Neutrophil elastase and granulocyte colony-stimulating factor receptor mutation analyses and leukemia evolution in severe congenital neutropenia patients belonging to the original Kostmann family in northern Sweden

Göran Carlsson
Andrew A.G. Aprikyan
Kim Göransdotter Ericson
Steve Stein
Vahagn Makaryan
David C. Dale
Magnus Nordenskjöld
Bengt Fadeel
Jan Palmblad
Jan-Inge Hentera

Background and Objectives. Severe congenital neutropenia (SCN) or Kostmann syndrome was originally reported to be an autosomal recessive disease of neutrophil production causing recurrent, life-threatening infections. Mutations in the neutrophil elastase gene (*ELA-2*) have previously been identified in patients with sporadic or autosomal dominant SCN.

Design and Methods. We studied 14 individuals (four patients with SCN and ten close relatives) belonging to the original Kostmann family in northern Sweden for mutations in the *ELA-2* and the granulocyte colony-stimulating factor (G-CSF) receptor genes.

Results. One patient belonging to the original Kostmann family harbored a novel heterozygous *ELA-2* mutation (g.2310T→A;Leu92His) that was not inherited from her parents. The mutation was identified in DNA isolated from both whole blood and skin fibroblasts, suggesting a sporadic *de novo* mutation. As a young adult this patient sequentially acquired two mutations in the gene for the G-CSF receptor (*G-CSFR*) and therefore recently received a hematopoietic stem cell transplant, due to the risk of evolution to leukemia. Moreover, another patient developed acute leukemia and was treated with transplantation. No pathogenic *ELA-2* or *G-CSFR* gene mutations were found in this patient or the other two patients, nor in any healthy relative.

Interpretation and Conclusions. Our data are the first to document leukemia evolution and *G-CSFR* gene mutations in the original Kostmann kindred. In addition, our findings indicate that *ELA-2* mutations are not the primary cause of SCN in the Swedish Kostmann family.

Key words: severe congenital neutropenia, Kostmann syndrome, *ELA-2*, G-CSF receptor, leukemia.

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From the Childhood Cancer Research Unit, Department of Woman and Child Health, Karolinska University Hospital Solna, Stockholm, Sweden (GC, KGE, J-IH); Department of Medicine, University of Washington, Seattle, WA, USA (AAGA, SS, VM, DCD); Clinical Genetics Unit, Department of Molecular Medicine, Karolinska University Hospital Solna, Stockholm, Sweden (KGE, MN); Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden (BF); Department of Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden (JP).

Correspondence:
Jan-Inge Henter, MD, PhD, Childhood Cancer Research Unit, Karolinska University Hospital, Q6:05, SE-171, 76 Stockholm, Sweden.
E-mail: jan-inge.henter@ki.se

Severe congenital neutropenia (SCN), also known as Kostmann syndrome, was first described by Rolf Kostmann in 1956 as an autosomal recessive disorder in a large kindred from northern Sweden.[1,2] The disease is characterized by an arrest of the maturation of neutrophil precursors at the promyelocytic stage of differentiation and 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 members from the original Kostmann family have survived, and we have recently reported on their clinical status and response to administration of G-CSF.[4] In addition, our recent studies have provided evidence of accelerated apoptosis of myeloid bone marrow progenitor cells from these individuals.[5] A lack of certain antibacterial peptides has also been observed in Kostmann patients.[6] However, the underlying molecular defect remains unknown. Mutations in the cytoplasmic region of the gene for the G-CSF receptor (*G-CSFR*) have been reported in a proportion of SCN patients in association with evolution to acute myeloid leukemia.[7-9] It is now understood that these mutations represent an acquired event and are not the underlying cause of the disease. Moreover, mutations in the Wiskott-Aldrich syndrome gene (*WAS*) may cause X-linked SCN, although these cases seem to be quite rare.[10] We have previously reported that all patients with cyclic neutropenia, a disease with a characteristic 21-day cycle of oscillating neutrophil numbers, and more than 80% of autosomal dominant and sporadic cases of SCN harbor either inherited or *de novo* mutations in the neutrophil elastase gene (*ELA-2*).[11-13] In contrast, three families with autosomal recessive SCN were recently reported to have no *ELA-2* mutations.[14] To address the issue of the underlying genetic cause of the SCN in the original Kostmann family, we performed mutation analyses of the candidate genes *ELA-2*, *WAS*, *GFI-1*, and *G-CSFR*, in the four surviving affected family members.

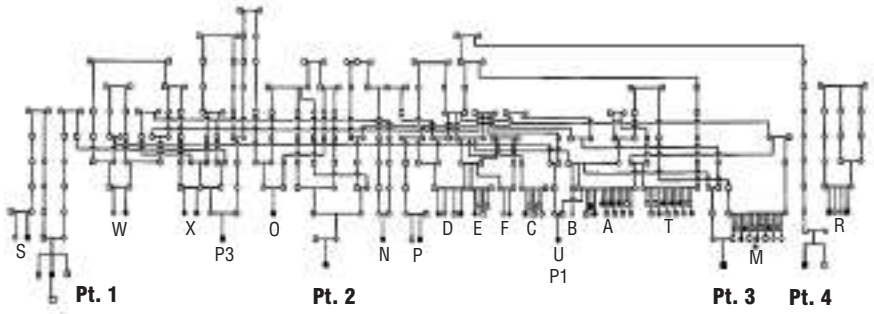

Figure 1. Pedigree of the original Kostmann family from northern Sweden. Modified from Carlsson & Fasth (2001) to include parents, siblings, and children of the four patients described in the present study. Filled squares and circles indicate affected individuals.

Design and Methods

Patients

Kostmann reported 22 patients with SCN 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, a maturation block at the promyelocyte-myelocyte level in the bone marrow, and a similar pattern of clinical signs and symptoms of recurrent fevers and infections. Based on family studies, Kostmann concluded that the congenital neutropenia was inherited in an autosomal recessive manner. There is some evidence that all these cases of SCN descend from a single founder, the likely place of origin being the parish of Överkalix in northern Sweden.[15] Four individuals, all linked to the large family initially described by Kostmann, and the healthy parents, siblings and children of three of these four individuals are the subjects of the present report (Figure 1). Patient 1, a 29-year old woman is the first of three children. Her two brothers are healthy. She is the mother of a healthy son, aged 2 years. Because of the *G-CSFR* mutations described herein, she received a stem cell graft from one of her brothers, who was HLA-identical. The families of both her parents are related to the large family described by Kostmann (Figure 2). Patient 2, a 22-year old woman is the only common child of parents originating from the parish of Överkalix, and also belongs to the original Kostmann family. Patient 3, a 21-year old man is the only child of parents who have relatives affected by Kostmann syndrome. He underwent a hematopoietic stem cell transplant at 7 months of age with the father as the donor. The brother of the maternal great-grandmother was also the father of Kostmann's index case identified in 1949. The father of patient 3 had a brother who died of Kostmann syndrome in the 1950s. Patient 4, a 12-year old boy is the second child of 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. In September 2005, at the age of 12 years and while in good clinical condition, a routine annual bone marrow biopsy revealed an acute leukemia with 30% blasts. His platelet count and hemoglobin level were normal but the absolute neutrophil count was only 0.55×10^9/L despite G-CSF therapy. The blasts expressed CD34, CD13, CD33, CD19, CD22, CD123, Tdt, CD79a, but were negative for

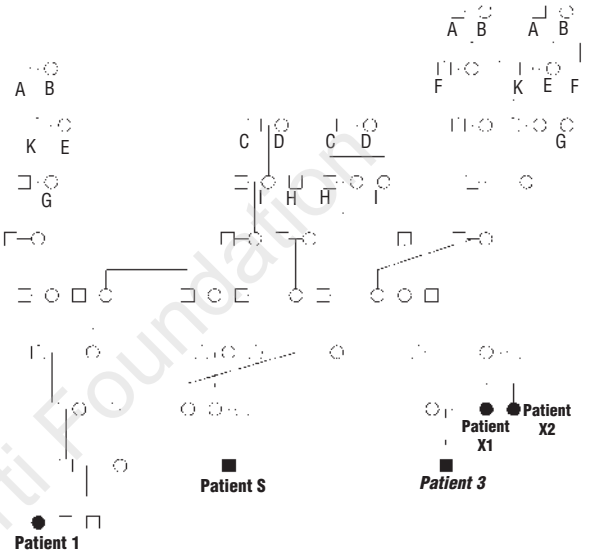

Figure 2. Pedigree of the family of patient 1. The letters mark different ancestors and show that the same ancestor (A, B, C, etc) may have several positions in the pedigree, thus demonstrating the consanguinity of the family. The pedigree also shows the relations to four other patients, three described by Kostmann in 1975 (patients S, X1 and X2),[2] and one by Carlsson & Fasth in 2001 (patient 3).[4] Their positions in Figure 1 are marked with S, X and P3, respectively.

CD117, myeloperoxidase, cytoplasmatic CD22, and CD65. Thus, the immune phenotype was difficult to interpret but it was considered as a pre-B-acute lymphocytic leukemia with aberrant expression of myeloid markers. Karyotyping using G-banding indicated a marker chromosome in five of 25 analyzed cells. The remaining 20 cells showed a normal male karyotype, 46,XY. However, the findings prompted us to perform further analyses by fluorescence *in situ* hybridization (FISH) and spectral karyotyping (SKY). The results from the FISH analysis indicated two extra chromosomes 21 in 74% of the investigated cells.

By SKY analysis the karyotype was further refined to 47,X,der(Y)t(Y;1),der(5)t(4;5),der(7)t(6;7),+i(21)(q10). The patient subsequently underwent hematopoietic stem cell transplantation from a matched unrelated donor. Detailed summaries of the previous clinical histories of these patients have been reported elsewhere.[4] The absolute neutrophil counts of the affected family members before

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

Patients and relatives	Year of birth	Sex	Age at diagnosis	WBC prior to G-CSF ($\times10^9$/L)	ANC prior to G-CSF ($\times10^9$/L)	Monocytes prior to G-CSF ($\times10^9$/L)	ANC with G-CSF ($\times10^9$/L)
*Patient 1**	*1976*	*F*	*2 weeks*	*3.9-15.6*	*0-0.3*	*0.5-3.0*	*3.6-12.6*
Mother	1956			8.4	5.7	0.7	
Father	1952			7.7	4.7	0.5	
Brother	1978			5.3	2.4	0.7	
Brother	1980			6.1	4.0	0.5	
Child	2003			12.6	8.2	0.9	
Patient 2	*1983*	*F*	*5 months*	*3.0-13.6*	*0-0.2*	*0.6-4.0*	*1.2-4.4*
Patient 3†	*1984*	*M*	*2 months*	*4.6-9.5*	*0-0.2**	*0.2-1.2*	*1.5-7.5*
Mother	1960			4.2	2.1	0.4	
Father	1953			4.0	2.1	0.3	
*Patient 4**	*1993*	*M*	*5 months*	*6.5-9.2*	*0.1*	*1.6-6.4*	*1.0-9.7*
Mother	1969			5.4	2.8	0.3	
Father	1971			6.3	3.8	0.6	
Brother	1991			5.4	2.7	0.4	

*ANC: absolute neutrophil count; WBC: white blood cell count; *Patients 1 and 4 have recently undergone HSCT (as described in the current report), and their ANC values are now normalized without treatment with G-CSF. †ANC prior to HSCT (performed in early childhood). ANC after HSCT but before initiation of G-CSF treatment varied between 0.2-2.0×10⁹/L (mostly <1.0×10⁹/L).*

and during G-CSF therapy, their parents and unaffected siblings are presented in Table 1. All parents, siblings, and children were hematologically healthy, and in contrast to the affected family members these individuals experienced no problems with recurrent infections.

Collection of samples

Blood samples from four patients, three sets of parents, siblings, and one child (of patient 1) were obtained using a routine venipuncture technique. Blood counts were performed using an automated whole blood cell counter and by examining Wright-stained blood smears. Bone marrow samples were collected in association with the annual control recommended by the Severe Chronic Neutropenia International Registry (SCNIR) due to the risk of leukemia evolution in these patients. All subjects participated in these studies after giving informed consent according to a protocol approved by the ethical committee of Umeå University, Sweden. Skin biopsies were performed using standard techniques and used for isolation of genomic DNA based on proteinase K digestion.

Mutation analysis

Genomic DNA isolated from peripheral blood mononuclear cells or skin fibroblasts was used for mutation analysis of the *G-CSFR*, *WAS*, *GFI-1*, and *ELA-2* genes as described previously.[8,10,12,16] Sequencing of polymerase chain reaction (PCR)-amplified products was performed using BigDye™ terminator chemistry on an ABI 3700 sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). To rule out PCR-introduced artifacts, each *ELA-2* mutation predicted to affect the protein structure, but not polymorphisms, was confirmed at least three times by independent PCR followed by sequencing across the region of interest. *G-CSFR* mutation analysis in patient 1 was also performed on bone marrow samples by sequence analysis of isolated cDNA clones, as previously described.[7,8]

Structural modeling

Co-ordinates of the neutrophil elastase (NE) protein were obtained from the Protein Data Bank and mutations were introduced using a previously reported program.[12] Homology modeling was carried out with tools available within the Molecular Operating Environment from the Chemical Computer Group, Montreal, Canada (*http://www.chemcomp.com*) as previously described.[12]

Results

Mutation analysis of the ELA-2 gene

Sequencing analysis of all five exons of the *ELA-2* gene revealed a heterozygous missense mutation in exon 3 and a sequence variant regarded as a polymorphism in exon 5 (Figure 3A). A nucleotide substitution that did not affect the protein was observed at codon 173 in three of the four patients, in four of six healthy parents, as well as in one of three healthy siblings examined (Figure 3B). A heterozygous missense mutation was identified in exon 3 in one of the four patients (Figure 3A,B). The mutation caused a nucleotide substitution, g.2310T→A resulting in an amino acid substitution of leucine to histidine at codon 92 (Leu92His). This mutation was not found in any of the parents or siblings of this patient, nor in her child or in healthy subjects. Mutation analysis revealed the presence of the same Leu92His mutation in genomic DNA isolated from skin fibroblasts of patient 1, whereas no mutation was identified in genomic DNA isolated from her healthy parents (*data not shown*).

Our current results for patient 1 are not attributable to misidentification of parents of the affected family members, since analysis of available genomic DNA from the parents confirmed that these individuals are her biological parents. It is also of interest to note the absence of neutropenia in the son of patient 1, which further supports the original assumption of Rolf Kostmann that the disease is inherited as a recessive trait. Moreover, this child has not inherited either the Leu92His mutation or the Ser173 sequence variant identified in his mother, indicating that these nucleotide alterations are on the same allele in patient 1.

Structural analysis of the Leu92His mutant protein

The tertiary structure of the NE protein, with an inhibitor molecule in its active site, is shown in Figure 4. Neutrophil elastase has two N-linked glycosylation sites, designated here as NG. The active site with a catalytic serine at codon 173 (Ser 173) is marked with an arrow. The Leu92His mutation identified in patient 1 is adjacent to the Asn95 glycosylation site (Figure 4) and may alter normal glycosylation-deglycosylation processes resulting in abnormal targeting of the mutant protease. The location of the Leu92His heterozygous mutation thus suggests that the normal protein processing and substrate

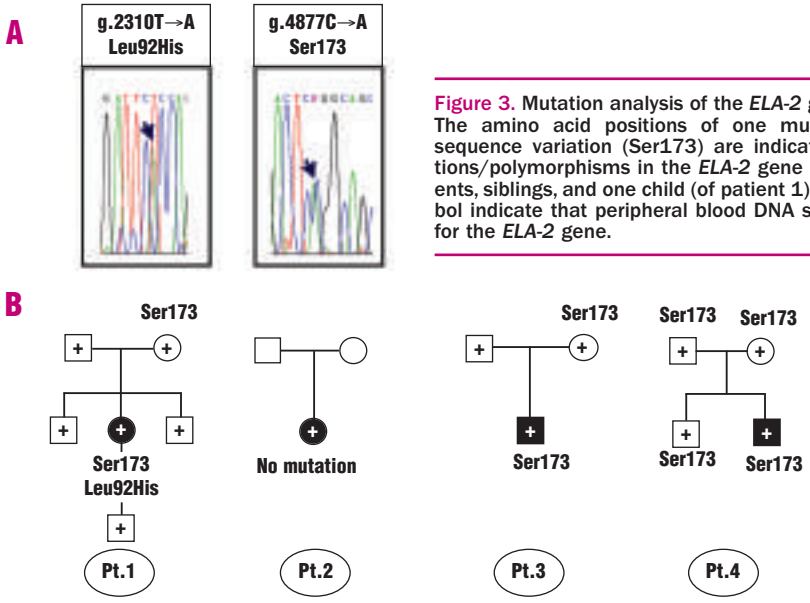

Figure 3. Mutation analysis of the *ELA-2* gene in the original Kostmann kindred. (A). The amino acid positions of one mutation (g.2310T→A; Leu92His) and one sequence variation (Ser173) are indicated with arrows. (B). Heterozygous mutations/polymorphisms in the *ELA-2* gene in Kostmann syndrome patients, their parents, siblings, and one child (of patient 1). Circles and squares marked with a + symbol indicate that peripheral blood DNA samples from the individual were analyzed for the *ELA-2* gene.

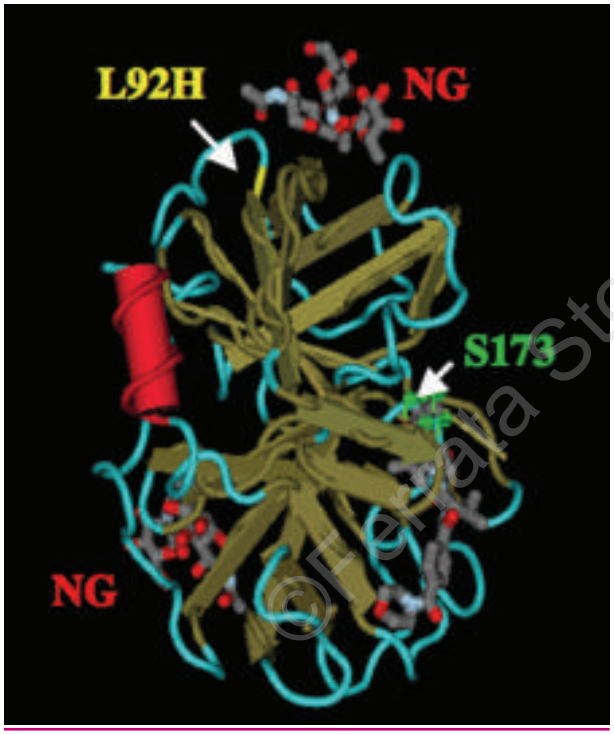

Figure 4. Molecular modeling of neutrophil elastase showing the tertiary structure of the protein with the position of the Leu92His mutation identified in Kostmann syndrome patient 1. The catalytic serine Ser173 is indicated by an arrow; NG denotes the position of N-linked glycosylation sites. The image was prepared from the X-ray crystallographic co-ordinates of NE as described in the Design and Methods.

specificity of this enzyme may be altered; alternatively, this mutation may confer resistance to NE-specific inhibitors, such as α_1-antitrypsin.

Mutation analysis of the G-CSFR, WAS, and GFI-1 genes

We then performed direct sequencing of the *G-CSFR* gene in the four patients and ten relatives. Initial mutation analysis of the intracellular portion of the *G-CSFR* gene did not reveal any aberrations in patients 1-3.[8] However, examination of a DNA sample from patient 1 acquired in January 2003 showed a g.2387C→T mutation, in seven out of 24 individual clones from the bone marrow, resulting in a premature stop codon at 718 (Gln718Stop) (Figure 5A). One year later (January 2004) this mutation was still detectable. In addition, a g.2410C→T mutation, which results in a premature stop codon at 726 (Gln726Stop), was also observed (Figure 5B). No *G-CSFR* gene mutations were identified in the other Kostmann patients or their healthy relatives; of note, there were no *G-CSFR* gene mutations in patient 4 either one or two years prior to the diagnosis of his leukemia or at the time when his leukemia was diagnosed. Moreover, analysis of the *WAS* gene revealed no mutations in the Kostmann patients (*data not shown*), and none of the patients reported herein harbored *GFI-1* gene mutations, which have been described in one patient with severe neutropenia and in some individuals with mild neutropenia.[16]

Discussion

We present the first molecular genetic data on affected and healthy members of the original Kostmann family in northern Sweden. Our studies show that one of four patients harbors a heterozygous *de novo* mutation in the *ELA-2* gene; these findings thus suggest that *ELA-2* mutations cannot explain autosomal recessive SCN or Kostmann syndrome. However, patient 1, with the Leu92His mutation in the *ELA-2* gene, was diagnosed earlier (at the age of 2 weeks) than were the children who did not display any mutations in the *ELA-2* gene.

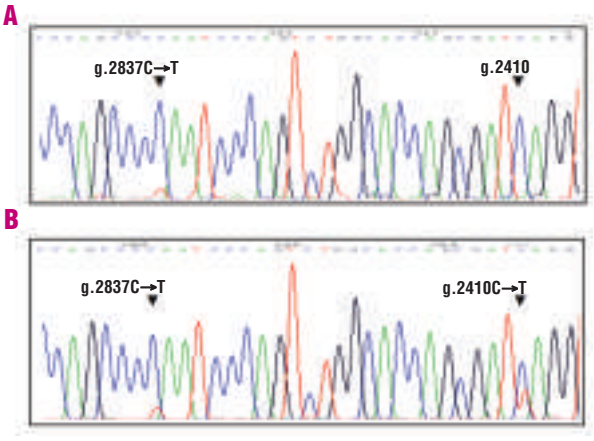

Figure 5. ***G-CSFR*** **receptor gene mutations in patient 1 from the original Kostmann family. Electropherograms depicting the results of analyses performed in January 2003 (A) and January 2004 (B). First, a g.2387C→T mutation resulting in a premature stop codon at 718 (Gln718Stop) was identified; subsequently, the patient acquired a g.2410C→T mutation, which results in a premature stop codon at 726 (Gln726Stop).**

Moreover, this patient has a more severe phenotype insofar as she has suffered from severe periodontal disease with alveolar bone loss despite G-CSF treatment and normalization of absolute neutrophil counts.[4,6] One may theoretically consider the possibility that patient 1 in our study is a phenocopy with an *ELA-2* mutation that occurred spontaneously but a pedigree of the family shows a relation between patient 1 and numerous patients affected with Kostmann syndrome consistent with an autosomal recessive model (Figure 2). Another missense mutation at codon 92 causing a leucine to a proline substitution (Leu92Pro) in the *ELA-2* gene was recently reported in another family with SCN; it was predicted that this codon is of structural importance for the human elastase protein.[17] Our findings support the notion that *ELA-2* mutations may contribute to the phenotype of SCN, and are thus in line with a recent report from the French Neutropenia Register indicating that mutations in the *ELA-2* gene correlate with more severe expression of neutropenia in SCN patients.[17]

Structural modeling of the NE molecule indicates that the Leu92His mutation, identified in patient 1, is positioned in immediate proximity to the glycosylation site Asn95. We hypothesize that this amino acid substitution may alter the availability of this asparagine residue and result in abnormal glycosylation/deglycosylation processing of the mutant protein. Moreover, we have found that the intracellular localization of the Leu92His mutant protein expressed in transfected HL-60 cells is not different from that of normal NE in these cells (*Aprikyan et al., unpublished observations*). Together, these findings suggest that the putative pro-apoptotic effect of NE mutants in transfected cells (discussed below) might be due to an altered substrate specificity rather than ectopic localization or mistrafficking of the protein within the cell. However, a Gly185Arg NE mutant was recently shown to localize predominantly to the nuclear and plasma membranes of HL-60 cells,[18] human myeloid leukemia cells differentiating to neutrophils but lacking secondary granules.[19] Benson *et al.* have provided evidence that *ELA-2* mutations do affect the subcellular localization of NE, at least upon expression in the rat basophilic/mast cell leukemia RBL cell line.[20] The latter findings suggest that NE mutants may display different patterns of subcellular localization depending on the specific mutation and/or the model system used. Normal NE has been shown to proteolytically cleave the G-CSFR and its ligand, thus suggesting a role for NE as a potential negative regulator of hematopoiesis.[21,22] Whether or not mutant NE displays a similar proclivity for neutrophil survival factor(s) and receptor(s) remains to be tested.

We have recently reported accelerated apoptosis of bone marrow myeloid progenitor cells from patients with SCN or cyclic neutropenia.[5,23] Moreover, we and others showed that expression of various NE mutants, but not normal NE, resulted in accelerated apoptosis of HL-60 cells.[13,18] The Leu92His mutant reported here can also trigger apoptosis upon transient overexpression using the same model system. In concordance with these findings, Massullo *et al.* have recently shown that retroviral transduction with a Gly185Arg mutant provokes premature apoptosis of HL-60 cells induced to differentiate with dimethyl sulfoxide, resulting in fewer mature (neutrophil-like) cells.[18] Collectively, these findings suggest that mutant NE may contribute to the classical maturation arrest at the promyelocytic/myelocytic stage in the bone marrow of SCN patients. This arrested maturation may occur either through a direct pro-apoptotic effect on neutrophil precursors or indirectly by altering the fate of myeloid differentiation to favor monocytopoiesis over granulocytopoiesis, possibly through dysfunctional interactions of mutant NE with the Notch signaling pathway.[24] However, it is important to note that accelerated apoptosis is a common feature of a range of disorders of granulocytopoiesis, including myelokathexis, cyclic neutropenia, and SCN, irrespective of the presence or absence of *ELA-2* mutations.[13,23,25] Indeed, we have recently shown that all surviving members of the original Kostmann family have decreased expression of the anti-apoptotic protein Bcl-2 prior to treatment with G-CSF and accelerated apoptosis of hematopoietic progenitor cells.[5] Elevated apoptosis and low expression of certain Bcl-2 family members was also documented in a recent study of German SCN patients not related to the original Kostmann family.[26] Therefore, further studies are necessary to elucidate the underlying mechanism of accelerated progenitor cell apoptosis in Kostmann syndrome.

Mutations in the *G-CSFR* gene may render individuals susceptible to the development of leukemia as well as high-risk myelodysplastic syndromes.[7,27] Ancliff and colleagues previously reported a *G-CSFR* gene mutation in a patient with autosomal recessive SCN who subsequently developed acute myeloid leukemia and died.[28] However, to the best of our knowledge, our study is the first to show the simultaneous occurrence of *ELA-2* and *G-CSFR* mutations in a patient with the autosomal recessive form of SCN. Importantly, patient 1 in the current study showed progression of acquired genetic

events since the *G-CSFR* mutations appeared sequentially over the course of 3-4 years, suggesting an underlying genetic instability. The karyotype and the morphology of the bone marrow of this patient showed no evidence of leukemic transformation during the course of the disease; however, we decided that the two *G-CSFR* mutations warranted a hematopoietic stem cell transplant, since the acquisition of additional *G-CSFR* mutations has been suggested to be a strong indicator of leukemic transformation.[8,29] Although the presence of *G-CSFR* gene mutations in patients with SCN correlates with progression to acute myeloid leukemia/myelodysplastic syndrome, there is little direct evidence that these mutations contribute to leukemogenesis. The patient is doing well at the time of writing of this report (12 months post-transplantation), without the need for G-CSF treatment. Notably, patient 4 in our report developed an acute leukemia, without the presence of concomitant *G-CSFR* gene mutations. This patient is also doing well, although only 2 months have passed since his transplant. Thus, three out of the four patients (75%) belonging to the original Kostmann kindred have now been transplanted, including the child transplanted in the 1980s before the availability of G-CSF.[4]

Finally, for clarification, we wish to mention that our group previously reported, in a preliminary study, that heterozygous *ELA-2* mutations were present in both patient 1 and patient 3 from the original Kostmann family.[30] However, repeated analyses of these patients confirmed the Leu92His mutation in patient 1 (described herein), but failed to confirm mutations in patient 3. Of note, patient 3 had a partially successful hematopoietic stem cell transplant at 7 months of age and is now a mosaic; therefore, it is theoretically possible that re-sequencing at different time-points might yield opposing results, or that the original data may have been due to cross-contamination of DNA or mislabeling of samples in the laboratory. In support of the possibility of mosaicism, a recent report provided evidence of mosaicism for an *ELA-2* mutation in the father of a child with SCN; the authors of this report suggested that precursor cells harboring the mutation are selectively lost during myelopoiesis or fail to develop into mature neutrophils.[31] With regard to the sequence variation observed at codon 173, it could be speculated that this polymorphism is a modifier gene but since the polymorphism does not change the protein sequence and was not found in all patients, and since it was also observed in approximately 10% of healthy volunteers (Aprikyan *et al.*, *unpublished observations*) it is unlikely that this sequence variation plays a role in the pathogenesis of SCN.

In conclusion, our studies show that mutations of the *ELA-2* gene are not the cause of autosomal recessive SCN in the original Kostmann family. Moreover, we found no *WAS* or *GFI-1* gene defects in these patients. Similarly, a study of one family with two affected children and mosaicism for an *ELA-2* mutation in the father, suggested that mutations in *ELA-2* are not sufficient to explain the phenotype of Kostmann-like SCN.[32] These observations contrast with recent reports on autosomal dominant and sporadic forms of SCN and cyclic neutropenia that identify mutations in the *ELA-2* gene as a causative factor in the majority of patients.[12,17] The mutations reported here may, instead, reflect an underlying genetic instability in this disease. Moreover, the finding of an *ELA-2* mutation and the sequential acquisition of mutations in the *G-CSFR* gene, a harbinger of malignant progression,[33] in one patient belonging to the original Kostmann family and the development of overt leukemia in another patient suggest that Kostmann syndrome may, in fact, be considered a premalignant condition.

GC and JIH initiated the Kostmann family project, and JP, DD, GC and JIH contributed to the conception of this study. GC was responsible for the patients studied and clinical data. AAAP performed and processed most experiments, assisted by SS and VM; KGE and MN separately performed and evaluated neutrophil elastase gene analyses. AAGA, JIH, BF, JP, DD and GC contributed to study design, data analysis and the final version of the manuscript, which was drafted by AAGA, BF and JIH, and approved by all authors.

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