was obtained from the local Institutional Review Board for Studies in Humans. Informed consent was obtained from each patient. Urine samples were aliquoted (1 mL) and frozen at −20 °C until being measured in parallel by the present HPLC method (sample dilution, 1:100 by volume; injection of 200-μL aliquots; flow rate, 1 mL/min; detection at 236 nm) and by the alkaline picrate method (sample dilution, 1:5 by volume), which was carried out on a Hitachi automated analyzer (Hitachi Ltd.) by a certified clinical institution. In this study, drugs were not determined in the urine of the patients.

Linear regression analysis between the creatinine concentrations measured by the present HPLC method (y) and those measured by the alkaline picrate method (x) yielded a mean (SD) slope of 1.4 (0.02) and y-intercept of 0.50 (0.14) mmol/L (r = 0.997) for the control samples and a slope of 1.49 (0.06) and y-intercept of −0.15 (0.59) mmol/L (r = 0.981) for the patient samples. The results were also compared by plotting the difference for creatinine measurements (HPLC − Jaffe) vs the mean concentration (Fig. 1B) according to the method of Bland and Altman (3). Analysis by the Jaffe method gave urinary creatinine concentrations that were lower than those measured by HPLC in the whole concentration range (0.2–33 mmol/L). Best agreement between the methods was observed at creatinine concentrations <3 mmol/L. The greatest disagreement between the HPLC and Jaffe methods was for analysis of the urine samples from the patients and for urinary creatinine concentrations >10 mmol/L (Fig. 1B). In accordance with the statement of the clinical institution at which the urinary creatinine was measured, all urine samples were diluted with distilled water by the same factor: 1:5 (by volume). We believe that the discrepancies noted at higher creatinine concentrations are attributable to interfering compounds in the Jaffe method, in which the extent of interference increased with increasing concentration of the interfering compounds.

We measured creatinine in serum samples from 17 hospitalized patients in duplicate by the present HPLC method with detection at 236 nm. The serum creatinine concentrations were determined with a mean imprecision (SD) of 4.5 (3.6)% and ranged between 58 and 219 μmol/L. In seven of these samples, creatinine was also measured by HPLC analysis with detection at 215 nm. The ratio (SD) of the respective creatinine concentrations measured at the two wavelengths (236 nm/215 nm) was 0.97 (0.09) with a CV of 9.3%. In these analyses, the ratio of the peak areas for creatinine at the two wavelengths (215 nm/236 nm) was 4.4 (0.4). This ratio agrees with that obtained for aqueous solutions of creatinine calibrators (see above). Thus, these findings suggest no interferences from coeluting substances in these serum samples.

The proposed HPLC method is simple, rapid, and suitable for the accurate measurement of creatinine in urine, plasma, and serum samples from healthy and severely diseased humans. It also offers the opportunity to analyze creatine in plasma or serum. The method’s simplicity and streamlined sample treatment make it suitable for automated analysis of creatinine.

References


DOI: 10.1373/clinchem.2003.024141

Multiplex Single-Nucleotide Primer Extension Analysis To Simultaneously Detect Eleven BRCA1 Mutations In Breast Cancer Families, François Révillon, Alain Verdière, Joëlle Fournier, Louis Hornez, and Jean-Philippe Peyrat (Laboratoire d’Oncologie Moléculaire Humaine, Centre Oscar Lambret, 3 rue Frédéric Combemale, BP 307, 59020 Lille Cédex, France; * address for correspondence: fax 33-3-2029-5535, e-mailjp-peyrat@o-lambret.fr)

Approximately 5% of breast cancers are considered as hereditary, and their development is associated with germline mutations of specific genes. The BRCA1 gene was isolated in 1994 (1) and is estimated to account for almost one-half of inherited breast cancers and three-fourths of inherited breast/ovarian cancers. BRCA1 is a large gene containing 5592 nucleotides spread over ~100 000 bases of genomic DNA.

More than 1200 different mutations have been found in BRCA1 associated with breast or ovarian cancer (2, 3). Mutations, recurring or unique in distribution, are dispersed throughout the coding sequence. Recurring mutations are either international or national. Six mutations have been observed commonly (3): 185delAG, 5382insC, 3007>T>G, 4446C>T, 4184delTCAA, and 3875delGTCT. Other frequent mutations have been localized to specific countries: 5149delCTAA (4) and 3958delCTCAAG (5) in France; 2804delAA in Holland (6, 7); 3960 C>T in northern France, Belgium, and Holland (8); and 3600del11 in northeastern France (9).

As no functional assays are available, detection of BRCA1 mutations must be carried out at the DNA level. Because the gene is large and the mutations are distributed throughout its length, systematic sequencing is certainly the “gold standard” method. However, this method is time-consuming and expensive.

We describe here a method (10), multiplex single-nucleotide primer extension (MSNPE) analysis, to simultaneously detect 11 international and regional recurring mutations.

The patients were treated at the Centre Oscar Lambret for breast and/or ovarian cancer and belonged to high-risk families. Written informed consent was obtained from each patient. Genomic DNA was extracted from 2 mL of citrate blood samples by use of the QIAamp DNA Blood Midi Kit (Qiagen).

Fragments of BRCA1 gene containing the 11 recurrent screened mutations were amplified by multiplex PCR.
The PCR mixture (total volume, 25 µL) contained 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM MgCl₂, 0.4 mM each of the deoxynucleotide triphosphates (Amersham Biosciences), 100 ng of genomic DNA, 2 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems), and the primers (see Table 1 in the online Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue1/) at 1.6 µM for exon 17, 0.8 µM for exons 2 and 13, 0.4 µM for exons 5 and 11H, 0.2 µM for exon 11F, and 0.12 µM for exon 20. After activation of AmpliTaq Gold DNA polymerase for 10 min at 95 °C, the thermocycling conditions were 55 cycles at 94 °C for 30 s, 54 °C for 20 s, and 72 °C for 2 min, with a final elongation step at 72 °C for 8 min.

PCR products were purified by use of Sephacryl 400 (Amersham Biosciences) in Costar microplates and digested by incubation for 1 h at 37 °C with exonuclease I (Amersham Biosciences) and shrimp alkaline phosphatase (Roche Diagnostics).

The MSNPE reaction was performed with the ABI Prism SnaPshot Multiplex reagent set (Applied Biosystems). The 11 purified primers (Genosys; Table 1), each

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>Minisequencing primer, 5’-3’&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primer Wild type</th>
<th>Mutant Wild type</th>
<th>Mutant size&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mutant extension&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>185delAG</td>
<td>(24)t-atgctatgcagaaatcttag</td>
<td>45</td>
<td>A</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>300T&gt;G</td>
<td>(28)t-cagaagagggctcctacag</td>
<td>49</td>
<td>T</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>11F</td>
<td>2804delAA</td>
<td>ctctgggtccttgaagaaaca</td>
<td>21</td>
<td>A</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>11H</td>
<td>3600del11</td>
<td>(4)t-ttagtgagtggtaaataaa</td>
<td>25</td>
<td>G</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>11H</td>
<td>3875delIGTCT</td>
<td>(36)t-cacggtgctacaggtgtct</td>
<td>57</td>
<td>C</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>11H</td>
<td>3958delCTCAGinsAGGC</td>
<td>(40)t-ttccctactaagttgat</td>
<td>61</td>
<td>C</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>11H</td>
<td>3960C&gt;T</td>
<td>(8)t-gtaatatggcaagacct</td>
<td>29</td>
<td>C</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>11H</td>
<td>4184delTCAA</td>
<td>(12)t-aacgggcttggaagaataa</td>
<td>33</td>
<td>T</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>4446C&gt;T</td>
<td>(32)t-tcctgtgccttggagactg</td>
<td>53</td>
<td>C</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>5149delCTAA</td>
<td>(16)t-gaaacaccacacatctcttaa</td>
<td>37</td>
<td>C</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>5382insC</td>
<td>(20)t-cagagcagcaaggaatccc</td>
<td>41</td>
<td>A</td>
<td>C</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The size of the gene-specific primers was 21 bases. They were then synthetically elongated at their 5’ end with a poly(T) tail, varying from 4 to 40, which facilitated the separation of the extension products by electrophoresis.

<sup>b</sup>Indicates the nucleotide added by extension of the primer and the color of the corresponding peak in the wild-type DNA sample.

<sup>c</sup>Indicates the nucleotide added by extension of the primer and the color of the corresponding peak in the mutated DNA sample.
specific for a screened mutation, bind to the complementary sequence in the presence of fluorescently labeled dideoxynucleotide triphosphates (ddNTPs), and the AmpliTaq DNA Polymerase extends the primer by adding a single ddNTP to its 3' end. Fluorescent dyes are assigned to the individual ddNTPs as follows: green for A, yellow for C (which for convenience is black on the electropherogram), blue for G, and red for T. The reaction mixture contained 3 μL of PCR products, 5 μL of multiplex reaction premixture (fluorescently labeled ddNTPs, AmpliTaq DNA polymerase, and reaction buffer), and 2 μL of primers (0.5 pmol for mutation 3875delGTCT; 5 pmol for 185delAG, 300T>C, 2804delAA, 3600delT1, 3960C>T, 4446C>T, and 5382insC; and 10 pmol for the others). The reaction was performed as follows: 27 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. Samples were treated with 1 U of shrimp alkaline phosphatase at 37 °C for 1 h, then diluted 10-fold with deionized formamide and denatured at 95 °C for 5 min. The fluorescently labeled fragments were resolved by capillary electrophoresis on an automated ABI Prism 310 Genetic Analyzer (Applied Biosystems).

The specificity of the multiplex PCR was checked by electrophoresis of the products on 4% agarose gels containing ethidium bromide (see Fig. 1 in the online Data Supplement).

The MSNPE reaction was performed on samples previously analyzed by direct sequencing. All mutations were easily detected. Fig. 1 shows the electropherograms of the wild-type DNA and the three most frequent BRCA1 mutations (3). Because all of the BRCA1 samples with mutated DNA were in a heterozygous state, two peaks were detected.

It is noteworthy that these two peaks did not overlap, although the sizes of their products were similar. This is a consequence of the electrophoretic mobility of these small final extension products, which depends not only on their lengths, but also on their nucleotide sequences and on the fluorescent dye used in the reaction.

To validate the procedure, we analyzed 48 DNA samples (24 samples with a BRCA1 mutation and 24 without). All of the mutations were always detected, and no false-positive results were observed in samples without mutations, demonstrating the reliability of the procedure. Repetitive analyses demonstrated the reproducibility of the procedure.

The present procedure was accurate: All 48 DNA samples already genotyped by direct DNA sequencing displayed concordant results. The 11 heterozygous mutations were easily detected when compared with the wild-type profile. The criteria of specificity for a “mutant” peak were as follows: (a) a peak was observed with the expected size and color; (b) the obtained peak was at least threefold higher than the background peaks of the same color; and (c) the wild-type peak was decreased.

For each screened mutation, the primer was designed to anneal immediately adjacent to the nucleotide at the mutation site, on either the sense or antisense DNA strand. The primer orientation allowed multiplex PCR in the absence of intra- and intercomplementarity. Despite
the purification of the primers, several small peaks appeared, which seem to be extension products coming from n-1 oligonucleotides for the higher molecular weight primers corresponding to the 3958, 3875, and 4446 loci. These peaks were easily identified on all electropherograms.

The present procedure allowed the detection of 36% of the cases identified in our region and 32% of the international mutations (3). It could also be adapted easily and quickly by other laboratories to take into account their local mutation frequencies. In this regard, this procedure could potentially be a useful tool for prescreening studies.

The MSNPE reaction developed here requires only one reaction per patient sample, and it is possible to analyze 96 DNA samples in 1.5 working days. The subsequent interpretation of peak patterns is simple. This method is sensitive, rapid, and offers reduced laboratory costs. The multiplex technique increases both the practicability and ease of handling in the laboratory. If multichannel capillary electrophoresis instruments are used, this procedure could also be automated for high-throughput genotyping. Finally, a similar strategy can easily be developed to detect the most frequent BRCA2 mutations.

This work was supported by the Ligue Nationale Contre le Cancer (Paris, France), its local Committee (Lille, France), and the A.R.C. (Villejuif, France). We are grateful to Dr. Dave Fernig (University of Liverpool, Liverpool, UK) for critical reading of the manuscript.

**References**


---

**Effects of Filtration on Glyceraldehyde-3-Phosphate Dehydrogenase mRNA in the Plasma of Trauma Patients and Healthy Individuals,** Timothy H. Rainer, Nicole Y.L. Lam, Nancy B.Y. Tsui, Enders K.O. Ng, Rossa W.K. Chiu, Gavin M. Joynt, and Y.M. Dennis Lo

The biology and role of circulating cell-free nucleic acids in the diagnosis and risk stratification of many critical diseases attracted much interest in the late 1990s. Although plasma DNA took early center stage in disease diagnosis, monitoring and risk stratification for many conditions, including pregnancy, cancer, transplantation, and trauma, recent publications suggest that the next stage of development of this field may involve plasma RNA.

Cell-free RNA exists in small quantities in the plasma of healthy individuals, and qualitative and quantitative studies have demonstrated increased plasma and serum concentrations of tumor-derived RNA in cancer patients. Although we have reported that plasma DNA increases in patients early after trauma, correlates with injury severity, and predicts mortality and other posttraumatic complications, there are no published studies investigating circulating mRNA in patients after trauma. Unlike nuclear DNA, cellular mRNA is present in much larger copy numbers, exists normally in the cytoplasm and ribosomes of cells, and may therefore be released earlier than nuclear DNA after trauma.

In this study, we investigated whether mRNA was detectable in plasma of trauma patients, whether concentrations correlated with injury severity and posttraumatic complications, and whether filtration might have an effect on the diagnostic interpretation of the results.

The study was approved by the Ethics Committee of The Chinese University of Hong Kong. Twenty-six trauma patients [mean (SD) age, 46 (18) years; 23 males] admitted to the resuscitation room of the Accident and Emergency Department of the Prince of Wales Hospital were recruited with informed consent. Using a well-established injury severity score (ISS), we divided patients into four groups: minor injury (ISS <9), moderate injury (ISS = 9–15), severe injury (ISS >15), and major complication (mortality or multiple organ dysfunction syndrome; MODS). Fifteen healthy age- and sex-matched controls were also recruited. Peripheral blood was withdrawn into EDTA tubes and centrifuged at 1600 g for 10 min at 4 °C. Median time from injury to sampling for the groups with minor, moderate, or severe injuries and the