

# Cyclic Neutropenia and Severe Congenital Neutropenia in Patients With a Shared *ELANE* Mutation and Paternal Haplotype: Evidence for Phenotype Determination by Modifying Genes

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**Background.** Cyclic neutropenia (CN) and severe congenital neutropenia (SCN) are disorders of neutrophil production that differ markedly in disease severity. Mutations of the *ELANE* gene (the symbol recently replacing *ELA2*) are considered largely responsible for most cases of CN and SCN, but specific mutations are typically associated with one or the other. **Procedure.** We performed *ELANE* genotyping on all individuals and paternal sperm in an SCN kindred with eight SCN progeny of a sperm donor and six different mothers. **Results.** One patient with CN had the same S97L *ELANE* mutation as seven patients with the SCN phenotype. The mutant allele

was detected in the donor's spermatozoa, representing 18% of the *ELANE* gene pool, but not in DNA from his lymphocytes, neutrophils, or buccal mucosa, indicating gonadal mosaicism. **Conclusions.** The coexistence of CN and SCN phenotypes in this kindred with a shared paternal haplotype strongly suggests both a role for modifying genes in determination of congenital neutropenia disease phenotypes, and the classification of CN and SCN within a spectrum of phenotypes expressing varying degrees of the same disease process. *Pediatr Blood Cancer.* 2010;55:314–317. © 2010 Wiley-Liss, Inc.

**Key words:** cyclic neutropenia; *ELANE* mutation; severe congenital neutropenia; sperm donor

## INTRODUCTION

Cyclic neutropenia (CN) is an autosomal dominant disorder (sporadic or inherited) characterized by regular oscillations of peripheral blood neutrophils from near normal to severely low levels, generally with 21-day periodicity. Absolute neutrophil count (ANC) nadirs are often below 200/mm<sup>3</sup> and are associated with fever, mouth ulcers, pharyngitis, sinusitis, or more serious infections (such as colitis with gram negative sepsis) [1–3]. CN usually presents early in childhood but may run an asymptomatic course. Transformation to myelodysplastic syndrome and acute myeloid leukemia has not been reported [4,5]. Symptomatic CN is responsive to granulocyte colony-stimulating factor (G-CSF; filgrastim), often at low doses, which typically shortens nadir duration and increases mean and nadir ANC but usually does not ablate cycling [6,7].

Severe congenital neutropenia (SCN) is a phenotypically distinct and usually more severe disease. It is characterized by recurrent, severe infections developing in the first months of life, with ANC's consistently below 200/mm<sup>3</sup>. Bone marrow examination characteristically shows maturation arrest of neutrophil precursors at the promyelocyte/myelocyte stage of development [3,8]. Unlike CN, SCN is characterized by transformation to myelodysplasia and acute myeloid leukemia [9,10]. Genetic transmission may be either autosomal dominant and recessive, with many more sporadic than inherited cases reported [11–14].

CN and SCN appear to derive largely from heterozygous germline mutations in the *ELANE* (*EL*astase–*Neutrophil Expressed*) gene, previously termed *ELA2*. Mutations have been found in 80–100% of CN cases [14–16] and in 35–63% of SCN cases [3,16,17], whether sporadic or inherited. The *ELANE* gene encodes neutrophil elastase, a chymotryptic serine esterase synthesized during the myeloblast to promyelocyte transition [18]. The mutant elastase likely induces accelerated apoptosis in differentiating myeloid cells [10,19,20]. One proposed model suggests that this accelerated apoptosis is due to the production of misfolded elastase proteins and subsequent activation of the unfolded protein response (UPR) [20,21]. However, further studies are needed to define the role of the *ELANE* gene and its protein product in CN and SCN pathogenesis.

While SCN and CN both appear to largely derive from *ELANE* mutations, studies have demonstrated that the type of *ELANE* mutation largely differs between these diseases. Specific mutations have been associated with both CN and SCN in unrelated patients [21], but the vast majority of mutations are associated with either CN or SCN, not both. SCN and CN-associated mutations tend to vary in genetic location and possibly, effect on elastase activity. For example, a mutation mapping study of 81 unrelated patients with SCN (n = 54) or CN (n = 27) found that CN *ELANE* mutations tend to be located in exons 2, 3, and 4 (which encode the mature polypeptide), while SCN *ELANE* mutations are spread over the entire five-exon sequence [16]. Although studies have suggested a relationship between individual *ELANE* mutations and disease severity (e.g., G185R is associated with a more severe phenotype than the R191Q mutation [21]), a more general genotype–phenotype correlation has not been established.

## METHODS

All procedures and consents were approved by the human subjects committees of the investigators' institutions. Peripheral blood mononuclear cells and granulocytes were purified by Dextran sedimentation and Ficoll/Hypaque centrifugation as previously

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described. Sperm was isolated by a previously described method [22]. DNA was isolated from leukocytes, buccal swabs, and spermatozoa using the Genra Puregene Core Kit A or QIAmp DNA Mini Kit (both from Qiagen, Inc., Valencia, CA) according to the manufacturer's recommended procedures.

PCR amplification of the affected sequence in the *ELANE* gene was performed with primer sets 8037F (5'-GCA CTC AAG CCA CAT CCA-3')/8706R (5'-GAT TAG CCC GTT GCA GAC-3') and 7799F (5'-TTG ATT GGC CCA CCT CAG C-3')/8706R (primer position numbers refer to the genomic sequence for *ELANE*, GenBank accession NG\_009627). Products of PCR amplification with HotStarTaq DNA Polymerase (Qiagen, Inc.) were purified from 1% agarose gels using Qiaquick Gel Extraction Kits (Qiagen, Inc.).

The amplicons were sequenced in the forward and reverse directions using BigDye Terminator V3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA) and a ABI3730xl 96 capillary DNA analyzer (Applied Biosystems). Maximum peak intensities were extracted manually from raw sequencing traces using Sequence Scanner v1.0 software (Applied Biosystems). The fraction of mutant (T) versus wild-type (C) allele representation was calculated using the formula  $T/(C+T) \times 100\%$  or  $A/(G+A) \times 100\%$  for forward or reverse traces, respectively. Averaging of values from forward and reverse traces produced quantitative data that accurately reflected the composition of known mutant and wild-type PCR product mixtures. The fraction of mutant allele in sperm DNA was calculated from a calibration curve generated from mixtures containing 0%, 12.5%, 25%, and 50% mutant alleles, with the process repeated in four independent amplification and sequencing runs.

## RESULTS

### Patients

A previously unreported patient was the product of conception with frozen sperm from the same donor described in our report of five SCN patients in Michigan, all progeny of the same sperm donor [13]. The current patient presented at 12 months of age with severe stomatitis and mouth ulceration. He was subsequently found to have

an ANC of zero and treated with antibiotics, after which his ANC began to rise. Within the following month he was readmitted for fever and neutropenia, again with stomatitis. Bi-weekly complete blood counts over the following three months showed periods of severe neutropenia ( $ANC < 500/mm^3$ ), separated by ANC peaks of 1,560 and 1,300/ $mm^3$ . The pattern included two cycling episodes with 21 days or less between ANC nadirs below 200/ $mm^3$ . Figure 1 illustrates two ANC cycles identified prior to G-CSF treatment.

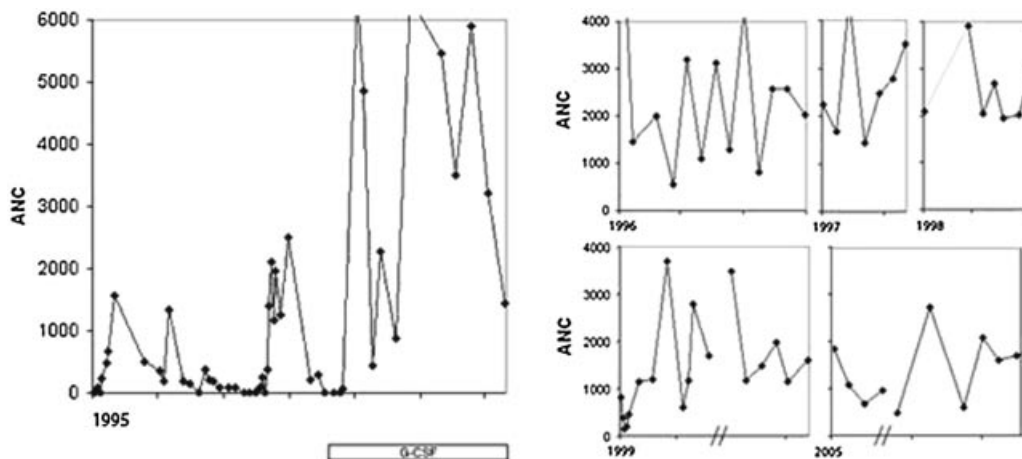
A bone marrow examination before G-CSF treatment showed normal cellularity with moderate myeloid hyperplasia and a left-sided myeloid series with myeloid maturation to, but not beyond, the metamyelocyte stage. An anti-neutrophil antibody test showed borderline positive signal for neutrophil-associated IgG in a flow cytometric assay. Due to his severe neutropenia and oral mucositis, the patient began G-CSF treatment and his ANC improved to values above 1,000/ $mm^3$  within 6 days. Over the following years, his ANC has continued to demonstrate cycling, with all nadirs (after the first two months of G-CSF treatment) greater than 500/ $mm^3$ . In 13 cycles monitored during G-CSF therapy, 8 have shown periods of 14 days or less (also shown in Fig. 1). He currently receives treatment with G-CSF at a dose of 1.2 mcg/kg subcutaneous daily.

Sequencing of the patient's *ELANE* gene showed a mutation in the fourth exon, resulting in the amino acid substitution S97L. This mutation is identical to that found to be the common cause of SCN in the previously reported progeny of the sperm donor. The affected amino acid is located on a surface loop, distant from the active site of the protease.

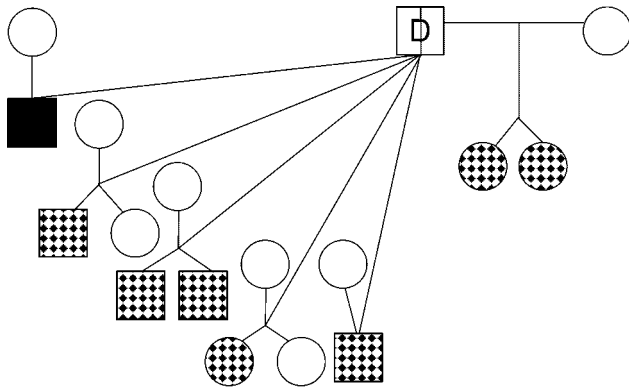
Further investigation has found that the donor has normal peripheral blood counts, and that his wife has borne twins with SCN. All mothers had *ELANE* genotyping, with no mutations identified. The resultant complete pedigree is shown in Figure 2.

### DNA Sequence Analysis

The missense mutation responsible for the S97L substitution is a C8284T transition [14] in exon 4 of the *ELANE* gene (GenBank NG\_009627). As shown in Figure 3, sequence analysis of mixtures of DNA from S97L mutant and normal peripheral blood leukocytes

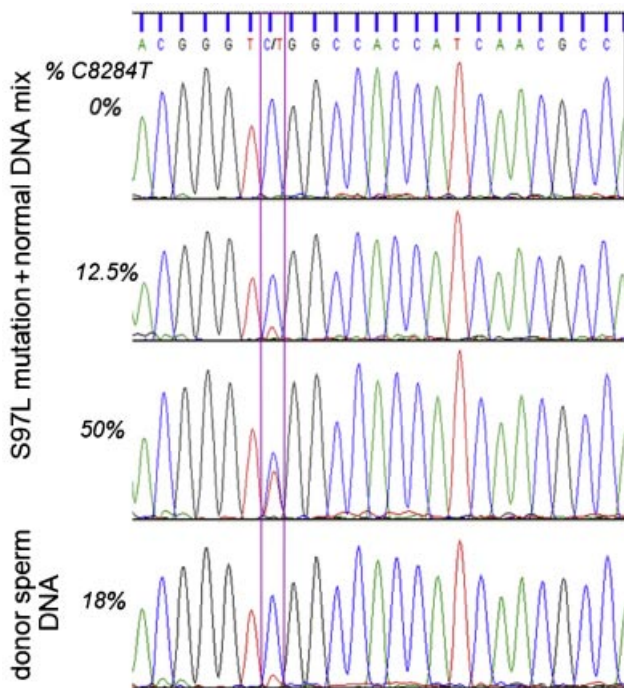


**Fig. 1.** CN phenotype: ANC cycling from age of 12 months to 12 years, before and during G-CSF treatment. The ordinates show ANC in units of cells  $\times 10^9/L$ . Tic marks on the abscissa indicate monthly intervals. Years with no identified cycling and periods lacking ANC data are not presented. **Left panel:** ANCs from age 12 to 18 months in 1995, with the period of G-CSF therapy indicated by the bar in the lower margin. **Right panel:** ANCs in the indicated years; all during therapy with G-CSF.



**Fig. 2.** Pedigree of kindred with CN and SCN phenotypes. Checked squares (male) and circles (female) represent members with SCN. The filled square represents the patient with a CN phenotype. The square with a vertical dividing line and letter “D” represents the sperm donor carrying the *ELANE* mutation. A horizontal relationship line is shown between the sperm donor and his spouse; the clustered diagonal lines indicate fertilizations by sperm donation.

showed the expected nearly equal representation of the C and T nucleotides at position 8284 (indicated by the purple box) in the mutant sample, only C in the normal, and a small but detectable



**Fig. 3.** DNA sequence chromatograms of donor sperm and control DNA samples. The top three lanes represent mixes of 0%, 25%, and 100% mixes of DNA from a normal donor and an SCN patient who is heterozygous for the autosomal dominant S97L mutation. The bottom lane shows sequence from the donor sperm. The numbers in italics, to the immediate left of the chromatograms, indicates the calculated (for the mixes) or experimentally determined percent representation of the C8284T allele in the DNA. The purple box outlines the position of nucleotide 8284 in the sequence, which demonstrates signals for both cytosine (blue) and thymidine (red).

T peak in the 25:75 mutant/normal mix. The donor sperm DNA shows a similar ratio of peaks, quantitatively determined to represent  $18.0 \pm 0.9\%$  (mean  $\pm$  SD,  $n = 5$ ) mutant sequence.

## DISCUSSION

This study examined a kindred that includes both a patient with a CN phenotype, as well as seven children with SCN, all of whom share the same paternal haplotype. The cyclic phenotype was established by multiple documented ANCs  $> 500 \times 10^9/L$  prior to G-CSF therapy, bone marrow demonstrating myeloid maturation to the metamyelocyte stage, and consistent cycling of neutrophil counts, both prior to and on G-CSF therapy. This patient also had a borderline positive anti-neutrophil antibody test, but this finding does not rule out congenital neutropenia [23]. As previously reported for five of the seven the half-siblings [13], those affected with classic SCN had consistent ANC levels  $< 200 \times 10^9/L$  prior to G-CSF therapy and bone marrows showing “maturational arrest” at the promyelocyte/myelocyte stage. The current and previously reported patients all share the S97L *ELANE* mutation. This specific mutation has been found to be associated with multiple phenotypes [16], but not in the same family or with a shared haplotype.

The coexistence of CN and SCN phenotypes in this kindred strongly suggests a role for modifying genes in the determination of congenital neutropenia disease phenotypes. Recent studies report that the transcription factors, LEF-1 and Gfi-1 are linked to SCN in mice and humans [24–26]. These and other genes associated with neutrophil production may interact with a specific *ELANE* mutation to determine the phenotype and severity of clinical disease.

Identification of modifying genes may eventually help in the classification of patients with characteristics of both CN and SCN. While CN and SCN are currently classified as two distinct diseases, clinical studies, and personal experiences of the authors suggest that CN and SCN represent phenotypes on a disease spectrum. For example, one of the sperm donor’s progeny with SCN has demonstrated (after starting treatment with G-CSF) four consecutive ANC oscillations with magnitudes ranging from 0 to 980 and periods ranging from 16 to 24 days, as did at least one patient on the initial study demonstrating efficacy of G-CSF for treatment of SCN [27]. ANC oscillations may reflect the extent of apoptosis in differentiating myeloid cells, and thus relate to disease phenotype. Further studies of modifying genes are needed to identify these determinants, as well as to create a non-reductionist disease classification based on both genetics and phenotype [28].

The finding of 18% mutant alleles in the paternal donor’s sperm explains the appearance of congenital neutropenia in eight children from six kindreds. His normal phenotype and lack of detectable mutant DNA in peripheral blood lymphocytes or buccal mucosal epithelial cells indicates that the mosaicism was likely limited to the germ cells, suggesting a new mutation during germ line development and resultant gonadal mosaicism. It is not clear whether the large number of affected offspring, from 20 or fewer fertilizations by the donor’s sperm, represents a statistical anomaly or, less likely, a selective advantage for spermatozoa bearing the mutant allele.

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