whether the protein itself can directly lower triglycerides when administered at physiologic concentrations.

We thank Dr. Holger Schilske, Dr. David Robbins, Nancy Hale, Paula Santa, and Jayne Talbot for their support.

References


Allele Dropout in PCR-Based Diagnosis of Wilson Disease: Mechanisms and Solutions, Ching-Wan Lam1* and Chloe M. Mak1,2 (1 Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong, China; 2 Division of Clinical Biochemistry, Queen Mary Hospital, Hong Kong, China; * address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong, China; fax 852-26365090, e-mail ching-wanlam@cuhk.edu.hk)

Background: We investigated the mechanisms leading to allele dropout—the nonamplification of 1 of the alleles—in PCR-based diagnosis of Wilson disease (WD).

Methods: We extracted genomic DNA from blood samples from 6 WD patients (P1–P6) with allele dropouts detected in a previous study of WD in a Hong Kong Chinese population. We amplified the ATP7B gene by PCR and performed direct DNA sequencing of all exons of the ATP7B gene. To support the proposed mechanism of allele dropout, we used proofreading DNA polymerase, primer design avoiding single-nucleotide polymorphism sites, and duplex PCR.

Results: Patients P1–P4 were all apparently homozygous for a known disease-causing mutation, c.2975C>T (p.P992L) in exon 13. Patient P5 was apparently homozygous for a novel mutation, c.2524G>A, and patient P6 was apparently homozygous for another known mutation, c.522_523insA (p.K175K-fs). In all cases, we determined that the patients were actually heterozygous for these mutations.

Conclusion: Our results confirm that allele dropout is the mechanism causing apparent homozygosity of heterozygous mutations in these WD patients.

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Wilson disease (WD) is an autosomal recessive inherited disorder of abnormal copper metabolism related to mutational inactivation of the ATP7B gene. DNA-based diagnosis of WD has been used for confirmatory diagnosis and carrier detection of WD. In a WD compound heterozygote, if the wild-type allele fails to be amplified by PCR, DNA sequencing or restriction analysis of the PCR products will show apparent homozygosity of the corresponding mutant alleles. This PCR artifact is called allele dropout. In this study, we investigated the mechanisms leading to allele dropout in 6 WD patients from a Hong Kong Chinese population.

After obtaining informed consent, we collected peripheral blood samples from patients P1–P6 and from the parents of P4. Genomic DNA was extracted by use of a QiAamp Blood Kit (Qiaegen). We amplified the coding exons and the flanking introns of the ATP7B gene by PCR using a touch-down approach: 94 °C for 12 min; 10 cycles of 94 °C for 45 s, 66 °C for 45 s (1 °C decrement for each cycle), and 72 °C for 45 s; 30 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s; and a final extension at 72 °C for 8 min. Each reaction mixture had a final volume of

DOI: 10.1373/clinchem.2005.061374
25 μL and contained 1× PCR buffer II, 2.5 mM magnesium chloride, 200 μM each of the deoxynucleoside triphosphates, 1 μM each primer (see Table 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol52/issue3), 0.625 U of AmpliTaq Gold polymerase (Applied Biosystems), and 100 ng of DNA template. PCR products were sequenced in the sense and antisense directions by cycle DNA sequencing (Applied Biosystems).

Direct DNA sequencing of all exons of the ATP7B gene indicated that patients P1–P4 were all apparently homozygous for a known disease-causing mutation, c.2975C>T (p.P992L), in exon 13 and heterozygous for a disease-causing mutation in other exons (see Table 2 in the online Data Supplement). The mother of P4 was also apparently homozygous for c.2975C>T (see Fig. 1a in the online Data Supplement). The mutation c.2975C>T abolished the only Btg1 site in the PCR products. The size of the undigested PCR products was 395 bp. In the presence of 2975C, the PCR products were digested into 176- and 219-bp fragments. In patients P1–P4 and the mother of P4, restriction analysis showed only a single fragment of 395 bp, indicating the presence of a homozygous c.2975C>T mutation in the PCR products (see Fig. 1b in the online Data Supplement). The restriction analysis findings were consistent with the sequencing results.

Because allele dropout was a possible explanation for the apparent c.2975C>T homozygosity in patients P1–P4, we reviewed the genomic sequence of the ATP7B gene in the National Center for Biotechnology Information (NCBI) human genome database. We found several single-nucleotide polymorphisms (SNPs) in the flanking introns of the ATP7B gene (Fig. 1). Interestingly, a IVS12-90G/T (NCBI accession no. rs2296246) is located at the most 3' position of the primer ATP7BE13F. The minor allele of the SNP, IVS12-90T, has an estimated frequency of 0.25 in the Hong Kong Chinese population. Because the primer ATP7BE13F contains the major SNP allele, it will have a mismatch with a DNA template having the minor SNP allele, and the disease-causing allele c.2975T will be preferentially amplified in a WD compound heterozygote with a genotype of IVS12-90G/T. To test this assertion, we determined, by direct DNA sequencing, the genotypes of patients P1–P4 at IVS12-90. We found that patients P1–P4 all had the genotype IVS12-90G/T. We also determined the genotypes at IVS12-90 in 3 other WD compound heterozygotes shown by the original PCR and DNA sequencing methods to be heterozygous for c.2975C>T. In all 3 WD compound heterozygotes, the genotype at IVS12-90 was G/G. This result is consistent with that predicted from the proposed mechanism of allele dropout.

We designed a pair of outer primers, ATP7BE13Fouter and ATP7BE13Router, that enclosed the entire region bounded by the primers ATP7BE13F and ATP7BE13R. Restriction analysis using the outer primer pair showed that patients P1–P4 were actually heterozygous for the c.2975C>T mutation (shown for patient P4 in Fig. 1b in the online Data Supplement). Using the primers ATP7BE13F and ATP7BE13R as amplification primers, we used DNA polymerase with 3' to 5' exonuclease activity to restore the amplification of the dropped wild-type exon 13 (see Fig. 1b in the online Data Supplement), a result that supports the proposed mechanism of allele dropout. Taken together, the results confirmed that allele dropout...
is the mechanism causing apparent homozygosity of c.2975C>T mutation in patients P1–P4.

Additionally, using the outer primers ATP7BE13Fouter and ATP7BE13Router for PCR and the inner primers ATP7BE13F and ATP7BE13R for sequencing, we found that sequencing the sense strand using the primer ATP7BE13F showed a homozygous c.2975C>T mutation, whereas sequencing the antisense strand using the primer ATP7BE13R showed a heterozygous c.2975C>T mutation (see Fig. 2 in the online Data Supplement). Therefore, allele dropout of the wild-type exon 13 can occur in cycle DNA sequencing.

On direct DNA sequencing of all exons of the ATP7B gene, we found that patient P5 was heterozygous for a known disease-causing mutation, c.2602delC (p.H11022A), and apparently homozygous for a novel mutation, c.2524G>A, which changes codon GAT to AAT, which in turn leads to the substitution of aspartic acid at residue position 842 by asparagine, i.e., p.D842N (see Fig. 3a in the online Data Supplement). The mutation c.2524G>A was not detected in 50 apparently healthy individuals, indicating that c.2524G>A is probably a disease-causing mutation. Patient P6 was heterozygous for a known disease-causing mutation, c.314C>A (p.S105X), and apparently homozygous for another known mutation, c.522_523insA (p.K175K-fs; see Fig. 3b in the online Data Supplement). Interestingly, the binding site of primer ATP7B13R was amplified by the wild-type c.522_523insA, is mutated by ATP7B10Rmutant, which had the c.2602delC mutation, and that of the primer ATP7B2BF, which amplifies the wild-type c.522_523insA, is mutated by c.314C>A.

To restore amplification of the dropped alleles of patient P5, we designed a mutant reverse primer, ATP7BE10Rmutant, which had the c.2602delC mutation, for duplex PCR. We set up a duplex PCR reaction using primers ATP7BE10R, ATP7BE10Rmutant, and ATP7BE10F. DNA sequencing of the duplex PCR products showed that patient P5 was actually heterozygous for c.2524G>A (see Fig. 3a in the online Data Supplement). In addition, we designed a new primer pair, ATP7BE10-11F and ATP7BE10-11R, to amplify the entire exons 10 and 11. DNA sequencing of the PCR products also showed that patient P5 was actually heterozygous for c.2524G>A mutation (see Fig. 3a in the online Data Supplement).

For patient P6, we designed a mutant primer, ATP7BE2BFmutant, which had the c.314A mutation, and sequencing of the duplex PCR products showed that patient P6 was actually heterozygous for the mutation c.522_523insA (see Fig. 3b in the online Data Supplement).

We found that 15 of the 21 exons of the ATP7B gene have SNPs within 250 bp of their 5’ and 3’ flanking regions in the NCBI human genome database (Fig. 1). Three exons—exons 10, 14, and 19—have SNPs in both the 5’ and 3’ regions. Primers having these SNP sites can cause single or double allele dropout of the wild-type or mutant alleles. In addition, we reviewed the primer sequences for PCR-based diagnosis of WD in the literature published since the ATP7B gene was identified (1, 2). We noted several differences between published primer sequences and the genomic sequence currently in the NCBI human genome database (Table 1). All changes in these primers are internal, and stringency conditions will determine whether allele dropout occurs. However, the primer sequence differences may contribute to the muta-

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Table 1. Corrections over published primer sequences.*

<table>
<thead>
<tr>
<th>Primer names used in the original report</th>
<th>Original primer sequences, 5’-3’</th>
<th>Corrections/Comments</th>
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<tr>
<td>Thomas et al. (1)</td>
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<td>Petrukhin et al. (2)</td>
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* Nucleotide positions of the mutated bases in the primer are underlined. The bases in bold font represent the SNP sites in the primer sequences. The frequencies of the SNPs can be found in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP).
Identification of mRNA Markers for Molecular Staging of Lymph Nodes in Colorectal Cancer, Liqiang Xi,1† William Gooding,2 Kenneth McCarty,3 Tony E. Godfrey,1,4 and Steven J. Hughes1† (Departments of 1 Surgery and 3 Pathology, and the 2 Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA; † current affiliation: Mount Sinai School of Medicine, New York, NY; * address correspondence to this author at: University of Pittsburgh, 497 Scaife Hall, 3550 Terrace Street, Pittsburgh, PA 15261; fax 412-648-2045, e-mail Hughess2@upmc.edu)

Background: One evolving approach to improved prognostication of cancer patients is the identification of previously occult disease by use of quantitative reverse transcription-PCR. Surprisingly, no systematic analysis of potential mRNA markers for colorectal cancer has been reported. We therefore performed an extensive mRNA marker survey for colorectal cancers.

Methods: We identified potential markers through literature and database searches. We analyzed all markers by quantitative reverse transcription-PCR on a limited set of primary tumors and benign lymph nodes. Selected markers were further evaluated on a larger tissue set with positive lymph nodes.

Results: We evaluated 43 markers and undertook further analysis of 6 in the secondary screening. Five gene markers—CDX1, carcinoembryonic antigen (CEA), CK20, TACSTD1, and Villin (VIL1)—provided perfect classification of lymph node status.

Conclusions: Several mRNA markers are capable of providing exceptionally accurate characterization of lymph node status in colorectal cancer. An automated, multimarker, quantitative reverse transcription-PCR assay for characterization of lymph nodes from colorectal cancer patients may be useful for improved staging and therapeutic decision making in colorectal cancer.