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Overexpression of PTP4A3 in ETV6-RUNX1 acute lymphoblastic leukemia

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ABSTRACT

Cell signalling, which is often derailed in cancer, is a network of multiple interconnected pathways with numerous feedback mechanisms. Dynamics of cell signalling is intimately regulated by addition and removal of phosphate groups by kinases and phosphatases. We examined expression of members of the PTP4A family of phosphatases across acute leukemias. While expression of *PTP4A1* and *PTP4A2* remained relatively unchanged across diseases, *PTP4A3* showed marked overexpression in ETV6-RUNX1 and BCR-ABL1 subtypes of precursor B cell acute lymphoblastic leukemia. We show that *PTP4A3* is regulated by the ETV6-RUNX1 fusion, but noticed no marked impact on cell viability either after *PTP4A3* silencing or treatment with a PTP4A3 inhibitor. Regulation of *PTP4A3* expression is altered in specific subgroups of acute leukemias and this is likely brought about by expression of the aberrant fusion genes.

1. Introduction

Acute leukemia is the most common cancer in childhood. *ETV6-RUNX1* (E/R) fusion gene is present in around 25% of cases of childhood precursor B cell acute lymphoblastic leukemia (pre-B-ALL) [1]. *E/R* fusion is generated by translocation between chromosomes 12 and 21 (t[12,21][p13;q22]) and creates an aberrant fusion transcription factor which induces repression of regulatory sequences and gene transcripts genome-wide [2]. Presence of *E/R* fusion is associated with good prognosis so that the overall survival exceeds 90% [3].

Adept regulation of cellular signalling is essential in controlling cellular proliferation. Kinases and phosphatases maintain a precise balance of intracellular signalling activity, and this balance is often deregulated in malignancies. The protein tyrosine phosphatase 4A (PTP4A) (also known as phosphatases of regenerating liver, PRL) are small, approximately 20 kDa proteins with dual specificity phosphatase activity [4]. The family consists of three members (PTP4A1-3) that share a high degree of sequence identity and harbour a phosphatase domain. Further, they contain a C-terminal consensus prenylation motif CaaX, which is important for localization to the plasma membrane and early endosomal compartments [4–7]. Phosphatases of PTP4A family participate in a wide range of cellular activities, including cell proliferation, migration and invasion [4].

In colorectal, breast, and gastric cancers, *PTP4A3* overexpression is correlated with poor prognosis and progression to metastasis [4]. In a murine model of colon carcinogenesis, *PTP4A3* knockout mice exhibited significantly fewer tumors than wild-type mice [8]. High expression of *PTP4A3* is associated with poor prognosis in acute myeloid leukemia (AML) with normal karyotype [9–11]. It is upregulated in human chronic myeloid leukemia (CML) cell lines, primary CML patient samples and Ph + ALL patients [12,13]. PTP4A3 is a downstream target of BCR-ABL1 fusion although the mechanism of action is unknown [13]. In multiple myeloma, *PTP4A3* is overexpressed and its knockdown inhibits cell migration [14]. Depending on expression level, *PTP4A3* may exhibit either positive or negative effects on cell cycle regulation [11].

Members of the PTP4A family of phosphatases have been associated with several hematological malignancies. This motivated us

Abbreviations: E/R, ETV6-RUNX1; pre-B-ALL, precursor B cell acute lymphoblastic leukemia; PTP4A, the protein tyrosine phosphatase 4A; PRL, the phosphatases of regenerating liver; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; shRNA, short hairpin RNA; GRO-seq, global nuclear run-on sequencing.

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to investigate their expression across acute leukemias, and specifically the role of PTP4A3 in ALL.

2. Materials and methods

2.1. Cell lines and cell culture

Previously created stable cell line models with an inducible *E/R* fusion in NALM-6 cells and *E/R* short hairpin RNA (shRNA) knockdown in REH cells were used [2]. Cells were cultured in RPMI (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 2 mM L-glut, 100 U penicillin, 100 μg/ml streptomycin and 10% Tet System Approved FBS (Clontech) (NALM-6-Tet-cells) or 10% FBS (Gibco) (REH, NALM-6) at 37 °C in 5% CO_2. The expression of *E/R* was induced with 500 ng/ml doxycycline (Clontech). The shRNA knockdown and the induced expression of *E/R* were confirmed with RT-qPCR (Ssofast EvaGreen, BioRad, Hercules, CA, USA) with fusion gene specific primers (Supplemental Table 1).

2.2. Quantitative PCR

Total RNA extractions were performed using either GeneJET RNA Purification Kit (Thermo Fisher Scientific) and DNA-free™ DNase Treatment & Removal kit (BioRad), or using PureLink™ RNA Mini Kit with On-Column PureLink® DNase Treatment Protocol (Ambion® by Life Technologies). 1 μg of RNA was used as a starting material to perform cDNA synthesis with iScpript (BioRad). RT-qPCR reactions were performed with SsoFast EvaGreen® Supermix (BioRad) according to manufacturer's instructions. BioRad CFX96™ Real Time System (BioRad) was run with the following program: initial denaturation at 96 °C for 30 s, 39 cycles of denaturation at 96 °C for 2 s, annealing at 60 °C for 5 s, and plate read. RT-qPCR measurements were repeated from three independent experiments performed in triplicate, and the relative $2^{-\Delta\Delta C}{}_T$ method was used for quantification [15]. ChIP-qPCR was performed in duplicate runs from two independent biological replicates and the percent input method was used to normalize the data (2% of starting material as input). All qPCR primer sequences are listed in Supplemental Table 1. The level of *PTP4A3* mRNA expression between cell lines was evaluated with Mann-Whitney *U* test.

2.3. Chromatin immunoprecipitation, GRO-seq assay, and microarray data sets

Cells were crosslinked and DNA-protein interactions were immunoprecipitated using two pooled antibodies against ETV6 (sc-166835, Santa Cruz Biotechnology Inc., Dallas, TX, USA; RRID:AB_2101020 and HPA000264, Atlas Antibodies, RRID:AB_611466) as described in Teppo et al. [2]. Nuclei were extracted and the global nuclear run-on (GRO-seq) reaction was performed as described in Heinäniemi et al. [16]. To study differential gene expression, a previously combined and processed database containing microarray samples was utilized [16]. Two other data sets containing gene expression profiles in patients with pediatric ALL, GSE47051 [17], and data from Pediatric Cancer data portal (St. Jude Children's Research Hospital) [18], were retrieved. Differential expression of *PTP4A3* in distinct subgroups was evaluated with Kruskal-Wallis *H* test and Mann-Whitney *U* test.

2.4. Cell viability assays and inhibition of PTP4A3

Fresh media was changed on the REH and NALM-6 cells, and cells were plated at 1 million/ml on 6 well plates. To induce *E/R* or luciferase expression in NALM-6 cells, doxycycline (Clontech) was added at 500 ng/ml. After incubating plated cells for 24 h, 10 000 cells per well were plated in 96 wells with the desired concentration of PRL-3 inhibitor I (Sigma, Lot # 047K4614V) concentrations. The doxycycline treatment was continued in E/R induction samples during the entire experiment. Cells were allowed to grow up to 96 h. 10 μl Alamar Blue reagent (Life technologies) was added to each well at each time points. After 2 h, fluorescence was measured with excitation of 560 nm and emission of 590 nm using the Tecan fluorometer Infinite 200 (Tecan, Switzerland). Three technical replicates per sample were included in each proliferation experiment. Difference in cell viability between inhibitor cultured cells and control cells was evaluated with Student's *t*-test.

3. Results

3.1. High expression of PTP4A3 in the t(12;21) and t(9;22) subtypes of ALL

We investigated expression of members of PTP4A family of phosphatases across acute leukemias and healthy cells of hematopoietic origin. To this end, a large collection of previously combined and processed microarray samples was utilized [16]. Subgroup consisting of leukemic and normal healthy samples was selected and a "leukemia-map" was generated by using t-SNE algorithm to visualize the data in two dimensions [19], and expression of the *PTP4A* family members was examined. As shown in Fig. 1A, *PTP4A1* was expressed indifferently across diseases and healthy cells, whereas *PTP4A2* showed increased expression among some T-ALL samples. In contrast, *PTP4A3* showed markedly increased expression among pre-B-ALL and a few T-ALL cases (Fig. 1A). Strong expression of *PTP4A3* co-occurred mostly with the fusion positive subtypes of ALL, namely the E/R and BCR-ABL1/t(9;22) cases.

The expression of *PTP4A3* in the E/R subtype was approximately three to four-fold higher as compared to other pre-B-ALL leukemias (Fig. 1B), and this difference was statistically significant (Mann-Whitney *U* test p = 1.894997e-52 when E/R is compared to other subtypes, see Supplemental Table 2 for details). As further validation, we retrieved two more leukemia data sets (GSE47051 [17] and PeCan [18]), and *PTP4A3* showed similarly high expression among E/R and BCR-ABL1 positive leukemias in both data sets (Fig. 1B). At the same time, MLL-rearranged leukemias exhibited low level of *PTP4A3* across all three data sets.

3.2. PTP4A3 expression is regulated by E/R fusion

We next sought to determine if *E/R* fusion directly regulates *PTP4A3*. To this end, a panel of leukemia cell lines was screened by RT-qPCR for the expression of *PTP4A3* (Fig. 2A). REH cells, which endogenously carry the *E/R* fusion, exhibited strongest expression while KOPN-8, 697 and healthy T-cells and B-cells showed moderate expression. Another pre-B cell line NALM-6 evidenced low expression along with majority of the tested T-ALL lines. We generated two additional cell line models: a NALM-6 cell line with an inducible *E/R* fusion (NALM-6-E/R) and a REH cell line with knockdown of endogenous *E/R* fusion (REH-shE/R) [2]. As shown in Fig. 2B, induction of *E/R* in NALM-6 cells increased *PTP4A3* mRNA level. Knockdown of endogenous *E/R* fusion in REH cells did not significantly decrease expression of *PTP4A3* at the level of mature mRNA transcript (Fig. 2C). However, when the transcription was examined at primary transcript level, as assayed by GRO-seq, the decrease in signal intensity was more evident (Fig. 3A). When leukemia patient samples were profiled using the GRO-seq assay, *PTP4A3* showed robust expression in E/R cases and also to varying degrees among other subtypes (Fig. 3A).

Our data suggested that *PTP4A3* expression may be regulated by the E/R fusion itself, either directly or indirectly, and this moti-