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Different Pattern of Gene Mutations in Iranian Patients with Severe Congenital Neutropenia (Including 2 New Mutations)

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ABSTRACT

Severe congenital neutropenia (SCN) is a rare primary immunodeficiency disease. Different genes are found to be associated with SCN, including *ELA2, HAX1, WAS, GFI1, G-CSFR and G6PC3.* The aim of this study was to find different gene mutations responsible for SCN in Iranian patients.

Twenty-seven patients with SCN referred to Immunology, Asthma and Allergy Research Institute during a five year priod 5 years (May 2007 and May 2012), were included in this study. Neutropenia related exons and flanking regions of *ELA2, HAX1, WAS, GFI1, G-CSFR and G6PC3* were amplified by PCR and the sequences were analyzed.

The results showed different mutations including 4 *ELANE* mutations, 11 *HAX1* mutations and 2 *G6PC3* mutations. None of the patients had *GFI1* mutation and also one mutation was found in *G-CSFR* in a patient with ELANE mutation. Ten patients had unknown genetic diagnosis which was compatible with other studies.

According to these results, most of the patients showed *HAX1* mutations and this finding which significantly differed from other reports, might be related to differences in Iranian ethnicity and also in high rate of consanguineous marriages in Iran.

Keywords: Neutropenia; Gene mutation; Severe congenital neutropenia

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INTRODUCTION

Severe congenital neutropenia (SCN) includes heterogeneous disorders characterized by severe neutropenia from early infancy with low absolute neutrophil counts (less than 500/μl), with increasing life threatening infections, and bone marrow maturation arrest in promyelocytes/myelocytes stages.[1] SCN is a multigenic disorder which is inherited by autosomal dominant, autosomal recessive, X-linked and also sporadic patterns. Mutations (autosomal dominant and sporadic) in *ElANE (ELA2*) gene which encode neutrophil elastase (NE) cause SCN and cyclic neutropenia (CN).[2] According to multiple studies, *ElANE* is the most common responsible gene causing SCN.[3-5] However, it is not clear how heterozygous mutations in the ELANE gene can cause both CN and SCN.[6] Recently, *HAX1* and *G6PC3* have been suggested to be related to autosomal recessive forms of SCN.[7-10] Moreover, few cases of SCN patients have been reported to have *HAX1* and *G6PC3* compound heterozygous mutations.[11,12]

Neurological abnormalities have been reported in several cases of SCN with *HAX1* mutations[7,13,14] and in *G6PC3* deficient SCN patients. Syndromic features of the disease which may associate with heart and/or urogenital anomalies have been documented.[10,15,16] There are rare reports of autosomal dominant heterozygous GFI1 mutations in SCN patients.[17,18] More than 90% of SCN patients respond to Granulocyte-Colony Stimulating Factor (G-CSF) and all responding patients require fewer antibiotics and experience lower frequency of infections.[19]

Some SCN patients develop myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). It has been reported in several studies that these disorders are due to the long term G-CSF administration and mutations in the cytoplasmic region of G-CSF Receptor (G-CSFR).[20,21]

The aim of the current study was to find gene mutations responsible for SCN in Iranian patients referred to Immunology, Asthma and Allergy Research Institute in Tehran University of Medical Sciences during the last five years.

PATIENTS AND METHODS

Twenty-seven patients with SCN referred to Immunology, Asthma and Allergy Research Institute for five years (May 2007 to May 2012) were enrolled in this study. All patients were registered in Iranian Primary Immunodeficiency Registry (IPIDR). Demographic data, family history and history of infectious diseases were recorded. Neutrophil counts and bone marrow studies were performed. The criteria to define SCN were low neutrophil counts (less than ($0.5x10^6$/μl) for 3 times in 3 consequent months, recurrent infections and myelocyte/promyelocyte and/or bone marrow maturation arrest. Patients with assumed cyclic neutropenia, were documented by at least two low neutrophil counts ($0.5x10^6$/μl) in 3 months with 21 day intervals and/or myelocyte/promyelocyte bone marrow maturation arrest.[22]

An informed consent was obtained from patients and/or their parents before entering the study.

Mutation Analysis

Genomic DNA of the patients was extracted from peripheral blood samples using standard procedures. Five exons of *ELANE* (ENSG00000197561), 7 exons of *HAX1* (ENSG00000143575), 6 exons of *G6PC3* (ENSG00000141349), 9 exons of *GFI1* (ENSG00000162676) and their respected flanking regions were amplified by PCR and sequenced. First, all patients were screened for *ELANE* mutations, then patients who had negative *ELANE* mutations were screened for *HAX1* mutations. Mutation in G6PC3 was considered for patients with syndromic neutropenia (cardiac and/or urogenital abnormalities). Analysis of *GFI1* was performed for patients who had no mutations in *ELANE, HAX1* or *G6PC3*. Finally *WAS* gene mutation was evaluated in 1 patient (patient 16). At the end of this study, the genomic DNA of intracellular domain of *G-CSFR* gene (ENSG00000119535) was amplified and sequenced to find any acquired *G-CSFR* mutations in all studied patients. Prenatal diagnosis for three siblings (patient 3, 16, and 20) was performed during the study. All sequencings were done by ABI 3730XL genetic analyzer (applied Biosystem).

RESULTS

Twenty-seven SCN patients entered the study (16 male and 11 female, mean age 10.3 years) and 24 of them were offspring of consanguineous marriages. Eleven HAX1 mutations were found including 9 *W44X* in exon 2, E59X in exon 2 and one V144X in exon 3.

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Four mutations in *ELANE* including Pro257Leu in exon 5, Val69Leu, Val72Leu two heterozygous single base change as sporadic mutations in exon 3 were documented. Two new mutations 597-597+3 del CGTA and 571-580delAGGGGCCGGC were also found in exon 4.

The sequencing results of intracellular domain of *G-CSFR* showed only one mutation in patient number 5. Analysis of *GFI1* in patients with no detectable *ELA2* and *HAX1* mutations did not show any mutations in known reported regions of this gene. Clinical features and genetic findings of the included SCN patients are shown in the table 1. Of these patients, 6 patients (P1, P2, P5, P9, P11, P16) had been reported previously.[22,23]

Table 1. Clinical features and genetic findings of the SCN patients

Patient	Sex	ANC	History of bacterial infections	Bone marrow maturation arrest	Gene mutation	Outcome at the time of research
1	M	470	Pneumonia, otitis, oral ulcers, gingivitis	Myeloid maturation arrest	Trp44X (HAX1)	12 Yrs, alive
2	F	140	Pneumonia, otitis, gingivitis	Myeloid maturation arrest	Trp44X (HAX1)	17 Yrs, alive
3	M	125	Recurrent fever	Myeloid maturation arrest	Trp44x (HAX1)	6 Yrs, alive
4	M	199	Oral ulcers, otitis	Normal	NF	18 Yrs, alive
5	M	300	Skin abscesses, oral &anal ulcers, urinary tract infections	Severe myeloid maturation arrest	V69L.V72L (ELANE) Q739P (G-CSFR)	14 Yrs, alive
6	M	432	Pneumonia,lung abscesses	Mild myeloid maturation arrest	Trp44X (HAX1)	12 Yrs, alive
7	M	82	Septicemia, neck& head abscess	Severe myeloid maturation arrest	NF	8 Yrs, alive
8	M	330-1660	Oral ulcers, gingivitis, lung abscesses	Mild myeloid maturation arrest	Trp44X (HAX1)	17 Yrs,alive
9	F	509	Oral ulcers, gingivitis	Myeloid maturation arrest	Gln59X (HAX1)	17 Yrs, alive
10	M	28	Pneumonia, otitis, skin abscesses, gingivitis	Myeloid maturation arrest	NF	18 Yrs, alive
11	M	20	Pneumonia	Myeloid maturation arrest	Asn313fs (G6PC3)	4 Yrs, alive
12*	M	270	Recurrent aphthae, recurrent respiratory infections	Myeloid maturation arrest	Trp44X (HAX1)	13Yrs, alive
13*	M	70	Recurrent fevers, recurrent respiratory infections, diarrhea	Myeloid maturation arrest	Trp44X (HAX1)	8 Yrs, alive
14	F	190	Recurrent aphthae	Normal	NF	12 Yrs, alive
15	F	280	Pneumonia	Myeloid maturation arrest	Pro257Leu (ELANE)	3 Yrs, deceased
16	M	40	Pneumonia, sepsis	Myeloid maturation arrest	Ser139Ile (G6PC3)	9 mths, deceased
17	M	320	Fevers	-	NF	3 Yrs, alive
18	M	30	Recurrent pharingitis infections	-	NF	9 Yrs, alive
19	F	208	Urinary tract infections	-	NF	12 Yrs, alive
20	F		Oral ulcers, sepsis	Myeloid maturation arrest	Val144X (HAX1)	2Yrs, deceased

Patient	Sex	ANC	History of bacterial infections	Bone marrow maturation arrest	Gene mutation	Outcome at the time of research
21**	F	140	Anal ulcers, eye and skin abscesses, gingivitis	Myeloid maturation arrest	NF	19 Yrs, alive
22**	F	60	Gingivitis	Amyeloid maturation arrest	NF	15 Yrs, alive
23**	M	100	Eye inflammation	Myeloid maturation arrest	NF	3 Yrs, alive
24	F	490	recurrent respiratory infections	Myeloid maturation arrest	597_597+3 del CGTA (ELANE)	5 Yrs, alive
25	F	?	Gingivitis, ear and foot abscesses, fevers, diarhea	?	571_580del AGGGGCCGGC (ELANE)	5 Yrs, alive
26	F	140	Recurrent abscesses, oral aphthae	Myeloid maturation arrest	Trp44X (HAX1)	18 Yrs, alive
27	M	60	recurrent respiratory infections	Myeloid maturation arrest	Trp44X (HAX1)	9 Yrs, alive

*Two siblings **Three siblings with congenital abnormal spine and abnormal growth rate
NF: Not Found ANC: Absolute neutrophil count ?: Missing data

Prenatal Diagnosis

In further experiments for prenatal diagnosis, sequencing results of chorionic villus sampling (CVS) of the sibling of patient number 3 showed that the embryo was heterozygote for W44X mutation in *HAX1*. Prenatal diagnostic tests for sibling of patient number 16 also confirmed that there was no mutation in G6PC3 exon 3. These siblings were followed up for 6 months after birth by visiting and absolute neutrophil counts monthly. They were reported to be healthy. However, prenatal diagnosis of sibling of patient number 20 showed the same mutation as documented in patient 20.

DISCUSSION

Multiple genes are associated with severe congenital neutropenia including *ELANE, HAX1, WAS*, *GFI1* and *G6PC3*. These gene mutations are reported to have variable frequencies in different populations and ethnicities. In North America, 44- 63% of SCN patients, and in France, 35% of SCN patients are reported to have *ELANE* mutations (18, 20, 24). However, in our center in Iran, among 27 SCN patients, only 4 patients (15%) had *ELANE* mutation (V69L, V72L and Pro257Leu respectively, 597-597+3 del CGTA and 571-580delAGGGGCCGGC). It seems *ELANE* mutation does not have a high rate in Iranian SCN patients; this may be partially because of the severity of the disease in SCN patients with *ELANE* mutation which causes death before referral.[24-26] No cyclic neutropenia regarding *ELANE* mutation has been referred to this center.This may be because this disease is less severe as most patients who are referred to this center are in critical condition.

Cyclic neutropenia is the least severe form and the bone marrow features are erratic over time and sometimes bone marrow is stringently normal. It is suggested that lack of enzymatic activity of mutated *ELANE* causes irregular intracellular trafficking and stimulation of the unfolded protein response through AP3 (cargo protein) inhibition protein binding.[27,28]

Although our study did not include all Iranian SCN patients, it seemed *HAX1* mutation was the most prominent mutation in the SCN patients; as it was different from most of the other studies, this could be the result of high rate of consanguinity in Iran.[29] The precise frequency of *HAX1* mutation is not distinguished, but it is documented to be lower than *ELANE* mutation and limited to the various geographical regions including Middle East and Sweden.[18,26] We found 11 *HAX1* mutations in our SCN patients including 9 Trp44X, 1 Glu59X, and 1Val144X.

Among our patients, only patient 8 had cyclic neutropenia This was the first report of cyclic neutropnia in a patient with *HAX1* deficiency. The HAX1 protein is ubiquitously expressed on mitochondria and is involved in signal transduction and cytoskeleton organization.[8] The *HAX1* gene has two different splice variants which represent isoform A and B. Mutations which affect isoform A include W44X, Glu59X, and Glu60fs that lead to SCN without neurological symptoms while mutations which influence both isoforms (Arg86X, Gln 123fs, Val144fs, Gln 190X) are related to extra neurologic disorders including developmental delay, epilepsy, mental retardations and seizure. Although, in a recent study, 2 cases of W44X mutation with mild to moderate developmental delay were reported,[30] in this study none of the patients with *HAX1* mutation were reported to have neurological symptoms.

Two patients with syndromic symptoms of neutropenia had *G6PC3* mutations. Patient number 11 had arterial septal defect (type 2), unilateral hydronephrosis and superficial venous pattern and his *G6PC3* variant was Asn313fs.[10] Patient number 16 had arterial septal defect (type2) and was diagnosed to have Ser139Ile mutation. Recent studies have declared a relation between glucose and apoptosis through signaling pathway involving Gsk3β (glucose synthase kinase) and MCI1 (a BCL-2 family member). An increase of Gsk3β activity has been demonstrated in *G6PC3* deficient patients resulting in degradation of MCI1 and neutrophil apoptosis.[8,10] None of our patients had *GFI1* mutation. GFI1 is a zinc finger transcription factor that controls the expression of ELANE.[17] Recent studies have proposed that GFI1 mutation should be deliberated in SCN patients with excessive monocytosis.[18]

Among our patients, only patient number 5 showed a mutation in G-CSFR after 5 years receiving G-CSF. It was a C/T transition causing Q739P substitution. Apparently this mutation has no effect on *G-CSFR* intracellular conserved tyrosin residues (Y704, Y729, Y744 and Y764). These residues are harbor sites for SH2-containing signaling molecules and are important in the control of myeloid progenitor's differentiation. Patients receiving long term G-CSF should be monitored for *G-CSFR* mutation to find any acquired mutation.[31]

Based on these data, the *HAX1* genotyping is suggested to be performed first in Iranian SCN patients and analysis of *ELANE* mutation might be done in all other patients without *HAX1* mutations regardless of family history and because of high consanguinity in Iran. For those without *HAX1* and *ELANE* mutations and recessive inheritance pattern of the disease, *G6PC3* genotyping can be performed based on clinical features especially if associated with cardiac and/or urogenital abnormalities. Because of a cyclic neutropenic patient with *HAX1* mutation among our patients, *HAX1* genotyping should be considered for patients with CN who did not show mutation in *ELANE*.

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