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Prepublished online Dec 9, 2009;
doi:10.1182/blood-2009-07-233965

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Running heads:

PATERSON et al

PLAU duplication in Quebec platelet disorder

Scientific section designation: PLATELETS AND THROMBOPOIESIS

Title: Individuals with Quebec platelet disorder have a tandem duplication of *PLAU*, the urokinase plasminogen activator gene

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Title Page Notes:

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The online version of this article contains methods and data supplements.

ABSTRACT

Quebec platelet disorder (QPD) is an autosomal dominant bleeding disorder linked to a region on chromosome 10 that includes *PLAU*, the urokinase plasminogen activator (uPA) gene. QPD increases uPA mRNA levels, particularly during megakaryocyte differentiation, without altering expression of flanking genes. As *PLAU* sequence changes were excluded as the cause of this bleeding disorder, we investigated if the QPD mutation involved *PLAU* copy number variation. All 38 subjects with QPD had a direct tandem duplication of a 78 kilobase genomic segment that includes *PLAU*. This mutation was specific to QPD as it was not present in any unaffected family members (n=114), unrelated French Canadians (n=221) or other individuals tested (n=90). This new information on the genetic mutation will facilitate diagnostic testing for QPD and studies of its pathogenesis and prevalence. QPD is the first bleeding disorder to be associated with a gene duplication event and a *PLAU* mutation.

INTRODUCTION

Quebec platelet disorder (QPD) is an autosomal dominant bleeding disorder with a unique, gain-of-function defect in fibrinolysis.^{1,2} The disorder is characterized by: delayed onset bleeding;³ markedly increased production and storage of urokinase plasminogen activator (uPA) in platelets,^{4,5} without systemic fibrinolysis or markedly increased plasma or urinary uPA;^{1,2,4,6,7} and uPA-triggered, plasmin-mediated proteolysis of diverse platelet α -granule proteins.^{5,7-11} QPD is strongly linked to *PLAU*.¹² Furthermore, QPD increases uPA mRNA levels, from the disease chromosome, by approximately 2-4 fold in saliva and CD34⁺ hematopoietic progenitor cells, and > 150-fold in megakaryocytes and platelets.¹² Mice that over express uPA in megakaryocytes have QPD-like abnormalities.¹³ However, extensive sequencing and Southern blot analysis of *PLAU*, and its regulatory elements, failed to identify QPD-specific mutations.¹² This led us to investigate if the QPD mutation is a *PLAU* copy number variation (CNV).

PATIENTS, MATERIALS AND METHODS

Studies were carried out in accordance with the revised Helsinki Protocol for research on human subjects, with ethics review board approval of McMaster University and Hamilton Health Sciences, and Centre Hospitalier Universitaire Sainte Justine. Samples were obtained with written informed consent (from parents for subjects < 18 years old).

All available individuals with QPD were studied (38 affected, 114 unaffected; all descendants of a common ancestor), including 14 new subjects (10 affected, based on increased platelet uPA and degraded platelet fibrinogen^{3,4,8}). Unrelated participants included 221 French-Canadians and 90 individuals from HapMap CEPH pedigrees of 30 Caucasian trio families from Utah.¹⁴

Molecular methods

Supplement 1 contains the details of the sample preparation and molecular methods. Samples from distantly related, QPD individuals were selected for CNV screening, fluorescence in situ hybridization (FISH) and G-band karyotyping of blood lymphocytes. Chromosome 10 locations were expressed as Build 36.3 positions from the NCBI database <http://www.ncbi.nlm.nih.gov/> (accessed May 29, 2009). Results were analysed using the Kruskal-Wallis non-parametric test, and the Fisher's exact test for disease associations. MatInspector 8.0 (Genomatrix Software GmbH, München, Germany) was used to analyze sequences, *in silico*, for transcription factor binding motifs.¹⁵

RESULTS AND DISCUSSION

QPD-1 (T/G SNP ~13.7 kb 5' of *PLAU*)¹² genotyping, of previously unstudied family members, confirmed that the G allele segregates with the disease, consistent with linkage to *PLAU*.

To screen for CNV mutations,^{16,17} 2 QPD subjects were genotyped using Illumina 1M BeadArray and analysed using BeadStudio. The assays failed to classify genotypes for SNPs immediately 3' of *PLAU* for QPD subjects (rs2461863, rs2675666, rs2688621 and rs2633321), but not for 1,304 Caucasians tested with the same assays for another study. Visual inspection of the SNP allele signal intensity plots near *PLAU* in QPD (example shown in Figure S1) suggested that there were copy number abnormalities (summarized in Table S1). For example, at rs2461863, QPD samples had A allele signal intensities similar to AA homozygous individuals, but G allele signal intensities similar to heterozygous individuals (Figure S1). Real-time analysis of more QPD family members for SNPs rs2461863, rs4065 and rs1815076 (Table S2) estimated that there was one additional copy of *PLAU* in QPD. Real-time, dosage analyses of non-polymorphic sequences estimated that the duplication boundaries on chromosome 10 were likely between nt positions 75,328,946-75,329,067 at the centromeric end and between 75,406,671-75,407,151 at the telomeric end (Table S3). FISH analysis suggested this was a local duplication as chromosomes from both QPD subjects (who had normal karyotypes) showed normal, single position, hybridization patterns with *PLAU* (10q22.2) and control chromosome 10 probes (Figure S2).

Polymerase chain reaction (PCR) assays, designed to assess the duplicated segment orientation (see strategy diagram, Supplement 1), amplified a novel, 930 bp-sized fragment from QPD, but not control, samples, using a primer set specific for direct, tandem, *PLAU* duplication. The breakpoint junction endpoints (Figure 1; determined by bidirectional sequencing of this fragment from two representative QPD subjects and confirmed for two additional subjects; GenBank[®]

accessions GQ246945-8) were at nt positions 75,329,022-4 (~11.87 kb 5' of *PLAU* transcription start site) and 75,406,960-2 (~59.69 kb 3' of *PLAU*), in agreement with real-time dosage estimates (Tables S1, S3). Alignments of normal and breakpoint sequences on chromosome 10 indicated that non-homologous recombination had produced a direct (head-to-tail), tandem repeat of a 77,938 nt region, containing *PLAU* (Figure 1) and *C10orf55* (unknown function; antisense to *PLAU*), but no other genes. There were no additional sequence changes at the junction (Figure 1) or duplication ends (not shown). Southern blotting (using probes for a region larger than previously evaluated¹²) confirmed the local duplication (Figure S3).

The specificity of the mutation was evaluated by multiplex, real-time and gel fragment size assays (Figure 2). These assays detected the duplication in all 38 QPD subjects, but not in any others tested (n=321, real time assay; n=425, fragment size assay)($p < 0.0001$), including all unaffected family members, suggesting that the tandem duplication is likely the causative mutation.

At present, QPD is the only bleeding disorder to be associated with a gene duplication event and a *PLAU* mutation. Our observation that QPD is associated with one additional copy of *PLAU* is consistent with the modest (2-4 fold) but significant increases in *PLAU* transcripts from the disease chromosome in QPD CD34⁺ hematopoietic progenitor cells and saliva.¹² It may prove challenging to determine why an extra copy of *PLAU* leads to greater increases (>150 fold) in the disease chromosome *PLAU* transcripts during megakaryocyte differentiation,¹² and if these

increases come from the centromeric and/or telomeric copies of *PLAU*. The breakpoint (located upstream of all characterized *PLAU* regulatory elements¹ and ~11.87 kb upstream of the transcription start site of the telomeric copy) introduces motifs for Forkhead domain factors which have roles in megakaryopoiesis,^{18,19} and removes binding motifs for heat shock transcription factors²⁰ and for some transcription factors not known to influence megakaryopoiesis (Figure S4). The duplication also alters the sequences further upstream of the telomeric copy of *PLAU* on the QPD chromosome, and this region on the QPD and normal chromosomes contains many motifs for binding megakaryocyte-expressed transcription factors, albeit in different locations (Figure S5). It is possible that the QPD duplication mutation further increases *PLAU* expression during megakaryopoiesis by other mechanisms, perhaps involving alternative transcription, splicing and/or histone binding. While determining the mechanism(s) is beyond the scope of this study the information on the genetic mutation will facilitate diagnostic testing for QPD and studies of its pathogenesis and prevalence as a cause of bleeding.

ACKNOWLEDGMENTS

This work was supported by the Canadian Institutes of Health Research (grant MOP 97942, C.P.M.H.), Heart and Stroke Foundation of Ontario (grant T5888 and Career Investigator Award) (C.P.M.H.), Bayer Canada (G.E.R.), Ontario Graduate Student Scholarship (M.D.), Canada Research Chairs in Molecular Hemostasis (C.P.M.H) and the Genetics of Complex Diseases (A.D.P.). The authors thank Ms. Francine Derome for sample collections and Mr. Barry Eng, Mr. Andrew McFarlane and Dr. Shilun Zheng for technical help.

AUTHORSHIP

Contribution: A.D.P. and C.P.M.H. jointly supervised the project, designed experiments, interpreted results and wrote the manuscript; C.P.M.H. and G.E.R. recruited subjects; J.M.R., B.B., J.B, I.W., M.D, J.S.W. and G.E.R. contributed to study design, result interpretation and manuscript writing; B.B., J.B., I.W., and M.D. also performed experimental work.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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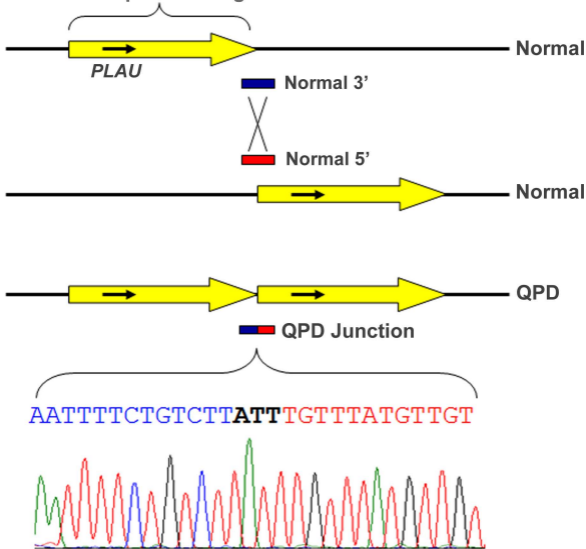
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Figure legends:

Figure 1. Illustration of the tandem duplication of *PLAU* in subjects with QPD and alignment of the junction sequence. The duplicated, ~78 kb region (yellow arrow) on chromosome 10 in QPD includes *PLAU* and all of its characterized regulatory elements (upper diagram, not to scale) and *C10orf55* (not shown) on the antisense strand to *PLAU*, a gene of unknown function. Alignment of the breakpoint junction between the copies (below) with the normal gene sequence indicates that the QPD duplication resulted from non-homologous recombination.

Figure 2. Genotyping assays for the tandem duplication of *PLAU* in QPD. Panels compare data for affected (Q) and unaffected (C) QPD family members. (A) Real-time assay findings compare the cycle thresholds (CT) of the normal (VIC) and duplicated (FAM) allele copies for 38 affected and 54 unaffected family members, tested on the same run. Ct values of 40 indicate an undetectable genotype. NTC indicates no template controls. (B) Multiplex, fragment size assays show the 207 bp and 165 bp fragments that correspond to the normal and duplicated copies of *PLAU* (representative subjects shown).

78 kb Duplicated Region



AATGGAATTTTCTGTCTTATTCCTTCTAGATTATTAGG 3'

|||||

AATGGAATTTTCTGTCTT**ATT**TGTTTATGTTGTTTAATA QPD

|| | | |||

CCAGGCTCTCTGGGGTAACATTTGTTTATGTTGTTTAATA 5'

Figure 1

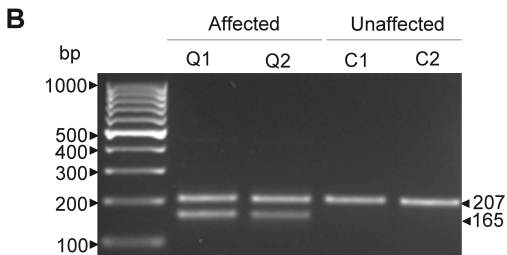
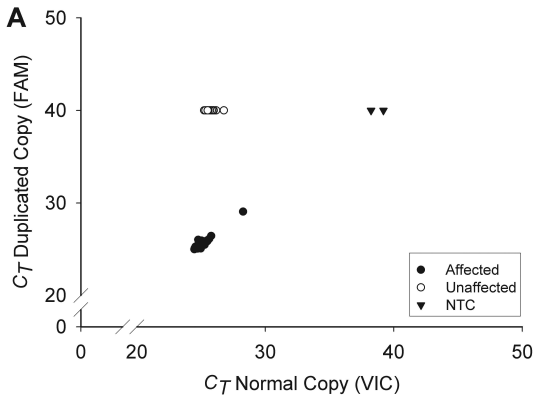


Figure 2