Cloning and characterization of a novel druggable fusion kinase in acute myeloid leukemia

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Supplementary Material

Next generation sequencing

Targeted resequencing of the *PDGFR(beta)* fusion transcript was performed using the FusionPlex® Heme v2 panel (ArcherDX, Boulder, CO) and 100 ng total RNA as input. The library was quantified in dilutions of $1:10^5$ and $1:10^6$ by qPCR using the NEBNext® Library Quant Kit for Illumina® as recommended on a CX96 qPCR cycler (BioRad, Hercules, CA). Concentrations were calculated from C_t values assuming a mean library size of 200 base pairs (bps). For sequencing, the library was diluted to a final concentration of 17.5 nM. Sequencing was performed on a MiSeq instrument (Illumina, San Diego, CA) with a standard v2 flowcell and a read length of 2x 150 bps with two index reads. Sequencing results were analyzed using the Archer® Analysis pipeline (version 5.1) with advanced settings (Error Correction: *ON*, Read Depth Normalization: *1,500,000*) and, apart from that, default parameters.

Chromosome banding analysis and fluorescence in situ hybridization

Chromosome banding analyses followed standard methods for patients with myeloid malignancies[1]. Whole chromosome painting was performed with probes for chromosomes 3 (red) and 5 (green) (MetaSystems, Altlussheim, Germany), interphase nuclei and metaphase were hybridized with a dual color break apart probe (3'*PDGFR(beta)* labeled in red, 5'*PDGFR(beta)* labeled in green, MetaSystems).

Flow cytometry, proliferation and cell viability

The expression of green fluorescent protein (GFP) in cell lines was measured using an LSRII cytometer with FACSDiva software version 6.1 (BD Becton Dickinson, Heidelberg, Germany). Proliferation of infected 32D cells after interleukin-3 (IL-3)

withdrawal was quantitated by trypan blue exclusion after seeding $1x10^5$ cells per ml into six-well plates (Greiner bio-one, Frickenhausen, Germany). Cells were counted 2,4,7 and 9 days after IL-3 withdrawal in a Neubauer counting chamber. Cell death of imatinib treated cells was assessed after 48 hours of treatment by flow cytometric quantitation of 4′,6-diamidino-2-phenylindole (DAPI)-positive cells. 10,000 events were analyzed for each sample.

Western Blotting

Preparation of whole cell protein extracts, SDS-PAGE and transfer were performed as previously described [2-4]. The following primary antibodies were used: anti-PDGFR(beta) rabbit antibody, clone C82A3 (Cell Signaling, Danvers, USA), anti-phospho-PDGFR(beta) (Tyr 751) rabbit antibody 3161 (Cell Signaling), anti-ZBTB11 rabbit antibody A303-240 (Bethyl Laboratories, Montgomery, TX, USA), anti-beta-actin mouse antibody, clone AC-74 (Sigma-Aldrich, Steinheim, Germany). The secondary antibodies were a horseradish peroxidase-conjugated goat anti-mouse and a horseradish peroxiadase-conjugated goat anti-rabbit antibody (both Dako Cytomation, Glostrup, Denmark).

Cytology

Cytological peripheral blood and bone marrow smears were stained by the panoptic method of Pappenheim and analysed using the Olympus BH-2 microscope (Olympus, Tokio, Japan), oil objectives, x60 and x 100.

Investigation of the oligomerization behavior of ZBTB11

To investigate the oligomerization behavior of the zinc-finger protein ZBTB11, we produced a truncated version N_{term}-K313, containing the BTB-domain, which we held

responsible for dimerization of the zinc-finger protein and, thus the fusion protein. Analytical size-exclusion chromatography coupled to multi-angle light scattering and refractive index analysis (SEC-MALS-RI)[5] was used to determine the absolute molecular weight free of any reference to standards. A model of the ZBTB11 homodimer was generated with Phyre2[6] and visualized with Pymol (http://www.pymol.org)).

Cell lines and cell culture

The 32D cell line was obtained from the `Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH' (DSMZ, Braunschweig, Germany) and maintained according to the suppliers' recommendations. The Phoenix eco cell line was obtained from G.P. Nolan, Stanford University, Palo Alto, California, United States of America (USA) and cultured according to the suppliers' recommendations.

All the cell lines were routinely tested for mycoplasma negativity.

Ethical approval

Human bone marrow and peripheral blood samples were obtained after written consent in accordance to the biobanking tissue collection policies as approved by the University Hospital Marburg.

Ethical approval was provided by the local ethics committee of the Philipps-university Marburg, faculty of medicine, file number 64/17.

Polymerase chain reaction (PCR) and nested PCR

RNA isolation was performed according to the manufacturers' instructions using the RNeasy Mini Kit (Qiagen, Hilden, Germany) for patient samples and the Nucleospin RNAII RNA isolation kit (Macherey-Nagel, Dueren, Germany) for cell lines. Reverse transcription was performed using the Omniscript RT Kit 200 (Qiagen).

Reverse transcription-PCR was performed in a total reaction volume of 25 µl containing 0.5 µM of the *glycerinaldehyd-3-phosphat dehydrogenase (GAPDH)* primers as housekeeping gene reference or 0.25 µM of all other primers and DreamTaq Green PCR 2x Master Mix (Thermo Fisher, MA, USA). PCR conditions were: 35 cycles of 94 °C for 15 s, 60 °C for 30 and 72 °C for 30 s. For nested PCR, products from the first PCR step were diluted 1:100 and used as template for second PCR.

The following primer pairs were used:

human (h) GAPDH: 5'-ctcctccacctttgacgctg-3' and 5'-accaccctgttgctgtagcc-3', *murine (m) GAPDH:* 5'-ctggtgtcttcaccacca-3' and 5'-cttcagtgggccctcaga-3', *ZBTB11-PDGFR(beta)*: 5'-acaggtgtaaagccacatgc-3' and 5'-ctcccacgtggagtcatagg-3' , *BTB flanking region*: 5'- tccaaaacattgtcaggctgt-3' and 5'-agctgcttctcttccattagc-3', *ZBTB11-PDGFR(beta)* nested (2nd): 5'-tcaatcacagaagcagttcca-3' and 5'-gctgagatcaccaccacctt-3'.

We have not yet assessed the exact sensitivity of our nested PCR-approach.

Quantitative *NPM1*-PCR for detection of minimal residual disease was performed as previously described[7].

Plasmids, cloning and retroviral transduction

Amplification of full-length ZBTB11-PDGFR(beta) open reading frame was performed from the patient's cDNA by using the following primers with inserted 5'- and 3'-EcoR1 restriction sites: 5'-gaattcatgtcaagcgagaaagctaccggg-3' and 5'-gaattcctacaggaagctatcctctgcttc-3'.

PCR was performed in a 25 µl volume containing 0.5 µM of each primer and Q5 High Fidelity 2x Master Mix (New England Biolabs, Ipswich, MA, USA). PCR conditions were: 35 cycles of 98 °C for 8 s, 65 °C for 20 s and 72 °C for 160 s. The PCR product was purified by the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Dueren, Germany) following the manufacturer's protocol. After A-tailing the product was cloned into pCR2.1-TOPO vector using the TOPO-TA cloning kit (Thermo Fisher, MA, USA). After plasmid amplification and EcoR1 digestion the *ZBTB11-PDGFR(beta)* product was cloned into pMSCV-IRES-eGFP (pMIG)[4] to generate pMSCV-ZBTB11-PDGFR(beta)-IRES-eGFP (pMIG-ZBTB11-PDGFR(beta)). Correct orientation and sequence of the insert were confirmed by sequencing with the following primers: 5'-cccttgaacctccctcgttcgacc-3' (MSCV), 5'-gaattcatgtcaagcgagaaagctaccggg-3', 5'-tggaagaattgggactagacct-3', 5'-aagctaatggaagagaagcagc-3', 5'-gctgaggatatttgtgccga-3', 5'-cagtacagtgcctctttgcg-3', 5'-ctctgacggccatgagtaca-3', 5'-atggacatgagcaaggacga-3', 5'-cggagagcatcttcaacagc-3' and 5'-ccaatgagggtgacaacgac-3'.

The deletion of the region coding for the BTB domain was generated by using reverse complementary primers with the following primer pairs: 5'-gaattcatgtcaagcgagaaagctaccggg-3' and 5'-atcagccatgctacagaactggttggaaag-3' for the part from the 5'- end to the start of the BTB (cDNA position 640 in transcript with accession number NM_014415.4) domain and 5'-ctttccaaccagttctgtagcatggctgat-3' and 5'-gaattcctacaggaagctatcctctgcttc-3' for the part from the end of the BTB domain

(cDNA position 846 in the same transcript) to the 3'-end. PCR was performed as described above with extension time of 30 s for the shorter 5'- fragment and 120s for the longer 3'- part. PCR products were purified as described above and used as template for amplification of the full length *ZBTB11-PDGFR(beta)* with deletion of the region coding for the BTB domain. This PCR, the subsequent cloning into pMIG to generate pMIG-ZBTB11-PDGFR(beta)-ΔBTB and the sequence confirmation were performed as described above. Retroviral transductions were performed as previously described[4]. 32D-MIG (32D-vector), 32D-ZBTB11-PDGFR(beta) and 32D-ZBTB11-PDGFR(beta)- ΔBTB cells were generated by transduction of 32D cells with pMIG, pMIG-ZBTB11-PDGFR(beta) or pMIG-ZBTB11-PDGFR(beta)- ΔBTB, followed by cell sorting of the green fluorescent protein (GFP)-positive cell fraction using a MoFlo Astrios cytometer (Beckman Coulter, Brea, CA, USA).

Statistical methods

Data were analysed with GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance of differences between groups of samples was determined by one-way analysis of variance with Bonferroni adjustment for multiple testing (significance level $\alpha=0.05$): $p>0.05$ not significant; $0.01< p\leq 0.05$ *; $0.001< p\leq 0.01$ **; $p\leq 0.001$ ***.

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