lier commercial assay, which also used B152 as the capture antibody, stated that diluting serum samples produced the expected results and that results for serum and plasma were identical (5). This assay has been used in several clinical studies. It remains to be seen whether earlier promising results on the clinical use of hCG-h can be verified and possibly improved by eliminating the variable effect of complement in serum.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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The Detection of Double Mutations in KRAS Depends on the Mutation-Detection Assay Used

To the Editor:

Epidermal growth factor receptor is frequently overexpressed in colorectal cancer, and targeted therapies have been developed to block this receptor. Monoclonal antibodies such as cetuximab and panitumumab have demonstrated effectiveness in increasing the survival rates for some metastatic patients. Patients with mutations in codon 12 or 13 of the KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) gene are not responsive to this therapy, however, and tumors from patients are systematically screened for somatic KRAS mutations before treatment. Several techniques have been developed to screen for KRAS mutations, and the differences in these techniques with respect to diagnostic sensitivity, diagnostic specificity, and efficiency for mutation detection have been described (1–4). The main potential weakness of direct sequencing is its low diagnostic sensitivity (approximately 20%). Other techniques, such as TaqMan® PCR–based assays and SNaPshot® (Applied Biosystems), have diagnostic sensitivities between 1% and 10%. In this study, we analyzed 1970 colorectal carcinoma tumors for KRAS mutations by direct sequencing. We detected 10 cases with double mutations at codons 12 and 13 via direct sequencing and compared the ability of single-nucleotide extension (SNE) and hydrolysis probe real-time PCR to detect these rare mutations. The nucleotide pairs implicated in the nucleotide changes are 34 and 35, 35 and 36, 35 and 37, 35 and 38, 37 and 38, and 38 and 39. Sequencing analysis did not allow us to determine whether the 2 mutations were located on the same allele.

With hydrolysis probe real-time PCR, we detected both mutations in only 1 case (c.35G>T and c.38G>A); 9 of the tumors were classified as wild type for codons 12 and 13. The lack of detection of either one of the double mutations implies that the 2 mutations are located on the same allele. Yet, the detection of mutations c.35G>T and c.38G>A suggests that these mutations are located on separate alleles, although the c.35G>T mu-

Clinical Chemistry 57:7 (2011) 1077
The sequencing corresponded to 1.3% of mutations at codons 12 and 13 by direct real-time PCR result (Table 1).

This finding indicated by SNE, was localized on a separate allele. For 1 case (c.38G>A, c.39C>T), the c.38G>A mutation was detected by both the forward and reverse primers, suggesting that the second mutation, c.39C>T, which was not screened by SNE, was localized on a separate allele. This finding indicated a discrepancy between the SNE result and the hydrolysis probe real-time PCR result (Table 1).

The detection of double mutations at codons 12 and 13 by direct sequencing corresponded to 1.3% of the KRAS mutations at codons 12 and 13. Although the rate of double mutations is low, it is important to detect such mutations; otherwise, these patients would be considered eligible for treatment with antibodies to epidermal growth factor receptor, which potentially would have no clinical benefit but could have adverse effects. The hydrolysis probe real-time PCR analysis reported codons 12 and 13 of KRAS as wild type for 9 of the 10 cases screened. The SNE technique was more successful because one of the 2 mutations was detected from the forward and/or reverse sequences. Although these mutations are rare events, they have been reported in the literature.

Metastatic colorectal carcinomas occur at a high frequency, and thus numerous cases are routinely tested for KRAS mutations. Given that patients need quick and efficient treatment, the testing procedure must be performed in the shortest time with the best available technique in terms of rapidity, diagnostic specificity, and diagnostic sensitivity. Different in-house methods (1–3) and commercially available kits [Signature® KRAS/BRAF Mutations Kit (Asuragen), Therascreen KRAS PCR Kit (Qiagen)] have been developed, some of which are based on the hybridization of mutation-specific probes. The detection of 1.3% KRAS double mutations located on the same allele must be kept in mind when choosing the KRAS mutation-detection method for routine clinical setting. It is essential not only that the test used be diagnostically sensitive but also that it be able to detect all possible mutations (even if rare) so that the patient can be offered the most appropriate treatment.

Table 1. Detection of double mutations in KRAS exon 2 in a case of colorectal adenocarcinoma tumor, according to the method of analysis.

<table>
<thead>
<tr>
<th>Site</th>
<th>Hydrolysis probe real-time PCR</th>
<th>SNE</th>
<th>Direct sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.38</td>
<td>WT*</td>
<td>G&gt;A (both strands)</td>
<td>G&gt;A</td>
</tr>
<tr>
<td>c.39</td>
<td>ND</td>
<td>ND</td>
<td>C&gt;T</td>
</tr>
</tbody>
</table>

*WT, wild type; ND, not done.

References


Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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Letters to the Editor

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

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 21A, 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% and 8.5%, respectively, of the alleles (200 unrelated individuals examined).

Because CYP21A2 MLPA-specific probes contain the wild-type sequences for the Del8bp, p.I172N, ClusterE6, and p.Q318X mutations, Cantürk et al. stated that "without a parallel CYP21A1P [sequencing] analysis, one would falsely infer a heterozygous duplication of CYP21A2 with a risk of 3.6%" (p.I172N) 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 and given the data of Cantürk et al., 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., however, we stress that the use of this methodology requires a deep knowledge of CYP21A2 genetics. 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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