Digenic mutations in severe congenital neutropenia

Manuela Germeshausen,[1] Cornelia Zeidler,[1] Manfred Stuhrmann,[2] Marina Lanciotti,[3] Matthias Ballmaier,[1] and Karl Welte[1]

[1]Dept. of Pediatric Hematology and Oncology/Molecular Hematopoiesis, Hannover, Germany; [2]Institute of Human Genetics, Hannover Medical School, Hannover, Germany; and [3]Dept. of Pediatric Hematology and Oncology, Giannina Gaslini Institute, Genoa, Italy

ABSTRACT

Severe congenital neutropenia a clinically and genetically heterogeneous disorder. Mutations in different genes have been described as causative for severe neutropenia, e.g. *ELANE*, *HAX1* and *G6PC3*. Although congenital neutropenia is considered to be a group of monogenic disorders, the phenotypic heterogeneity even within the yet defined genetic subtypes points to additional genetic and/or epigenetic influences on the disease phenotype. We describe congenital neutropenia patients with mutations in two candidate genes each, including 6 novel mutations. Two of them had a heterozygous *ELANE* mutation combined with a homozygous mutation in *G6PC3* or *HAX1*, respectively. The other 2 patients combined homozygous or compound heterozygous mutations in *G6PC3* or *HAX1* with a heterozygous mutation in the respective other gene. Our results suggest that digenicity may underlie this disorder of myelopoiesis at least in some congenital neutropenia patients.

Key words: *ELANE*, congenital neutropenia, *HAX1*, myelopoiesis.

Citation: Germeshausen M, Zeidler C, Stuhrmann M, Lanciotti M, Ballmaier M, and Welte K. Digenic mutations in severe congenital neutropenia. Haematologica 2010;95:1207-1210. doi:10.3324/haematol.2009.017665

Introduction

Severe congenital neutropenia (CN) is a clinically and genetically heterogeneous disorder. Most of the sporadic or autosomal dominant inherited cases of congenital neutropenia harbor heterozygous mutations in the gene *ELANE* (previously: *ELA2*) coding for neutrophil elastase.[1] The underlying defect in autosomal recessive congenital neutropenia (Kostmann syndrome) has been recently identified as *HAX1* gene mutation.[2] However, there are additional congenital neutropenia patients, partly with syndromic forms, with rare mutations in other genes, e.g. *G6PC3* (CN4),[3] *TAZ* (Barth syndrome),[4] *ROBLD3* (p14-immunodeficiency),[5] *WAS* (X-linked CN),[6] *GFI1* (CN2),[7] and others.

Congenital neutropenia has been considered to be a monogenic disorder. We still do not have a complete understanding of the variance in the phenotype of diseases caused by seemingly the same or similar genotype, suggesting additional genetic and/or epigenetic influences on the disease phenotype. For example, there is a fundamental clinical difference between patients who suffer from congenital neutropenia or from cyclic neutropenia, both diseases caused by similar or same mutations within the *ELANE* gene. In congenital neutropenia patients, only gene mutations in one of the candidate genes have been reported so far. However, the phenotypical heterogeneity of congenital neutropenia may point to a genotypical variety with a possible contribution of different gene mutations to the disease phenotype. In this report we describe 4 patients clinically and genetically diagnosed with congenital neutropenia harboring mutations in two candidate genes.

Design and Methods

Mutation analysis of candidate genes was performed as previously described.[2,3,8] During screening of congenital neutropenia patients for mutations in the candidate genes *ELANE*, *HAX1* and *G6PC3* (n=203), we identified 4 patients with mutations in two genes. All patients fulfilled the diagnostic criteria for congenital neutropenia and were the only family members affected by neutropenia. Normal karyotypes with no numerical or structural aberrations were reported for all patients in this study. Molecular and clinical findings of all patients are summarized in Table 1. The inheritance of mutations detected in congenital neutropenia patients was verified in all cases and is specified in Table 1. All novel mutations reported here have been assessed in ethnically matched healthy individuals (Table 1). The study was approved by the institutional review board of Hannover Medical School. Informed consent was provided according to the declaration of Helsinki.

Funding: the study was funded in part by grants from the Federal Ministry of Education and Research (German Network on Congenital Bone Marrow Failure Syndromes). Acknowledgments: the authors would like to thank all patients and families who participated in the study. We thank Delphine Lees, Queen Mary's School of Medicine and Dentistry, Barts, UK, for helpful discussions on HAX1 transcript variants.
Manuscript received on September 24, 2009. Revised version arrived on December 9, 2009. Manuscript accepted on December 24, 2009.
Correspondence: Manuela Germeshausen, Dept. of Pediatric Hematology and Oncology/Molecular Hematopoiesis, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany. E-mail: germeshausen.manuela@mh-hannover.de

Table 1. Molecular and clinical findings in the patients of this study.

Patient[a]	Gene mutations[b]	Inheritance	Hematologic findings[c]	Other findings
#1 (Turkish / 24 / f)	ELANE: **p.Ala166Thr (Exon 4)** (het)	mother	neutropenia (ANC: 200-700/µL) no eosinophils, thrombocytopenia	constitutional developmental delay; hypogonadotropic hypogonadism; type II atrial septal defect; mild mitral and tricuspid insufficiency; prominent superficial venous pattern
	G6PC3: **p.Met116Lys (Exon 3)** (hom)	mother / father		
#2 (Arabian / 0.5 / f)	ELANE: **p.Ala25Val (Exon 2)** (het)	father	neutropenia (ANC: 170-230/µL)	recurrent infections; no signs of neurodevelopmental delay yet
	HAX1: p.Val144GlyfsX5 (Exon 3) (hom)	mother / father		
#3 (Caucasian) (20 / m)	G6PC3: p.Gly260Arg (Exon 6) (hom)	mother / father	neutropenia (ANC 0-30/µL), no eosinophils, thrombocytopenia	height and weight below 3rd percentile; cryptoorchidism; genital dysplasia; microcephaly; inner-ear hearing loss; hypogammaglobulinemia; type II atrial septal defect; prominent superficial venous pattern
	HAX1: **p. Val172Ile (Exon 4)** (het)	mother		
#4 (Caucasian) (5 / m)	HAX1: p.Val144GlyfsX5 (Exon 3)	father	neutropenia (ANC 300-350/µL)	neurodevelopmental abnormalities
	p.Leu130Arg (Exon 3) (compound het)	mother		
	G6PC3: **p.Arg189Gln (Exon 5)** (het)	father		

[a]in parentheses: ethnicity/current age in years/sex; [b]Novel mutations are marked by bold letters. New mutations in G6PC3 and HAX1 were not present in 400 alleles from healthy controls (including 90 each from Turkish and Arabian individuals), new ELANE mutations were not present in 860 alleles from healthy controls (including 200 each from Turkish and Arabian individuals). c ANC: absolute neutrophil count before start of G-CSF treatment; Abbreviations: het: heterozygous, hom: homozygous.

Results and Discussion

Patient #1 is the first live born child of consanguineous parents (2nd grade cousins) after 3 miscarriages and one stillbirth. Beside severe neutropenia and moderated thrombocytopenia, the patient was affected by hypogonadotropic hypogonadism and constitutional developmental delay, by mild mitral and tricuspid insufficiency and a type 2 atrial septal defect, and by a prominent superficial venous pattern. During the screening of congenital neutropenia patients for mutations in *ELANE*, we detected a novel heterozygous missense mutation in exon 4 (p.Ala166Thr). Due to the peculiar phenotype of the patient, we also screened for mutations in *G6PC3*.[3] Interestingly, we found a novel homozygous missense mutation in exon 3 (p.Met116Lys).

Although an *ELANE* mutation had been detected in this patient, the later found *G6PC3* mutations explained the clinical phenotype much better. Cardiac and urogenital abnormalities, as well as a conspicuous pattern of subcutaneous veins, have been described as typical features of patients with G6PC3 deficiency.[3] One might argue that the *ELANE* mutation in patient #1 does not have any effect on the disease. Indeed, the mutation is predicted to be benign by different prediction algorithms,[9,10] and did not cause congenital neutropenia in the patient's mother from whom the mutation was inherited. However, the pathophysiologic mechanism of *ELANE* mutations in congenital neutropenia is still unclear and hence the consequence of different *ELANE* mutations. Effects on proteolytic activity, on cellular trafficking and on unfolded protein response (UPR) have been demonstrated only for subgroups of *ELANE* mutations, respectively.[11-14] Furthermore, other *ELANE* mutations described as causative for congenital neutropenia, e.g. p.Val72Met[1] were also categorized as benign by the prediction algorithms. One could speculate that the *ELANE* mutation is able to worsen the phenotype in terms of severity of neutropenia and additional hematologic features.

Mutational analysis of patient #2, born with severe neutropenia, revealed a novel heterozygous mutation in exon 2 of *ELANE* leading to an amino acid exchange in amino acid 25 of the signal peptide (p.Ala25Val). As in patient #1, the *ELANE* mutation was predicted as benign by prediction algorithms. Further analysis revealed a homozygous *HAX1* mutation in exon 3 (p.Val144GlyfsX5) which not only affects the full length transcript *HAX1-001* but also the transcription variant *HAX1-004*[15] and which was associated with a neurological phenotype in other congenital neutropenia patients.[16] Nevertheless, the baby did not yet show apparent signs of neurodevelopmental abnormalities which might develop in the future.

The clinical phenotype of patient #3 was characterized by severe neutropenia and intermittent thrombocytopenia, a type 2 atrial septal defect, cryptoorchidism and genital dysplasia, microcephaly and inner-ear hearing loss, height and weight below 3rd percentile, hypogammaglobulinemia, and a prominent superficial venous pattern. Compatible with the clinical phenotype, we detected a homozygous mutation in exon 6 of *G6PC3* (p.Gly260Arg). Additionally we detected a novel heterozygous mutation in exon 4 of the *HAX1* gene

(p.Val172Ile). The symptoms observed in patient #3 were in accordance with the G6PC3 deficiency, proven by the detection of a homozygous mutation predicted to be deleterious.[3] The patient additionally harbors a heterozygous *HAX1* mutation which does not apparently seem to contribute to the phenotype: (i) *HAX1* mutations are acting in a recessive way. (ii) The mutation is predicted to be tolerated with a score of 0.36 by SIFT.[9]

However, considering the extreme rarity of recessive forms of congenital neutropenia associated with *G6PC3* or *HAX1* mutations, the likelihood of a coincidence of both *G6PC3* and *HAX1* mutations is extremely low as deduced from Hardy-Weinberg calculated heterozygote rates of below 1% for each of these genes and from the analysis of healthy controls. Although individuals heterozygously affected from *HAX1* mutations are completely healthy, it cannot be excluded that heterozygous mutations in *HAX1* may worsen the clinical phenotype of *G6PC3* deficiency.

Patient #4 was characterized by severe neutropenia from birth and marked neurodevelopmental abnormalities. Molecular analysis of the *HAX1* gene revealed compound heterozygous mutations (p.[Val144GlyfsX5]+[Leu130Arg]),[17] both affecting not only the full length transcript *HAX1-001* but also the alternative splice product *HAX1-004*,[15] confirming our observation that mutations affecting *HAX1-004* are associated with a neurological phenotype in congenital neutropenia.[16] Additionally, we found a novel paternally inherited heterozygous mutation in *G6PC3* (p.Arg189Gln) which is predicted to be tolerated (SIFT score of 0.41).[9] Similar to patient #3, the heterozygous mutation in the second gene is not predicted to contribute to the disease phenotype, but again a statistically extremely unlikely coincidence of *HAX1* and *G6PC3* mutations occurs in this patient.

None of the novel mutations has been identified in a large number of ethnically matched controls (Table 1).

This report blurs the seemingly clear picture of genetic subtypes in congenital neutropenia and suggests that di- or oligogenicity may underlie what so far has been considered to be a monogenic disorder of myelopoiesis, at least in some congenital neutropenia patients. Particularly congenital neutropenia patients with *ELANE* mutations demonstrate a considerable overlap of genotype with phenotype, even within families. The enormous phenotypical variability in congenital neutropenia and unclear inheritance patterns in some families may indicate that the classification of the diseases according to the mutated alleles as either dominant or recessive may be an oversimplification.

There are various examples of clinical disorders in which mutations in a second gene are necessary for the disease phenotype (digenic diseases) or worsen the phenotype caused by a primary mutation such as Emery-Dreifuss muscular dystrophy (*EMD, LMNA*),[18] idiopathic hypogonadotropic hypogonadism (*FGFR1*, *NELF* or *GNRHR*, respectively),[19] retinitis pigmentosa (*RPGR*, *CRX*),[20] Hirschsprung disease (*RET*, *GDNF*),[21] and many others. We are now beginning to understand the molecular basis of oligogenicity in these diseases: in retinitis pigmentosa, the two mutants combine to prevent the formation of complexes which are important for the integrity of photoreceptors.[20] Hirschsprung disease is characterized by mutations in receptor-ligand pairs.[21] In contrast to these molecularly well characterized disorders, a possible interaction of mutated gene products in congenital neutropenia is not understood. Two mutant proteins may act at different levels of the same pathway that becomes progressively compromised by two mutations. One common pathway might be the stress induced UPR within the endocytoplasmatic reticulum. Indeed, UPR has been documented for *ELANE* mutations[13] and *G6PC3* mutations[3] and might be also induced by *HAX1* mutations, since HAX1 is involved in the regulation of intracytoplasmatic calcium[22] necessary for proper protein folding. Interestingly, all patients, independent of the genetic subtype, demonstrate common downstream pathomechanisms such as lack of LEF1, C/EBPα and ELANE expression.[23,24]

Our results also challenge the proposed algorithms for mutational analyses in congenital neutropenia. With the common procedure of first testing the *ELANE* gene as the most frequent candidate gene in congenital neutropenia, patients #1 and 2 would have been assigned to the group of patients with *ELANE* as the disease causing gene. We suggest the sequencing of other candidate genes in congenital neutropenia even in patients with an ELANE mutation if: (1) this mutation is also present in an unaffected parent; or (2) if the patient exhibits an exceptional clinical phenotype, such as for instance developmental abnormalities in combination with severe neutropenia. In at least a small subgroup of congenital neutropenia patients we have to consider a cooperative action of mutations in ELANE with those in other genes. Since there is a considerable percentage of congenital neutropenia patients without mutation in any of the known candidate genes,[25] we also expect that other yet unknown genes may play a role in complex patterns of di- or oligogenic inheritance in congenital neutropenia.

Authorship and Disclosures

MG designed the study and performed the molecular analyses; CZ, MG and KW collected clinical data; ML performed molecular analyses of *HAX1* and *ELANE* and collected clinical data of patient 3; MS provided DNA from healthy controls; MG, MS, MB analyzed data; MG, MS, MB and KW wrote the manuscript.

The authors declare no competing financial interests.

References

1. Dale DC, Person RE, Bolyard AA, Aprikyan AG, Bos C, Bonilla MA, et al. Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. Blood. 2000;96(7):2317-22.
2. Klein C, Grudzien M, Appaswamy G, Germeshausen M, Sandrock I, Schaffer AA, et al. HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease). Nat Genet. 2007;39 (1):86-92.
3. Boztug K, Appaswamy G, Ashikov A, Schaffer AA, Salzer U, Diestelhorst J, et al. A syndrome with congenital neutropenia and mutations in G6PC3. N Engl J Med. 2009;360(1):32-43.
4. Bione S, D'Adamo P, Maestrini E, Gedeon AK, Bolhuis PA, Toniolo D. A novel X-linked gene, G4.5. is responsible for Barth syndrome. Nat Genet. 1996;12(4):385-9.
5. Bohn G, Allroth A, Brandes G, Thiel J, Glocker E, Schaffer AA, et al. A novel human primary immunodeficiency syndrome caused by deficiency of the endoso-

mal adaptor protein p14. Nat Med. 2007;13(1):38-45.
6. Devriendt K, Kim AS, Mathijs G, Frints SG, Schwartz M, Van Den Oord JJ, et al. Constitutively activating mutation in WASP causes X-linked severe congenital neutropenia. Nat Genet. 2001;27(3):313-7.
7. Person RE, Li FQ, Duan Z, Benson KF, Wechsler J, Papadaki HA, et al. Mutations in proto-oncogene GFI1 cause human neutropenia and target ELA2. Nat Genet. 2003;34(3):308-12.
8. Germeshausen M, Schulze H, Ballmaier M, Zeidler C, Welte K. Mutations in the gene encoding neutrophil elastase (ELA2) are not sufficient to cause the phenotype of congenital neutropenia. Br J Haematol. 2001; 115(1):222-4.
9. Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res. 2003;31(13):3812-4.
10. Ramensky V, Bork P, Sunyaev S. Human non-synonymous SNPs: server and survey. Nucleic Acids Res. 2002;30(17):3894-900.
11. Massullo P, Druhan LJ, Bunnell BA, Hunter MG, Robinson JM, Marsh CB, et al. Aberrant subcellular targeting of the G185R neutrophil elastase mutant associated with severe congenital neutropenia induces premature apoptosis of differentiating promyelocytes. Blood. 2005;105(9):3397-404.
12. Benson KF, Li FQ, Person RE, Albani D, Duan Z, Wechsler J, et al. Mutations associated with neutropenia in dogs and humans disrupt intracellular transport of neutrophil elastase. Nat Genet. 2003;35(1):90-6.
13. Kollner I, Sodeik B, Schreek S, Heyn H, von Neuhoff N, Germeshausen M, et al. Mutations in neutrophil elastase causing congenital neutropenia lead to cytoplasmic protein accumulation and induction of the unfolded protein response. Blood. 2006; 108(2):493-500.
14. Li FQ, Horwitz M. Characterization of mutant neutrophil elastase in severe congenital neutropenia. J Biol Chem. 2001;276(17):14230-41.
15. Lees DM, Hart IR, Marshall JF. Existence of multiple isoforms of HS1-associated protein X-1 in murine and human tissues. J Mol Biol. 2008;379(4):645-55.
16. Germeshausen M, Grudzien M, Zeidler C, Abdollahpour H, Yetgin S, Rezaei N, et al. Novel HAX1 mutations in patients with severe congenital neutropenia reveal isoform-dependent genotype-phenotype associations. Blood. 2008;111(10):4954-7.
17. Lanciotti M, Indaco S, Bonanomi S, Coliva T, Mastrodicasa E, Caridi G, et al. Novel HAX1 gene mutations associated to neurodevelopment abnormalities in two Italian patients with severe congenital neutropenia. Haematologica. 2010;95(1):168-9.
18. Ben Yaou R, Toutain A, Arimura T, Demay L, Massart C, Peccate C, et al. Multitissular involvement in a family with LMNA and EMD mutations: Role of digenic mechanism? Neurology. 2007;68(22):1883-94.
19. Pitteloud N, Quinton R, Pearce S, Raivio T, Acierno J, Dwyer A, et al. Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. J Clin Invest. 2007;117(2):457-63.
20. Jin ZB, Mandai M, Yokota T, Higuchi K, Ohmori K, Ohtsuki F, et al. Identifying pathogenic genetic background of simplex or multiplex retinitis pigmentosa patients: a large scale mutation screening study. J Med Genet. 2008;45(7):465-72.
21. Parisi MA, Kapur RP. Genetics of Hirschsprung disease. Curr Opin Pediatr. 2000;12(6):610-7.
22. Vafiadaki E, Arvanitis DA, Pagakis SN, Papalouka V, Sanoudou D, Kontrogianni-Konstantopoulos A, et al. The anti-apoptotic protein HAX-1 interacts with SERCA2 and regulates its protein levels to promote cell survival. Mol Biol Cell. 2009;20(1):306-18.
23. Skokowa J, Cario G, Uenalan M, Schambach A, Germeshausen M, Battmer K, et al. LEF-1 is crucial for neutrophil granulocytopoiesis and its expression is severely reduced in congenital neutropenia. Nat Med. 2006;12(10):1191-7.
24. Skokowa J, Fobiwe JP, Dan L, Thakur BK, Welte K. Neutrophil elastase is severely downregulated in severe congenital neutropenia (CN) independent of ELA2 or HAX1 mutations but dependent on LEF-1. Blood. 2009;114(14):3044-51.
25. Dale DC, Link DC. The many causes of severe congenital neutropenia. N Engl J Med. 2009;360(1):3-5.