Disease cohorts

ORIGINAL ARTICLE

Genetic analysis of inherited bone marrow failure syndromes from one prospective, comprehensive and population-based cohort and identification of novel mutations

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ABSTRACT

Introduction Inherited bone marrow failure syndromes (IBMFSs) often have substantial phenotypic overlap, thus genotyping is often critical for establishing a diagnosis.

Objectives and methods To determine the genetic characteristics and mutation profiles of IBMFSs, a comprehensive population-based study that prospectively enrols all typical and atypical cases without bias is required. The Canadian Inherited Marrow Failure Study is such a study, and was used to extract clinical and genetic information for patients enrolled up to May 2010.

Results Among the 259 primary patients with IBMFS enrolled in the study, the most prevalent categories were Diamond–Blackfan anaemia (44 patients), Fanconi anaemia (39) and Shwachman–Diamond syndrome (35). The estimated incidence of the primary IBMFSs was 64.5 per 106 births, with Fanconi anaemia having the highest incidence (11.4 cases per 106 births). A large number of patients (70) had haematological and non-haematological features that did not fulfil the diagnostic criteria of any specific IBMFS category. Disease-causing mutations were identified in 53.5% of the 142 patients tested, and in 16 different genes. Ten novel mutations in SBDS, RPL5, FANCA, FANCG, MPL and G6PT were identified. The most common mutations were nonsense (31 alleles) and splice site (28). Genetic heterogeneity of most IBMFSs was evident; however, the most commonly mutated gene was SBDS, followed by FANCA and RPS19.

Conclusion From this the largest published comprehensive cohort of IBMFSs, it can be concluded that recent advances have led to successful genotyping of about half of the patients. Establishing a genetic diagnosis is still challenging and there is a critical need to develop novel diagnostic tools.

INTRODUCTION

Inherited bone marrow failure syndromes (IBMFSs) are complex genetic disorders characterised by physical malformations and haematopoietic failure, which result in single or multiple lineage cytopenias.1,2 Specific categories of IBMFSs also carry a high risk of developing malignant diseases such as myelodysplastic syndrome (MDS), leukaemia, particularly acute myeloid leukaemia, and solid tumours.1-3 It is often impossible to establish a diagnosis based solely on clinical features because of substantial overlap of haematological and non-haematological manifestations and late development of characteristic features of individual conditions.4 Therefore the study of genes mutated in IBMFSs is critical for establishing a diagnosis and providing proper treatment and genetic counselling.

Multiple genes have been associated with IBMFSs. These include genes involved in ribosome biogenesis, such as those associated with Diamond–Blackfan anaemia (DBA)5 and Shwachman–Diamond syndrome (SDS);6 genes involved in telomere maintenance such as the dyskeratosis congenita (DC) genes;7 and genes involved in recombination DNA repair such as the Fanconi anaemia (FA) genes.8,9 Aside from direct application of genetic knowledge to the clinical management of patients, the discovery of IBMFS genes helps to conceptualise the biological pathways involved in the pathogenesis of IBMFSs and to develop novel therapeutic strategies.

Several groups have analysed specific genes in individual IBMFSs. However, cohorts limited to one disease may be biased by non-inclusion of atypical or mild cases. Published data from several registries that include patients with several common IBMFSs suffer from substantial selection bias and inclusion of retrospectively analysed cases.3,9 Owing to substantial progress in understanding the clinical and laboratory aspects of the IBMFSs over the last 10–15 years, retrospective surveys of IBMFSs diagnosed many years ago may suffer from inclusion or exclusion biases. Therefore the relative prevalence of mutated IBMFS genes among the whole population of IBMFSs, the types of mutations, and the clinical characteristics of patients who are difficult to diagnose at the genetic level is not known. Such information is pertinent for a rational approach to the genetic work-up of new patients suspected of having an IBMFS, but whose clinical manifestations overlap with several other syndromes or do not fit the clinical diagnostic criteria of known syndromes. Such information about the spectrum of genetic alterations causing IBMFSs can only be inferred from comprehensive and population-based cohorts in whom all patients
with the disorders are enrolled in a prospective manner. The Canadian Inherited Marrow Failure Study (CIMFS) is such a study, which aims to enrol all patients with IBMFSs in Canada. We analysed 259 cases of patients with IBMFSs who were prospectively enrolled in the study over almost 10 years. We investigated the spectrum of genetic alterations causing IBMFSs and the clinical characteristics of the patients. We hypothesised that, because of genetic discoveries over the last decade, a large proportion of patients with IBMFS can now be genotyped. We also hypothesised that, because of the mixed ethnic origin of the Canadian population, a proportion of the patients in this cohort carry novel mutations. Further, we hypothesised that a population-based study would reveal different frequencies of mutations from previously published non-population-based studies.

**METHODS**

The Canadian Inherited Bone Marrow Failure Study

The CIMFS is a multicentre study, which was approved by the institutional ethics board of all the participating institutions. Patients have been prospectively enrolled since January 2001 after written consent was obtained from the patients or guardians. The CIMFS is population-based, since the participating centres care for >98% of the eligible paediatric IBMFS population in Canada. More than 90% of the patients in the present study are from centres that, according to data provided by the site co-investigator, enrol more than 80% of the patients visiting their institutions. Patient information at presentation, study entry and yearly follow-up appointments was collected and included demographics, diagnosis, symptoms, family history, physical malformations, laboratory tests, genetic tests and imaging studies, treatment and outcomes.

**Inclusion criteria and classification**

Individuals who fulfilled the diagnostic criteria for an IBMFS depicted in box 1 were recruited by haematologists at each participating centre. In short, the criteria included two of the following: (1) evidence of chronic bone marrow failure (≥5 months); (2) evidence of an inherited disorder determined by either family history or physical malformation or presentation earlier than 1 year; (3) positive genetic testing. Each case was categorised with a specific syndrome (eg, FA and DBA) according to published diagnostic criteria (phenotypic and laboratory findings). The criteria for the main IBMFSs are provided in online supplementary table 1. Cases that fulfilled the diagnostic criteria of an IBMFS but not the diagnostic criteria for any known conditions were defined as unclassified IBMFS.

In the present analysis we included only cases of primary IBMFS—that is, disorders in which hypo-productive cytopenia is a major component of the syndrome and occurs in the majority of the patients such as FA and DBA. Bone marrow failure was defined as reduced production caused by either hypoplasia (eg, DC and FA), ineffective haematopoiesis with or without maturation (eg, congenital dyserythropoietic anaemia and Kostmann/severe congenital neutropenia (K/SCN)) or bone marrow release defects (eg, myelokathexis). Patients with these conditions are routinely asked if they will enrol in the study at the participating centres. Patients with a diagnosis of acquired aplastic anaemia, de novo MDsS, de novo leukaemia or haemoglobinopathies were excluded. Patients with an unclassified syndrome who developed leukaemia without preceding bone marrow failure or MDS were also excluded. Patients who were seen in more than one centre (nine patients in total) were identified by the CIMFR office coding information and were enrolled only once.

**Box 1 Criteria for a diagnosis of an inherited bone marrow failure syndrome**

Fulfil one of the following criteria

1. Fulfil the criteria for one of the inherited marrow failure syndromes

2. Have at least two of the following:

i. Signs of chronic bone marrow failure as defined by having at least two of the following:

a. Chronic cytopenia(s) detected on at least two occasions over at least 3 months.*

b. Reduced marrow progenitors or reduced clonogenic potential of haematopoietic progenitor cells or evidence of ineffective haematopoiesis.**

b. Increased increase in haemoglobin F (over 3 months apart).

d. Persistent red blood cell macrocytosis (over 3 months apart) (not caused by haemolysis or a nutritional deficiency).

ii. At least one of the following features which suggest an inherited aetiology of chronic bone marrow failure:

a. First-degree relative of bone marrow failure.

b. Anomalies involving multiple systems to suggest an inherited syndrome.

iii. Positive genetic testing for an IBMFS gene.

**Cytopenia was defined as follows: neutropenia, neutrophil count <1.5×10⁹/l; thrombocytopenia, platelet count <150×10⁹/l; anaemia, haemoglobin concentration <2 SDs below mean, adjusted for age.**

**Haemoglobinopathies with ineffective erythropoiesis and high haemoglobin F were excluded.**

**Specific screening test for IBMFSs**

Chromosomal fragility testing was performed by metaphase cytogenetics of stimulated peripheral blood cells in a clinically certified laboratory at the treating centre or the Hospital for Sick Children, Toronto. Telomere length was measured by fluorescence in situ hybridisation (FISH) of telomere repeats in the Repeat Diagnostic Laboratory, Vancouver, Canada. Testing for red blood cell adenosine deaminase activity was performed by spectrophotometry in Stanford Clinical Laboratory, Stanford, California, USA.

**Genetic testing**

The majority of the genetic tests were performed in clinically certified laboratories (mentioned in the Results section next to each gene); a minority of the tests were performed in research laboratories (YD, Hospital for Sick Children, Toronto) (online supplementary table 2). Genetic testing was performed at the discretion of the treating physician based on clinical phenotype, family history, test availability, family wishes, insurance coverage and results of screening tests such as adenosine deaminase, telomere length and chromosomal fragility. Genetic testing was not required for enrolment in the CIMFS.

After DNA extraction, most targeted genes were analysed by bidirectional sequencing of individual exons and flanking intronic regions after PCR amplification (eg, SBDS, DKC1 and ELA2). In the case of FA, complementation groups were usually determined by screening with retro-vectors carrying the various genes, followed by sequencing of the respective gene.
Constitutional karyotype and FISH analysis
Testing for constitutional karyotype was performed by metaphase cytogenetics of stimulated peripheral blood T lymphocytes, skin fibroblasts or buccal smears.

Statistical analysis
The χ² or Fisher exact test was used to determine statistical significance of the differences in discrete clinical or demographic parameters between groups. The Student t test was used for analysis of quantitative variables. Non-parametric one-way analysis of variance was also used to determine significance of ethnic distributions and IBMFSs. p<0.05 was considered significant. Statistical analysis was conducted through GraphPad Prism 4 software.

RESULTS
The most prevalent IBMFSs are DBA, FA and SDS
During the period January 2001 to May 2010, 274 patients were enrolled in the CIMFS. Of these, 259 had primary IBMFSs. The other 15 had conditions that were occasionally associated with bone marrow failure or have not been previously reported to be associated with bone marrow failure, and were excluded from the current analysis. Of the patients with primary IBMFSs, 41 belonged to 18 multiplex families and had relatives enrolled in the study. Overall, the male to female ratio in the CIMFS was 1.05:1. The gender ratios for the various syndromes are shown in figure 1.

In contrast with estimations from literature reviews,² ¹¹ or registries in which enrolment was not prospective,⁹ the most prevalent condition was DBA (17%). The other common conditions were FA (15.1%), SDS (13.5%), K/SCN (6.2%) and DC (5.9%) (figure 1). The remaining classified patients were diagnosed as having 11 different disorders, which included glycogen storage disease 1b, thrombocytopenia with absent radii, familial thrombocytopenia, congenital dyserythropoietic anaemia and Barth syndrome. Interestingly, a large group of patients with primary IBMFSs in our study were unclassified (27%). These cases were deemed unclassifiable because they had a constellation of clinical manifestations and laboratory tests that did not fit the diagnostic criteria of any of the known IBMFSs. An example family with an unclassified IBMFS includes a father with aplastic anaemia and a son with pancytopenia since birth, Tourette syndrome, no physical malformations, normal chromosome fragility, moderately short telomeres, normal exocrine pancreatic function and negative mutation analysis for multiple genetic testing including genes for DC, SDS, congenital amegakaryocytic thrombocytopenia, DBA and mitochondrial disease.

The IBMFSs with the highest incidence are FA, DBA and SDS
We calculated the incidence of the IBMFS cases (diagnosed relatively early in childhood; in the first 9 years of life) per 10⁶ births based on the average birth rate in Canada (http://www.indexmundi.com/canada/birth_rate.html). To do so, we selected the cases enrolled in the CIMFS during their first 9 years of life, as this correlated with analysis of data from the first 9 years of the CIMFS (between January 2001 and December 2009). This approach eliminates a potential bias caused by exclusion of children who had been diagnosed earlier and died. To eliminate bias from centres that are at early stages of enrolment of patients, we included all cases from large paediatric centres (Toronto, Halifax, Ottawa and Winnipeg), which enrolled >80% of the patients. The total population served by these four centres is 11 million (http://www.citypopulation.de/Canada-MetroEst.html) with an average of 10.64 births per 1000 people per year (http://www.statcan.gc.ca/). The annual disease birth rate was calculated as the total number of children born with the diseases (67) divided by the average births in this population per year divided by the number of years of the analysis (9). The overall annual incidence of IBMFSs was 64.5 per 10⁶ births. Using this analysis, the most common IBMFS was FA, with an annual incidence of 11.4 per 10⁶ births, followed by DBA and SDS, with an incidence of 10.4 per 10⁶ and 8.5 per 10⁶ births, respectively (table 1).

The diagnosis of IBMFSs is often delayed
The age of presentation with haematological or non-haematological features of an IBMFS ranged from birth to 206 months (median age 1.75 months); however, a definitive diagnosis of a specific category of an IBMFS was not established until later at
Figure 2  Age at presentation and diagnosis of the common inherited bone marrow failure syndromes. (A) Fanconi anaemia (FA); (B) Diamond—Blackfan anaemia (DBA); (C) Shwachman—Diamond syndrome (SDS); (D) dyskeratosis congenita (DC); (E) Kostmann/severe congenital neutropenia; (F) unclassified syndromes.

Most frequent delays in diagnosis were DC (55.6%), SDS (34.3%), FA (26.3%), DBA (20.5%) and K/SCN (18.8%) (p<0.05).

Family history helps to establish a diagnosis of an IBMFS in one-quarter of the patients

Comprehensive data on family history were available for 255 patients from 241 families (figure 3). Of the 255 patients, 60 had a first- or second-degree family member with a history of an IBMFS (54 first-degree relatives and six second-degree relatives). Of the 165 families for whom information about consanguinity was available, 16 pairs of parents were consanguineous. Upon analysis of familial aggregation between the most prevalent conditions, 56% of K/SCN, 37% of SDS, 23% of unclassified IBMFS, 21% of FA and 11% of DBA patients indicated a family history of an IBMFS.

Genotype was determined in about one-half of the patients tested

Targeted gene analysis was performed on 142 patients; 76 (53.5%) returned positive results. Thus, of the total population included in the study (259), a mutation was identified in 29.3% of patients. In 11 cases, genotyping solved diagnostic dilemmas; however, no discrepancies were identified between clinical diagnosis and genetic results.

A total of 16 genes were found to harbour mutations in our study. Of the 66 patients who had had genetic testing but no mutations were found, 30 had classified IBMFSs (ie, DBA, FA and SDS) and 36 had an unclassifiable IBMFS. The classified patients had typical features of the disease. This included, for example, a patient with DC who had pancytopenia, leukoplakia, developmental delay and dystrophic nails, but no mutations in...
Disease cohorts

Figure 4  Distribution of mutated inherited bone marrow failure syndrome genes in the study population.

the DKC1, TINF2, TERT, TERC, NOP10 and NHP2 genes. Another classified patient had DBA with pure red cell aplasia, transfusion dependence since birth and short stature, but was negative for RPS19, RPL5, RPL11, RPL35a, RPL24, RPS7, RPL26, RPL10 gene mutations.

Figure 4 shows the prevalence of the various mutated genes among the patients with positive genetic testing. Figure 5 illustrates the prevalence of anaemia, neutropenia, thrombocytopenia and pancytopenia among the various mutated genes. By far the most prevalent mutated gene among the patients in this study was SBDS, which was mutated in 26 of the 76 patients with IBMFSs, followed by FANCA. To correct for possible bias due to differences in the percentage of patients with complete testing among the various IBMFSs, we multiplied the proportion of the gene frequency by the total number of patients with a specific disorder (as if all patients with the disorder were tested). Using this calculation, the most common IBMFS genes were still SBDS, FANCA and RPS19, with predicted frequency among the primary IBMFSs of 20%, 13.6% and 7.8%, respectively.

The most common mutations were nonsense (51 alleles), splice site (28 alleles) and missense (18 alleles). Deletions (small or large), small insertions and indels were rare (table 2).

Of the 142 patients who had genetic testing, 67 tested negative for 257 tests, and no causative mutations were identified (online supplementary data, table 3).

Figure 5  Distribution of mutated inherited bone marrow failure syndrome genes among patients with anaemia, neutropenia, thrombocytopenia and pancytopenia.

Mutations in SBDS

As mentioned above, SBDS was the most commonly mutated gene in our series. All SDS patients with SBDS mutations were compound heterozygous. The most common disease-related alleles were c.258+2T→C (p.C84fsX3), c.183_184TA→CT (p.K62X) and c.183_184TA→CT+c.258+2T (p.K62X+p.C84fsX3) (table 2).

We identified two novel SBDS mutations. The first patient had a novel mutation, c.388G→T (p.V130L), on one allele and a common mutation, 183_184TA→CT (p.K62X), on the other allele. The novel mutation is in the second domain of the SBDS protein. This patient presented with classic features of SDS, which included low neutrophil concentration and pancreatic insufficiency (table 3, figure 6).

The second patient had a novel mutation, c.621+1G→A, on one allele and a common mutation, c.258+2T→C (p.C84fsX3), on the other allele. This mutation is predicted to cause splice site mutation, frameshift and truncation eight amino acids downstream (p.C207fsX8). This patient had an atypical presentation with no apparent extra-haematological features of SDS, but had a significantly lower than normal neutrophil count (table 3, figure 6).

Mutations in ribosome protein genes

Of the 22 clinically diagnosed patients with DBA who had had genetic testing, six (27%) had mutations in RPS19 (table 2). Three of the 10 patients with wild-type RPS19 were found to have mutations in RPL5. On the basis of these limited numbers, the estimated prevalence of RPL5 is 22% of the whole DBA population. All mutations in the ribosome protein genes were identified by direct sequencing, except for the RPL35a deletion, which was found by metaphase cytogenetics. We identified two novel RPL5 mutations. One of the patients had a novel frameshift mutation in the RPL5 gene: c.63_86delCTGAsntTG. The mutation is predicted to cause frameshift and early truncation of the protein (p.T27MfsX15). This patient also had severe clinical phenotype with severe anaemia, developmental delay, cleft lip and hypoplastic radii.

Often, measurement of red blood cell adenosine deaminase activity could not be performed in the patients who were eventually diagnosed as having DBA. This was mainly because they required urgent transfusion at presentation and frequent transfusions thereafter. Another reason was that, once the clinical and genetic diagnosis was made, the test was not required for the management of the patients. Of the 15 patients with DBA who had testing while not receiving transfusions, 10 had positive results. None of the eight patients who were eventually deemed not to have DBA had raised red blood cell adenosine deaminase activity (online supplementary data, figure 1). The patients tested for adenosine deaminase activity predominantly had anaemia at least one stage of their disease.

Mutations in the FA pathway proteins

Among the FA population, six belonged to the FA-A group, five to FA-C, two to FA-G, and one to FA-E. Four novel mutations in FA genes were identified (table 2). Among these, a novel homozygous c.1561G→T (p.G521X) mutation was identified in exon 12 of the FANCG gene. This patient presented with characteristic features of FA, which included thumb anomalies,
Table 2  Genetic abnormalities found in our cohort of patients

<table>
<thead>
<tr>
<th>Gene</th>
<th>Disease</th>
<th>Inheritance</th>
<th>cDNA</th>
<th>Protein</th>
<th>Mutation type</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
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<td>SBDS</td>
<td>Shwachman–Diamond</td>
<td>Compound heterozygous</td>
<td>c.183_184TA→CT+c.258+2T→C / c.258+2T→C</td>
<td>p.K62X/p.C84fsX3</td>
<td>Nonsense/splice site</td>
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<td>Shwachman–Diamond</td>
<td>Compound heterozygous</td>
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<td>p.C84fsX3/p.C84fsX3</td>
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<td>Frameshift/splice site</td>
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<td>p.C84fsX3/p.F207IfsX8</td>
<td>Splice site/site/splice site</td>
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<td>p.F1263X/p.F1263X</td>
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<td>c.39G→T</td>
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<td>c.97C→L</td>
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<td>p.225X</td>
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<td>c.597G→A</td>
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<td>G6PT</td>
<td>Glycogen storage 1b</td>
<td>Compound heterozygous</td>
<td>c.464del10ins3C/c.1211delICT</td>
<td>p.G155fsX/p.A400X</td>
<td>Insertion/deletion</td>
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<tr>
<td>G6PT</td>
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<td>Compound heterozygous</td>
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<td>p.L348fsX/p.F340fsX5</td>
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<tr>
<td>G6PT</td>
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<td>Unavailable</td>
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<td>Amegakaryocytic</td>
<td>Homozygous</td>
<td>*c.304C→T</td>
<td>p.R102C</td>
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<td>WHIM syndrome</td>
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<td>C1HR1</td>
<td>Cohen’s syndrome</td>
<td>Compound heterozygous</td>
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<td>p.R502X/p.V224fsX17</td>
<td>Nonsense/splice site</td>
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</table>

Unavailable denotes mutations that were identified but the exact mutation was not provided to the CIMFR. WHIM, Warts, Hypogammaglobulinemia, Immunodeficiency, Myelokathexis syndrome.

anaemia, thrombocytopenia and failure to thrive (table 3, figure 6). Two patients had a novel mutation in FANCA: c.105ST→C (p.L56P). One patient presented with café au lait spots and anaemia, thrombocytopenia and neutropenia. An additional patient with FA had two novel mutations in FANCA: c.1827?-_1900+7del and c.3239+1_3239+2insG. This patient did not show any typical extraehaematological manifestations of FA (table 3, figure 6). Six patients had positive complementation testing suggesting mutations in FANCA, FANCC, FANCG and FANCE genes, but the exact mutations were unavailable at the
time of data analysis. In three other cases, repeated attempts to establish lymphoblastoid cell lines for complementation grouping from the patients were unsuccessful because of poor cell growth or the death of the patient before completion of the testing.

Eighty patients had chromosome fragility testing because of complete or partial phenotype that may be consistent with FA (online supplementary data, figure 2). Chromosome fragility is rarely increased in patients who do not have FA. In our series, four non-FA patients had increased chromosome fragility. Chromosome fragility was increased after diepoxybutane treatment (in four cases), with mytomycin C (one case) or spontaneously (in four cases). Mytomycin C can reduce chromosome fragility in patients with FA by inhibiting the activity of the NF-κB pathway. In contrast, diepoxybutane increases chromosome fragility in patients with FA by activating the NF-κB pathway. The discovery of more DC genes is necessary to determine whether such patients have DC of a different disease. It is noteworthy that patients with IBMFSs other than DC can also have very short telomeres,12 like one of our patients with FA in this study.

### Mutations in telomere maintenance genes

Four of the 10 patients with a clinical diagnosis of DC had mutations in TINF2; all were from different families. All identified TINF2 mutations were in exon 6 and clustered in the end of the TRF-1 binding domain: p.P283S, p.R282C, p.L287P and p.R282H (table 2). TINF2 mutations seem to be de novo, since all the parents of these severely affected children were healthy and had normal blood counts. In two of the families, the parents were tested for mutations in TINF2 and were found to be negative. In addition, of the patients with DC who were tested for the remaining DC-related genes, only two male patients had a mutation in the DKK1 gene, and none had mutations in TERC, TERT, NHP2 or NOP10. Seventeen patients had telomere length testing because of a complete or partial phenotype that might be consistent with DC (online supplementary data, figure 3). Three patients with DC had telomere studies, which showed very short telomeres (lower than the first centile of the normal distribution range for age) of peripheral blood lymphocytes or cord blood. Peripheral blood lymphocytes from an additional seven of 14 patients with unclassified IBMFSs who were found to have very short telomeres; however, these patients remain unclassified, as they did not have the classic clinical manifestations of DC, and genetic testing of DC genes was negative. Patients with unclassified IBMFSs who were found to have very short telomeres were managed as DC patients even though the causative mutation was not identified. The discovery of more DC genes is necessary to determine whether such patients have DC of a different disease. It is noteworthy that patients with IBMFSs other than DC can also have very short telomeres,12 like one of our patients with FA in this study.

### Mutations in neutropenia-associated genes

Of the 11 patients with K/SCN (ie, severe neutropenia and maturation arrest at the bone marrow promyelocyte/myelocyte), seven were tested for mutations in the ELA2 gene; four of them had heterozygous mutations (table 2). Two of the patients with cyclic neutropenia tested positive for ELA2. The gene was analysed in an additional 10 patients with unclassified inherited neutropenia; all were negative. HAX1 was analysed in two patients with K/SCN and in an additional nine patients with unclassified inherited neutropenia; all were negative. Other mutated neutropenia genes identified in our patient cohort included TAZ (6), GéPT (4), COH1 (1) and CXCR4 (1).
The second patient presented with a cystic fourth ventricle and patients with congenital amegakaryocytic thrombocytopenia. Most do not have any extrahaematological features, similar to most novel mutation: c.304C>T.

Two patients with congenital amegakaryocytic thrombocytopenia had mutations in cMPL. Interestingly, both had the same novel mutation: c.304C>T (p.R102C). One of the patients did not have any extrahaematological features, similar to most patients with congenital amegakaryocytic thrombocytopenia. The second patient presented with a cystic fourth ventricle and Dandy–Walker syndrome (table 3, figure 6).

Mutations in haematopoietic receptor genes

Two patients with congenital amegakaryocytic thrombocytopenia had mutations in cMPL. Interestingly, both had the same novel mutation: c.304C>T (p.R102C). One of the patients did not have any extrahaematological features, similar to most patients with congenital amegakaryocytic thrombocytopenia. The second patient presented with a cystic fourth ventricle and Dandy–Walker syndrome (table 3, figure 6).

Mutations in patients with leukaemia or severe phenotype

Of the 74 patients with identified mutations, nine developed leukaemia or refractory cytopenia with excess blasts (eight acute myeloid leukaemia/refractory cytopenia with excess blasts and one acute lymphoblastic leukaemia). Two of these patients had SDS: one carried a c.183_184TA (splice site/splice site) mutation and the other had a c.258+2T>CT (splice site/splice site) mutation. Of the other patients who did not have identifiable mutations, there was one with K/SCN with combined mutations in ELA2 (c.498delG) and in GCSFR (c.850C>A), two with FA with unknown mutations, and four with unclassified IBMFSS.

We also calculated the correlation between nonsense, missense or splice mutations and severity of the cytopenia (defined as haemoglobin <70 g/l or need for transfusions; platelets <20×10^9/l or need for transfusions; neutrophils <0.5×10^9/l or a need for treatment with granulocyte colony stimulating factor). No significant correlations were found.

**DISCUSSION**

This is the first study to analyse the genetic basis of a comprehensive cohort of patients with IBMFSS. Our study shows that the IBMFSS group comprises common known categorised syndromes such as DBA, SDS and FA, less common categorised syndromes such as congenital amegakaryocytic thrombocytopenia, as well as a large group of unclassified cases. Recent advances in understanding the genetic basis of the IBMFSS were evident from our ability to positively genotype over 50% of the patients. Sixteen genes were mutated, and nine novel mutations were identified. It is noteworthy that deletions of ~1 kb to 5 Mb in chromosome areas of IBMFSS genes may not be detectable by conventional sequencing and metaphase cytogenetics. For example, the finding of only heterozygous mutations in autosomal recessive diseases may be due to missed deletion of the other allele.

Among the patients with positive genetic testing, we found mutations in 16 genes, which are involved in a variety of biochemical pathways. SDS, presently the only known SDS gene, was far more common than any other gene in this study. This is in sharp contrast with previous studies, in which fewer patients with SDS were included. Possible reasons for this difference include the population-based or prospective enrolment design of our study and differences in study populations and referral patterns. SDS is probably a multifunctional gene that plays a role in ribosome biogenesis, cell survival, and cell death and growth. In addition, this study is probably a multifunctional gene that plays a role in ribosome biogenesis, cell survival, and cell death and growth. It is noteworthy that deletions of ~1 kb to 5 Mb in chromosome areas of IBMFSS genes may not be detectable by conventional sequencing and metaphase cytogenetics. For example, the finding of only heterozygous mutations in autosomal recessive diseases may be due to missed deletion of the other allele.

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Disease cohorts

RPS19, which is mutated in ~25% of the patients: this was also seen among the Canadian DBA population in our study. RPL5 is the second most commonly mutated gene in DBA (12–21%). Based on small numbers, we estimated the prevalence of mutations in RPL5 among the tested Canadian population to be 22%; however, this needs to be validated after testing a larger number of patients with DBA. Both novel RPL5 mutations (p.T277MfsX10 and p.R1125fsX15) appeared in patients with severe haematological and non-haematological manifestations. This is in agreement with a previous report. An additional patient had deletion of one RPL5a allele due to a larger deletion of chromosome 3q27-29. Other DBA genes are mutated in a small proportion of the patients.

FA is another genetically heterogeneous disease. The FA genes may have a variety of functions, but all belong to the FA homologous recombination DNA repair pathway. FANCA and FANCC were the most commonly mutated genes in our study, which is consistent with the literature. Four novel mutations in FANCA and FANCC were identified in our series. The novel FANCA mutation, c.1827-?1900+7del/c.3259+1_3259+2insG, appeared in a patient without extrahaematological manifestations, which underlines the importance of testing patients with isolated bone marrow failure for chromosomal fragility before the start of treatment. There are certain problems when genetically testing patients with FA, including early death of the patient and failure to establish lymphoblastoid cell lines for complementation testing. Direct sequencing without prior complementation screening should be considered; however, it is laborious and costly, and thus was attempted in only a small number of patients in this series.

Multiple genes have been associated with DC. The most common DC gene in our study was TINF2, with a relatively high prevalence of 40%. The prevalence in the literature is 11–25%. Recruitment of additional patients will be important to validate the high frequency of TINF2 mutations among the Canadian DC population and to determine relative frequencies of the other DC-related genes. In the present series, two of the 10 DC patients had mutations in DKC1. The incidence of the X-linked disease was originally estimated to be >50%, but with the identification of more DC genes, the true incidence seems lower and was recently estimated to be 50%.

Another genetically heterogeneous IBMFS is the K/SCN group. ELA2 was mutated in most patients with K/SCN who were tested. The prevalence of ELA2 mutations seems to be higher in North America than in Europe, and was reported in about 40–80% of the K/SCN patients. In the present series, the patients with ELA2 mutations had classical K/SCN features, with severe neutropenia and differentiation arrest at the promyelocyte stage. Of the 10 patients with inherited neutropenia without bone marrow promyelocyte differentiation arrest, none had ELA2 mutations. ELA2 mutations may occur in patients with severe congenital neutropenia and maturation arrest at the promyelocyte stage, but rarely in patients without this feature. HAX1 was reported to be mutated in 40% of patients with K/SCN in a European study, but in none of the patients in the American studies and in none of the patients in the present study. This suggests that HAX1 mutations are more common in specific European ethnic groups. One patient in our study had digenic germline mutations in ELA2 and the extracellular domain of G-CSFR and was published previously. Other K/SCN genes are rare.

Identification of the genetic cause positively affected patient management in several aspects. In all cases, genotyping led to the determination of transmission mode (ie, autosomal dominant, autosomal recessive, de novo, etc), which allowed accurate assessment of the risk of other family members carrying the same genetic alteration. Family history and genetic testing solved diagnostic dilemmas in 11 cases. Importantly, identifying the disease-causing alterations permitted accurate prediction of future offspring risk and facilitated prenatal diagnosis. These were particularly relevant to three of the patients in the study who reached adulthood at the time of the analysis. In two cases, the disease was confidently ruled out in sibling donors for haematopoietic stem cell transplantation. In some cases it was possible to better explain specific phenotypes; for example, the three patients with a RPL5 mutation had severe non-haematological manifestations such as cleft lip and palate (two patients), thumb anomalies (two patients) and growth hormone deficiency (one patient). We found that establishing a diagnosis of IBMFS is often delayed, sometimes for over a year after presentation. Future studies are necessary to determine whether the delay is related to the progressive nature of certain disease features until the respective diagnostic criteria are evident (eg, cytopenia in FA or nail dystrophy in DC), lack of sufficient diagnostic screening tools (eg, for patients with pure cell aplasia who are receiving chronic transfusions and consequently the red blood cell adenosine deaminase testing is not informative), lack of sufficient genetic testing, or a need for acuity on the part of treating physicians.

Establishing a diagnosis based solely on clinical features is often impossible because of substantial overlap of haematological and non-haematological manifestations and late development of characteristic features of individual conditions. Therefore the study of genes mutated in IBMFSs is critical for establishing a diagnosis and providing proper treatment and genetic counselling.

Owing to the comprehensive, population-based design of our study with prospective enrolment, the relative prevalence of the IBMFSs was different from non-population-based or non-prospective studies. Also, several important differences were noted between prevalence and incidence of diagnoses per births. FA had the highest incidence per birth followed by DBA and SDS. However, FA had lower prevalence than DBA. This is probably due to a higher mortality from FA than DBA among patients diagnosed in childhood. Indeed, among the patients who were diagnosed before the age of 18 years, 15.4% of those with FA died compared with none of those with DBA (p<0.01). The most common causes of death in our FA population were transplant-related mortality (typically multiorgan failure with acute respiratory distress syndrome, cardiomyopathy, renal failure, liver dysfunction or fungal infections) and post-transplant graft versus host disease. Other explanations for differences between the incidence and prevalence of an IBMFS are also possible and include changes in birth rates as a result of prenatal diagnostic tools and loss of follow-up due to spontaneous amelioration of phenotype or cure. Another interesting difference between incidence and prevalence was in DC. The incidence of DC was one-third of that of FA, 57% of that of DBA, and 45% of that of SDS, but the prevalence was much lower. Again, this is probably related to high mortality of patients with DC diagnosed in the first decade of life. Among patients diagnosed before the age of 18, the crude mortality for DC was 40% compared with 0% for DBA (p<0.01). The difference in crude death rate between FA (15.4%) and DC (40%) children did not reach significance (p=0.12), probably because of the relatively small number of patients with DC. Among our DC cohort, the most common causes of death were massive gastrointestinal bleeding, pulmonary fibrosis and post-
transplant lymphoproliferative disorder. It is noteworthy that the birth incidence was calculated on the basis of diagnosed cases in the first 9 years of life, since opening of the study in 2001 until 2009. Although the majority of our patients were diagnosed at this stage, this is an underestimate. The magnitude of underestimation may prove to be even higher if advances in diagnostic tools reveal that a large proportion of the patients present in adulthood.

The present research is the largest population-based, prospective and comprehensive analysis of all the IBMFS and gives a broad perspective on their genetic basis. However, one of its limitations is the relatively small population size of specific categories of IBMFSs. Therefore the spectrum of mutations in a specific category of IBMFSs needs validation by future recruitment of additional patients from each category. As the CIMFS database is a registry of information collected from multiple centres, it reflects genetic discoveries made in the clinical setting and clinical application of genetic knowledge. Although information on the application of scientific knowledge to clinical practice is critically important, it has the limitation of incomplete testing of all patients because of differences in clinical practice and financial coverage of tests, as well as a relatively small number of patients who are tested for recently identified genes, such as RPS7, which is mutated in patients with DBA, NOP10 in patients with DC, and G6Pc3 in patients with congenital neutropenia. As these genes were reported to be mutated in a very small proportion of patients with IBMFSs, together they probably account for less than 5–10% of the patients in our study. Therefore the lack of information on these genes probably does not affect the conclusions of the study about the prevalence of the major genetic groups. Analysis of all the IBMFS-causing genetic alterations awaits the completion of the continuously growing list of known IBMFS genes.

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Competing interests None.

Ethics approval This study was conducted with the approval of the research ethics board at the Hospital for Sick Children and other contributing centres.

Contributors ET performed research, analysed data, and wrote the paper. RK is a study co-investigator, contributed vital data, and wrote the paper. CVF is a study co-investigator, contributed vital data, and wrote the paper. RY is a study co-investigator, contributed vital data, and wrote the paper. ES is a study co-investigator and contributed vital data. JC is a study co-investigator and contributed vital data. MS is a study co-investigator and contributed vital data. JB is a study co-investigator and contributed vital data. BM is a study co-investigator and contributed vital data. SA is a study co-investigator and contributed vital data. SA is a study co-investigator and contributed vital data. JS is a study co-investigator, contributed vital data, and wrote the paper. KA is a study co-investigator and wrote the paper. NB is a study co-investigator. UA is a study co-investigator and wrote the paper. LL is a study co-investigator. JPJ is a study co-investigator. JG is a study co-investigator and contributed vital data. PC contributed clinical data. CA performed research and analysed data. JB is a study co-investigator and contributed vital data. MC contributed clinical data. CMR designed and performed research. YD designed research, analysed data, and wrote the paper.

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627

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E Tsangaris, R Klaassen, C V Fernandez, et al.

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