Molecular basis of congenital neutropenia

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In this issue of The journal, Beel and Vandenberghe[1] describe 2 patients with an activating mutation in Wiskott-Aldrich syndrome (WAS) who developed myelodysplastic syndrome and acute myeloid leukemia, associated with mutations in G-CSF receptor (*CSF3R*) and monosomy 7. This report is interesting in various aspects. First, it adds a new variant of congenital neutropenia to the growing list of pre-leukemic syndromes. Second, it illuminates the clinical diversity of congenital neutropenia syndromes and their long-term complications. And third, this observation follows up on a potential link between control of the actin cytoskeleton and cytogenetic stability by documenting that a constitutive active *WASp* mutation predisposes to MDS/AML.[2]

In 1950, Rolf Kostmann discovered autosomal recessive severe congenital neutropenia (SCN) and thus described the first hereditary human immunodeficiency syndrome.[3,4] For more than 50 years, the molecular etiology has remained enigmatic. Marshall Horwitz and David Dale opened the field of molecular genetics in SCN research when they identified heterozygous mutations in the gene encoding neutrophil elastase (*ELANE/ELA2*) in patients with cyclic neutropenia[5] and in patients with severe congenital neutropenia.[6] Ten years later, more than a dozen genes have been involved in the molecular pathophysiology of congenital neutropenia. And the number continues to grow. Based on these recent genetic insights, many patients with SCN can now be classified according to the underlying mutation.

From a clinical perspective, congenital neutropenia may be an isolated hematologic finding or may be part of a syndrome associating abnormalities in other organs, such as the skin, the brain, the heart, the pancreas, or the urogenital tract. The clinical presentation may offer important clues to the genetic diagnosis (Table 1). Even in the era of molecular medicine, the importance of the clinical approach and systematic physical examination cannot be underestimated.

Patients with congenital neutropenia typically present with recurrent bacterial infections due to a lack of sufficient numbers of neutrophils. In addition to quantitative abnormalities, many variants of congenital neutropenia also comprise a spectrum of qualitative aberrations, such as defective production of bactericidal proteins or defective activation. A prominent feature in many, but not all, variants of severe congenital neutropenia is a so-called *maturation arrest* in the bone marrow. Myeloid maturation appears *arrested* at the stage of promyelocytes. Congenital neutropenia must be differentiated from immune-mediated neutropenia or benign ethnic neutropenia, two conditions rarely associated with severe infections. Benign ethnic neutropenia, seen in patients of African or Arabic descent, has recently been linked to a polymorphism in the gene encoding the Duffy antigen receptor for chemokines (*DARC*).[7] This Duffy Null polymorphism (SNPrs2814778) is also associated with protection against *plasmodium vivax malaria*, yet both of these mechanisms remain unknown.

In Central Europe and Northern America, approximately 50% of SCN patients have heterozygous mutations in *ELANE/ELA2*. Patients either inherit a mutation in an autosomaldominant pattern of inheritance or acquire mutations during embryogenesis. If the mutations are transmitted into the hematopoietic system, they may cause cyclic or severe congenital neutropenia. ELANE is exclusively expressed in neutrophil granulocytes and monocytes and functions as a serine protease cleaving a wide array of cellular and extracellular proteins. Cells expressing mutated ELANE are more likely to undergo apoptosis. While the exact molecular mechanism accounting for the phenotype of increased apoptosis is still under investigation, recent studies suggest that mutated ELANE cause increased stress in the endoplasmic reticulum (ER), initiating apoptosis.[8,9] Increased ER-stress elicits a cellular response collectively termed the *unfolded protein response*. Three ER-localized protein sensors are known: IRE1alpha (inositol-requiring 1alpha), PERK (double-stranded RNA-dependent protein kinase (PKR)-like ER kinase), and activating transcription factor 6 (ATF6).[10] In cases of ER-stress, these sensors are being activated and trigger a complex series of events destined to maintain the homeostasis of the ER and to promote protein folding, maturation, secretion, and ER-associated protein degradation. In cases in which these rescue mechanisms fail, the unfolded protein response initiates an apoptosis program to protect cells and the organism from dysfunctional or toxic proteins.

Autosomal-recessive SCN can be caused by biallelic mutations in *HAX1* (HCLS1 associated protein X-1).[11] HAX1 is an antiapoptotic molecule which is predominantly, but not exclusively, localized in the mitochondria. Initially identified as as a binding partner of HS1, a protein kinase involved in BCR-mediated signaling, HAX1 has emerged as a ubiquitously expressed protein promiscuously interacting with multiple partners, apparently including proteins controlling endocytosis, nuclear factors, and even RNA.[12] No unifying theory has been proposed that would explain this diversity of networks. With respect to the function of HAX1 in mitochondria, a murine knockout model has provided some mechanistic insights.[13] Murine Hax1 interacts with Parl, thus controlling processing of HtrA2/Omi, a factor involved in mitochondrial apoptosis pathways. However, Hax1-deficient mice only partly mirror the phenotype of HAX1-deficiency in human patients. Hax1$^{-/-}$ mice are not neutropenic and develop a severe lethal neurodegenerative disease. Interestingly a subset of HAX1-deficient patients associate severe congenital neutropenia and neurological

Table 1. Organ involvement in congenital neutripenia syndromes.

Congenital neutropenia variant	Congenital neutropenia	Osteopenia	Skeletal system (Growth delay/ dysmorphic features)	Skin/ Hair	Neurological system	Cardiovascular system	Urogenital system	Gastrointestinal system	Endocrine system	Adaptive immune system	Increased risk of malignancies	Mutated gene
SCN1 (OMIM 202700)	•	•									yes	*ELA2*
SCN2 (OMIM 600871)	•	•								•	?	*GFI1*
XLN (OMIM 300299)	•									•	yes	*WAS*
SCN3 (OMIM 610738)	•	•			•						yes	*HAX1*
Reticular dysgenesis (OMIM 267500)	•				•					•	?	*AK2*
Glycogen storage disease type Ib (OMIM 232220)	•	•	•			•		•	•		no	*SLC37A4*
SCN4 (OMIM 612541)	•		•	•		•	•	•			no	*G6PC3*
Barth syndrome (OMIM 302060)	•					•					no	*TAZ*
Shwachman-Diamond syndrome (OMIM 260400)	•	•	•		•	•		•		•	yes	*SBDS*
Cartilage-hair hypoplasia (OMIM 250250)	•	•	•	•	•			•		•	yes	*RMRP*
Chediak-Hiagashi syndrome (OMIM 214500)	•		•	•	•					•	no	*LYST*
Griscelli syndrome type 2 (OMIM 607624)	•			•						•	no	*RAB27A*
Hermansky-Pudlak syndrome type 2 (OMIM 608233)	•		•	•						•	no	*AP3B1*
P14-deficiency (OMIM 610798)	•		•	•						•	?	*MAPBPIP*
Cohen syndrome (OMIM 216550)	•		•	•							no	*COH1*
Poikiloderma with neutropenia (OMIM 604173)	•		•	•							?	unknown
Charcot-Marie-Tooth disease (OMIM 606482)	•				•						?	*DNM2*
Pearson syndrome (OMIM 557000)	•				•	•	•	•	•		?	Mitochondrial DNA

SCN: severe congenital neutropenia; XLN: X-linked severe congenital neutropenia.

symptoms such as cognitive dysfunction or developmental delay and epilepsy, depending on whether the mutation affects a HAX1 isoform expressed in neuronal cells or not.[14]

Reticular dysgenesis (RD) is the most severe variant of severe combined immunodeficiency (SCID), associating an early differentiation arrest in the myeloid lineage associated with severe lymphopenia due to impaired lymphoid development. Furthermore, affected patients suffer from sensorineural hearing loss. Mutations in adenylate kinase 2 (*AK2*) have recently been identified by two independent groups.[15,16] Like HAX1, AK2 is localized in the mitochondrial and is important in mitochondrial energy metabolism and control of apoptosis.

Growth factor independent (GFI)-1 is a zinc finger molecule with transcriptional repressor and splice control function. GFI1 regulates differentiation of hematopoietic and non-hematopoietic cells. Heterozygous mutations in the zinc finger domain have been described in rare patients with SCN.[17] The hematologic phenotype of GFI1-mutant patients resembles Gfi1$^{-/-}$ mice characterized by decreased maturation of mature neutrophils and an increased number of immature myeloid cells.[18,19] A great variety of target genes is under the control of GFI1, so the exact mechanisms accounting for congenital neutropenia are still not completely clear. Recent studies in a mouse model system indicate that the particular mutation in *GFI1* found in SCN patients confers a dominant negative block in granulopoiesis associated with dysregulation of *CSF1* and its receptor.[20]

The most recent asset to the list of the genes causing congenital neutropenia is glucose-6-phosphatase catalytic subunit 3 (*G6PC3*),[21] encoding a paralog of glucose-6-phosphatase. G6PC3 is ubiquitously expressed and localized in the endoplasmic reticulum. Biallelic mutations in *G6PC3* abrogate its function, leading to premature apoptosis of neutrophils and associated morphological and biochemical evidence of ER-stress. Originally identified in a religious minority, G6PC3 deficiency has now been recognized in many ethnic groups. In addition to severe congenital neutropenia, affected patients display a wide array of constitutional problems involving structural heart defects (e.g. ASD-II, pulmonary stenosis) and urogenital malformations (e.g. cryptorchidism, urachal fistula). Furthermore, many patients have a characteristic appearance of superficial veins.

It has been known for many years that a subtype of glycogen storage disease, GSD-Ib, is characterized by signs of deficient metabolic alterations due to glcycogen storage and by congenital neutropenia.[22] The underlying

mutations affect the endosomal transporter for glucose-6-phosphate, glucose-6-phosphate translocase (G6PT), encoded by *SLC37A4*. The discovery of human G6PC3 deficiency has shed light on the unexplained finding of neutropenia in glycogen storage disease type Ib. Although the stoichiometric and biochemical relationships between glucose-6-phosphate translocase and glucose-6-phosphatase remain to be resolved, both proteins appear to be functionally linked. The G6PC1/G6PT complex (in liver, kidney and intestine) regulates glucose levels, and the ubiquitous G6PC3/G6PT complex appears to be of critical importance in the differentiation and homeostasis of mature neutrophil granulocytes. Nevertheless, both clinical presentation and neutrophil function assays differ between G6PT and G6PC3 deficiency, suggesting that their epistatic relationship is complex.

Classically, mutations in *WASP* cause Wiskott-Aldrich syndrome characterized by microthrombocytopenia, lymphoid and myeloid immunodeficiency, and eczema or X-linked thrombocytopenia. This disease is caused by a loss of function of WASP, leading to reduced WASP levels and/or activity and diminished actin polymerization. As a consequence, many aspects of hematologic and immune cell function are perturbed, including signal transduction, migration, and phagocytosis.[23,24] WASP is subject to autoinhibition by adopting a conformation which prevents its C-terminal VCA domain from interacting with the actin-related protein 2/3 (ARP2/3) complex. Therefore, certain mutations may abolish conformation-dependent autoinhibition. In fact, such gain-of-function mutations have been described in patients with X-linked congenital neutropenia.[25-27] In addition to congenital neutropenia, affected patients show reduced numbers and function of lymphocytes. Previous studies have reported that X-linked neutropenia patients may have a susceptibility to myelodysplastic syndromes[25] and elegant experimental strategies have confirmed that murine hematopoietic stem cells engineered to express a mutant WASP (I294T) showed enhanced and delocalized actin polymerization throughout the cell, decreased proliferation, and increased apoptosis. Cells became binucleated, suggesting a failure of cytokinesis, and micronuclei were formed, indicative of genomic instability.[2] It remains to be shown whether other genetic defects leading to congenital neutropenia may also involve aberrant regulation of the actin cytoskeleton.

There is preliminary evidence that at least two additional genetic defects cause autosomal recessive severe congenital neutropenia, nourishing hope that discovery of novel genes controlling differentiation and function of neutrophil granulocytes will help us understand molecular pathways more comprehensively. Furthermore, these insights may shed light on general principles that determine clonal expansion and onset of myelodysplasia or leukemia.

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Defective ribosome biogenesis in myelodysplastic syndromes

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Malignant disorders are poorly understood at the biological level in general, and myelodysplastic syndromes (MDS), a heterogeneous clonal stem cell disorder, in particular, are further confounded by representing a group of diseases rather than a single entity with clinical and biological heterogeneity within each subtype. The basis for grouping these assorted diseases under one MDS umbrella is the clinical presentation of variable cytopenias despite a generally cellular and dysplastic marrow. Diagnosis is based on bone marrow morphology, percentage of blasts, unexplained cytopenias and cytogenetics. These components have been used to classify the disease (French-American British[1] or FAB and World Health Organization[2] or WHO classifications) and to predict prognosis (International Prognostic Scoring System[3] or IPSS). The typical patient is elderly; thus with the increase in our aging population, the incidence of MDS has now surpassed that of acute myeloid leukemia (AML). Approximately 30% of MDS patients will progress to AML, but the majority die from infection or bleeding due to an increasing profundity of the cytopenias. The paradox of MDS is that despite peripheral cytopenia, the marrow is most often hypercellular. The initial breakthrough in understanding the biological basis of this paradox came with the demonstration that, in MDS, bone marrow cells were not only rapidly proliferating, but also undergoing excessive apoptosis.[4-7] As a result, the maturing hematopoietic cells are eliminated in the marrow and do not reach the peripheral blood, accounting for the cytopenias in the presence of a cellular marrow composed of mostly apoptotic cells. Furthermore, it was demonstrated that multiple cytokines involved in mediating apoptosis and proliferation are deregulated in MDS marrows.[7-9] While the MDS cell itself probably contributes, recent insights suggest that it is the bone marrow microenvironment that is also responsible for potentiating disease pathology and progression through cytokine imbalance. In summary then, MDS appears to be a disease of both the *seed and the soil* (the cell and its microenvironment) where peripheral cytopenias appear to be the result of an increased proliferation-increased apoptosis in the clonal cells and deregulated proapoptotic and trophic cytokines in the marrow.

Approximately half the patients with MDS present with recurring cytogenetic abnormalities most commonly affecting chromosomes 5, 7, 8, and 20. These have been shown to greatly affect prognosis and survival.[3] Despite the presence of these well recognized karyotypic aberrations, genetic mutations that accompany them have not been well understood and, in fact, mutation in a specific biological pathway that is common to multiple MDS subtypes has not been identified. In short, apoptosis has remained the sole unifying biological characteristic of the disease.

Recently, however, a possible cohesive genetic explanation for this increased apoptosis has been taking shape. Initial hints began with the study of patients who present with the specific karyotype abnormality of del(5q) in one allele. This is the most common chromosomal abnormality (10-15%) in MDS with two regions of deletions in the long arm of chromosome 5.[10] The more telomeric deleted region (the commonly deleted region or CDR) is found in a subtype of MDS; the 5q- syndrome. Patients with 5q- syndrome rarely transform to AML and have long survival. The well mapped CDR,[10] a 1.5MB interval flanked by marker D5S413 and the gene *GLRA1*, contains 40 genes, including two ribosomal genes, *RBM22* and *RPS14*. While no mutation in the undeleted allele was found in any of these genes, Boultwood *et al.*[11] noted that their expression was down-regulated in patients with 5q- syndrome (haplo-insufficiency). They speculated that deregulation of these genes and the pathway that they were part of, could be the causative abnormality in 5q- syndrome patients. This idea was supported by previous findings that genetic defects of ribosomal genes were implicated in congenital anemias including dyskeratosis congenita,[12] cartilage-hair hypoplasia,[13] Diamond-Blackfan anemia[14] and Shwachman-Diamond syndrome.[15,16] The importance of *RPS14* haplo-insufficency as a causative event in 5q- syndrome was then shown by Ebert et al. using RNAi to selectively inhibit each of the 40 genes within the deleted region.[17] Suppression of *RPS14* in normal CD34$^+$ cells resulted in a 5q- syndrome like phenotype while forced expression in the hematopoietic cells of patients with 5q- syndrome rescued the phenotype. Additionally, expression of multiple genes associated with ribosome biogenesis was found to