High PDGFRA expression does not serve as an effective therapeutic target in ERG-deleted B-cell precursor acute lymphoblastic leukemia

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Supplemental information for

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Short title: PDGFRalpha as therapeutic target in ERG-deleted BCP-ALL.

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Supplementary Online Methods

Methods

Primary leukemic cells, cell lines, and mesenchymal stromal cells

The study comprised children with newly diagnosed BCP-ALL with age at diagnosis ranging from 0 to 18 years. Written informed consent was obtained from parents or guardians, and institutional review boards approved the use of excess diagnostic material for research purposes. Clinical characteristics were kindly provided by the Dutch Childhood Oncology Group (the Hague, Netherlands). These studies were conducted in accordance with the Declaration of Helsinki. Mononuclear cells were isolated using density gradient centrifugation with Lymphoprep (Axis Shield, Norway) as previously described.1 All samples contained at least 90% leukemic blasts. Cytogenetic subtypes were determined by karyotype, fluorescence in-situ hybridization (FISH), and/or fusion-gene specific PCR. BCR-ABL1-like cases were identified using microarray gene expression profiling by the means of a 110 probeset classifier.2 Animal experiments were approved by the animal ethics committee (EMC 2863 (103-12-08)). Xenografts of BCP-ALL cells from patient D were established as previously reported (Steeghs et al., submitted manuscript) in NOD.Cg-Prkdc<sup>c<sup>scid<sup>/Il2rg<sup>tm1Wjl</sup>/Szl</sub></sup> (NSG) mice (Charles River, France) using intra-femoral injection. Leukemic cells were isolated from the spleen, frozen as viable cells, and subsequently thawed for protein isolation and ex vivo coculture assays. Leukemic cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) and routinely validated by DNA fingerprinting. Mesenchymal stromal cells (MSCs) were isolated from the bone marrow aspirate taken from a BCP-ALL patient after consolidation therapy as previously described.3 MSCs were negative for hematopoietic markers and positive for mesenchymal markers.3,4

Gene expression profiling and RT-qPCR

RNA and DNA was isolated using Trizol reagent (Life technologies) according to manufacturer’s instructions and RNA subsequently used for gene expression profiling and RT-qPCR. Gene expression profiling was performed using Affymetrix U133 plus 2.0 GeneChips. Detailed methods have been reported previously5, and data is deposited at NCBI’s Gene Expression Omnibus with accession numbers GSE13351 and GSE13425. High PDGFRA expression was defined based on the probe set 203131_at, with a cutoff at 2<sup>log</sup>-expression value >5.9 (see also Figure 1A). cDNA was generated using the Sensifast cDNA synthesis kit (Bioline), and RT-qPCR was performed in a standard SYBR green-based assay (ThermoFisher) on the ABI-PRISM 9700HT Fast Real-Time PCR System. Primer sequences were as follows: exon 3 forward: 5’-TTGGGGAGAGTGAAGTGA-3’, exon 3/4 reverse: 5’-TGGGTCTGGCACATAGAT-3’, exon 12 forward: 5’-GCTGCCATGACTCAAG-3’, exon 14 reverse: 5’-GCTCCCAGCAAGTTTACA-3’, exon 17 forward: 5’-CCGAGGAATGGGAGTTTTT-3’, exon 18 reverse: 5’-TGCCCCTCGACACATAGTT-3’.

Genetic aberrations of B-other cases

Genetic aberrations were determined by array-based comparative genomic hybridization (aCGH) and multiplex ligation-dependent probe amplification (MLPA) assays as previously reported.6 In brief, the SALSA P335 ALL-IKZF1 (a3) and the SALSA P202 Multiplex Ligation-dependent Probe Amplification (MLPA) assays (MRC-Holland) were
used to identify lesions in \textit{IKZF1}, \textit{CDKN2A}, \textit{CDKN2B}, \textit{ETV6}, \textit{PAX5}, \textit{RB1}, \textit{BTG1} and \textit{EBF1}. Copy number analysis with aCGH was performed using Agilent SurePrint G3 Hmn 4x180K arrays (Agilent Technologies, Amstelveen, the Netherlands). \textit{CRLF2} status was determined using gene expression array data: As described previously, cases with a signal intensity of the probe set 208303_s_at above the 90th percentile of the total BCP-ALL group were classified as \textit{CRLF2}-high.\textsuperscript{2}

**RT-PCR for detection of known fusion genes**

RT-PCR was performed on cDNA of 49 BCP-ALL cases from the B-other and \textit{BCR-ABL1}-like subtype, including 24 of the 26 patients with high \textit{PDGFRA} expression identified by microarray profiling. Primer sequences are reported in Supplemental Table 1. Forward primers were used as previously reported, except for an adaptation in BCR-ex7 forward (new sequence: 5'-TACAAGCCTGTGGACCGTGT-3').\textsuperscript{7-11} Forward primers for known fusion partners of \textit{PDGFRA} were confirmed to be functional in an RT-PCR using a reverse primer located within the partner gene. All forward primers were combined with \textit{PDGFRA} exon 14 reverse (see above), but an additional PCR run with \textit{FIP1L1} forward combined with \textit{PDGFRA}-R2 as published by Cools et al.\textsuperscript{9} PCR products were amplified as follows: 2min 50°C, 10min 95°C, five times 8 cycles of 15s 95°C and 1min at 60°C, 57°C, 54°C, 51°C, or 48°C, followed by 30s at 72°C. PCR products were separated on agarose gel and evaluated for bands by visual inspection under UV light.

**Fluorescence \textit{in situ} hybridization (FISH)**

FISH was performed using the FIP1L1/CHIC2/PDGFRA deletion probes by Cytocell (Cambridge, UK). Cytospins were made from freshly isolated cells or from viable frozen cells after thawing. They were dried and stored at -80°C until used. Prior to hybridization, samples were treated with 20 µg/mL pepsin in 0.01 M HCl at 37°C for 10 min and fixated in PBS containing 1% formaldehyde and 200 g/L MgCl\textsubscript{2} for 10min. Denaturation was performed for 5 min at 75°C and probes hybridized at 37°C overnight. Nuclei were stained with DAPI antifade and results evaluated and recorded with a Leica AF6000 workstation and Leica LAF LS imaging software version 2.0.0 build 1929 (Wetzlar, Germany). Visibility of blue DAPI signals in Figure 2A were enhanced using Adobe Photoshop CS6.

**Targeted locus-amplification (TLA)**

The precise methods for targeted locus amplification have been published previously.\textsuperscript{12} Viable frozen cells of BCP-ALL cases were thawed and directly used for the procedure. Following cross-linking in viable cells, digestion, and proximity-based re-ligation, targeted amplification was performed from a so-called anchor or viewpoint in the \textit{PDGFRA} gene and the amplification product was sequenced. Crosslinking and DNA isolation were performed according to manufacturer’s instruction. Custom primers were designed by Cergentis BV (Utrecht, Netherlands) to amplify regions of \textit{PDGFRA} that were previously reported to be involved in structural aberrations, and PCR products sequenced by Illumina paired-end sequencing. Raw reads in FASTQ format were aligned to GRCh37 using BWA mem. Whole genome coverage in a sliding window of 10 kb was calculated using Samtools view, and per base pair coverage near the \textit{PDGFRA} gene using the GATKs DepthOfCoverage tool. Coverage plots in a sliding window of 10 kb were then generated using R language.
Protein isolation and western blot

Primary BCP-ALL cells and cell lines were treated with recombinant human PDGF-BB (#8912, Cell Signaling, Danvers, USA) and inhibitors imatinib (10 µM) and CP673451 (1 µM, both Selleckchem, Munich, Germany) for the indicated time span and lysed using caspase lysis buffer (150 mM NaCl, 50 mM Tris, 10 mM EDTA, 1% Triton-X, 1mM DTT, 1mM PMSF, 2µg/ml Aprotinin, 1mM Sodium-Pervanadate, and 10mM Sodium-Fluoride, all Sigma Aldrich, St. Louis, USA). Nalm6 cells were serum starved for 2 hours, which was omitted in primary BCP-ALL cells to avoid massive cell death. Protein concentrations were quantified using a BCA assay (Thermo Fisher Scientific, Waltham, USA), and 25µg loaded and separated on precast polyacrylamide gels (BioRad, Hercules, USA). Proteins were transferred to nitrocellulose membranes using 1.3 A and 25 V for 13 minutes in the Bio-Rad Trans-blot Turbo system. Membranes were blocked with 5% BSA (w/v) in TBS containing 0.1% Tween-20 (TBST) for 2 h and probed with anti-PDGFRα #3174, anti-phospho-PDGFRα (Y762) #12022, (both Cell Signaling) or anti-β-actin (ab6276, Abcam, Cambridge, UK). Fluorescent-labeled secondary antibodies were obtained from Li-COR (Lincoln, USA), and blots were scanned using the Odyssey system (Li-COR). Odyssey software version 3.0 was used for image acquisition and signal quantification.

Cell culture, ex vivo drug sensitivity and co-culture model

Cell lines were cultured in RPMI-1640 GlutaMAX supplemented with penicillin, streptomycin, and fungizone (all Life technologies, Bleiswijk, Netherlands) and 10% (Nalm6) or 20% (EOL1) FCS. Primary BCP-ALL cells were cultured in RPMI-1640 Dutch modified supplemented with glutamine, insulin, transferrin, selenium, penicillin, streptomycin, fungizone, gentamycin, and 20% FCS. Ex vivo cytotoxicity assays of prednisolone, L-asparaginase, and vincristine were performed as previously described. In brief, freshly isolated primary ALL cells were incubated with a concentration range of prednisolone, vincristine, and L-asparaginase. After four days, cell viability was evaluated by adding MTT and measuring formazan-conversion with optical density measurement. LC50-values were calculated (concentration at which 50% conversion activity was measured relative to no-drug control cells) and scored according to the cut-offs defined by den Boer et al. For ex vivo co-culture assays, MSC were seeded one day prior to start. Leukemic cells were cultured at a concentration of 2*10⁶/mL in presence or absence of MSC, in presence or absence of 50 ng/mL recombinant human PDGF-BB, and with three different concentrations of Imatinib (12.5 µM, 1.6 µM, 0.2 µM) or CP673451 (1.25 µM, 0.16 µM, 0.02 µM). After four days, cells were harvested and analyzed by flow cytometry on a MACSquant analyzer 10 (Miltenyi Biotec GmbH, Bergisch Gladbach). Viable leukemic cells were quantified within a fixed volume by combined staining for CD19-APC (positive), Annexin-V-FITC (negative, both BioLegend, San Diego, USA), and propidium iodide (negative, Sigma Aldrich). The gating strategy was as previously reported.
Supplemental Tables and Figures

Table S1: Overview of fusion gene specific RT-PCR primers for detecting PDGFRA-rearrangements.

Table S2: Recurrent cytogenetic aberrations in PDGFRA high BCP-ALL.

Figure S1: RT-qPCR for PDGFRA.

Figure S2: Ex vivo drug sensitivity towards the 3 major therapeutic agents with prognostic relevance.

Figure S3: PDGFRA gene expression in ERG-wildtype and –deleted B-other cases.

Figure S4: Complete FISH results of tested BCP-ALL samples.

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Supplemental tables

Table S1: Overview of fusion gene specific RT-PCR primers for detecting PDGFRA-rearrangements.

<table>
<thead>
<tr>
<th>Fusion partner</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Tested samples (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIP1L1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>ACCTGGTGCTGATCTTTCTGAT</td>
<td>TGAGAGCTTTTTTCACTGGA</td>
<td>49</td>
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<tr>
<td>FIP1L1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>ACCTGGTGCTGATCTTTCTGAT</td>
<td>GCTCCGCAAGTTTACA</td>
<td>49</td>
</tr>
<tr>
<td>STRN&lt;sup&gt;11&lt;/sup&gt;</td>
<td>TCTGAGTTAACAAGATTCTGCTC</td>
<td>GCTCCGCAAGTTTACA</td>
<td>49</td>
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<tr>
<td>BCR&lt;sup&gt;10&lt;/sup&gt;</td>
<td>TACAAGCCTGGGACGGTGAT</td>
<td>GCTCCGCAAGTTTACA</td>
<td>49</td>
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<tr>
<td>CDK5RAP2&lt;sup&gt;7&lt;/sup&gt;</td>
<td>CAAAGGAGACTGCACCATCCG</td>
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<tr>
<td>ETV6&lt;sup&gt;11&lt;/sup&gt;</td>
<td>ACTGTAGACTGCTTTGGGAATTAC</td>
<td>GCTCCGCAAGTTTACA</td>
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<tr>
<td>KIF5B&lt;sup&gt;8&lt;/sup&gt;</td>
<td>TGAACACAGATCCAGAGCCATA</td>
<td>GCTCCGCAAGTTTACA</td>
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Table S2: Recurrent cytogenetic aberrations in PDGFRA high BCP-ALL.

MLPA and aCGH data used to generate Figure 1D. “Total” indicates the number of cases for which data was available, ”cases” indicates the number of cases in which the respective feature was identified. Frequencies were compared using Fisher’s exact test and Odds ratios are given with a 95% confidence interval. p-values <0.05 are printed in bold. Odds ratio is only given for significant associations. Chromosome 21 aberration: partial or complete amplification of chromosome 21, including germline trisomy. High CRLF2: High expression of CRLF2 by gene expression array as described above.

<table>
<thead>
<tr>
<th>Cytogenetic Feature</th>
<th>PDGFRA high</th>
<th></th>
<th>PDGFRA low</th>
<th></th>
<th>Fisher’s Exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (n)</td>
<td>Cases (n)</td>
<td>Frequency (%)</td>
<td>Total (n)</td>
<td>Cases (n)</td>
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<tr>
<td>ERG intragenic deletion</td>
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<td>63%</td>
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<td>Chromosome 21 aberration</td>
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<td>0%</td>
<td>84</td>
<td>23</td>
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<td>High CRLF2</td>
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<td>0%</td>
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<td>9p-deletion</td>
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<td>0%</td>
<td>83</td>
<td>19</td>
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<td>ETV6 deletion</td>
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<td>0%</td>
<td>87</td>
<td>23</td>
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<td>PAX5 deletion</td>
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<td>3</td>
<td>14%</td>
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<td>36</td>
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<td>CDKN2A or B deletion</td>
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<td>5</td>
<td>23%</td>
<td>87</td>
<td>43</td>
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<td>IKZF1 deletion</td>
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<td>2</td>
<td>9%</td>
<td>85</td>
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<td>0%</td>
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<td>RB1 deletion</td>
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<td>5%</td>
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<td>6</td>
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<td>BTG1 deletion</td>
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<td>1</td>
<td>5%</td>
<td>86</td>
<td>8</td>
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<tr>
<td>TCF3 deletion</td>
<td>16</td>
<td>1</td>
<td>6%</td>
<td>71</td>
<td>19</td>
</tr>
</tbody>
</table>
Supplemental figures

Supplemental Figure S1: RT-qPCR for PDGFRA.

RT-qPCR for PDGFRA mRNA levels in primary BCP-ALL samples, cell lines, and primary MSC with high (grey background) or low (white background) expression levels measured on gene expression arrays. Expression values are given relative to RPS20 housekeeping gene. Numbers on x-axis represent patient identifiers with reference to the samples used in in vitro experiments. Samples marked by roman numerals (patients I to V) were used in ex vivo cytotoxicity assays in Figure 2. Patient I* marks the xenograft sample derived from cells of patient I.
Supplemental Figure S2: *In vitro* drug sensitivity towards the 3 major therapeutic agents with prognostic relevance.

LC50-values for prednisolone, vincristine, and L-asparaginase in primary B-other BCP-ALL cases with high or low PDGFRA expression. *Ex vivo* sensitivity was determined by means of an MTT assay as previously described and compared using a Mann-Whitney U test.1 **: p<0.01
Supplemental Figure S3: Complete FISH results of tested BCP-ALL samples.

A EOL-1 and Nalm-6 cell lines. B 25 of 26 patient samples with high PDGFRα expression. C 10 representative cases of BCP-ALL cases with low PDGFRα expression.
**Supplemental Figure S4: PDGFRA gene expression in ERG-deleted B-other cases.** PDGFRA gene expression was determined by the same probe sets as presented in Figure 1, and groups separated according to the deletion status of ERG.

A

**PDGFRA (215305_at)**

- **log-expression (a.u.)**
- **no ERG deletion (n = 77)**
- **ERG deleted (n = 10)**

B

**PDGFRA (203131_at)**

- **log-expression (a.u.)**
- **no ERG deletion (n = 77)**
- **ERG deleted (n = 10)**
Supplemental Figure S5: Effect of PDGFR inhibition on primary BCP-ALL cells cultured with and without ligand.

Nalm6 and three primary BCP-ALL samples with high PDGFR expression (patients I, IV, and V) were tested in mono- and co-culture for sensitivity towards PDGFR inhibitors as in Figure 2C, but with or without recombinant ligand (50ng/mL PDGF-BB). Each dot represents one patient, matched samples with or without PDGF-BB are connected by lines. Dashed line indicates 100% survival relative to the untreated control, ‡ indicates PDX sample of patient I, which was used for western blot analysis in Figure 2A.
References


