Severe developmental delay and epilepsy in a Japanese patient with severe congenital neutropenia due to HAX1 deficiency

HAX1 deficiency has recently been identified as a cause of severe congenital neutropenia (SCN), but little is known about the phenotype. We described an SCN patient with a homozygous 256C-to-T transition causing an R86X mutation in the *HAX1* gene. Notably, the patient has been complicated by epilepsy and severe delay of motor, cognitive, and intellectual development; each developmental quotient was 21-26 at 7 years old. Growth failure and dental development delay were also noted. Neurodevelopmental delay in this patient expands the clinical phenotype of HAX1 deficiency and suggests an important role of HAX1 on neural development as well as myelopoiesis.

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Severe congenital neutropenia (SCN) is a rare disorder of myelopoiesis resulting in recurrent life-threatening infections due to a lack of neutrophils.[1] Individuals with SCN have characteristic bone marrow findings of myeloid hypoplasia with arrest of myelopoiesis at the promyelocyte/myelocyte stage.[1] This disorder was first described in Kostmann's seminal paper in 1956.[2] Recent evidence has indicated that SCN is a heterogeneous disorder involving mutations in various genes, including those encoding granulocyte colony stimulating factor receptor (G-CSFR) (*CSF3R*),[3] neutrophil elastase (*ELA2*),[4] Wiskott-Aldrich syndrome protein (*WAS*),[5] and *GFI-1*.[6] More recently, Klein et al. identified homozygous *HAX1* mutations as a cause of an autosomal recessive SCN in 20 Middle Eastern patients and 3 classical Kostmann family members.[7] They suggested that HAX1 is critical for maintaining the inner mitochondrial membrane potential and protecting against apoptosis in myeloid cells.[7]

HAX1 has been initially described as an interacting partner with HS-1, a signal-transduction protein in hematopoietic cells.[8] HAX1 mRNA is ubiquitously expressed,[8,9] and therefore its deficiency could theoretically result in dysfunction of several organs other than hematopoietic systems. Given that the precise function of HAX1 is not fully defined, clinical phenotypes of patients with deficiency of the responsible gene may be helpful. Here, we described an SCN patient caused by homozygous R86X mutations in *HAX1*, who presented with severe developmental delay followed by refractory epilepsy. This case may expand the phenotype of HAX1 deficiency in human and may provide a novel function of this gene.

Design and Methods

A 9-year-old boy was investigated because of congenital neutropenia with presentation of fulminant left otomastoiditis at two months old. The patient was born without asphyxia from nonconsanguineous parents at 39 weeks' gestation. There was no family history of immunodeficiency. Initial investigations demonstrated an absolute neutrophil count (ANC) of 0-0.09x10^9/L and marked monocytosis. Bone marrow examinations showed myeloid hypoplasia with maturation arrest at the promyelocyte/myelocyte stage. Cytogenetic analysis was normal. Under the diagnosis of SCN and otomastoiditis, the patient was treated with parenteral antibiotics and G-CSF. A higher dose of G-CSF at 10 μg/kg/day for 9 days was required to achieve ANC over 1x10^9/L. He was discharged with hearing impairment at a threshold of 50 dB on audiograms of the left ear. Details on his early clinical course were published previously.[10]

After discharge, he was treated with subcutaneous administration of G-CSF at 5-10 μg/kg two to three times per week and prophylactic use of sulfamethoxazole/trimethoprim. Six months after discharge, the frequency of G-CSF administration was reduced to once per week. Although ANC has remained low at 0.05-0.1x10^9/L under this therapy, there has not been any recurrent, invasive bacterial infection for more than 9 years. Annual follow-up bone marrow examinations have not shown any findings compatible with myelodysplastic syndrome or acute myelogenous leukemia.

Neurological development of the patient has been delayed. Months at which the patient achieved each developmental milestone were as follows; head control (4 months), sit alone (9 months), stand with support (12 months), stand without support (15 months), walk alone (18 months), and speak single words (24 months). Developmental retardation became more pronounced in later periods. The patient became bowel and bladder trained at 5 years old. He can not construct complete sentences and can not read at the time of this manuscript preparation (9 years old). Using the New Edition of the Kyoto Scale of Psychological Development, a standardized test widely used in Japan,[11] we assessed his neurodevelomental status at 36 months and 93 months of age, indicating severe delay in motor (developmental quotient, DQ; 49 and 26, respectively), cognitive (DQ; 62 and 21, respectively), and verbal and social development (DQ; 61 and 23, respectively). Plasma amino acids distributions and plasma concentrations of lactic and pyruvic acids were normal. Computed tomography (2 months and 2 years of age) and magnetic resonance images (6 and 8 years of age) of the brain did not demonstrate any abnormalities. At 4 years old, the patient demonstrated a brief, repetitive loss of consciousness. Electroencephalographic findings led to the diagnosis of symptomatic generalized epilepsy presenting with atypical absence. Epileptic symptoms were partially controlled with combined administration of valproric acid, clobazam, and ethosuximide, and remained under observation. Physical growth has failed to thrive. At 9 years old, body height and weight was 121.7 cm

(-1.8 SD) and 21.0 kg (-1.5 SD), respectively. Dental development has also been delayed – first mandibular permanent molars erupted at 8 years of age, and of 8 permanent incisors, only 3 have erupted at 9 years of age.

Mutational analysis and immunoblot analysis of the genes responsible for SCN

We obtained written informed consent for analysis from both parents. All study centers received approval from the institutional review boards to perform genetic analysis. Mutations in *CSF3R*, *ELA2*, and *WAS* were analyzed by previously described procedures.[12-15] For sequencing analysis of *HAX1*, genomic DNA was extracted from the peripheral blood leukocytes. Polymerase chain reaction (PCR) was performed with primers that span all exons of *HAX1* (5'-CGTTTAC-GACAGTGTCAGGATCG-3' and 5'-TGACAAACTGA-CATGGCCCCAG-3') using the Expand High Fidelity PCR System (Roche Diagnostics GmbH, Mannheim, Germany). The PCR products were sequenced using 9 primers (primer design will be provided upon request). These were then analyzed by cycle sequencing using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 genetic analyzer (Applied Biosystems).

To perform immunoblot analysis of HAX1, cell extracts of peripheral blood leukocytes were subjected into SDS-PAGE. The proteins were transferred to polyvinyldifluoride membranes (Immobilon-P, Millipore, Bradford, MA) using a semi-dry electroblotter (ATTO Corp., Tokyo, Japan). Following the transfer, immunoblots were incubated with a monoclonal antibody against HAX1 (BD Biosciences Pharmingen, Franklin Lakes, NJ). After washing the membranes, the bound secondary anti-mouse antibody was labeled for visualization using SuperSignal (Pierce Biotechnology Inc., Rockford, IL) by enhanced chemiluminescence (FUJIFILM Corp., Tokyo, Japan).

Results and Discussion

Sequence analysis of the patient demonstrated wild type for *CSF3R*, *ELA2*, and *WAS*. Next, we analyzed HAX1 mutations of the patient, and identified a homozygous single-base pair substitution (256C-to-T) leading to a stop codon at codon 86 (R86X). This deficiency was confirmed by the absence of HAX1 protein in immunoblot analysis. The site of this mutation was consistent with that reported in one of 23 SCN patients in the previous study.[7] Although neither parent showed a detectable phenotype, both parents had a heterozygous R86X mutation with the expression of HAX1 proteins.

HAX1 has recently been identified as a cause of SCN by Klein *et al.*, who analyzed 23 *ELA2* mutation-negative SCN patients.[7] According to the mutation sites, the 23 patients were classified into 3 groups; W44X in 19 patients mainly in Kurdish and Turkish populations, R86X in 1 Iranian patient, and Q190X in 3 Swedish patients from an original Kostmann family.[7] One implication of this finding might be that mutation sites are linked to ethnicity. Our patient is the second patient identified as harboring R86X mutation, and the first patient with HAX1-deficient SCN in the Japanese population.

Although not fully understood, HAX1 seems to play a critical role in apoptosis and the organization of cytoskeleton.[8,16-18] *HAX1* is a ubiquitously expressed human gene,8 and therefore symptoms other than myelopoietic disorder could be present in patients with this deficiency. Indeed, some of the 23 Klein's patients were associated with conditions other than low ANC.[7] These included splenomegaly (n=8), growth failure (n=2), muscular hypotonia (n=1), and tricuspid insufficiency (n=1).[7] Of these, splenomegaly is a common feature with SCN irrespective of the underlying genetic defects.[1] Our patient has failed to thrive, a condition concordant with the finding in the first patient showing the same R86X mutation.[7] Together with dental development delay, this may be a reflection of a malfunction in cytoskeleton organization due to HAX1 deficiency.[8]

Of special note is the finding that our patient showed severe delay in motor and intellectual milestones. Several explanations of such developmental delay exist. One possibility is derived from hearing impairment due to sequelae at the first presentation of otomastoiditis. However, since hearing impairment was moderate and unilateral, it is unlikely that this was the cause of severe developmental delay, and is, at least, unrelated to the delay of motor function. The second possibility is that infection due to initial otomastoiditis might have extended to the central nervous system. However, this possibility is unlikely because the patient's general condition had remained well and brain CT scans at that time did not show any abnormalities. Thus, the most plausible explanation is attributable to *HAX1* mutation. This explanation is supported by the report[19] by Carlsson and Fasth, who described neurological impairments in 3 of 6 SCN patients of the original Kostmann pedigree; some of these patients were later demonstrated to be HAX1-deficient.[7] Of the three, one had mild mental retardation, the second showed speech and gross motor retardation, and the third demonstrated attention deficit hyperactivity syndrome plus epilepsy.[19] Furthermore, an experimental study showed that HAX1 is maximally expressed in brain tissues on Western blot analysis.[9] N2a cells, a mouse neural cell line, also contained significant amount of *HAX1* mRNA with subcellular localization in the cytoplasm and mitochondria.[9] Collectively, HAX1 deficiency may potentially affect neural development, reflecting severe developmental retardation in our patient. However, whether neurological complication in our patient is linked to mutation sites in *HAX1* or to HAX1 deficiency itself is not clear.

In addition, since diverse proteins have recently been identified to interact with HAX1,[8,16-18] some of these or other yet to be determined genetic factor(s) may be involved in this condition. Further accumulation of similar patients is needed to clarify this question.

In conclusion, we presented a sporadic SCN patient with a nonsense mutation in *HAX1*. Importantly, this is the first case in the Japanese population and the second patient with an R86X mutation. Furthermore, this patient demonstrated severe developmental delay and epilepsy.

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