ADAMTS13 Mutational Analysis in Chinese Patients with Chronic Relapsing Thrombotic Thrombocytopenic Purpura

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Abstract
Mutational analysis of ADAMTS13 gene was performed on three Chinese children with chronic relapsing thrombotic thrombocytopenic purpura, who all showed severe deficiency (<5% activity) of von Willebrand factor-cleaving protease (vWF-CP) and lack of inhibitors. In two patients, three new mutations, namely G194V, R349C and G1181R, were identified along with a fourth mutation A596V recently described in a French patient. No significant ADAMTS13 gene defect was detected in one patient, in whom the presence of low titer or non-neutralizing antibodies to vWF-CP was not excluded. The prevalence of G194V and G1181R mutations was 1.5% and 5.8% among normal Chinese subjects. They may constitute genetic susceptibility factors for thrombosis in the population.

Key words ADAMTS13; Mutation; Thrombotic thrombocytopenic purpura; vWF-cleaving protease

Introduction
Thrombotic thrombocytopenic purpura (TTP) is a life-threatening clinical disorder characterised by the pentad of microangiopathic haemolytic anaemia, thrombocytopenia, fever, renal failure and neurological dysfunction. Major recent advances in understanding the pathophysiology of TTP indicates that this disorder is etiologically linked to deficiency of a specific von Willebrand factor cleaving protease (vWF-CP), which normally cleaves the Tyr-Met bond at A2 domain of the vWF molecule and limits the propagation of platelet thrombus (reviewed in 1). The vWF-CP has been purified by chromatographic methods and shown to belong to the ADAMTS family of metalloproteases. Based on this information, the cDNA and gene structure of the ADAMTS13 gene encoding for vWF-CP is quickly determined. Similar conclusions are obtained independently through positional cloning of familial TTP cases.1 The vWF-CP deficiency that leads to microvascular platelet thrombi formation is due either to autoantibody inhibition of vWF-CP activity in sporadic acquired TTP, or constitutive
deficiency of vWF-CP as a result of ADAMTS13 gene defects in familial TTP.2

Familial or hereditary TTP, also known as Upshaw-Schulman syndrome and much less common than the acquired form of disease, shows autosomal recessive inheritance. It usually manifests at the neonatal period or early childhood, is associated with severe vWF-CP deficiency, and runs a chronic relapsing course. Mutational analysis of the ADAMTS13 gene in hereditary TTP patients and their family members has detected more than 50 mutations believed to be causative for the disorder, together with many single nucleotide polymorphisms (SNPs).1,3-12 We report results of ADAMTS13 gene mutation detection on family study of three Chinese paediatric patients with chronic relapsing TTP in Hong Kong.

Materials and Methods

Patients

Three paediatric patients of Chinese ancestry who presented with chronic relapsing TTP were studied. Citrated blood samples were collected from the patients and family members with informed consent. The plasma was used for vWF-CP activity assay whereas the buffy coat was saved for DNA extraction.

vWF-CP Activity Assay

vWF-CP activity was determined based on proteolytic degradation of vWF substrate by patient vWF-CP as reflected by impaired collagen binding,13 with the modification of cryoprecipitate substituting pooled normal plasma as the source of substrate. Briefly, vWF-CP of the cryoprecipitate was inhibited by EDTA and Pefabloc SC, and dialysed in Visking cellulose tubing. The endogenous vWF of patient plasma was partially degraded by BaCl2 and the supernatant was collected for incubation with substrate. The residual collagen binding activity of the digested substrate was measured by ELISA method (Gradiopore), using peroxidase conjugated anti-human vWF antibody and detected by chromogenic reaction. The calibration curve was prepared with various dilutions of pooled normal plasma so that a fitted sigmoidal curve was constructed. Detection of vWF-CP inhibitors was performed by 1:1 mixture of patient and pooled normal plasma, followed by vWF-CP activity assay as described above.

ADAMTS13 Mutation Detection

We performed mutation detection of ADAMTS13 gene defects through a direct nucleotide sequencing approach, in accordance with the primers as published by Kokame et al. Genomic DNA was extracted from peripheral blood by phenol chloroform method. All exons of the ADAMTS13 gene, including intron-exon boundaries, were amplified by GeneAmp® 9700 PCR system (Applied Biosystems, CA, USA). The PCR mixture comprised: 1 X PCR buffer, 0.2 mM dNTPs, 1.5-2.0 mM MgCl2, 0.2 μM of each primer, 0.5 U Taq polymerase (MBI Fermentas, Lithuania) and 100 ng template DNA in a final volume of 20 ml. PCR products underwent pre-sequencing treatment with ExoSAP-IT® (Amersham Pharmacia Biotech Inc., Sweden) at 37°C for 15 minutes to remove excess primers and dNTPs. The enzymes were subsequently heat-inactivated at 80°C for 15 minutes. Products were sequenced in both directions by DYEnamic™ ET terminator cycle sequencing system (Promega, MD, USA) and a 377 DNA sequencer (Applied Biosystems) according to instructions of the manufacturer. Sequencing results were analysis with reference to NCBI published genomic sequences (GenBank accession nos. AL158826 and AC002325) and mRNA sequences (AB069698, AF414401 and AY055376). Nucleotide sequence was numbered such that the initiator ATG was denoted as +1.

Restriction Fragment Length Polymorphism (RFLP) Study on Normal Subjects

Amplified PCR products for exon 6, exon 16 and exon 25 were digested with Hin1I (MBI Fermentas), MspA1I (New England Biolabs Inc., Beverly, USA) and SmaI (MBI Fermentas) respectively for determination of their prevalence in Chinese subjects. Overnight digestions were carried out with 1U of restriction enzymes supplement with 0.1 mg/ml BSA per 5 μl PCR products at either 30°C or 37°C respectively. For G581T, fragments sizes were 262 bp and 376 bp for allele G, and 638 bp for allele T after digested with Hin1I at 37°C. For C1787T, fragments sizes were 90 bp and 297 bp for allele C, and 387 bp for allele T after digested with MspA1I at 37°C. For G3541A, fragment sizes were 47 bp, 221 bp and 461 bp for allele G, and 268 bp and 461 bp for allele A after digested with SmaI at 30°C. All digestion products were analysis on 2% agarose gel.

Results

The clinical features, vWF-CP activity, and results of ADAMTS13 mutational analysis were summarised in Table 1. On screening, they were all negative for
## Table 1: Mutational analysis of ADAMTS13 in chronic relapsing TTP

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex/Age (years)</th>
<th>Clinical features</th>
<th>vWF-CP activity</th>
<th>ADAMTS13 mutations</th>
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<tr>
<td><strong>Patient 1</strong></td>
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| Patient       | F/10            | Presented at age 2 with petechiae and red urine. Found to have microangiopathic haemolysis and thrombocytopenia but no neurological manifestation. Eight attacks from 1996-1999, and all precipitated by mild infections. Recovery usually spontaneous or on response to FFP ± steroids. | <5%             | Exon 6: nt 581 G→T; Gly194Val [#AY532929]  
Exon 9: nt 1045 C→T; Arg349Cys [#AY532930]  
Exon 25: nt 3541 G→A; Gly1181Arg [#AY532931] |
| Father        | M/40            | Asymptomatic                                                                       | 60%             | Exon 9: nt 1045 C→T; Arg349Cys                                                   |
| Mother        | F/40            | Asymptomatic                                                                       | 38%             | Exon 6: nt 581 G→T; Gly194Val  
Exon 25: nt 3541 G→A; Gly1181Arg                                                   |
| **Patient 2** |                 |                                                                                   |                 |                                                                                   |
| Patient       | F/4             | Presented at age 3 as petechiae after flu-like illness. Found anaemic and markedly thrombocytopenic with microangiopathic blood picture. Five relapses from 2001-2003, usually triggered by mild infections, with no neurological manifestation. Recovery was either spontaneous or on response to FFP. | <5%             | SNPs and silent polymorphisms only                                               |
| Father        | M/37            | Asymptomatic                                                                       | 60%             | SNPs and silent polymorphisms only                                               |
| Mother        | F/33            | Asymptomatic                                                                       | 100%            | SNPs and silent polymorphisms only                                               |
| **Patient 3** |                 |                                                                                   |                 |                                                                                   |
| Patient       | F/2.5           | History of severe NNJ requiring exchange transfusion. Presented at 5 months with petechiae due to thrombocytopenia and managed as ITP. Relapsed at age 2 with thrombocytopenia and microangiopathic haemolysis precipitated by influenza B infection. Recovered on FFP infusion. | <5%             | Exon 6: nt 581 G→T; Gly194Val  
Exon 16: nt 1787 C→T; Ala596Val [#AY532932]  
Exon 25: nt 3541 G→A; Gly1181Arg                                                   |
| Younger brother | M/0.5         | Presented as NNJ and developed microangiopathic haemolysis associated with haemoglobinuria. Exchange transfusion performed. Given FFP infusion on day 2 of life with normalisation of platelet count and resolution of haemolysis. | <5%             | Exon 6: nt 581 G→T; Gly194Val  
Exon 16: nt 1787 C→T; Ala596Val  
Exon 25: nt 3541 G→A; Gly1181Arg                                                   |
| Father        | M/35            | Asymptomatic                                                                       | 48%             | Exon 6: nt 581 G→T; Gly194Val  
Exon 25: nt 3541 G→A; Gly1181Arg                                                   |
| Mother        | F/32            | Asymptomatic                                                                       | 42%             | Exon 6: nt 581 G→T; Gly194Val  
Exon 16: nt 1787 C→T; Ala596Val  
Exon 21: nt 2708 C→T; Ser903Leu                                                      |

Key: vWF-CP, von Willebrand factor-cleaving protease; #, GenBank Accession number; FFP, fresh frozen plasma; NNJ, neonatal jaundice; ITP, immune thrombocytopenic purpura
Patient 1 was heterozygous for 3 new mutations (Figure 1), namely exon 6 nt 581 G→T; Gly194Val (GenBank accession no. AY532929), exon 9 nt 1045 C→T; Arg349Cys (AY532930), and exon 25 nt 3541 G→A; Gly1181Arg (AY532931). These mutations occurred at the metalloprotease, disintegrin-like and thrombospondin1-8 domains respectively, and involved changes in charge and solubility of amino acids. Single nucleotide polymorphisms (SNPs) were also detected. Besides intronic variations, two previously reported silent polymorphism exon 15 nt 1716 G→A and exon 19 nt 2280 T→C were identified in this patient. The G194V and G1181R mutations were detectable by PCR restriction analysis with enzymes HinII and Smal respectively, and their prevalence was 1.5% and 5.8% among 197 and 172 control samples from healthy Chinese subjects.

Patient 2 showed known SNPs only with no new mutations detected, and included 3 silent polymorphisms at coding regions, namely exon 5 nt 420 T→C, exon 15 nt 1716 G→A and exon 19 nt 2280 T→C, were identified in addition to intronic variations.

Patient 3 was heterozygous for G194V and G1181R mutations as encountered in the first patient. She was also heterozygous for exon 16 nt 1787 C→T; Ala596Val mutation (AY532932) (Figure 1). This mutation occurred at the spacer domain and was recently reported in a French family. The A596V was amendable for detection by PCR restriction analysis with MspAI, but not was detected in 164 control samples. Known SNPs present in this case were.

**Figure 1** Sequence chromatograms of respective PCR amplified fragments, with arrows indicating: A) exon 6 nt 581 G→T (G194V) mutation; B) exon 9 nt 1045 C→T (R349C) mutation; C) exon 25 nt 3541 G→A (G1181R) mutation, complementary sequencing result; and D) exon 16 nt 1787 C→T (A596V) mutation. The mutations are all present in heterozygous form, and a control sequence is included for comparison.
Discussion

We performed mutational analysis on three Chinese children with chronic relapsing TTP and identified three new ADAMTS13 gene mutations, namely G194V, R349C and G1181R, together with a fourth mutation A596V just described in a French patient.11 All patients showed severe vWF-CP deficiency and the disease course was chronic relapsing in nature. Recurrent thrombocytopenia and bleeding diathesis featured prominently in all cases, though with differing frequency and severity. We encountered five cases of childhood chronic relapsing TTP over the past 10 years based on data from the Hong Kong Paediatric Haematology & Oncology Study Group, three of whom were described in this report. With a childhood (age <15 years) population of 1.15 million in Hong Kong, the estimated annual incidence is around 0.4 / million children / year. This however may be an underestimation. Since TTP is less common in children than adults, paediatricians may not be familiar with the clinical manifestations and haematological abnormalities of this disorder. The patient may often be mislabeled as immune thrombocytopenic purpura or Evan's syndrome, and a diagnosis of TTP may thus be missed unless a high level of clinical suspicion is available.7 Careful serial examination of the blood film for red cell fragmentation is mandatory, since management decisions are usually imminent and have to be made before the availability of investigation results. However, to confirm the diagnosis of TTP and to differentiate between the inherited and acquired forms, assessment of vWF-CP activity, screening for inhibitors, and mutational analysis of the ADAMTS13 gene have to be performed.

Our patients all showed severe vWF-CP deficiency (i.e. <5%) which is considered to be specific for TTP.14 This observation was confirmed in another study on childhood TTP.7 On mutational analysis, nevertheless, patient 2 showed only SNPs and silent polymorphisms so that ADAMTS13 gene defect was not detected. In this particular case, the possibility of low titer or non-neutralizing antibodies to vWF-CP has not been excluded,15 especially in the absence of a similarly affected sibling. This notwithstanding, the vWF-CP activity and inhibitor assay is useful in identifying patients with inherited form of TTP for further mutational analysis. The diagnosis if confirmed in these cases justifies the use of regular fresh frozen plasma infusion, for example on a monthly basis, this obviating the risks associated with plasma exchange.

The G194V (non-hydrophobic to hydrophobic), R349C (highly charged group to non-polar) and G1181R (non-polar to highly charged group) mutations as identified in our patients all involve significant amino acid changes and are located at domains with previously reported causative mutations. Given that the G194V and G1181R mutations are also present in normal healthy control, their role in TTP pathogenesis may be doubted. The situation, however, may be analogous to the P475S mutation that is present in 9.6% of the Japanese population but shows reduced in-vitro activity when transiently expressed in HeLa cells.3 The P475S mutation is recently described in the Chinese population16 but is not encountered in the present series. As direct experimental evidence supporting a pathogenic role is lacking, the pathophysiological effect of the mutation as reported in our patient cohort remains to be defined. It would be of interest to document whether ADAMTS13 polymorphism might constitute genetic susceptibility factors for thrombotic disorders in the population.

Note: This study was presented in part at the 45th Annual Meeting of the American Society of Hematology, San Diego 2003; abstract number 2970.

References

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