Phosphatase Sequencing of Pediatric Acute Myeloid Leukemia Reveals a Novel Mutation in the Phosphatase Gene PTPN4

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https://doi.org/10.31083/j.jmcm.2018.01.001

Abstract

It has been well established that dysregulated activation of kinases is essential for the development of acute myeloid leukemia (AML). In contrast, little is known about the role of their dephosphorylation counterparts, the tyrosine phosphatases. Here we performed whole tyrosine phosphatome sequencing in 15 pediatric AML samples and found a somatic P394L mutation in the FERM-adjacent region of PTPN4. In the absence of a crystal structure of PTPN4, bioinformatics analysis with the software tool PROVEAN (Protein Variation Effect Analyzer) revealed that this P394L mutation is expected to inflict a deleterious effect on the phosphatase activity of PTNP4. Exploring the frequency of this PTPN4 mutation in 227 acute leukemia samples uncovered an additional silent A364A mutation in exon 13 of PTPN4. No additional mutations were found, which further emphasizes the low mutation burden in pediatric AML. Further functional studies are warranted to explore the actual impact of this P394L mutation on the structure and/or function of PTPN4.

Keywords

Phosphatases; PTPN4 mutation; Acute myeloid leukemia

Submitted: October 22, 2017; Accepted: November 6, 2017

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease caused by genetic alterations, resulting in enhanced proliferation and impaired differentiation. Unraveling the molecular characteristics of AML in the past decades has provided important insights leading to a better understanding of the molecular basis of this disease and patient stratification for risk-group directed personalized therapy [1]. However, current treatments protocols are still hampered by side effects and despite complete remissions in the majority of patients, a substantial number of patients relapse (depending on age and AML subtype), hence posing a major obstacle towards curable treatment [2]. Therefore, the development of novel tumor-specific treatments is in burningly needed, including small molecules directed against AML-specific mutations. An example of a well-characterized mutation is an ITD in the receptor tyrosine kinase FLT3, which is one of the most common genetic abnormalities associated with pediatric AML correlating with poor prognosis [3, 4]. Recent high-throughput genome wide sequencing has shed light on the role of somatic mutations, including signal transduction molecules involved in AML [5]. These mutations generally involve protein tyrosine kinases (PTKs) that confer upon these cells a proliferative advantage. The proteins which act in concert with PTKs to regulate various cellular processes are protein tyrosine phosphatases (PTPs). Genetic aberrations as well as up- or down-regulation of these genes are involved in different types of cancer [6]. Although PTPs are crucial counterparts of PTKs, their physiological function and relevance in human cancer have not been investigated to the same extent in AML. In this respect, the human tyrosine phosphatome comprises 107 genes and is divided into four groups based on their amino acid sequence and catalytic domains [7]. A comprehensive mutational analysis has been performed on the tyrosine phosphatome in colorectal cancer, which revealed somatic mutations in six PTPs [8].

In the current study we investigated the possible involvement...
Table 1. List of sequenced tyrosine phosphatases (PTPs) and their aberrations in various leukemias

| PTP family                  | Genes                     | Aberrant gene | Type of leukemia | Type of abberation | reference |
|-----------------------------|---------------------------|---------------|------------------|--------------------|-----------|
| Class I Non-receptor PTPs   | PTPN1-6, PTPN7, PTPN9     | PTPN2         | T-ALL            | Deletion           | [9]       |
| (17 genes)                  | PTPN11, PTPN14, PTPN18, PTPN20, PTPN21-23 | PTPN2 | AML              | High mRNA expression | [10] |
|                             |                           | PTPN4         | AML              | Low mRNA expression | [11] |
|                             |                           | PTPN6         | AML              |                      |           |
|                             |                           | PTPN7         | T-ALL            |                      |           |
|                             |                           | PTPN11        | AML              | High mRNA expression | [10] |
|                             |                           | PTPN22        | AML              | Low mRNA expression | [12, 13] |
|                             |                           | PTPN22        | AML/ALL/JMML     |                      |           |
|                             |                           | PTPN7         | AML              | Overexpression      | [14] |
|                             |                           | PTPN11        | ALL              |                      |           |
|                             |                           | PTPN22        | ALL              |                      |           |
|                             |                           | PTPN22        | CLL              |                      |           |
| Class I Receptor PTPS       | PTPRA-H, PTPRJ-K, PTPRM-N-N2-O, PTPRQ-Z | PTPRC | AML              | High mRNA expression | [10] |
| (21 genes)                  |                           | PTPRC         | T-ALL            | Mutation            | [21]     |
|                             |                           | PTPRE         | AML              | High mRNA expression | [11] |
|                             |                           | PTPRG         | B-ALL            | Methylation         | [22]     |
|                             |                           | PTPRJ         | AML              | Inactivation        | [10]     |
|                             |                           | PTPRK         | ALL              | Methylation         | [23]     |
|                             |                           | PTPRM         | ALL              | Methylation         | [23]     |
|                             |                           | PTPRO         | ALL              | Methylation         | [23]     |
|                             |                           | PTPRT         | T-LGL            | Mutation            | [25]     |
|                             |                           |               | AML              | Mutation            | [26]     |
| Class I VH1 like or Dual specificity PTPs | MK-STYX, STYX, RNGTT, EPM2A, PTP4A1-3, PTP9Q22, CDKN3, SSH1-3, TIP, TPTE, TNS | PTP4A1-2 | AML              | High mRNA expression | [10] |
| (61 genes)                  |                           | PTP4A3        | CML              | Uregulation         | [27]     |
|                             |                           | PTEN          | T-ALL            | Mutation, deletion  | [28]     |
|                             |                           | PTEN          | AML              | High mRNA expression | [29] |
|                             |                           |               |                  | Deletion            | [30]     |
|                             |                           | DUSP1         | AML              |                      |           |
|                             |                           | DUSP2         | AML              |                      |           |
|                             |                           | DUSP6         | AML              |                      |           |
|                             |                           | DUSP7         | AML              |                      |           |
|                             |                           | DUSP10        | ALL              |                      |           |
|                             |                           | DUSP21, DUSP22 | MTMR6                   | AML              | High mRNA expression | [11] |
|                             |                           | DUSP24, DUSP27 | MTMR11               | AML              | Overexpression | [32] |
|                             |                           | MTMR1-15      | AML              | Low mRNA expression | [11] |
| Class II and III (4 genes)  | ACP1, CDC25A-C            | ACP1          | AML              | High mRNA expression | [10] |

of PTP mutations in pediatric AML by performing whole tyrosine phosphatome sequencing using high throughput Sanger sequencing.

2. Materials and Methods

2.1. Patient samples and DNA isolation

Genomic DNA from 15 pediatric samples, used for the primary screening of tyrosine phosphatome, was isolated by QIAamp DNA Blood Mini Kit as recommended by the manufacturer (Qiagen, Germantoen, USA). Samples were selected based on normal karyotype, carrying no known type I or type II abnormalities, and having more than 90% blast cells. Genomic DNA of the remaining samples was isolated from cryopreserved cytopsins by overnight incubation with proteinase K (Roche Diagnostics Gmbh, Germany) at 52°C. Aqueous phase was transferred following the addition of a mixture of phenol: chloroform: isoamyl alcohol (Sigma life science, St Louis, MO, USA) and centrifugation for 10 min at 12,000xg. DNA precipitation was performed using 100% ethanol. Pellets were washed twice with 70% ethanol, air-dried and resuspended in water.

This study was approved by the individual Institutional Review Boards in the Netherlands and the Hannover Medical School Ethical Board according to national law and regulations and written informed consent was obtained for all patients.

2.2. Tyrosine phosphatome primers and amplification

PTPs were selected from a comprehensive published list [7].
Fig. 1. Mutational analysis of the **PTPN4** gene
(A) Schematic representation of **PTPN4** exons (top), domains (middle) and sequence data (bottom) of reference and mutated samples showing the mono-allelic **PTPN4-P394L** and **PTPN4-A364A** mutations. FERM domain: 4.1 protein Ezrin Radixin Moesin, PDZ domain: Post synaptic density protein Drosophila disc large tumor suppressor Zonula occludens-1 protein, PTP domain: protein tyrosine phosphatase. (B) Mutational analysis of **PTPN4** exon 13 by HRM in **PTPN4-P394L** and **PTPN4-A364A** mutated patients (red lines; in duplicate) and negative controls (blue lines). (C) HRM analysis is depicted for myeloid (red lines; in duplicate) and lymphoid (blue lines; in duplicate) compartments of both the **PTPN4-P394L** and **PTPN4-A364A** mutated patient following cell sorting compared to other patients (blue lines), which are negative for mutation in exon 13.

(Alonso) on the basis of the presence of a phosphatase domain. Unprocessed transcripts such as PTPRV were excluded. All genes included in this study are listed in **Table 1**. Exons of each PTP gene were divided into 300-500 bp, and in total, 1527 amplicons (all coding exons) were created by using the Limstill (LIMS for Identification of Mutations by Sequencing and TILLING) program (<http://limstill.niob.knaw.nl>). A complete list of (~6100) primers is available upon request. To design primers, we also used the LIMSTILL program in which parameters were set to design primers with an optimal melting temperature of 58°C. Two sets of primers located in the outer region of the amplicon were designed in order to perform a nested PCR on each amplicon in 384-well plates (GeneAmp9700, Applied biosystems). PCR reaction mixtures contained 0.2 µM of each forward and reverse primers, 200 µM of each dNTP, 25 mM Tricine, 7% Glycerol (w/v), 1.6% DMSO (w/v), 2 mM MgCl₂, 85 mM ammonium acetate (pH 8.7), and 0.2 U Taq polymerase in a total volume of 10 µl. 8 ng genomic DNA were used for the first PCR reaction and 1 µl out of the 400× diluted first PCR product were used as a template for the second PCR reaction, which contained 0.1 µM of each forward and reverse primers, 100 µM of each dNTP, 25 mM Tricine, 7% glycerol (w/v), 1.6% DMSO (w/v), 2 mM MgCl₂, 85 mM ammonium acetate (pH 8.7) and 0.1 U Taq polymerase in a total volume of 10 µl. Several reactions of each amplicon were tested on a 1% agarose gel containing ethidium bromide for the presence...
of the proper PCR fragment.

2.3. Sequencing reactions, purification and analysis

1 µl of 5× diluted second PCR product was used as template for the sequencing reaction in a total volume of 5 µl. This reaction contained BigDYE (v3.1; Applied Biosystems), 2.5× dilution buffer (Applied Biosystems) and 0.4 µM forward and reverse primers, which were implicated for the second PCR reaction. Pipetting steps were performed by Tecan genesis RSP200 liquid handling robots (with integrated TeMo96 and Velocity11 Vprep with BenchCell 4×). Thermocycling was performed for 40 cycles of 10 sec at 92°C, 10 sec at 50°C and 2 min at 60°C. Final products were purified by ethanol precipitation in 384-well plates as recommended by the manufacturer (Applied Biosystems) and analyzed on 96-capillary 3730XL DNA analyzer (applied biosystems) (Supplementary Fig. S1).

In order to identify single nucleotide substitutions, Poly-Phred program [9] was used to compare fluorescence-based sequences among the reference and patient sequences. For this reason, all patient sequences for a certain phosphatase gene were aligned with the reference sequence obtained from the Ensemble database. Poor quality sequences were excluded by the PolyPhred program automatically. Mutated positions were manually inspected afterwards and candidate mutations were introduced to LIMSTILL program. Any potentially interesting mutations identified using this method were verified using additional targeted Sanger sequencing.

2.4. High resolution melting analysis

Screening of PTPN4 exons to detect single nucleotide substitutions was carried out with high resolution melting (HRM) analysis by Lightcycler 480 II (Roche, Woerden, the Netherlands). The fluorescent dye intercalates into double stranded DNA and following melting of double stranded DNA, the fluorescence signal decreases and in case of single nucleotide change, a shift in the melting curve will be observed. The difference in the melting temperature is caused by the loss of integrity due to the nucleotide substitution. Applied amplification mix was used as recommended by the manufacture’s protocol (Roche). 5 µM forward and reverse primers were used for this amplification. Analysis was done by LightCycler 480 Software 1.5 (Roche). All HRM analyses were examined on gel for validation of the correct single PCR product. Any relevant change in HRM curve was verified using Sanger sequencing.

2.5. Flow cytometry sorting of myeloid and lymphoid cells

In order to isolate myeloid and lymphoid cells from patient samples, which are positive for a PTPN4 mutation, cells were FACs sorted (FACSaria, BD Biosciences, Breda, The Netherlands). Viable cells were gated based on 7AAD-PerCP. Leukemic blasts were characterized and sorted based on the expression of CD34-FITC (BD) and CD45-FTTC as well as low side scattering value. CD33 and CD13-APC, CD7-PE and CD19-APC-H7 were used to distinguish the normal lymphocytes. Following sorting of myeloid blast and lymphoid compartments, genomic DNA was isolated and HRM was performed. All antibodies were purchased from BD Biosciences.

3. Results and Discussion

Known SNPs were excluded and re-sequencing was performed to confirm candidate mutations. Analyzing the AML specimens sequence data revealed several sequence variations, from which a non-conserved point mutation (C>T) located at amino acid 394 (amplicon 13) of a non-receptor PTP, PTPN4, was confirmed. This mono-allelic mutation resulted in substitution of Proline to Leucine due to conversion of Cytosine to Thymine (Fig. 1A). The patient carrying this PTPN4-P394L mutation belonged to FAB-M2 subtype. PTPN4, also known as PTP megakaryocyte-1 (UniProtKB: P29074) encodes for a 924 amino acid protein and is composed of N-terminal FERM, central PDZ and C-terminal catalytic phosphatase domains. The FERM domain is divided into N-terminal, central, C-terminal PH-like and adjacent domains. While the FERM domain is involved in localizing the protein to the plasma membrane, the PDZ domain mediates protein-protein interactions within signal transduction complexes. The discovered PTPN4 mutation is located at a FERM-adjacent region, which regulates the activities of its neighboring FERM domains. In the absence of a complete crystal structure of PTPN4, we aimed at evaluating the possible impact of the P394L mutation on the function of this phosphatase using bioinformatics tools. Towards this end, we used the software tool PROVEAN (Protein Variation Effect Analyzer; http://provean.jcvi.org/index.php) which indicated that the PTPN4 P394L mutation is predicted to have a deleterious impact on the catalytic activity of this phosphatase with a PROTEAN score of -3.541. Furthermore, using the ConSurf server tool for the identification of functional regions in proteins, P394 emerged as a water-exposed residue.

Although many PTPN proteins are associated with different types of cancers, data about the functional role of PTPN4 in cancer is still lacking [10]. From a physiological perspective, PTPN4 has been shown to function in T-cell receptor and glutamate-delta2 receptor signaling, learning, and cerebellar synaptic plasticity [11–13]. Regarding its plausible role in cancer, it has been shown that PTPN4 silencing protects glioma cells against apoptosis [14]. Interestingly, it has recently been demonstrated that PTPN2, a non-receptor PTP belonging to the same family as PTPN4, is deleted in T-cell acute lymphoblastic leukemia [15].

Although we selected a homogenous group of normal karyotype AML cells to perform this sequencing study, the PTPN4 mutations may not be mutually exclusive from other aberrations. To explore the incidence of PTPN4 mutations, we screened all 26 PTPN4 amplicons in 106 randomly selected initial pediatric AML patient samples including all commonly occurring karyotypes and currently established AML-related mutations. HRM analysis was employed for performing large scale PTPN4 screening following optimization steps for each amplicon. Interestingly, we only found a silent PTPN4 A364A mutation (A>G also in amplicon 13) in one additional patient (Fig. 1B), that harbored a normal karyotype and belonged to the FAB-M2 subtype. Although it is not certain what the actual impact of this mutation on the phosphatase activity PTPN4 is, it has been described that even silent mutations can have substantial consequences [16]. The PTPN4 mutations did not coincide with known AML related mutations since we searched for FLT3/ITD, FLT3/TDK, NPM1, cKIT, N-and K-Ras and in CEBPα in these two samples.

Since we had no access to normal tissue or remission samples of the mutated patients, the myeloid and lymphoid compartments were FACs sorted in order to assess whether or not the identified mutation was a somatic mutation. We first selected viable white blood cells and then isolated CD45high and FSC/SSClow for lyn-
phocytes, whereas CD45dim and FSC/SSCmedium were employed for the sorting of myeloid blasts. The PTPN4 P394L mutation was only present in the myeloid cells and not in the normal lymphocytes, indicating that this mutation was somatically acquired during the process of leukemogenesis (Fig. 1C). This result was confirmed by direct sequencing of amplicon 13 in the myeloid and lymphoid compartments. In order to determine the frequency of this PTPN4 P394L mutation in other leukemic patients we screened four other cohorts, consisting of 64 initial and 48 relapsed pediatric AML, 125 adult AML and 32 pediatric ALL samples. However, the HRM analysis did not reveal any mutation.

Considering the importance of both tyrosine kinases and phosphatases in central cellular processes under physiological and pathological conditions, one would anticipate the presence of additional PTP mutations. This may be explained, at least in part, by the fact that kinase mutations are sufficient (and mutually exclusive) and many other mechanisms have been described to result in gain or loss of PTP function, which are associated with leukemia (Table 1). Thus far, exome sequencing of normal karyotype AML had only revealed a single PTP mutation in PTPRT, which appeared to be the same PTP mutation in colorectal cancer [8, 17, 18]. Although the frequency of the present PTPN4 somatic mutation is low in our study, this phosphatase might have relevance in AML. This is particularly true since it was shown that PTPN4 is one of 11 phosphatases, which are deregulated in AML [19]. Further investigation is warranted to determine the possible functional role of PTPN4 in AML.

Acknowledgments
This study was supported by KIKA (Children Cancer Free).

Conflict of Interest
The authors do not have any conflict of interest related to this manuscript.

Author Contributions
D.C., M.I., E.dB., Y.H., M.A., R.A.J.F.B., N.H., E.R.A., J.P. Z.K. performed research; M.I., G.J.L.K., T.K.vdB., C.M.Z., M.M.vdH-E., E.S.J.M.dB., E.C. J.C. designed the research study; G.J.L.K., V.dH., D.R., C.M.Z., M.M.vdH-E., E.S.J.M.dB. contributed patient materials and clinical data; M.I., E.dB., E.C. and J.C. analysed the data; D.C. M.I., Y.H. G.J.L.K., C.M.Z., M.M.vdH-E. and J.C. wrote the paper.

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