Different substitutions at residue D218 of the X-linked transcription factor GATA1 lead to altered clinical severity of macrothrombocytopenia and anaemia and are associated with variable skewed X inactivation

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GATA1 is the X-linked transcriptional activator required for megakaryocyte and erythrocyte differentiation. Missense mutations in the N-terminal zinc finger (Nf) of GATA1 result in abnormal hematopoiesis, as documented in four families: the mutation V205M leads to both severe macrothrombocytopenia and dyserythropoietic anaemia, D218G to macrothrombocytopenia and mild dyserythropoiesis without anaemia, G208S to macrothrombocytopenia and R216Q to macrothrombocytopenia with β-thalassemia. The three first GATA1 mutants display a disturbed binding to their essential transcription cofactor FOG1, whereas the fourth mutant shows an abnormal direct DNA binding. In this study, we describe a new family with deep macrothrombocytopenia, marked anaemia and early mortality, if untreated, due to a different GATA1 mutation (D218Y) in the same residue 218 also implicated in the above mentioned milder phenotype. Zinc finger interaction studies revealed a stronger loss of affinity of D218Y–GATA1 than of D218G–GATA1 for FOG1 and a disturbed GATA1 self-association. Comparison of the phenotypic characteristics of patients from both families revealed that platelet and erythrocyte morphology as well as expression levels of the platelet GATA1-target gene products were more profoundly disturbed for the hemizygote D218Y mutation. The D218Y allele (as opposed to the D218G allele) was not expressed in the platelets of a female carrier while her leukocytes showed a skewed X-inactivation pattern. We conclude that the nature of the amino acid substitution at position 218 of the Nf of GATA1 is of crucial importance in determining the severity of the phenotype in X-linked macrothrombocytopenia patients and possibly also in inducing skewed X inactivation.

INTRODUCTION

GATA transcription factors contain highly conserved C4-type zinc fingers which mediate sequence-specific binding to a consensus motif (A/T)GATA(A/G) (1,2). GATA1, the founding member of the GATA transcription factor family, is expressed at high levels in erythroid, megakaryocytic, mast and eosinophilic cells (3,4). Virtually all characterized erythroid-specific genes (i.e. α- and β-globin) and megakaryocytic-specific genes (i.e. GPIIb, GPIbα, GPIbβ, GPIX, PF4 and mpl) contain GATA motifs in critical cis-regulatory elements (4,5). The essential role of GATA1 in erythroid and megakaryocyte differentiation has been illustrated in two mouse models. Homozygous disruption of GATA1 causes embryonic lethality due to a severe erythroid defect (6,7). In contrast, mice with a mutation in the upstream region of GATA1 (GATA1 knockdown mice) show a milder erythroid defect but suffer from severe thrombocytopenia due to absent GATA1 expression in the megakaryocytic lineage (4).

GATA1 contains two zinc fingers; the C-finger, sufficient for sequence-specific direct DNA binding, and the N-finger (Nf), which plays a role in the stabilization of GATA1 binding to DNA (8). The Nf can regulate transcription by binding to a GATA1-interacting nuclear zinc-finger protein FOG1, friend-of-GATA1. FOG1 is coexpressed with GATA1 during embryonic and hematopoietic development (9). FOG1 knockout mice die during mid-embryonic development with severe anaemia and show a specific ablation of the megakaryocytic lineage (10). Recently, it was shown that the Nf can bind GATC sites in isolation and stabilizes binding to double GATA sites (11). The Nf also seems implicated in GATA1 self-association by interacting with the C-finger. Although there is a clear involvement in transcription regulation, the exact biological role of this self-association in gene activation is not certain (12,13).

Recently, different missense mutations in the Nf of GATA1 were found in four families, leading to a variable degree of macrothrombocytopenia with or without abnormalities in the
erythrocyte lineage. Two related patients with X-linked
dyserythropoietic anemia and macrothrombocytopenia carry a
V205M mutation, which leads to a reduced interaction of the
Nf of GATA1 with FOG1 (14). We originally described a
family with mutation D218G in the same Nf of GATA1,
showing isolated X-linked macrothrombocytopenia and some
features of dyserythropoiesis but without marked anemia (15).
Another family with macrothrombocytopenia was described
carrying mutation G208S, and finally a family with both
macrothrombocytopenia and β-thalassemia carried mutation
R216Q (16,17). The V205M, D218G and G208S mutations
reduce the interaction of GATA1 with FOG1, although to a
different degree, whereas the R216Q mutation results in a
reduced interaction with a GATA DNA recognition site
(11,14–17).

In the present study, we describe a new family with a
different missense mutation at position D218. We compare the
clinical severity in both our families and show that the pheno-
type not only depends on the position of the GATA1 mutation
but also on the nature of the substituted amino acid. The
GATA1 self-association was checked for both D218–GATA1
mutants. We determined the degree of skewed X inactivation
in female carriers, because some X-linked genetic disorders in
constitutional or in some specific lineages are known to give
rise to skewing of X inactivation (18). Finally, we also studied
the expression of the mutant GATA1 in the platelets of
obligate carriers from both families.

RESULTS

Family description and mutation analysis

We describe a new family characterized by pronounced X-linked
dyserythropoietic anemia and deep macrothrombocytopenia,
in which six affected boys died before the age of 2 years
(Fig. 1A). The only affected member still alive (patient III:1) is
a 1-year-old boy with deep transfusion-dependent anemia and
macrothrombocytopenia. The hematological data of this
patient and his mother (obligate carrier) are listed in Table 1.
The peripheral blood smear of patient III:1 shows a small
number of normal to giant platelets, dysmorphic erythrocytes
and has a normal platelet number
(15). Bone marrow
examination by light and electron microscopy reveals strong
dyserythropoiesis with dysmorphic erythroblasts (anisocytosis and poikilocytosis) and the presence of some
normoblasts. Electron microscopy of his platelets displays the
typical paucity of α granules and the presence of clusters
composed of smooth endoplasmic reticulum (15). Bone marrow

The mutation (652 G→T) is located immediately adjacent to
our previously reported missense mutation (653 A→G) and
results in a different amino acid substitution at the same
position 218 (D218Y versus D218G) of the Nf of GATA1.

In vitro effects of the GATA1 mutation

Although their mutations are located in the same residue of
GATA1, the differences in clinical severity between the
patients in our previously reported family carrying the D218G
mutation and the patients of the present family carrying the
D218Y mutation are striking. Nf GATA1 interaction studies
and expression studies of the GATA1-target gene products in
platelets were therefore undertaken.

In vitro binding studies with either the FOG1 fingers 5–9 or
FOG1 finger 1 were performed with the different GATA1 Nfs
(Fig. 2A). D218Y–GATA1 had a weaker affinity for FOG1
than D218G–GATA1; its affinity was similar to that of the
V205M mutant. Furthermore, the interaction of the different
GATA1 Nfs with the complete wild-type GATA1 protein was
studied. The binding of the D218 GATA1 mutants was
decreased compared to that of the wild-type or of the V205M
GATA1 Nf. We infer that the interaction with FOG1 not only
depends on the position of the mutation as had been suggested
by Fox et al. (19) and Crispino et al. (20), but also on the nature
of the amino acid substituted. We also conclude that both D218
GATA1 mutations hamper self-association of the GATA1
protein.
The normal values (in parentheses) are also indicated.

sedimentation rate. [Carrier IV:4 is described by Freson et al. (15).

Table 1. Hematological profiles of patient III:1 and obligate carrier II:1 with the D218Y–GATA1 mutation and obligate carrier IV:4 with the D218G–GATA1 mutation

<table>
<thead>
<tr>
<th></th>
<th>Hgb (g/dl) (10.5–16)</th>
<th>Hct (%) (35–45)</th>
<th>RBCs (×10^12/µl) (3.9–5.2)</th>
<th>RDW (%) (11.5–14.5)</th>
<th>LDH (U/l) (150–460)</th>
<th>Plts (×10^12/µl) (150–400)</th>
<th>ESR (mm/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III:1</td>
<td>9.7</td>
<td>33.5</td>
<td>3.42</td>
<td>17.2</td>
<td>812</td>
<td>–</td>
<td>12</td>
</tr>
<tr>
<td>II:1</td>
<td>14.9</td>
<td>43</td>
<td>4.73</td>
<td>13.6</td>
<td>310</td>
<td>255</td>
<td>–</td>
</tr>
<tr>
<td>IV:4</td>
<td>12.6</td>
<td>39</td>
<td>4.31</td>
<td>13.3</td>
<td>–</td>
<td>297</td>
<td>–</td>
</tr>
</tbody>
</table>

Hgb, hemoglobin; Hct, hematocrit; RBCs, red blood cells; RDW, red blood cell distribution width; LDH, lactate dehydrogenase; Plts, platelets; ESR, erythrocyte sedimentation rate. [Carrier IV:4 is described by Freson et al. (15).]

The normal values (in parentheses) are also indicated.

Direct binding of the GATA1 mutants to DNA, studied as previously described by Freson et al. (15), was not disturbed (Fig. 2B).

**In vivo effect of the GATA1 mutation**

To approach the in vivo effects of the two D218 mutants, we studied the GATA1-dependent gene regulation in the patients’ platelets of both families. Because we could not obtain enough mRNA from the platelets of patient III:1, only expression at the protein level was studied. Expression levels of GATA1-target gene products (GPIbα and GPIbβ) and of a non-directly GATA1-regulated gene product (Gsig) were analyzed. Western blot analysis showed undetectable levels of GPIbα and GPIbβ in the platelets of patient III:1 (with mutation D218Y), in contrast to the reduced but detectable levels of GPIbβ in the patient with the D218G mutation (Fig. 2B) (15). The Gsig protein was studied as a marker for incomplete platelet maturation since it is upregulated during megakaryocyte differentiation and has no known GATA1 regulatory element in its promoter (21). Compared to the slightly weaker expression in the patient with mutation D218G, the Gsig expression is only 50% in the platelets of patient III:1, suggestive of incomplete platelet maturation. Similar to what was observed in the carriers of the D218G–GATA1 mutation, no abnormalities were found in the platelets of the D218Y carrier.

**Study of the X-inactivation pattern and analysis of abnormal GATA1 message in platelets of female carriers**

The X-inactivation pattern in an obligate carrier from both families was analyzed. Carrier IV:4 with the D218G–GATA1 mutation (15) is informative for the androgen receptor (AR) polymorphism and showed slightly skewed X-chromosome inactivation (Fig. 3A). PCR fragments derived from one of the two alleles did not selectively disappear in either HhaI or CfoI digested samples. In contrast, carrier II:1 with the D218Y–GATA1 mutation was informative for the monoamine oxidase A (MAOA) polymorphism and showed clear skewed X inactivation (Fig. 3A). After digestion with either HpaII or SmaI, fragments amplified from one of the two alleles almost completely disappear.

The expression of the two D218–GATA1 mutations was also studied in the platelets from both carriers using RT–PCR and sequencing. Carrier IV:4 from the D218G–GATA1 family was heterozygous for the mutation at the mRNA level in her platelets (Fig. 3B). In the case of carrier II:1 of the D218Y–GATA1 family, the abnormal GATA1 allele was not detected at the mRNA level (Fig. 3B).

**DISCUSSION**

We showed that the severity of the phenotype of X-linked macrothrombocytopenia and dyserythropoiesis with or without anemia varies with the kind of missense mutation at a particular position and correlates with the degree of disturbed interaction of the mutant GATA1 with FOG1.

In contrast to position 218, residue 205 was considered at least in vitro to be important for GATA1:FOG1 zinc fingers interaction. It is now obvious that residue 218 also is crucial for the interaction in vitro and in vivo; moreover, the degree of disturbance upon mutation depends on the amino acid residue it is mutated to.

Biochemical studies have demonstrated that the zinc finger domains of GATA1 are also involved in the GATA1 self-association (12). Recently, it became clear that self-association is mediated by the Nf of GATA1 making intramolecular contact with the C-finger (13). A deletion mutant of the GATA1 Nf affects this association and interferes with its ability to activate transcription in transfection studies, especially from multi-GATA1 site promoters. Mackay et al. (13) also speculated on the exact biological role of the self-association; it would increase the local concentration of GATA1 and therefore extend its potency as a transcriptional activator. Since many erythroid promoters contain multiple GATA sites, it could be involved in the ordered assembly of higher order complexes at such promoters. The possible contribution of a disturbed GATA1 self-association by the D218 mutation to the clinical phenotype is not clear. However, our work shows for the first time that a missense mutation in the Nf can lead to a disturbed GATA1 self-association.

We were also interested in the consequences of the GATA1 mutation for the carriers from both families. GATA1 is an X-linked gene and a molecular defect can be involved in preferential cell selection and this results in skewed X inactivation in peripheral blood leukocytes. Such observations were found in other X-linked disorders as in X-linked agammaglobulinemia, incontinentia pigmenti type 2, Wiskott–Aldrich syndrome and dyskeratosis congenita (22–25). The carriers of both the D218G and the D218Y mutation had a normal platelet count but sporadically had a large-sized platelet in the peripheral blood smear. We analyzed isolated platelets from both carriers for the presence of mutant GATA1 mRNA. Carrier...
IV:4 (D218G) is heterozygous in her mRNA for the GATA1 mutation whereas in platelets from carrier II:1 with the more severe D218Y mutation, no mutant GATA1 mRNA could be detected although she is heterozygous at the genomic level. Although carrier IV:4 has platelets with abnormal GATA1 message, only a few large-sized platelets were detected and the mean platelet volume was within the range of controls, however, at the upper limit. Why are there not two distinct platelet populations in this carrier? A possible explanation is that patients with a GATA1 mutation also have some normal-sized platelets, so at least part of the megakaryocytes with mutant GATA1 seem to still undergo a normal maturation. Carrier II:1 of the severe D218Y mutation has a normal peripheral blood count, agreeing with no mutant GATA1 expression in her platelets. The absence of mutant GATA1 message could be due to the loss of large platelets during the centrifugation step for platelet isolation or to the lack of sensitivity of our RT–PCR analysis, which may not detect a subset of abnormal platelets expressing mutant GATA1. More likely, the absent or at least strongly reduced expression of the mutant GATA1 allele in the platelets of carrier II:1 is related to the observed nearly complete skewing of the X inactivation in leukocytes. This
suggested that the severe D218Y mutation impedes embryonic cell growth, whereas clones derived from a single stem cell or stem cell precursors with a normal GATA1 have a proliferative advantage. However, stochastic inactivation could also account for the observed skewing in this carrier (18). Skewing can be complete in random individuals (18). Nevertheless, in these two carriers, the X-inactivation patterns correlate with the clinical observations. Unfortunately, we did not have access to more samples.

In conclusion, our data provide further evidence that there indeed is a link between the severity of clinical features associated with GATA1 mutations and the extent of reduction in FOG1:GATA1 association. The clinical and biochemical differences between the two D218 GATA1 mutants are reflected in the variable loss of expression of GATA1-dependent genes in the patients’ platelets. Even in the asymptomatic carriers, a functional difference between the two D218–GATA1 mutants remains since it results in, respectively, a completely skewed X inactivation in leukocytes for D218Y and in a random X inactivation for D218G.

MATERIALS AND METHODS

RNA isolation and cDNA synthesis

Total RNA was extracted from platelets using TRIzol reagent (Gibco BRL) according to the manufacturer’s protocol. Approximately 1 µg RNA was used for oligo(dT)-primed first-strand cDNA synthesis using M-MLV reverse transcriptase (Gibco BRL).

Genetic analysis of GATA1

The following primer set was used to generate a full-size GATA1 cDNA fragment: GATA1-F1 (CCGAGAATTCTCC-CGAGAAGCTCATGGGAG) and GATA1-R1 (GGCCCTCT-GATTGTGCCTCTCAGCTGAGC). PCR was performed by adding 4 µl cDNA aliquots to the reaction mixture containing 25 pmol of each primer, 10 mM Tris–HCl buffer, pH 8.7, 1 mM MgCl₂, 50 mM KCl, 0.01% gelatine, 1% DMSO, 200 mM dNTP, 1 U Ampli-Taq polymerase (Perkin Elmer Cetus). PCR was performed in a PTC-100™ programmable thermal controller (MJ Research Inc., Biozym) using the following conditions: 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 1.5 min, and final extension at 72°C for 10 min. Primers GATA1-F1, GATA1-F2 (GGTGTACCCATTGCTCAACTGTATG), GATA1-R1 and GATA1-R2 (GTTTGACTGAAATCAGGCGCCTTC) were used for sequencing PCR generated cDNA fragments from patients using BigDye terminator chemistry (Perkin-Elmer Cetus) on an ABI310 (Perkin-Elmer) sequencer. The resulting sequences were aligned and compared with the published sequence in GenBank (accession no. XM 010214). PCR and sequencing of genomic DNA from leukocytes were performed with primers GATA1-F2 and GATA1-R2 using the same conditions as described above.

GST pull-down assay with GATA1 peptides

GATA1 Nf, GATA1 Nf D218G, GATA1 Nf D218Y and GATA1 Nf V205M (residues 197–251)–GST fusion proteins, coupled to glutathione–agarose beads, were prepared as described earlier by Freson et al. (15). Experiments with either in vitro translated S³⁵-labeled FOG1 fragments [consisting of finger 1 (241–291) or fingers 5–9 (587–997)] or S³⁵-labeled complete GATA1 were carried out as described previously (15–17). Primer sequences are available on request.

GST fusion protein/DNA-binding assay

DNA-binding studies were performed using a single GATA recognition site (the mouse α-globin GATA site (GATTCG-CGGCAACTGATAAGGATTCCCTG) with a biotinylated 5’ end) and the assay as described by Freson et al. (15).

Platelet immunoblot analysis

Proteins were extracted from platelets of patients, a carrier, and a normal individual using a modified lysis buffer (1% ipegal CA-630 (Sigma Chemical Co.), 2 mmol/l Na₂VO₃, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 2 mmol/l DTE, 1% aprotinin and 2 mmol/l NaF) by four cycles of freezing (–80°C) and thawing (37°C). Lysates were cleared of insoluble debris by centrifugation at 14 000 g for 20 min at 4°C. Protein expression was assessed by western blot analysis. Platelet protein fractions were mixed with Laemmli sample buffer (5% SDS reducing buffer), resolved by SDS–PAGE on 7% for GPIbα, 12.5% (for GPIbβ) or 10% (for Gατ) acrylamide gels, and transferred to Hybond ECL-nitro-cellulose membrane (Amersharm Pharmacia Biotech). The blots were blocked for 1 h at room temperature in Tris-buffered saline with tween (TBS-T; 0.1% Tween-20) supplemented with 5% non-fat dry milk. Incubation with primary (overnight at 4°C) and secondary antibody (2–3 h at room temperature) was performed in TBS-T with 5% non-fat milk. The primary antibodies were produced in our laboratory and used at 50 µg/ml. Blots were revealed with a monoclonal anti-Gsα antibody (α3), a monoclonal anti-GPIbα antibody (G27C9) or a polyclonal anti-GPIbβ antibody. The secondary antibody was conjugated with HRP and staining was performed with the western blotting ECL detection reagent (Amersharm Pharmacia Biotech).

X-chromosome inactivation analysis

The AR X-inactivation assay has been described elsewhere (26,27). In brief, treatment of genomic leukocyte DNA with the methylation-sensitive enzymes HpaII and CfoI results in digestion of alleles residing on the active X chromosome. Subsequent PCR with primers flanking the restriction sites and a polymorphic CAG repeat results in amplification of alleles on the inactive X only. In females heterozygous for the AR polymorphism, the two amplification products can be compared directly. We have used this technique and analyzed the PCR products on an A.L.F. DNA sequencer (Amersharm Pharmacia Biotech) to determine the X inactivation pattern quantitatively.

Similarly, a VNTR polymorphism at the 5′ end of the MAOA gene coupled to a differentially methylated CpG island was used (28). For that purpose we used methylation-sensitive enzymes HpaII and SmaI. Digestion with a non-methylation-sensitive enzyme (MspI) served as a control.
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