Severe congenital neutropenia-associated \textit{JAGNI} mutations unleash a calpain-dependent cell death program in myeloid cells

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EXTENDED METHODS

\textit{Clinical histories}

\textit{Patient 1}: A boy of Iraqi origin born in 2006, the youngest child with four healthy siblings to parents of consanguineous marriage, was admitted to the Karolinska University Hospital, Stockholm at the age of 2 years. During the neonatal period, he had an umbilical infection and during the first years of life frequent episodes of bronchitis and pneumonia. Neutropenia (ANC<0.5x10^9/L) was observed and bone marrow examination showed maturation arrest of the myelopoiesis, but this was initially interpreted as autoimmune neutropenia. However, our evaluation showed that pro-LL-37 was absent in plasma, which is seen only in SCN. The patient was diagnosed with SCN. Mutational analyses of the \textit{ELANE} and \textit{HAX1} genes showed no causative defects. Treatment with recombinant G-CSF commenced at 2½ years of age and ceased at the age of 9 years due to increased resistance to treatment. Homozygous mutations in \textit{JAGNI} were identified by exome sequencing and verified by direct sequencing (as described in the present study). In a recent study, we reported on neutrophil extracellular traps or NETs produced by \textit{ex vivo} activated neutrophils from the patient (Khandagale et al., 2018). In 2018, the patient was bone marrow transplanted with his haploidentical brother as donor due to resistance to G-CSF and problems with infections including severe periodontitis. Unfortunately, the patient currently suffers from liver GVHD and secondary diabetes mellitus, but his chronic neutropenia has been resolved. \textit{Patient 2}: A boy of Roma origin born in 1989, the youngest son of consanguineous parents, was first seen at the Children’s Memorial Health Institute in Warsaw at 8 years of age. When he was 6 years old, he was diagnosed as having...
chronic severe neutropenia at Great Ormond Street Hospital in London and treated with G-CSF due to neutropenia (ANC consistently <0.5x10^9/L). Bone marrow examination revealed a maturation defect (i.e., granulocytic series left-shifted to metamyelocytes). Congenital neutropenia was suspected due to severe chronic neutropenia, the bone marrow biopsy findings, absence of antigranulocytic antibodies, and recurrent bacterial infections. G-CSF treatment was, however, stopped as the patient no longer experienced severe infections and was doing well on antibiotic prophylaxis. During the observation period that lasted up to 20 years of age his ANC was between 0.18 to 2.2x10^9/L. Higher ANC values were observed after 13 years of age, initially only during bouts of bacterial infections, but eventually also when he was free of infection. Mutational analyses performed in Stockholm excluded mutations in the \textit{ELANE} and \textit{HAX1} genes, but homozygous mutations were identified in the \textit{JAGN1} gene, as described in the present report. His mother carries a heterozygous mutation of \textit{JAGN1}. Unfortunately, it was not possible to test the father who is deceased nor the brother who suffers from epilepsy. The patient is doing well and suffers only from nephrolithiasis. He has two healthy children. 

Patient 3: A boy of Roma origin born in 2003, the son of consanguineous parents was admitted at the Children’s Memorial Health Institute in Warsaw at 2 years of age due to chronic neutropenia. His ANC varied from 0.06 to 0.6x10^9/L and he presented with high monocytosis (up to 50%). He suffered from recurrent, severe bacterial infections, chronic gingivitis and mouth ulcers. Because of low height (below 10 percentiles for age) and failure to thrive, Shwachman-Diamond syndrome was considered as a differential diagnosis, but molecular diagnostics did not disclose any \textit{SBDS} mutations. Bone marrow examination showed maturation arrest at the myelocyte stage typical of SCN. G-CSF treatment was initiated, and some improvement was observed, but his ANC increased to 1.0 x 10^9/L only after high doses of G-CSF (approx. 40 \mu g/kg/d). After a few months, GSF-treatment ceased due to poor tolerance (i.e., bone pain). Instead, the patient was put on antibiotic prophylaxis and vaccinated against encapsulated bacteria. Bone marrow transplantation was considered, but his parents did not approve of such a treatment. Currently, the patient is 15 years old, and his ANC varies from 0 to 0.4 x 10^9/L, accompanied by monocytosis. With time, his height and weight increased over 25 percentiles. He is doing well on antibiotic prophylaxis. He had no severe infections for many years, apart from severe periodontitis with loss of most of his teeth. Mutational analyses performed in Stockholm excluded defects in the \textit{ELANE} and \textit{HAX1} genes, but homozygous mutations were identified in \textit{JAGN1}, as described here. It has subsequently been found that patients 2 and 3 are related.
**Exome sequencing**

To obtain a molecular diagnosis, genomic DNA was isolated from peripheral blood of the patients (Puregene Blood Core Kit C, Qiagen). Libraries for sequencing on Illumina HiSeq2000 (Illumina) were prepared from DNA samples and exome sequences enriched with Agilent SureSelect Human All Exon 50M (Agilent), according to the manufacturer’s instructions. Post-capture libraries were sequenced as 2x100 bp paired end reads on the Illumina sequencer. Reads were base called using Illumina OLB (v 1.9, Illumina). Sample library preparation, sequencing, and initial bioinformatics up to base calling and demultiplexing were performed at the Science for Life Laboratory, Stockholm. For patient 1, 123 million read pairs were obtained, 56% uniquely aligned read pairs after duplication removal, for a final mapped exome target average coverage of 74x, with 88% of target bases at 10x coverage or more. For patient 2, 42 million read pairs were obtained, 69% aligned uniquely, for a final uniquely mapped exome target average coverage of 34x, with 80% of target bases at 10x coverage or more. An in-house pipeline was used to process reads, call variants and annotate them (freely available under a GPL license: http://github.com/dnil/etiologica). Briefly, reads were mapped to the human reference genome (hg19) using Mosaik (v.1.0.1388) (Lee et al., 2014). Duplicate read pairs were removed using Mosaik DupSnoop. Variants were called using the samtools package (v.0.1.18) (Li et al., 2009). These were quality filtered (Q>=20), and further annotated using ANNOVAR (v. 2013 Feb 21) (Wang et al., 2010) to note allele frequencies as well as evolutionary conservation and segmental duplication status in available databases [1000 genomes (Abecasis et al., 2010), 6500 ESP (NHLBI GO Exome Sequencing Project, ESP) (https://evs.gs.washington.edu/EVS/), 134 local exomes, and dbSNP 137 (Sayers et al., 2012)]. The pathogenicity of the identified mutations was predicted by PredictSNP 1.0 (Bendl et al., 2014).

**Sanger sequencing**

DNA was extracted from blood samples followed by PCR amplification of exon 1 of *JAGN1* by PCR with gene specific primers (*JAGN1* fw 5’-CCCCCTCTGTAAGTGTTGGAC-3’ and *JAGN1* rv 5’-CTGGATCCCTCAAGGCAC-3’) and enzymatically purified (ExoSAP-IT, GE Healthcare). Fragments were sequenced (Big Dye Terminator v 3.1 Cycle Sequencing kit, Applied Biosystems) and the sequencing reactions were analyzed by capillary electrophoresis on an ABI 3730 DNA Analyzer. Data were analyzed using SeqScape v. 2.5 (Applied Biosystems).

**JAGN1 expression analysis**


Data on the expression of *JAGN1* mRNA across different human tissues was extracted from the public *in silico* transcriptomics (IST) database that contains data from almost 10,000 microarray gene expression analyses of normal tissues, cancer tissues, and other diseases (Kilpinen et al., 2008).

**Yeast two-hybrid screening**

The yeast two-hybrid screening was carried out by Creative BioLabs (Shirley, NY). The bait construct for yeast two-hybrid screening was made by subcloning the *JAGN1* cDNA into the vector pGBKT7 (Clontech). The bait construct was transformed into the strain AH109 (MATα, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS–Gal1TATA–His3, GAL2UAS–Gal2TATA–ADE2, URA3::MEL1UAS–MEL1TATA–lacZ) using standard procedures. The absence of self-activation was verified by transformation of the bait alone to select on minimal medium lacking the amino acids tryptophan, leucine and histidine (selective medium). In the yeast two-hybrid screening, the baits were mating with a Human Bone Marrow Library in Y187 (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, URA3::GAL1UAS–GAL1TATA–lacZ). The potential positive transformants were tested for His, Ade and LacZ activation and 26 positive clones showed His, Ade and/or LacZ activation. The identity of the positive interactors was determined by sequencing. For immunoprecipitation, mouse anti-FLAG antibody (Sigma Aldrich) was added to cell lysates of HL-60 cells transfected with FLAG-tagged *JAGN1* at 4°C overnight, and protein G Sepharose (GE Healthcare, Piscataway, NJ) was added for 4 h. After centrifugation, beads were removed by heating and precipitated immune complexes were re-suspended in SDS sample buffer for analysis.

**HL-60 cell experiments**

The human acute promyelocytic leukemia cell line HL-60 (American Type Culture Collection) was maintained in phenol red-free RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) (Sigma) in 5% CO₂ at 37°C with the highest density never exceeding 10⁶ cells/mL. HL-60 cells were transiently transfected with N-terminal FLAG-tagged *JAGN1* (myc-DDK-tagged-human jagunal homolog 1, JAGN1) (Origene). Three FLAG-tagged constructs including two patient-derived point mutation-expressing (G14S and E21D) and one wild type (WT) expressing *JAGN1* constructs were used. Cells were seeded in 24-well plates (200,000 cells/well) and the following day 4 µl of Lipofectamine 3000 reagent (Invitrogen) and 2 µg of plasmid DNA containing the specific human *JAGN1* sequence were each diluted in 100 µl Opti-MEM (Invitrogen) separately and incubated at RT for 5 min;
additionally, 2 µl of p3000 reagent (Invitrogen) was added. The solutions were then combined
and incubated at RT for 20 min. Subsequently, the Lipofectamine-DNA complexes were added
to the medium and cells were incubated for 24–48 h. To silence endogenous \textit{JAGN1}, HL-60
cells were seeded in 24-well plates one day prior to transfection, and cells were then transfected
with either negative control siRNA (\#4404021) or siRNAs 1 and 2 (\#s39100 or \#s39101, Life
Technologies, 25 nM each per well) against \textit{JAGN1} by using Lipofectamine 3000 (Invitrogen).
The medium was changed the following day and cells were maintained for 24 h prior to analysis.

\textbf{RT-PCR}

In order to determine the efficiency of \textit{JAGN1} silencing, HL-60 cells were lysed, and mRNA
was purified using the QIAGEN RNeasy Mini Kit according to the manufacturer’s protocol.
Then, mRNA was quantified with Nanodrop (Thermo Scientific) and the Revert Aid-H Minus
First Strand cDNA Synthesis Kit was used to perform reverse transcription loading 200 ng of
total mRNA. RT-PCR with the Power SYBR Green PCR Master Mix was performed using an
Applied Biosystems 7500 Real-Time PCR System and the data was analyzed using the Applied
Biosystems 7500 Real-Time PCR software v.2.3. The primers utilized were: \textit{GAPDH} fw: 5’-
CCC CTT CAT TGA CCT CAA CTA C-3’; \textit{GAPDH} rv: 5’-GAG TCC TTC CAC GAT ACC
AAA G-3’; \textit{JAGN1} fw: 5’-TGC ACT ACC AGA TGA GTG TGA-3’; \textit{JAGN1} rv: 5’-GCT AAT
GTT GTT GCG GGG AA-3’. Data are shown as relative mRNA levels normalized to \textit{GAPDH}.

\textbf{TEM}

Peripheral blood neutrophils were isolated as described (Khandagale et al., 2018) and
resuspended in fixation buffer and kept at 4°C until further processing. For TEM, cells obtained
from patient 1 or from a healthy control were fixed in 2.5% glutaraldehyde in 0.1 M phosphate
buffer, pH 7.4 at room temperature and further fixed over night at 4°C. After fixation cells were
rinsed in 0.15 M sodiumcacodylate buffer and centrifuged. The pellets were then post-fixed in
2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4 at 4°C for 2 h, dehydrated in ethanol
followed by acetone and embedded in LX-112 (Ladd Research Industries). Ultrathin sections
(50-60 nm) were obtained using a Leica EM UC 6 microtome. The sections were contrasted
with uranyl acetate followed by lead citrate and examined in a Tecnai 12 Spirit Bio TWIN
transmission electron microscope (FEI) at 100 kV. Digital images were captured using a Veleta
camera (Olympus).

\textbf{Immunofluorescence}
HL-60 cells, transfected with FLAG-tagged wt or mt JAGN1 constructs, were seeded at a density of 2x10^5 cells/mL in 6 well poly-L-lysine coated slides (ibidi, Germany). After 2 h cells were fixed with 4% (w/v) paraformaldehyde in PBS for 25 min and then permeabilized with PBS containing 0.1% Triton X-100. Samples were blocked with PBS containing 5% BSA at room temperature for 40 min and subsequently incubated with rabbit polyclonal anti-human calnexin antibody (Thermo Fisher Scientific) for 1 h. Cells were then washed in PBS and incubated for 1 h either with FITC-conjugated anti-mouse FLAG or Alexa Fluor® 594-conjugated anti-rabbit IgG secondary antibodies (Life Technologies). MitoTracker™ Deep Red FM and LysoTracker™ Red DND-99 (Molecular Probes, Life Technologies) were utilized to stain for mitochondria and lysosomes, respectively. The slides were mounted using ProLong Gold Antifade Mountant. Visualization of the samples was carried out using a ZEISS LSM510META confocal microscope and the images were processed using LSM510 software.

**Western blot analysis**

We used a mouse monoclonal anti-FLAG (Sigma Aldrich) and fluorescently labelled anti-mouse antibody from LI-COR. HL-60 cell lysates were obtained by lysis using CellLytic lysis buffer (Sigma Aldrich). Proteins were separated by electrophoresis under reducing conditions and blots were probed overnight at 4°C followed by secondary antibody incubation and washing. Blots were scanned and imaged using LI-COR following the manufacturer’s instructions.

**Cell cycle/apoptosis analysis**

Cells were fixed with 70% cold ethanol on ice for 30 min. Following fixation cells were washed and re-suspended in DNA extraction buffer (0.2 M Na₂HPO₄ and 0.1% triton x100; pH 7.8) for 5 min. Samples were then washed and stained with 0.002% propidium iodide containing 200 µg/mL RNase in PBS for 30 min at RT in the dark. Cells were analyzed by flow cytometry using a BD Accuri™ cytometer (BD Biosciences). 10,000 events were analyzed for each sample and cell debris were gated out on the basis of forward scatter/side scatter prior to analysis. For bone marrow progenitor cells, cell death was monitored annexin V staining, which enables the detection of externalized phosphatidylserine (PS) on the cell surface (Sanmun et al., 2006). Labeling with propidium iodide and annexin V-FITC (Calbiochem) was performed prior to analysis with a FACS scan flow cytometer (Becton Dickinson) operating with CellQuest software (Becton Dickinson).

**Caspase-3-like enzyme activity**
Caspase-3-like activity was monitored in whole cell lysates by using a fluorometric Caspase-3 Assay Kit (Sigma Aldrich). Cleavage of the DEVD-AMC substrate was monitored by AMC liberation in a TECAN Infinite® 200 plate reader (Labsystems, Stockholm, Sweden) using 355 nm excitation and 460 nm emission wavelengths. Fluorescence units were converted to nmol of AMC cleavage per minute based on a standard curve generated with free AMC (Jitkaew et al., 2009).

**Mitochondrial membrane potential**

To evaluate the dissipation of mitochondrial transmembrane potential ($\Delta \psi_m$), cells were incubated for 20 min at 37°C in cell culture medium with the fluorescent probe, tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, 25 nM). Cells were then spun down, washed once in PBS, and resuspended in HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2) containing 25 nM TMRE. As a positive control, cells were incubated for 10 min with the uncoupling agent, carbonyl cyanide 3-chlorophenylhydrazone (100 μM) (Sigma). Samples were analyzed on a BD Accuri™ cytometer.

**Cytosolic and mitochondrial calcium**

Cells were collected and washed in Ca²⁺/Mg²⁺-free HBSS and incubated with 2 μM Fluo-4-acetoxyethyl ester (Fluo-4 AM; Molecular Probes) in 3% dimethylsulfoxide (final concentration of DMSO, 0.06%) at 37°C for 30 min. Fluo-4 AM is a high-affinity calcium indicator for the detection of cytoplasmic calcium. After incubation, cells were washed twice and re-suspended in Ca²⁺/Mg²⁺ free HBSS. The fluorescence in each sample was analyzed using a BD Accuri™ cytometer. The calcium ionophore, A23187 (2.5 μM) (Sigma) was used as a positive control. Mitochondrial calcium levels were measured using Rhod2-AM (Invitrogen). The fluorescent Rhod2-AM dye has a net positive charge, facilitating its sequestration into mitochondria. Briefly, cells were loaded with 5 μM Rhod-2/AM at 4°C for 30 min to allow the dye to accumulate in the mitochondria and washed further before flow cytometric analysis, as above.

**LDH release assay**

The LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific) was used to determine cell death. Cells were suspended at a density of 0.5x10⁶ cells/mL in 24-well plates and samples lysed in lysis buffer at 37°C for 45 min were used to determine maximum LDH release. LDH release
was measured by using the Tecan Infinite® F200 plate reader operating with Magellan v7.2 software.

**Statistical analysis**
Two-way analysis of variance (ANOVA) with Newman-Keuls post-test or Tukey's multiple comparisons test was applied when multiple groups were compared, and two-tailed Student's t-test was used for analysis of two groups. For non-parametrically distributed data, the two-tailed Mann-Whitney test was used. Statistical tests were performed using GraphPad Prism or R-studio.

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