Letters to the Editor

Multiplex Ligation-Dependent Probe Amplification Analysis Is Useful for Diagnosing Congenital Adrenal Hyperplasia but Requires a Deep Knowledge of CYP21A2 Genics

To the Editor:

We read with great interest the recent report in Clinical Chemistry by Cantürk et al. (1). These authors affirmed that the CYP21A1P (cytochrome P450, family 21, subfamily A, polypeptide 1 pseudogene) genotype interferes with quantitative multiplex ligation-dependent probe amplification (MLPA) analysis of the CYP21A2 (cytochrome P450, family 21, subfamily A, polypeptide 2) gene. They also reported that the p.I172N and p.Q318X mutations were absent in 3.6% (p.I172N) with an risk of 3.6% (p.Q318X) with an MLPA method, or 8.5% for p.Q318X.

From our experience, we believe that such assertions are incorrect. Indeed, it is not possible to establish the CYP21A2 copy number from the signal of only 1 specific gene probe (the exon 4 or exon 8 CYP21A2 probe in this case). When MLPA analysis is performed, it is necessary to consider the value of all specific gene probes (2). If CYP21A2 duplication were actually present, all 5 specific probes would show a ratio >1.3. That occurs when all CYP21A2 alleles are wild type for the following mutations: exon 3 8-bp deletion, p.I172N, clusterE6, and p.Q318X. On the contrary, if a duplicated CYP21A2 allele is present and carries the p.Q318X mutation, only the exon 8 probe would show a typical ratio (0.7–1.3) (2).

Furthermore, if the p.I172N or p.Q318X mutation is absent in CYP21A1P, the CYP21A2-specific probes for exon 4 or exon 8 would also bind the wild-type CYP21A1P pseudogene sequence, giving a ratio >1.3. For this reason, we think it more correct to affirm that exon 4 and exon 8 CYP21A2 probes may give a ratio >1.3 in 3.6% and 8.5% of the cases, respectively. Therefore, when that occurs, it is incorrect to immediately assume gene duplication, but it is necessary to consider the value of all specific CYP21A2 probes for a definitive interpretation of the MLPA analysis. In this case, pseudogene sequencing can also be performed to strengthen the diagnosis.

Quantitative CYP21A2 diagnosis could provide an incorrect result when the CYP21A1P pseudogene lacks all of the following mutations: Del8bp, p.I172N, ClusterE6, and p.Q318X. In fact, in this case all specific CYP21A2 gene probes might also recognize the pseudogene sequence showing a ratio >1.3. To the best of our knowledge, that has never been reported.

Our group first used MLPA analysis for the diagnosis of congenital adrenal hyperplasia (2). Other groups with considerable experience have used this technique successfully (3, 4), and it is currently used for routine analysis of congenital adrenal hyperplasia as a valid alternative to the Southern blot.

As previously reported (2), we confirm that MLPA analysis is a very informative tool for the molecular diagnosis of congenital adrenal hyperplasia. Considering also the findings of Cantürk et al., we stress that the use of this methodology requires a deep knowledge of CYP21A2 genes. The CYP21A2 gene, which encodes a 21-hydroxylase, has a complex structure and is considered one of the most polymorphic of human genes.

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References

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1 Human genes: CYP21A1P, cytochrome P450, family 21, subfamily A, polypeptide 1 pseudogene; CYP21A2, cytochrome P450, family 21, subfamily A, polypeptide 2.
To the Editor:

Cardiac troponin is the marker of choice for evaluating myocardial injury (1). High-sensitivity assays improve analytical detection limits, thereby allowing concentrations to be measured in the majority of healthy individuals. This capability allows an assessment of biologic variation (BV)1 to determine what constitutes a clinically important change in the cardiac troponin concentration, a critical metric for identifying acute events. Such an event is often a myocardial infarction, but any acute cardiac injury can cause increasing and/or decreasing values (1). Accordingly, we evaluated BV for a recently developed high-sensitivity cardiac troponin I (hs-cTnI) assay (2) from Beckman Coulter. We performed this study with the same cohort used to define BV for the high-sensitivity cardiac troponin T (hs-cTnT) assay (3) and according to a protocol approved by our institutional review board.

For assessment of short-term BV, we collected blood into serum separator tubes (Becton Dickinson) at 0, 1, 2, 3, and 4 h, centrifuged the tubes, and stored the serum samples immediately at −70 °C. Samples are stable under these conditions (2). For the long-term study, samples were obtained biweekly for 8 weeks and processed similarly. After thawing the samples, we analyzed the samples without recentrifugation in duplicate on the Access analyzer (Beckman Coulter). The limit of blank for this assay is 1.03 ng/L, the limit of detection is 2.06 ng/L, the lowest concentration with an imprecision (CV) <10% is 8.66 ng/L, and the 99th percentile value for serum is 8.00 ng/L, as previously reported (2). Reference change values (RCVs) were calculated as previously described (4) according to the method of Fokkema et al. ANOVA (generalized linear model procedure in statistical analysis software; SAS Institute) was used to calculate the sums of squares for the analytical and biological components. Total (SDT), analytical (SDA), and the interindividual (SDG) variances were determined by the maximum-likelihood approach. Values were averaged across participants; variances were homogeneously distributed. The Cochran test was used to identify outliers; no values were removed. The index of individuality was computed as \[
\frac{\left( CV_A^2 + CV_I^2 \right)^{1/2}}{CV_G}\]
where CV_A is the analytical CV, CV_I is the intraindividual CV, and CV_G is the interindividual CV. Because the data were skewed, RCVs were calculated after lognormal transformation. A 95% CI was used for short- and long-term RCVs.

The mean age of the study participants was 39 years (range, 25–56 years); the median age was 36 years (interquartile range [IQR], 31.3–46.3 years). Sixty percent were women. None of the participants had a history of cardiovascular disease or other conditions known to affect cardiac troponin, and none were taking cardiovascular medications. One individual had only 2 initial blood draws in the short-term study, and 4 individuals missed 1 time point during the long-term evaluation.

No participants had values below the limit of blank. Baseline values ranged from 1.04 ng/L to 11.01 ng/L. The median value for short-term BV was 2.20 ng/L (IQR, 1.51–2.80 ng/L), and the median values for the short-term and long-term studies were similar [2.20 ng/L (IQR, 1.52–2.80 ng/L) and 2.19 ng/L (IQR, 1.5–2.82 ng/L), respectively]. The RCVs were +45.2%/−15.8% and 14.0%/−10.6% for short-term and long-term BV, respectively (Table 1). There were no significant differences between the sexes according to the t-test.

These results provide index data for a research hs-cTnI assay. The results are similar to those obtained for another hs-cTnI assay (4) but lower than those observed with the hs-cTnT assay (3). The high degree of BV in our hs-cTnT data has been suggested to be due to underlying cardiovascular comorbidities (5). The present data are more consistent with the hypothesis that the differences are related to differences in the precision of the assays at very low troponin concentrations.

**Letters to the Editor**

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1 Nonstandard abbreviations: BV, biologic variation; hs-cTnI, high-sensitivity cardiac troponin I; hs-cTnT, high-sensitivity cardiac troponin T; RCV, reference change value; IQR, interquartile range.