ELECTRONIC LETTER

Spectrum and clinical implications of syntaxin 11 gene mutations in familial haemophagocytic lymphohistiocytosis: association with disease-free remissions and haematopoietic malignancies

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Objective: To determine the frequency and spectrum of mutations in the gene encoding syntaxin 11 (*STX11*) in familial haemophagocytic lymphohistiocytosis (FHL), a rare autosomal recessive disorder of immune dysregulation characterised by a defect in natural killer cell function.

Methods: Mutational analysis of *STX11* by direct sequencing was done in 28 FHL families that did not harbour perforin mutations, previously identified in some FHL patients. A detailed investigation of clinical features of these patients was also undertaken.

Results: Two different *STX11* mutations were identified, one nonsense mutation and one deletion, affecting six of 34 children in four of 28 unrelated *PRF1* negative families. Both mutations have been reported before. Three patients experienced long periods (⩾1 year) in remission without specific treatment, which is very uncommon in this disease. Despite the milder phenotype, some children with *STX11* mutations developed severe psychomotor retardation. Two of the six patients harbouring *STX11* gene defects developed myelodysplastic syndrome (MDS) or acute myelogenous leukaemia (AML).

Conclusions: *STX11* gene mutations were found in 14% of the *PRF1* negative FHL families included in the present cohort. These results suggest that *STX11* gene mutations may be associated with secondary malignancies (MDS/AML), and that there is segregation of specific clinical features in FHL patients with an underlying genotype.

Familial haemophagocytic lymphohistiocytosis (FHL) is an autosomal recessive condition characterised by immune dysregulation with hypercytokinaemia and defective natural killer (NK) cell function.[1 2] Patients display fever, hepatosplenomegaly, pancytopenia, hypertriglyceridaemia, hypofibrinogenaemia, and neurological abnormalities, ranging from irritability and hypotonia to seizures, cranial nerve deficits, and ataxia. Haemophagocytosis (that is, the ingestion of erythrocytes and sometime platelets and leucocytes by macrophages) is a prominent feature of the disease, and a non-malignant infiltration of macrophages (histiocytes) and activated T lymphocytes in lymph nodes, spleen, and other organs is also found.[1] FHL is typically rapidly fatal in the absence of treatment[3]; current treatment protocols are based on a regimen of etoposide, ciclosporine A, and corticosteroids until stem cell transplantation (SCT) can be undertaken.[4]

Linkage analysis has shown that FHL is linked to four different disease loci: 9q21.2–22 (FHL1), 10q21 (FHL2), 17q25 (FHL3), and 6q24 (FHL4). While the gene defect responsible for FHL1 remains unknown, mutations in the perforin gene (*PRF1*) are found in FHL2 patients,[5] and *Munc13-4* mutations result in FHL3.[6] In addition, mutations in the gene encoding syntaxin 11 (*STX11*) were recently identified in FHL4 patients.[7] Perforin is a major constituent of NK cell and cytotoxic T lymphocyte (CTL) granules and is important for initiation of apoptosis of target cells, whereas Munc13-4 is required for priming of granules for membrane fusion and exocytosis.[6] Mutations in the *PRF1* gene have been identified in 20–50% of FHL patients, with some variation between populations of different ethnic background.[8–10] The overall contribution of mutations in the *Munc13-4* gene to FHL is more uncertain; some studies have indicated a frequency of approximately 20–30%.[11 12] Very recent studies have disclosed mutations in a gene encoding a so called SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) protein, syntaxin 11 (*STX11*), in six FHL families, and showed that this was absent in mononuclear cells from these patients.[7] The precise role of syntaxin 11, a protein expressed most prominently in phagocytes and antigen presenting cells,[13] in the pathogenesis of FHL is not well understood; however, the fact that Munc13-4 is involved in priming of granules for membrane fusion and exocytosis, whereas syntaxin 11 is involved in vesicle trafficking, suggests that defects in the granule secretory pathway may constitute a common aberration in FHL patients.[12]

The frequency of *STX11* mutations in FHL patients has not been established to date. In addition, previous studies have not determined any consistent correlations between genetic subtypes of FHL and clinical features of these patients.[8 11 14] In the present study, we aimed to determine the relative frequency and the clinical implications of *STX11* gene mutations in a large cohort of families with FHL.

METHODS

Patients

We studied 34 patients from 28 unrelated families with FHL in which at least one sibling fulfilled the diagnostic criteria for FHL developed by the Histiocyte Society[15]; four children with familial disease had been diagnosed in an early phase without fulfilling all the diagnostic criteria, and in all of these another child in the family did fulfil all the criteria. Familial

Abbreviations: AML, acute myelogenous leukaemia; CTL, cytotoxic T lymphocyte; FHL, familial haemophagocytic lymphohistiocytosis; MDS, myelodysplastic syndrome; NK, natural killer; *PRF1*, perforin; SCT, stem cell transplantation; *STX11*, syntaxin.

Table 1 Primers for amplification and sequencing of the *STX11* gene

Primer sequence	Annealing site	Annealing temperature
F 5′-CAAAATTGGGACTGCTGAGTAAAA-3′	5′ 391	57°C
R 5′- AGTTGGTGTCGCGCTTGAT-3′	3′ 740	57°C
F 5′-CCCGCTTCCTCACGTCCAT-3′	5′ 705	60°C
R 5′-CCACTTACCCTGCTCGAACATGTC-3	3′ 1031	60°C
F 5′-GCACGACTACAACCAGGCCGAGAT-3′	5′ 925	62°C
R 5′-CCGGCTTTGGTGCGTCCTTC-3′	3′ 1411	62°C
F 5′-GAAGAACCCCTGCCGGACC-3′	5′ 1309	57°C
F 5′-TCGCTCAAAATGGAACAGAACCAG-3′	3′ 1491	57°C

disease was demonstrated in 10 of the families. In addition, all patients were sequenced for mutations in the coding regions of the *PRF1* gene as described previously,[8] with negative results (data not shown). The majority of the families were of Turkish origin (n = 19), the remaining being mainly of European and Arabian descent. Clinical histories of the patients with *STX11* mutations are described in detail below.

The study was approved by the ethics committee at Karolinska Institutet, Stockholm.

PCR and mutation detection

Genomic DNA was isolated from peripheral blood or cultured fibroblasts by standard procedures. Primers were designed for amplification and direct DNA sequencing of the coding sequence of *STX11* (table 1). For primer pair annealing, temperatures were set between 57° and 62°C. Genomic DNA was amplified as described previously.[8] For direct DNA sequencing the forward primer contained the −21M13 complement and the reverse primer the M13 complement. Polymerase chain reaction (PCR) amplification conditions were: 10 minutes at 95°C, 30 seconds at 95°C, 50 seconds at annealing temperature, 30 seconds at 72°C (40 cycles), and finally two minutes at 72°C. The PCR products were then analysed on 2% agarose gels. For the sequencing reaction we used the ABI BigDye primer cycle sequencing ready reaction kit (Applied Biosystems, Foster City, California, USA). For cycle sequencing the following protocol was used: 10 seconds at 96°C, 5 seconds at 55°C, one minute at 70°C (20 cycles), 10 seconds at 96°C, and one minute at 70°C (20 cycles). All PCR reactions were done on an MJ Research PTC-225 Peltier thermal cycler (Bio-Rad Laboratories, Waltham, Massachusetts, USA). The sequencing reactions were analysed on an ABI genetic analyser 310 (Applied Biosystems).

RESULTS

STX11 mutational analysis in FHL patients

The complete 861 base pair open reading frame of the *STX11* gene was sequenced in all 28 FHL families. Mutations in *STX11* were identified in four unrelated families (table 2). Family A has been described, in part, in a previous report.[7] We thus confirmed the deletion in this family and identified this deletion also in another unrelated family (family B) of Turkish origin. This mutation deletes AG at the nucleotide position 369_370 and CGC at the nucleotide position 374_376 in exon 2 of *STX11*, causing a frameshift and a premature termination codon (table 2). The AG and CGC deletion were found to co-segregate on the same allele and were present in a homozygous state in all affected individuals investigated. Several branches in family A have experienced unexplained infant deaths with febrile episodes. We sequenced presumed carriers in this family, including some previously not investigated, and showed that the [c.369_370delAG; c.374_376delCGC] mutation co-segregated completely in a heterozygous state in obligate carriers available for analysis (fig 1). In the third and fourth family (family C and family D) a nonsense mutation was identified. The patients in these families were homozygous for a C to T nucleotide substitution, changing a glutamine to a premature termination at codon 268 (table 2).

The relative frequency of *STX11* gene mutations in FHL patients

Altogether four *STX11* gene mutations were detected in the 28 families studied, corresponding to 14% of all non-*PRF1* FHL families. The corresponding figures in the Turkish population were four of 19 (21%). In other words, no family of non-Turkish origin carried *STX11* mutations in the present cohort. It is therefore likely, as has been shown previously for *PRF1*,[8] that *STX11* mutations are an uncommon cause of FHL in the northern European population. In addition to the mutations reported here, the mother of a patient not included in the study was found to be heterozygous for the mutation reported in family A and family B. Unfortunately, DNA extraction from the patient and the father, both of Turkish origin, was unsuccessful and mutational analysis of the *STX11* gene was therefore not possible (data not shown).

Clinical investigation of *STX11* defective patients

Family A

Patients A1, A2, and A3 are brothers, aged three months, one year, and three years, respectively, at presentation (table 2), and their clinical history has been reported previously.[16] They were born in Sweden to consanguineous parents of Turkish (Kurdish) origin; of note, there was a family history of several unexplained infant deaths in other branches of the kindred (fig 1). The oldest brother (A1) died shortly after the diagnosis was made. A liver biopsy and bone marrow examination showed an increased lymphohistiocytic cell population and haemophagocytosis, but genetic analyses could not be done as the DNA was degraded. The second brother (A2) presented at 12 months of age with rhinitis, dry cough, and fever. The work up was consistent with a diagnosis of FHL and in 1982 combined treatment with vinblastine, methotrexate, hydrocortisone, and intravenous gamma globulin was initiated. Clinical and histological remission was gradually achieved, and despite the fact that no maintenance therapy was given the disease remained inactive until he relapsed three years after the initial diagnosis. He was given reinduction therapy with teniposide, prednisolone, and intrathecal injections of methotrexate, and remission was instantaneous. He then received intravenous maintenance therapy which was gradually discontinued 1.5 years after the relapse, after which he was given oral maintenance treatment including etoposide. He was then once more off treatment for more than a year until he experienced a third bout of the disease.

The third brother (A3) was admitted to hospital only two months after the first relapse of his older brother (A2). The clinical picture at the time of presentation bore similarities to that of his older brother, with a dry cough and four days of fever, but in addition he also had abdominal pain and conjunctivitis. He was started on the treatment regimen used during his older brother's relapse, responded well, and attained remission, but he relapsed three years after the diagnosis, after more than one year off treatment. The third brother developed MDS six years after the diagnosis of FHL, as reported previously.[17] Chromosome analysis showed a normal male karyotype. He had been given etoposide (cumulative doses of 6.9 g/m^2 intravenously and 13.6 g/m^2 orally) and teniposide (3.4 g/m^2 intravenously), but no other systemic antineoplastic drugs. He and his older brother (A2) both subsequently underwent SCT, 7 and 10 years after their

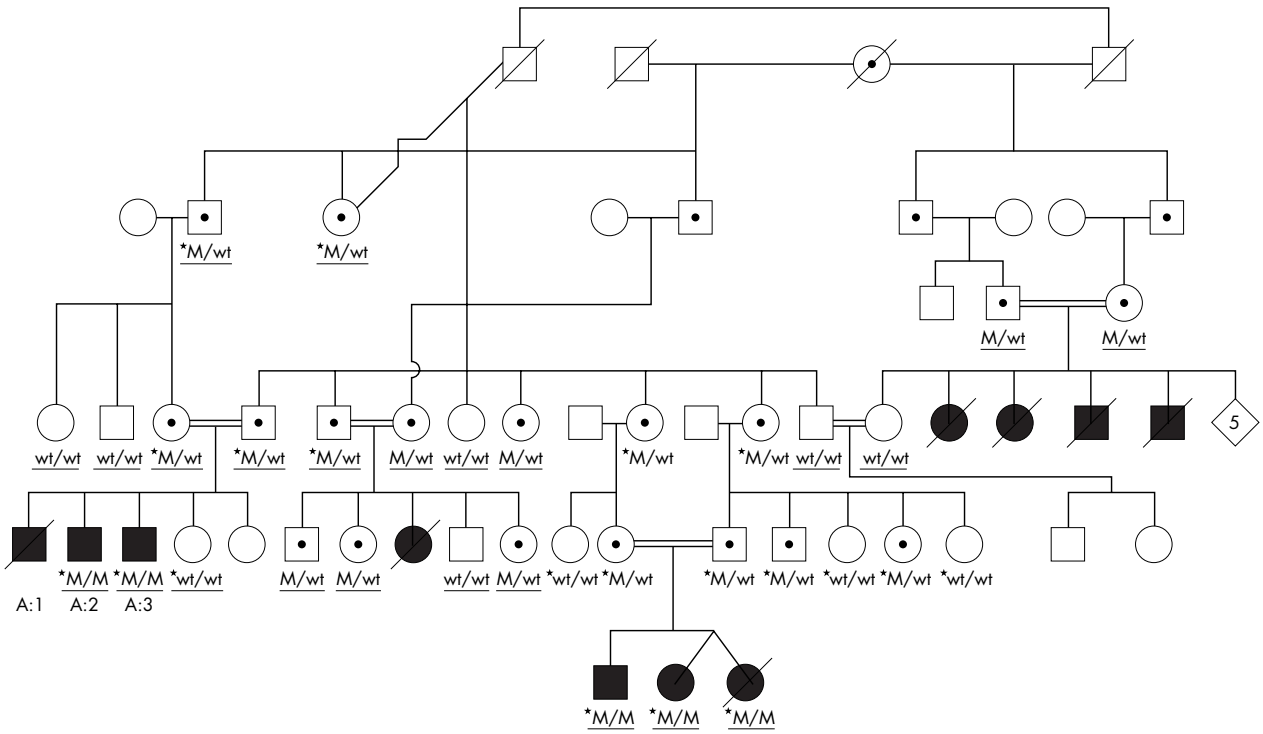

Figure 1 Pedigree of a large family (family A) with autosomal recessive familial haemophagocytic lymphohistiocytosis (FHL) in which mutations in the *STX11* gene were identified. Several branches of the family have experienced unexplained infant deaths with febrile episodes. The [c.369_370delAG; c.373_375delCGC] mutation is found in a homozygous state in all affected individuals, and was present in a heterozygous state in all obligate carriers that were available for analysis. Findings marked with an asterix were reported in the original presentation of syntaxin mutations,[7] and underlined results were acquired in the present study. Symbols with a dot indicate mutation carriers. M, mutation; wt, wild type.

Table 2 Familial data, clinical findings at diagnosis, and mutational data in patients identified with STX11 gene mutations

	Family A			Family B		Family C	Family D
Patient	**A:1**	**A:2**	**A:3**	**B:1**	**B:2**	**C:1**	**D:1**
Country of origin	Turkey	Turkey	Turkey	Turkey	Turkey	Turkey	Turkey
Familial disease	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Consanguinity	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Sex	Male	Male	Male	Female	Male	Female	Male
Mutation*	ND	V124Fs	V124Fs	V124Fs	V124Fs	Q268X	Q268X
Homozygocity	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Age at onset (months)	3	12	39	3	6	1	84
Fever >38°C	+	+	+	+	+	+	+
Splenomegaly	+	+	+	+	+	+	+
Hepatomegaly	+	+	+	+	+	+	+
Anaemia	+	+	+	+	+	+	+
Neutropenia	+	+	−	+	+	−	+
Thrombocytopenia	+	+	−	+	+	+	+
Hypertriglyceridaemia	ND	+	+	+	+	+	ND
Hypofibrinogenaemia	ND	ND	−	+	−	+	+
Raised ferritin	ND	ND	ND	+	+	+	+
Haemophagocytosis	+	+	−	+	+	+	+
Neurological disease†	−	−	−	+	+	−	ND
Treatment protocol	–	Other, see text	Other, see text	HLH-94‡	HLH-94	HLH-94	Other, see text
Remission	−	+	+	+	+	+	+
Remission >1 year off all Rx	−	+	+	−	−	−	+
Relapse	−	+	+	+	−	+	+
Secondary malignancy	−	−	MDS	−	−	AML M3	−
Stem cell transplant	−	+	+	−	+	−	−
Outcome	Deceased	Alive and well	Alive and well	Deceased	Alive with CNS sequelae	Alive and well	Deceased

For a detailed clinical description of the patients, see Methods.
A plus sign (+) indicates presence, a minus sign (−) indicates absence.
*V124Fs = [c.369_370delAG;c.374_376delCGC], Q268X = 802C>T
†Neurological disease relates to the entire course of the disease.
‡HLH-94 protocol.[4]
AML, acute myelogenous leukaemia; CNS, central nervous system; HLH, haemophagocytic lymphohistiocytosis; MDS, myelodysplastic syndrome; ND, no data; Rx, treatment.

diagnosis, respectively. They were both alive and well at the time of writing.

Family B

Two Turkish siblings (patient B1 and B2), a sister and a brother of consanguineous parents, presented with similar clinical findings in Turkey at the age of five months and six months, respectively. There is one healthy older sister. Both affected children developed severe psychomotor retardation. The sister (B1) was initiated on the HLH-94 protocol established by the Histiocyte Society[4] and her hepatomegaly decreased from 11 cm to 4 cm below the costal margin and the splenomegaly from 10 cm to 3 cm, but she had residual hypertriglyceridaemia and a raised ferritin level. When her symptoms had partially resolved she was released from the hospital but her psychomotor development was very delayed and at follow up at 12 months she could sit only with support and could not crawl, could not imitate words, and had developed social smiling only to her mother. The girl remained in good health (except for the developmental delay) for two months after the last visit to the hospital but then, at 14 months of age, she developed fever and cough and was found dead at home in her bed within a couple of days. Her younger brother (B2) was admitted at six months of age, at which time he was not able to control his head movements. He had low NK cell activity and was started on HLH-94 treatment. He improved, underwent SCT, and at the time of writing was alive with sustained full chimerism. He has no fever, normal blood counts, and normal fibrinogen, triglyceride, and ferritin levels, but the liver and spleen both extend 3 cm below the costal margin, which we cannot explain. He still has a developmental delay, and at 18 months of age he sits only with support and cannot crawl, and he cannot use expressive language.

Family C

Patient C1 is the first and only child of a consanguineous family in Turkey. She had fevers from the age of one month. At four months she was diagnosed and had clinical findings typical of FHL, including haemophagocytosis in the bone marrow. The patient was started on the HLH-94 treatment protocol and remission was achieved with no signs of active disease. The cumulative dose of etoposide the patient had received at that time was 3.15 g/m^2 intravenously. However, she developed AML (type M3) two years after the initiation of HLH-94 treatment. Chromosome analysis has revealed at (15,17) (data not shown). At the time of writing, the patient was still alive and in remission from AML and HLH, and had been off HLH treatment for four months.

Family D

Patient D1 was the forth child of a consanguineous family in Turkey. He had two elder sisters who died of a similar disease at two and six months of age. In 1993 he was referred for jaundice, fever, and pneumonia. His liver was palpable 9 cm and the spleen 7 cm below the costal margin. He also had lymphadenopathy and was treated with antibiotics. Haemophagocytosis was not observed in the first bone marrow aspiration; however, two months later, it was present in a lymph node biopsy. He was initially treated with high dose methylprednisolone and etoposide for two months, after which his family discontinued the treatment for financial reasons. One year later he was readmitted to the hospital with fever and hepatosplenomegaly, and HLH-94 treatment was given. Between 1995 and 1998 he was treated at a local hospital, receiving etoposide in addition to corticosteroids during several bouts of high fever and one episode of splenomegaly lasting four weeks. Apart from this treatment, his mother gave methylprednisolone at home when the patient developed a fever. He was admitted to the intensive care unit in 1998 at the age of 12 years and died a short time afterwards from bleeding.

DISCUSSION

This study, undertaken on a large cohort of FHL families, shows that mutations in *STX11* are present in a subset of these patients, in particular in those of Turkish origin. Hence, we identified two different *STX11* mutations, both previously reported,[7] in a group of six patients from four unrelated kindreds. As three of the patients in the present study (A:2, A:3, and C:1) had been reported previously,[7] a total of 13 individuals representing eight families has hitherto been shown to carry *STX11* mutations. Furthermore, the relative frequency of *STX11* mutations, based on direct DNA sequencing, in *PRF1* negative FHL families was found to be approximately 14% in all families and 21% in our cohort of Turkish FHL families. Clinical investigations revealed long periods of disease-free remission in the absence of treatment and an unexpectedly high incidence of secondary MDS/AML in patients with *STX11* gene mutations. This report thus shows that there may be an association between a specific genotype (*STX11*) and certain clinical features.

STX11 mutations have so far been found only in families of Turkish origin, which we cannot explain at present and which deserves further investigation. An attempt to evaluate potential common ancestors by analysis of polymorphisms in the coding sequence was unsuccessful as no polymorphisms were detected (unpublished observations). We have previously also reported that *PRF1* mutations are more common in Turkish FHL families than in families of non-Turkish origin, representing 30% and 7% of FHL cases, respectively.[8] An even higher proportion of *PRF1* mutations (25/43, 58%) has been reported in the USA, and many of the affected patients were of African-American ancestry.[10] Finally, a recent Japanese study reported 11 of 57 patients (19%) to have *PRF1* mutations and as many as eight of 24 *PRF1* negative patients (33%) tested for *Munc13-4* had mutations in this gene, suggesting that *Munc13-4* mutations may be a common cause of FHL.[11] Thus there is a marked difference in various ethnic groups in the causative gene in FHL families. Nevertheless, for many ethnic groups a majority of the affected families can now benefit from a molecular based diagnosis as well as prenatal diagnosis.

We initially hypothesised that defects affecting the perforin-granzyme B pathway may account for the NK dysfunction and immune dysregulation evidenced in FHL patients.[18] Indeed, mutations in the gene encoding perforin, a protein known to be involved in cellular cytotoxicity, were described soon afterwards in a subset of FHL patients,[5] and subsequent investigations have elucidated the functional consequences of these inherited mutations at the cellular level.[19] Moreover, *PRF1*-deficient mice have an impairment of cytotoxic activity of CTLs and NK cells, and they develop an uncontrolled cytokine driven expansion of lymphocytes, resembling the phenotype seen in children with FHL, if infected with lymphocytic choriomeningitis virus.[20] Furthermore, Clementi and colleagues[21] have recently reported that a significant proportion of patients with either Hodgkin or non-Hodgkin lymphoma harbour mutations in the *PRF1* gene. For comparison, previous studies of *PRF1* deficient mice have provided evidence for perforin mediated protection against spontaneous lymphoma.[22] Overall, these findings support the view that mutations affecting components of the cytolytic pathway may contribute to the development of haematopoietic malignancies through an impairment of immune surveillance for transformed cells.

As mentioned above, mutations in the *Munc13-4* gene were recently described in some FHL patients.[6 23] *Munc13-4* is

thought to be important for the vesicle-plasma membrane fusion during exocytosis of cytotoxic granules containing perforin and granzymes from CTLs and NK cells.[6] Therefore, mutations in *Munc13-4* impair the function of cytotoxic cells in FHL patients, despite a normal expression of perforin. While *Munc13-4* is involved in the exocytosis of cytotoxic granules, the function of *STX11* is not well understood but one may hypothesise that *STX11* is implicated in the trafficking of vesicles from intracellular compartments to the cell surface. However, it has been proposed that *STX11* has a regulatory role rather than being involved in the membrane fusion process.[24] One might therefore speculate that *STX11* is involved in the regulation of cytotoxic cells by affecting the interaction of these cells with dendritic cells. In fact, studies in recent years have shown that NK cells participate directly in adaptive immune responses, mainly by interacting with dendritic cells; such interactions can positively or negatively regulate dendritic cell activity. Reciprocally, dendritic cells regulate NK cell function.[25] The fact that NK dysfunction in FHL patients can be divided into several subcategories[26 27] suggests that various different underlying mechanisms (perhaps corresponding to distinct underlying genetic defects) may be involved. Detailed cellular and molecular studies are warranted to delineate the putative role of *STX11* in the regulation (direct or indirect) of cellular cytotoxicity.

FHL is characterised by a remarkable degree of genetic heterogeneity, yet previous studies have not disclosed any consistent genotype–phenotype correlations.[8 11 14] Some differences in terms of age at onset of disease were discernible between patients with nonsense and missense *PRF1* gene mutations[11] and between FHL2 (*PRF1* deficient) and non-FHL2 patients,[14] but further distinctions could not be made. In the current study, patients with *STX11* gene mutations fulfilled the diagnostic criteria for FHL, and the clinical symptoms at onset were identical to those of other patients with FHL. However, in some respects the clinical features of these children appear to be milder than for other FHL patients. First, three of the six children were one year of age or older at the time of onset. In addition, three of the six children with *STX11* mutations experienced long periods (one year or more) of remission without any specific treatment, which is highly unusual for familial forms of this disease. Two patients (B1 and B2) developed severe developmental delay, but as CNS involvement is found also in FHL patients with mutations in other genes,[6 8 11 14] and as B1 and B2 had the same mutation as patients A1, A2, and A3, all without neurological symptoms, it is probable that the neurological disease is not specific to the *STX11* mutation. A striking feature in the clinical presentation of the *STX11* defective patients is the development of myelodysplastic syndrome and acute myelogenous leukaemia in two of six patients (33%). To our knowledge, only three other children younger than 10 years of age with haemophagocytic lymphohistiocytosis and subsequent MDS/AML have been reported previously.[4 28 29] In fact, the HLH-94 registry at Karolinska Institutet currently includes 344 patients for whom HLH-94 treatment has been initiated, yet only two (0.6%) have developed MDS/AML (neither A:3, whose treatment was started in 1986, nor C:1 was registered in HLH-94).[4 29] Although epipodophyllotoxin administration may have influenced the development of malignancies in the patients reported here,[30] not least because patient A:3 received a very high dose of epipodophyllotoxins, it is noteworthy that two patients with secondary MDS/AML were found to carry mutations in the *STX11* gene. Indeed, the recent association between *PRF1* deficiency and lymphoma,[21] and the current finding that *STX11* mutations are linked to MDS/AML, suggest that genetic deficiencies targeting cellular cytotoxicity pathways in FHL patients may also result in an impairment of immune surveillance for malignant cells.

Conclusion

Our study shows that mutations in the *STX11* gene are associated with FHL, a rare and fatal disorder in children, and are present in approximately 20% of the Turkish FHL families tested. These findings may thus facilitate genetic counselling and prenatal diagnosis in affected families. Importantly, while the clinical phenotype in patients with *STX11* mutations in some respects is milder than in other FHL patients, with long disease-free remissions, these children may have an increased risk of developing MDS/AML. Additional studies should aim to clarify the specific molecular mechanism through which *STX11* gene mutations cause FHL.

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