Neutrophil Elastase Mutations in Severe Congenital Neutropenia Patients of the Original Kostmann Family

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Running title: Molecular Studies of Kostmann Syndrome

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Abstract

Severe congenital neutropenia (SCN), or Kostmann syndrome, was originally reported as an autosomal recessive disease of neutrophil production and recurrent bacterial infections. Heterozygous mutations in the neutrophil elastase (NE) gene have previously been identified in patients with sporadic or autosomal dominant SCN. Here we present cellular and molecular studies of all 4 surviving affected members of the original "Kostmann family" and their healthy parents and siblings. One of the patients had no mutation in the NE gene. Three other patients had one silent NE mutation that was also present in at least one of the healthy parents. Two of these 3 patients also displayed different heterozygous substitution mutations in the NE gene that were not inherited from parents. Sequencing analysis of genomic DNA from skin fibroblasts revealed an NE mutation in one of these patients, whereas no mutation was observed in the other patient. Expression of mutant NE cDNAs identified in these SCN patients, but not of normal NE, resulted in accelerated apoptosis of human promyelocytic HL-60 cells. Subcellular localization studies of NE in conjunction with molecular modeling of NE tertiary structure suggest that these substitution mutations might affect the substrate specificity of the enzyme. These data imply that NE mutations may contribute to the phenotype in some of the patients in this study and suggest that mutations in other genes may cause autosomal recessive SCN. Additional genetic studies are thus needed to further elucidate the cause of SCN in the original "Kostmann family".

(Word count: 244).
Introduction

Severe congenital neutropenia (SCN), also known as Kostmann syndrome, was first described by Kostmann in 1956 as an autosomal recessive disorder in a large kindred from northern Sweden [1,2]. The disease is characterized by a maturation arrest of neutrophil precursors at the promyelocytic stage of differentiation and by extremely low levels of mature neutrophils in peripheral blood. Before the availability of granulocyte colony-stimulating factor (G-CSF) therapy these patients were treated with antibiotics, but many affected family members died from recurrent bacterial infections [3]. A few affected family members from the original “Kostmann family” have survived and their clinical status and response to G-CSF administration was recently reported [4].

Mutations in the cytoplasmic region of the G-CSF receptor have been reported in a proportion of SCN patients in association with acute myeloid leukemia [5,6]. However, it is now understood that these mutations represent an acquired event and not an underlying cause of the disease. We have previously reported that all patients with cyclic neutropenia (CN), an autosomal dominant disease with a 21-day cycle of oscillating neutropenia, and more than 80% of SCN patients harbor either inherited or acquired mutations in the neutrophil elastase (NE) gene [7,8]. In contrast, three families with autosomal recessive SCN were recently reported to have no mutation in the NE gene [9]. NE is synthesized in promyelocytes and promonocytes and is normally released from neutrophil granules at the sites of inflammation [10]. Gain-of-function mutations in the NE gene have been suggested to interfere with the production of neutrophils in the bone
marrow [11]. We report herein the results of mutational analyses of the NE and G-CSF receptor genes in the four surviving affected family members related to the original “Kostmann family” and their parents, as well as the survival characteristics of promyelocytic HL-60 cells transfected with mutant NE identified in two of these patients.
Patients and Methods

Clinical data

Kostmann reported 22 patients with severe chronic neutropenia originating from the same region in northern Sweden [1,2], and we recently described five additional patients from the same area [4]. These individuals all had severe persistent neutropenia, bone marrow aspiration showed a maturation block at the promyelocyte/myelocyte level, and the clinical signs and symptoms in these patients were similar [4]. Based on family studies, Kostmann suggested that this was a congenital neutropenia with an autosomal recessive inheritance. In 1984, evidence was presented for a single founder for these patients, the likely place of origin being the parish of Överkalix in northern Sweden [12]. All but two of these 27 patients are part of the same extended family originally described by Kostmann, including the five most recently reported individuals [4]. Four of the 27 patients are alive and these four individuals, all linked to the large family initially described by Kostmann, are the subjects of the present report (Table 1), as are the healthy parents and siblings of three of these four individuals (Figure 1).

Patient 1, a 25-year-old woman is the first child of three. Her two brothers are healthy. Both parents’ families were related to the large family described by Kostmann. Patient 2, an 18-year-old woman is the only common child of parents originating from the parish of Överkalix, that also belongs to the original “Kostmann family”. Patient 3, a 17-year-old man is the only child of parents that have siblings affected with Kostmann syndrome. The brother of the maternal great-grandmother was also the father of Kostmann’s index case.
admitted in 1949 [1]. The father of patient 3 had a brother who died of Kostmann syndrome in the 1950s. Patient 4, an 8-year-old boy is the second child to parents from a neighbouring parish. His brother is healthy. Both parents have ancestors from the parish of Överkalix and are linked to the extended “Kostmann family” as far back as the seventeenth century. Detailed clinical summaries and treatment responses for these patients have been reported elsewhere [4].

**Patient sampling**

Blood samples were obtained by routine venopuncture, and heparinized marrow aspirates were obtained from the posterior iliac crest under general anesthesia by standard techniques. Blood counts were performed using automated whole blood cell counting and examination of Wright-stained blood smears. All subjects participated in these studies after giving informed consent under a protocol approved by the ethical committee of Umeå University, Sweden. The skin biopsies were performed using standard techniques followed by isolation of genomic DNA based on the proteinase K digestion.

**Mutational analysis**

Genomic DNA isolated from peripheral blood mononuclear cells or skin fibroblasts, and total RNA from bone marrow mononuclear cells were used for mutational analysis of the NE, G-CSF receptor, and WAS genes as described previously [7,13,14]. Sequencing of PCR-amplified products was performed using big dye terminator chemistry on an ABI 3700 sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). To rule out PCR-introduced artifacts, each mutation was confirmed at least three times by independent PCR followed by sequencing across the region of interest.
Structural modeling

Coordinates of the NE protein were obtained from the Protein Data Bank [15] and mutations were introduced using the program previously reported [16]. Homology modeling was carried out with tools available within the Molecular Operating Environment from the Chemical Computer Group, Montreal, Quebec as previously described [7].

In vitro cell culture

The human HL-60 promyelocytic leukemia cell line was kindly provided by Dr. Steven Collins (Fred Hutchinson Cancer Research Center, Seattle, WA) [17]. Cells were maintained at 37°C in a CO₂-incubator in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (BioWittaker, Walkersville, MA).

cDNA constructs

NE cDNA was amplified by RT-PCR using total RNA derived from U937 cells [18] and NE-specific primers containing the BamHI and EcoRI restriction sites. The cDNA was cloned into the pcDNA3.1 expression vector under control of the CMV promoter (Invitrogen, Carlsbad, CA). The mutant L92H and R191Q NE cDNAs were obtained by site-directed mutagenesis using corresponding mutant NE-specific primers. To verify the preservation of an open reading frame and absence of PCR-introduced artifacts, each construct was sequenced across the entire coding region.

Transient expression and survival assay

For transient transfection experiments, 15 µg of plasmid DNA containing mutant or intact
NE cDNA were used to electroporate 5×10⁶ HL-60 cells. To monitor the transfection efficiency and focus on transfected cells, 5 µg of the pEGFP-C3 plasmid that directs expression of an enhanced green fluorescent protein (EGFP) under control of the CMV promoter (Clontech Laboratories, Inc., Palo Alto, CA), were co-transfected with NE cDNA. Following 48 h of transfection, an aliquot of the cells cultured in RPMI 1640 supplemented with 10% FBS, was incubated for 20 h in serum-deprived medium (0.5% FBS) in a CO₂-incubator at 37°C, labeled with PE-conjugated annexin V (Pharmingen, San Diego, CA), and analyzed by flow cytometry. At least 10,000 events were assayed per sample and the cells positive for GFP were analyzed using Multi-Plus (Phoenix Flow System Inc., San Diego, CA) or CellQuest (Becton Dickinson, Mountain View, CA) software. Results are reported as the percentage of cells undergoing apoptotic cell death, i.e. the percentage of annexin V-positive cells within the GFP-gated population.

**Immunolocalization**

The cytospins of HL-60 cells transfected with normal or mutant NE cDNA or with empty plasmid were prepared using standard techniques. The cells were fixed in 4% paraformaldehyde for 5 minutes at room temperature, and washed 3 times in PBS. The slides were incubated with a blocking solution (5% donkey serum in RPMI) for 2 h at room temperature to prevent nonspecific antibody binding, followed by overnight incubation at 4°C in the presence of NE-specific monoclonal antibody AHN-10 (Research Diagnostics, Flanders, NJ). The slides were washed 4 times with blocking solution and then incubated at room temperature for 30 minutes with Rho-conjugated donkey anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA).
in fresh blocking solution. The slides were washed 4 times in PBS and stained with 4′6-diamidino-2-phenylindole (DAPI ) at 1 µg/mL in PBS for 5 minutes. After a further wash with PBS, the staining of Rho-labeled NE (red) and DAPI-stained nuclei (blue) was observed using a wide-field deconvolution fluorescence microscope (DeltaVision Imaging System, Applied Precision, Issaquah, WA). The Delta Vision system utilizes broad field optics coupled with computerized deconvolution of the optical image using Fourier transformation. A Z-stack of optical sections through 3.2 µ can be viewed as a three dimensional image with a resolution of approximately 90 nm.

**Western blot analysis**

Transfected HL-60 cells were lysed in the presence of a cocktail of serine protease inhibitors including 5 mM PMSF and 10 µg/ml of aprotinin and leupeptin (Sigma, St. Louis, MO). The cell lysates were then separated on a 9% polyacrylamide gel and blotted onto nitrocellulose membrane under semi-dry conditions. After blocking with 5% non-fat milk, membranes were incubated with rabbit NE-specific polyclonal antibodies (Research Diagnostics, Flanders, NJ) or antibodies against β-actin (Sigma, St Louis, MO) followed by secondary goat anti-rabbit antibodies conjugated with horseradish peroxidase (Research Diagnostics). The protein bands were visualized using chemoluminescence substrate (Life Science Products, Boston, MA) according to the manufacturer’s recommendations.
Results

Patients

The family pedigree of the original “Kostmann family” and the patients reported herein is presented in Figure 1. This pedigree shows that the Kostmann patients participating in this study belong to the same extended family, but there is, unfortunately, insufficient information to trace all surviving family members to the same ancestor. We acquired material for molecular analyses from all surviving family members with severe neutropenia [4] and from three sets of parents and siblings. Specimens from the parents of the fourth patient could not be obtained because of their remote geographic location. Patient 3, who is currently 18 yr old, underwent bone marrow transplantation at 7 months of age with the father as the donor, and is a mosaic as described previously (4). The absolute neutrophil counts (ANC) for the affected family members before and during G-CSF therapy, their parents and unaffected siblings are presented in Table 1. Prior to treatment all patients were severely neutropenic (ANC 0-300/µL). With administration of G-CSF, all patients exhibit ANCs within the normal range or higher. All parents and siblings were hematologically healthy, and displayed normal ANC values (Table 1). In contrast to the affected family members, these individuals experienced no problems with recurrent infections.

Mutational analysis

NE-specific primers and peripheral blood genomic DNA were used for amplification and sequencing of all 5 exons of the NE gene as previously described [7,8].
analysis revealed two missense and one silent mutation in 3 of 4 patients studied (Figure 2A). The silent mutation at the catalytic S173 residue was identified in 3 of 4 patients as well as in 4 of 6 healthy parents examined. Two of 4 patients had different heterozygous missense mutations resulting in substitution of Leu in position 92 to His (L92H) and Arg in position 191 to Gly (R191Q), respectively (Figures 2A and 3). Sequencing of the corresponding RT-PCR products across the region containing these mutations was confirmatory, indicating that the mutant transcripts are expressed in the bone marrow cells of these patients (data not shown). None of the parents or siblings harbored missense mutations (Figure 3). Concomitant mutational analysis of the intracellular portion of the G-CSF receptor or the Wiskott-Aldrich syndrome (WAS) gene revealed no missense or truncation mutations in the patients, the siblings or their parents (data not shown). Mutational analysis revealed the presence of a L92H missense mutation in the genomic DNA isolated from skin fibroblasts of patient 1, whereas no mutation was identified in the genomic DNA isolated from skin biopsy material of patient 3, or from their healthy parents (data not shown).

**Structural analysis**

The tertiary structure of the NE protein with an inhibitor molecule in its active site is presented in Figure 2B. Normally, NE has two N-linked glycosylation sites, designated here as NG. The proteolytically active center with a catalytic serine S173 residue is marked with a green arrow. The L92H mutation can be seen adjacent to the N95 glycosylation site (Figure 2B, left panel), whereas the R191Q is located on the side chain loop involved in the formation of the binding pocket of neutrophil elastase (Figure 2B,
right panel). The location of both heterozygous substitution mutations thus suggests that these mutations may alter the normal protein processing and substrate specificity of this enzyme, or confer resistance to elastase-specific inhibitors.

**Functional studies**

Based on previous studies demonstrating impaired cell survival of myeloid progenitor cells in cyclic neutropenia patients harboring NE mutations [19], we examined whether the expression of NE mutants identified in these patients would result in a similar cellular phenotype. For this purpose, both L92H and R191Q mutants, identified in patients 1 and 3, or normal NE was co-expressed with EGFP in the human promyelocytic HL-60 cells. Following 48 h of transfection, cells were subjected to overnight *in vitro* culture in serum-deprived medium and subsequently labeled with PE-conjugated annexin V, a marker for apoptotic cells [20]. As seen in Figure 4A, expression of each mutant NE cDNA resulted in approximately 2-fold higher rate of apoptosis of HL-60 cells compared with the cells transfected with normal NE cDNA. Specifically, the level of apoptosis observed for the L92H mutant NE transfected cells was 51+7% and for R191Q mutants – 49+7%, compared with 20+3% apoptosis for normal NE cDNA-transfected cells (mean ± SD, n=3) (Figure 4A). These numbers represent mean values of apoptotic cell death in 3 independent experiments ± standard deviation. The expression level of mutant and normal NE in transfected HL-60 cells is comparable as determined by Western blot analysis of the cell lysates with NE-specific and β-actin monoclonal antibodies (Figure 4B). These data demonstrate that mutations in the NE gene can, indeed, lead to accelerated apoptotic cell death.
Subcellular localization

To determine whether the apoptotic cell death of transfected cells is due to an aberrant subcellular distribution of mutant NE protein, the immunolocalization studies were performed in HL-60 cells transfected with NE mutants or normal NE cDNA. The DeltaVision Imaging System that allows optical sections throughout the cells was used to visualize the localization of the NE in these cells (Figure 5). Immunostaining of mock-transfected cells with human NE-specific mouse monoclonal antibody followed by Rho-conjugated anti-mouse antibody revealed primarily clustered granular localization of the NE (Figure 5, panel A). Immunolocalization analysis revealed no difference in the subcellular distribution of the L92H mutant, the R191Q or the normal NE as evidenced by a predominantly cytoplasmic localization of NE in corresponding transfected HL-60 cells (Figure 5, panels B-D).
Discussion

Kostmann syndrome was originally described more than 50 years ago as an autosomal recessive disease, yet in the intervening years many sporadic or acquired as well as inherited cases with an autosomal dominant mode of inheritance have been reported [21,22]. Kostmann reported clinical histories and blood counts in the original description of this disorder discovered in a sparsely settled area of Sweden [1]. The autosomal recessive inheritance in the original “Kostmann family” is supported by geographic studies of the spatial distribution of these cases [12] as well as by an updated pedigree of the family [4]. In the present study, we present the first molecular data on the affected and healthy members of this family.

The mutational analysis of the NE gene for the surviving members of the “Kostmann family” affected with severe neutropenia and their healthy parents and siblings revealed two different heterozygous mutations in 2 of 4 patients examined, but not in healthy family members including the parents. Importantly, both these NE mutations were confirmed by sequencing PCR-amplified genomic DNA and reverse-transcribed bone marrow RNA from the patients. The structural modeling of the NE molecule indicates that the L92H mutation, identified in patient 1, is positioned in immediate proximity to the glycosylation site N95. We hypothesize that this amino acid substitution alters the availability of the asparagine N95 residue and results in abnormal glycosylation/deglycosylation processing of the mutant protein. The other mutation, R191Q, identified in patient 3, was previously identified in a number of unrelated
families with autosomal dominant cyclic neutropenia [8]. This mutation appears to be located on a side chain of NE forming a binding pocket of the active site. We hypothesize that this mutation results in an acquired resistance to NE-specific inhibitors and/or an alteration of the substrate specificity of NE. In addition to these substitution mutations, a silent S173 mutation was identified in 3 of 4 patients examined, as well as in healthy parents and a healthy sibling. This polymorphism in the NE gene was observed in approximately 10% of healthy volunteers examined (unpublished observations), thus suggesting that this sequence variation does not play a role in the pathogenesis of severe congenital neutropenia. We found no mutations in the cytoplasmic domain of the G-CSF receptor gene in patients or family members participating in the present study (data not shown). Moreover, there is also no evidence to date of secondary leukemia in the original “Kostmann family” [4]. Interestingly, both patients with NE mutations (pts. 1 and 3) were diagnosed earlier (at the age of 2 weeks and 2 months, respectively) than the children without a mutation in the NE gene (patients 2 and 4, diagnosed at 5 months of age) (Table 1) [4]. This probably reflects an earlier onset of disease, but the current material is small and this finding needs to be confirmed in a larger study. Once G-CSF therapy was initiated, no differences in the clinical course of the patients with and without NE mutations were identified.

We have recently observed an accelerated apoptosis of bone marrow myeloid progenitor cells from cyclic neutropenia patients [18]. In order to test the hypothesis that the expression of mutant NE results in poor cell survival, we co-transfected normal or mutant NE with EGFP in human HL-60 cells and determined the survival of these cells by flow
cytometric analysis of annexin V labeled cells. Enhanced apoptotic cell death with approximately 50% of mutant NE-transfected cells positive for annexin V compared with 20±3% apoptosis in cells transfected with normal NE, was observed (Figure 4). The proportion of cells undergoing apoptotic cell death under these experimental conditions is presumed to be even higher because not all GFP-positive cells may express normal or mutant NE cDNA. Nevertheless, the observed 2-fold increase in the number of cells undergoing apoptosis indicates that expression of either L92H or R191Q mutant NE is sufficient for impaired survival of human myeloid cells. It is noteworthy that the endogenous HNE in HL-60 cells is localized in granules whereas the transfected protein is distributed in the cytoplasm (Figure 5). The regulation of neutrophil elastase expression, its processing and cellular distribution in the primary bone marrow progenitor cells of these patients is a subject of future investigations. The fact that the intracellular localization of each mutant NE protein expressed in transfected cells is not different from the pattern of normal NE protein in the transfected cells (Figure 5) suggests that the pro-apoptotic effect of NE mutants might be due to an altered substrate specificity rather than aberrant subcellular localization. Further studies are warranted to determine whether mutant NE exerts a similar role in the pathogenesis of cyclic or severe congenital neutropenia. It is important to be aware that accelerated apoptosis is a common feature of different disorders of granulopoiesis, independent of the presence of ELA2 mutations, and that further studies are also necessary to elucidate how mutated elastase induces accelerated apoptosis.

The current results are at variance with those of Li and Horwitz, who found no evidence that mutant forms of NE are cytotoxic [23]. However, these investigators
utilized the rat RBL basophilic/mast cell leukemia and the mouse 32D myoblast-like cell lines, two cell types that may not accurately reflect the effect of mutant NE on human cells of the myeloid lineage. Taken together, these findings emphasize the importance of additional tissue- and lineage-specific studies of mutant NE.

If the 4 patients participating in the present study are representative of the patients originally described by Kostmann, then the heterozygous mutations in the NE gene cannot explain the autosomal recessive pattern of inheritance of severe neutropenia in the original “Kostmann family”. However, the current results are not attributable to misidentification of parents of the affected family members, since analysis of genomic DNA from the family members confirmed that both parents are the biological parents of the 2 patients with missense mutations in the NE gene (data not shown). The presence of an NE mutation in the skin fibroblasts of patient 1 confirms that the L92H mutation was acquired very early during embryogenesis, whereas in patient 3 the R191Q mutation was present only in hematopoietic tissue, indicating that this mutation occurred in a progenitor cell already committed to the hematopoietic lineage. Unfortunately, hematological specimens from the original Kostmann cases are not available. Therefore, we cannot determine whether NE mutations were present in these individuals. Taking into account that the heterozygous L92H and R191Q mutations were present only in the patients but not in healthy parents or siblings, the fact that the heterozygous R191Q mutation was previously identified in autosomal dominant cyclic neutropenia [8], and the fact that L92H substitution was seen in a non-related SCN patient with no family history, we assume that the NE mutations identified in these patients are acquired rather than
inherited mutations. There is no obvious difference between the patients with and without ELA2 mutations, neither in the clinical course nor in the in vitro data. However, the patients with ELA2 mutations were diagnosed at an earlier age (2 weeks and 2 months) as compared to those without ELA2 mutations (5 months in both children) (Table 1). Based on these data, one may conclude that there is a heterogeneity in the original “Kostmann family”, or alternatively, that the causative gene for autosomal recessive severe neutropenia in this family is not NE, or not NE alone. Severe neutropenia in 2 of 4 patients examined may be due to acquired NE mutations, whereas in other patients including patients with autosomal recessive SCN, other genes may be responsible for the phenotype. Interestingly, during the preparation of this manuscript, Ancliff and associates reported three patients with autosomal recessive SCN, none of whom displayed NE mutations [9]. These observations thus support the notion that the autosomal recessive form of SCN may result from other underlying molecular aberrations than the ones observed in autosomal dominant SCN and cyclic neutropenia. Recently, Germeshausen et al. suggested that the heterozygous NE mutation is not the single cause of severe neutropenia and other genetic or epigenetic event may be necessary for expression of the neutropenic phenotype [24]. At least one of the patients (pt 3) is a mosaic for the described NE mutation. In a recent report Ancliff and coworkers described another patient with a paternal mosaicism, and found that precursors containing the mutation were selectively lost during myelopoiesis, or failed to develop into neutrophils, in their patient (25). Unfortunately, it is impossible to determine whether our patient was mosaic before his bone marrow transplant because of a lack of specimens taken prior to the transplantation.
To conclude, we have identified NE mutations in some, but not all surviving severe neutropenia patients of the original “Kostmann family”, and have provided evidence that expression of mutant NE in hematopoietic cells can stimulate apoptotic cell death. However, additional genetic and functional studies are clearly needed to provide a complete understanding of the underlying molecular causes of this congenital bone marrow disorder.
Acknowledgements

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REFERENCES


encoding neutrophil elastase are present in most patients with sporadic severe congenital neutropenia but only in some patients with the familial form of the disease. Blood. 2001;98:2645-2650.


Table 1. Clinical data and laboratory findings in Kostmann patients and family members

<table>
<thead>
<tr>
<th>Patients and relatives</th>
<th>Year of birth</th>
<th>Sex</th>
<th>Age at diagnosis</th>
<th>WBC prior to G-CSF (x10⁹/L)</th>
<th>ANC prior to G-CSF (x10⁹/L)</th>
<th>Monocytes prior to G-CSF (x10⁹/L)</th>
<th>ANC with G-CSF (x10⁹/L)</th>
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<td>4.7</td>
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<td></td>
<td></td>
<td>5.3</td>
<td>2.4</td>
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*ANC before BMT. ANC after BMT but before G-CSF varied between 0.2-2.0x10⁹/L (mostly <1.0x10⁹/L)
Figure legends

Figure 1. Pedigree of the original “Kostmann family” (modified from [4] to include parents and siblings of the 4 patients participating in the present study). Filled squares and circles indicate affected individuals.

Figure 2. Mutational analysis of the NE gene in the “original Kostmann family”. A - The amino acid positions of both substitution and silent mutations are indicated with an arrow. B - Tertiary structure of NE with position of mutations identified in Kostmann patients. The catalytic serine S173 is indicated with an arrow; NG indicates the position of N-linked glycosylation sites. The image was prepared from the x-ray crystallographic coordinates of NE as described in Materials and Methods.

Figure 3. Mutations in the NE gene identified in Kostmann patients, their parents and siblings. Circles and squares with + mark indicate that peripheral blood or bone marrow samples from the individual was available for mutational analysis of the NE, G-CSFR, and WAS genes.

Figure 4. Accelerated apoptosis of human promyelocytic HL-60 cells transfected with normal or mutant NE as assessed by flow cytometric analysis of annexin V-labeled cells (A). Normal (wild type) and 2 mutant L92H and R191Q mutant NE cDNA constructs were expressed. Data shown are
representative of three independent experiments. **B** - Expression level of normal and mutant NE in transfected cells as determined by Western blot analysis using NE or β-actin specific antibodies, as described in Methods.

**Figure 5.** Subcellular localization of neutrophil elastase (red) in human promyelocytic HL-60 cells. The mock-transfected cells (panel A) or cells transfected with NE mutants L92H (panel B), R191Q (panel C) or normal NE (panel D) were stained with primary NE-specific monoclonal antibody followed by labeling with Rho-conjugated secondary antibody. The nuclei were stained blue using DAPI, a DNA-binding dye, as described in Methods section.
Figure 1.
Figure 3.

- **Pt. 1**: S173, L92H
- **Pt. 2**: No mutation
- **Pt. 3**: S173, R191Q
- **Pt. 4**: S173
Figure 4.

A

B
Figure 5.
Neutrophil elastase mutations in severe congenital neutropenia patients of the original Kostmann family

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