Identified a critical role in human hematopoiesis anddescribe a role as oncosuppressor in chronic myeloid leukemia (CML).

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Abstract

Protein tyrosine phosphatase receptor gamma (PTPRG) is a ubiquitously expressed member of the protein tyrosine phosphatasefamily known to act as a tumor suppressor gene in many different neoplasms with mechanisms of inactivation includingmutations and methylation of CpG islands in the promoter region. We identified a critical role in human hematopoiesis anddescribe a role as oncosuppressor in chronic myeloid leukemia (CML). We have described PTPRG expression in various tissuesand recently developed a monoclonal antibody capable of recognizing the native antigen of this phosphatase by flow cytometry: weconfirmed PTPRG protein downregulation in CML patients at diagnosis in the Philadelphia-positive myeloid lineage (includingCD34+/ CD38bright/dim cells). After effective tyrosine kinase inhibitor (TKI) treatment, its expression recovered in tandem withthe return of Philadelphia-negative hematopoiesis. Of note, PTPRG mRNA levels remain unchanged in tyrosine kinase inhibitors(TKI) non-responder patients, confirming that downregulation selectively occurs in primary CML cells. We have also identified anovel regulative loop involving CTNNB1 gene. The availability of this unique antibody permits its evaluation for clinical applicationincluding the support for diagnosis and follow-up of these disorders. Evaluation of the role of PTPRG in health and disease isfacilitated by the availability of a specific reagent capable to specifically detect its target in various experimental conditions.

keywords: β-catenin, PTPRG, chronic myeloid leukemia, methylation, tumor suppressor, tyrosine phosphatase, DNMT, 5-azacytidine

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Introduction:

Chronic myeloid leukemia (CML) is a myeloproliferative disease affecting approximately 1 per 200,000 persons per year in industrialized countries. Many treatment improvements have been achieved recently, especially in the development of new drugs, but a mortality rate of 2%–3% per year remains. A distinctive feature of CML is the reciprocal translocation, originating in hematopoietic stem cells (HSCs), between the long arms of chromosomes 9 and 22, i.e., t (9;22) (q34;q11). This genomic aberration generates a new fusion gene, BCR-ABL1, which encodes for a tyrosine kinase held accountable for the neoplastic transformation of these cells by affecting normal cellular pathways essential for tissue homeostasis, and thus causing the alteration of crucial cellular processes, such as apoptosis, cell cycle and autophagy. In this context, one primary goal of the research is to identify the regulatory mechanisms antagonizing the kinase activity of BCR-ABL1 and, possibly, of other vital effectors intersecting this pathway, as players other than BCR-ABL1 have been involved in the pathogenesis of the disease. Protein tyrosine phosphatase receptor type γ (PTPRG) is a member of the protein tyrosine phosphatase (PTP) family, featuring an extracellular and a single transmembrane region and two tandem intracytoplasmic catalytic domains.

PTPRG is widely expressed in human tissues and is involved in the regulation of cell growth, differentiation, mitotic cycle, and oncogenic transformation . he gene encoding for this phosphatase is located in a chromosomal region (3p21-p14), frequently deleted in renal cell and lung carcinoma, where PTPRG acts as a tumor suppressor. Epigenetic events, such as hyper-methylation of its promoter region, negatively regulates the transcription of PTPRG, as demonstrated in CML and childhood acute lymphoblastic leukemia Re-expression of this protein occurs in the leukocytes of CML patients following targeted therapy Once activated, PTPRG can reduce the phosphorylation level of BCR-ABL1 and some of its key targets, such as CRK-L and STAT5. β-catenin (CTNNB1), the most critical nuclear effector of Wnt signaling, is an essential component of cadherin-based adherents junctions. The cellular levels of β-catenin are regulated by its phosphorylation and consequent binding to the destruction complex, including APC, Axin1, glycogen synthase kinase-3 (GSK3β) and casein kinase IOnce stabilized, β-catenin moves to the nucleus and binds TCF4/ LEF (T-cell factor/lymphoid enhancer factor) transcription factors, thereby promoting the transcription of genes involved in neoplastic transformation, such as MYC, Cyclin D1 (CCND1) and the DNA (cytosine-5-)-methyltransferase 1 This methyltransferase, with a preference for hemimethylated sites, causes the down-regulation of different tumor suppressor genes. BCR-ABL1 phosphorylates β-catenin on Tyrosine 654, thus increasing its stability and nuclear translocation. Imatinib mesylate (IM), a specific inhibitor of BCR-ABL1 activity, causes a decrement of β-catenin expression, both in established cell lines and in primary cells derived from CML patients in blast crisis. We found that, in CML cells, higher

expression of PTPRG correlates with dephosphorylation and increased β-catenin-degradation. Moreover, we demonstrated that they are part of the same molecular complex. At the same time, β-catenin promotes DNMT1 transcription, causing PTPRG silencing through the hypermethylation of its promoter region, thus indicating a pivotal role in the BCR-ABL1 activatedpathway.

Result:

K562 cells were stably transfected with PTPRG cDNA (K562 γ1), to restore the expression of PTPRG in this CML cell line. We also subcloned LAMA-84 (another CML cell line) cells and isolated high and low PTPRG-expressing clones, as demonstrated by qRT-PCR and immunoprecipitation experiments. We analyzed the expression of total and tyrosine-phosphorylated BCR-ABL1 and β-catenin proteins in K562 cells expressing or not PTPRG and in both LAMA-84 clones after the treatment with a PTPRG inhibitorat a concentration of 2 and 10 μM. We demonstrated that, in PTPRG-positive cells, the inhibition of PTPRG leads to the BCR-ABL1 Y245 and β-catenin Y654 phosphorylation increment, in tandem with a raised level of total protein Opposingly, PTPRG-negative cells were not responding to the treatment with PTPRG IN, in terms of the effect on phosphorylation or total expression levels for these two proteins: they remain highly-expressed at levels comparable to K562 γ1 after the inhibition of PTPRG. We confirmed that inhibition of BCR-ABL1 activity following treatment with imatinib mesylate in K562 cells causes the dephosphorylation of β-catenin Y654 and consequent protein degradation, as demonstrated by Gambacorti-Passerini's group in 2008. The interference with BCR-ABL1 activity by PTPRG is further supported by the finding that, in K562 γ1 cells, β-catenin and its Y654 phosphorylated form are strongly reduced in comparison with the mock control clone, making the evaluation of relative phosphorylation level challenging to assess. According to the data described above, the knock-down of PTPRG through RNA interference in unfractionated LAMA-84 cells resulted in an evident increase of phospho- and total- β-Catenin, as demonstrated by Western blotting and immunofluorescence experiments. Previous studies reported that BCR-ABL1 increases β-catenin stability through its phosphorylation activity in CML cells It can thus reasonably be expected that BCR-ABL1, β-catenin, and PTPRG belong to the same protein complex. So, we first evaluated the presence of β-catenin and PTPRG in a multi-molecular complex, by performing pull-down assay experiments. As a "bait" protein, we used the inactivated PTPRG substrate-trapping mutant recombinant protein immobilized on nickel-agarose beads and challenged with protein extracts from K562 (BCR-ABL1 positive) and U937 (BCR-ABL1 negative) cell lines. Co-immunoprecipitation experiments in cells in vivo expressing the substrate trapping form of PTPRG (K562 D1028A) confirm that β-catenin also binds PTPRG in a fully native condition As the additional confirmation of β-catenin-proteolysis events driven by PTPRG, we inhibited β-catenin degradation using the proteasome 26S subunit inhibitor, MG-132, in K562 cells expressing, or not, PTPRG. The proteasome inhibitor blocked the degradation of the protein, but dephosphorylation still occurred in the presence of active PTPRGindeed suggesting that PTPRG dephosphorylates β-catenin. To further strengthen this finding, we treated the unfractionated PTPRG-positive LAMA-84 for two hours with the proteasome inhibitor MG-132 (10 μM) alone, and with the two inhibitors PTPRG IN (10 μM) and MG-132 (10 μM) combined, respectively. In keeping with the proposed mechanism, restored expression of the total β-catenin protein was associated with an increased level of Y654 phosphorylation in the sample treated with both inhibitors

We next evaluated whether β-catenin transcriptional targets were vicariously affected by PTPRG expression in leukemic cells, as should be expected by the previous findings.

Since we have previously demonstrated that PTPRG expression is linked to the promoter methylation levels we hypothesize that DNMT proteins might negatively affect the transcription of this phosphatase. We focused on DNA (cytosine-5)-methyltransferase 1 (DNMT1), a DNA-binding enzyme responsible for the down-regulation of many tumor suppressor genes through hypermethylation of their promoter regions and a downstream effector of APC/β-catenin/TCF4 signaling. It has been reported that the inhibition of β-catenin/TCF4 transcriptional activity, through the N-terminal deletion dominant-negative mutant, decreases DNMT1 mRNA levels. Interestingly, DNMT1 transcript inversely correlates with PTPRG expression, as shown by the differential expression level in K562, LAMA84, and PTPRG-silenced LAMA84. Moreover, in K562 cells expressing β-catenin (K562 mock), the β-catenin signal disruptor PNU-74654, which prevents β-catenin binding to TCF4/LEF transcriptional co-factor, leads to the down-regulation of DNMT1 mRNA

Materials & Methods:

Cell Lines and Transfection: The CML cell lines K562 (American Type Culture Collection, Manassas, VA, USA), LAMA-84 and the human histiocytic lymphoma cell line U937 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) were cultured in RPMI 1640 medium, supplemented with 10% Fetal Bovine Serum (FBS) and 2 mmol/L of Ultraglutamine 1-(Lonza, Milan, Italy). Full-length human PTPRG cDNA, mutant PTPRG D1028A, or empty vector (pCR 3.1, Thermo Fisher Scientific, Monza, Italy), used to transfect K562 cells, were previously described.

Inhibitors: K562 and LAMA-84 cell lines were treated with the PTPRG inhibitor (from now on named PTPRG IN) 3-(3,4-dichlorobenzylthio) thiophene-2-carboxylic acidat 0.05 to 50 μM and with imatinib mesylate (IM) (Selleckem, Houston, TX, USA), 1 or 5 μM, for 2 h at 37 °C. The proteasome 26S unit inhibitor, MG-132, (Santa Cruz Biotechnology Inc, Heidelberg Germany) was used at 10 μM for 2 h at 37 °C. Moreover, 5-azacydine (MilliporeSigma, Milan, Italy) was used at 10 μM for 72 h at 37 °C. PNU-74654 (MilliporeSigma, Milan, Italy) was used at 20 μM for 12 h at 37 °C.

Clonogenic Assay: LAMA-84 and K562 cells were resuspended in IMDM (Thermo Fisher), with 2% FBS and 0.3% NaHCO3 (MilliporeSigma, Milan, Italy), at a concentration of 16.5 × 103

or 33 × 103 cells/mL. Then, 50 µL of this cellular dilution were mixed with Methocult (StemCell Technologies Germany GmbH, Cologne, Germany) and PTPRG IN at different concentrations (0.005–0.1–0.2 µM) or DMSO and transferred in 24-well plates. After 8 days, cell colonies in each well were stained with 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (MilliporeSigma, Milan, Italy) and, after 3 h at 37 °C, captured and quantified with ImageQuant LAS 4000 colony counting module

Small Interfering RNA (siRNA) Transfection: Small interfering RNA (siRNA) targeting PTPRG (siPTPRG, No s-11550), siRNA targeting DNMT1 (siPTPRG, No s-4215), and negative control (scrambled sequence) were purchased from Applied Biosystems, Thermo Fisher Scientific, Milan, Italy. PTPRG-negative K562 and LAMA-84 cells were transfected at a concentration of 1 × 105/mL with 30 nM of siRNAs, using siPORT™ NeoFX™ Transfection Agent (Applied Biosystems®, Thermo Fisher Scientific, Milan, Italy), according to the manufacturer's instructions. Cells were cultured for 72 or 96 h, washed twice with cold TBS, and lysed.

Pull-Down Assay: For the pull-down assay, 3 mg of total bacterial protein lysates were conjugated to 30 µL of HIS-Select Nickel Affinity Gel (MilliporeSigma, Milan, Italy) and incubated, after extensive washing, with 500 µg of protein lysates from K562 and U937 cell lines for 3 h at 4 °C, with gentle rocking. The beads were collected, washed twice with lysis buffer and once with cold TBS, and then subjected to SDS-PAGE andWestern blotting.

Western Blotting: Cells were lysed in Lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 10% Glycerol and 0,5% Nonidet P40), or in boiling Sample Buffer. Then, 10–30 µg of proteins were loaded onto 6 to 12% polyacrylamide, 0.1% SDS gels, and electro-blotted on nitrocellulose membranes (GE Healthcare Europe GmbH, Milan, Italy). Blocking solution was obtained using TBS-0.05% Tween®20/BSA 5% or fat-free milk 5%. All antibodies were diluted in TBS-0.05% Tween®20/BSA or fat-free milk 1%–5%. The membranes were washed and assayed with ECL (MilliporeSigma, Milan, Italy) after overnight incubation at 4 °C, three times washing with TBS-0.05% Tween®20 (TBS-T) and incubation with appropriate HRP-conjugated secondary antibodies.

Statistical Analysis:

The data analysis was performed using GraphPad 8.3.0 Instat software (GraphPad Software, San Diego, CA, USA). The Student's two-tailed unpaired t-test was applied to qRT-PCR and Colony assay (number and volume of colonies) experiments. For qRT-PCR analysis, we considered absolute values, even though the graphs show just the fold-change, representing the data more clearly. Also, the student's two-tailed unpaired t-test was applied to the densitometric comparisons depicted inEach PTPRG IN condition was compared to the DMSO control. The analysis was done separately for each different protein employing ImageJ software Results with a p-value < 0.05 were considered statistically significant.

Biography:

Claudio Sorio has completed his MD from University of Verona, Italy and his PhD and Postdoctoral studies from Thomas Jefferson University, Philadelphia, USA. Hespecilized in Surgical Pathology at the same university. He is the Director of Cystic Fibrosis Traslational Research Laboratory "D Lissandrini" at the University of Veronaand Head of the Biomarker Laboratory at the same institution. He has published more than 74 original papers and several reviews and book chapters in reputed journalsand has been serving as an Editorial Board Member of reputed journals.