6	0.118
	3, 4	12	4	
Elevated serum LDH (lactate dehydrogenase) levels				
	No	7	7	0.148
	Yes	10	3	
Eastern Cooperative Oncology Group (ECOG) score				
	0, 1	16	10	0.434
	2, 3, or 4	1	0	
Number of extranodal sites of disease				
	1	7	9	0.013
	>1	10	1	
IPI (International Prognostic Index)				
	0-1	5	7	0.040
	2, 3, or 4-5	12	3	

Supplemental Figures

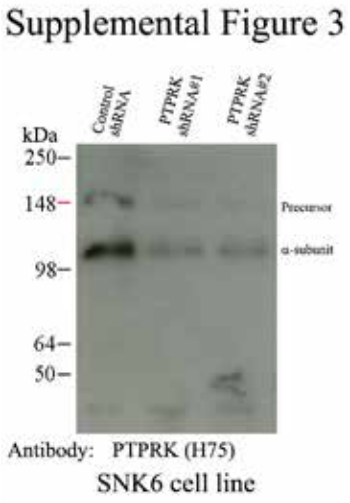
Supplemental Figure 1. Flow cytometric analyses showing the viral transduction efficiency. The transduction efficiency was measured as the percentage of cells with florescent protein expression 3 days after the viral transduction process. The retroviral transduction efficiencies of *PTPRK* in (A) KHYG and (B) NKYS cells were measured by the expression of GFP while (C) the lentiviral transduction efficiency of shRNA#2 targeting *PTPRK* in SNK6 was measured by the expression of RFP. The histograms at the left panel of each row represent controls with no viral transduction, while those in the middle and right panels represent populations with retroviral/lentiviral transductions. The figures are representative of at least 3 different experiments.



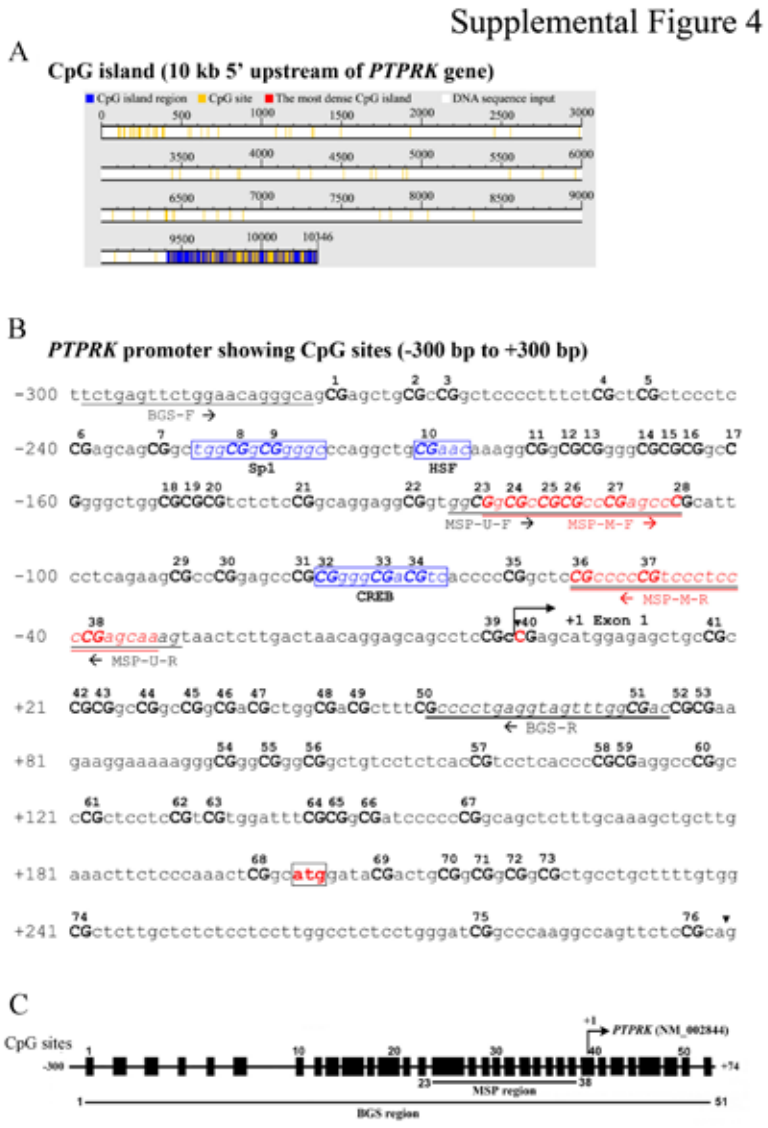
Supplemental Figure 2. Validation of reconstituted PTPRK protein expression. (A) The Western blot images show precursor and mature subunits of PTPRK using both a rabbit polyclonal PTPRK (H75) antibody raised against amino acids 27-101 mapping within an N-terminal extracellular domain of human PTPRK and an anti-DDK tag M2 (DDK tag at the C-terminus) antibody after the transduction of *PTPRK* cDNA into NKYS cells. (B) Reconstituted PTPRK was localized to the cellular membrane and cytoplasmic region after the transduction of *PTPRK* into NKYS cells. Immunofluorescence staining of *PTPRK*-transduced NKYS cells using an anti-PTPRK N-terminus antibody (H75; green fluorescence, cellular membranous staining) and an anti-DDK tag M2 antibody (green fluorescence cellular membranous and cytoplasmic staining) is shown. Hoechst 33342 dye was used to stain the nuclei (blue fluorescence nuclear staining). Original magnification 400x. (C) Immunofluorescence staining of normal NK cells using an anti-PTPRK N-terminus antibody (H75; green fluorescence, cellular membranous staining). Hoechst 33342 dye was used to stain the nuclei (blue fluorescence nuclear staining). Original magnification 400x. (D) Western blot images indicate the expression of PTPRK in normal NK cells and PTPRK-transduced NKYS cells using a PTPRK (H-75) antibody.



Supplemental Figure 3. Validation of knockdown of PTPRK protein expression. A Western blot image shows the precursor and α-subunit of PTPRK in PTPRK-expressing SNK6 cells after partial knockdown of the gene with shRNA#1 and shRNA#2 using an anti-PTPRK N-terminus antibody (H75) antibody.



Supplemental Figure 4. *PTPRK* promoter showing CpG sites and the position of the MSP and BGS primers used in this study. (A) The schematic diagram illustrates a CpG island in the 10-kb sequence 5' upstream of the *PTPRK* sequence. (B) The sequence of the *PTPRK* promoter and the first exon is presented (-300 bp to +300 bp). The 76 CpG sites in the *PTPRK* promoter and first exon are highlighted in bold. The positions of the forward and reverse primers used for the BGS and MSP analyses are underlined. The boundaries of the first exon of *PTPRK* are indicated by inverted arrowheads. The transcription initiation site is indicated by a bent arrow. The translation start site (ATG) is boxed. Within the region selected for BGS, 3 important predicted transcription factor binding sites (Sp1, HSF, and CREB) were identified (boxes), each containing 1-3 CpG sites. (C) The schematic diagram illustrates the CpG sites in the *PTPRK* promoter and the regions amplified by MSP and BGS. The transcription initiation site is indicated by a bent arrow.



Supplemental Figure 5. PTPRK potentially modulates JAK3 phosphorylation at Tyr980. Western blot images from preliminary *in vitro* experiments show changes in phospho-JAK3^{Tyr980} levels after the restoration of PTPRK in NKYS cells and partial knockdown of PTPRK with shRNA#1 and shRNA#2 in SNK6 cells.