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Breast Cancer Susceptibility Gene mRNAs Quantified by Microarrays with Electrochemical Detection, Hong Xie, Yuan Hong Yu, Fang Xie, Yuan Zhi Lao, and Zhigang Gao (Institute of Bioengineering and Nanotechnology, Singapore, Republic of Singapore; *address correspondence to this author at: Institute of Bioengineering and Nanotechnology, 51 Science Park Road, Singapore 117586, Republic of Singapore; fax 65-6874-9341, e-mail zqgao@ibn.a-star.edu.sg)

Abnormalities in the expression of specific genes have been linked to a large and increasing number of diseases. Quantification of gene expression is a promising basis for early diagnosis, but analysis at the mRNA level has shown to be difficult because of the limited sensitivity of existing nucleic-acid-detection techniques. The most commonly used methods for quantification of gene expression include Northern blotting (1), ribonuclease protection assays (RPAs) (2), and reverse transcription-polymerase chain reaction (RT-PCR) (3, 4). The main limitation of the first two techniques is their relatively low sensitivity. RT-PCR can theoretically amplify a single nucleic acid molecule millions of times, but optimization of primer sets prolongs the assay time, and different genes in a starting mRNA mixture may not be present in the same amounts in the final RT-PCR products because of selective and nonlinear target amplification (5). These limitations affect the precision and quality of the resulting data and often provide distorted information on gene expression. Sensitive, reliable gene detection is one of the challenges in molecular diagnostics.

Electrochemical detection provides a simple, accurate, and inexpensive platform for molecular diagnostics. Despite the enormous progress made in electrochemical nucleic acid biosensor research in the past 5 years, to be one step closer to commercialization, this research must overcome several important hurdles. The first is validation of the biosensor results on a statistically large population of real samples rather than the commonly reported relatively short synthetic oligonucleotides (6). Another challenge is to multiplex the electrochemical biosensors into useful sensor arrays. Typically, arrays of 30–100 sensors are needed for diagnostic purposes. For example, breast cancer screening requires the testing of 20–30 cancer susceptibility genes plus positive and negative controls (7). Of the many proposed electrochemical detection schemes, only a few attempts have been made to detect gene expression at the mRNA level (8).

Here we describe an ultrasensitive method for direct detection of expression of breast cancer susceptibility genes in human breast tissues on an 8 × 8 sensor array. The human breast tissues were stored in liquid nitrogen immediately after surgery until mRNA extraction. Tissues were mechanically homogenized, and mRNA was extracted by use of a Dynabeads® mRNA DIRECT™ Kit (Dynal ASA) according to the manufacturer’s protocol. Labeling of the mRNA was carried out with cisplatin-coupled biotin conjugates (Biotin-Chem-Link™, Roche Diagnostics) according to the manufacturer’s recommended procedure. All solutions were treated with diethyl pyrocarbonate, and surfaces were decontaminated with RNaseZap (Ambion).

To fabricate the sensor array, we evaporated a titanium adhesion layer (25–50Å) on a glass slide by use of an electron beam, followed by 2500–3000 Å of gold. A patterned 1-mm thick adhesive spacing/insulating layer with a screen-printed Ag/AgCl layer and a hydrophobic layer were assembled on the top of the slide (Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue7/). The diameter of the individual sensor was 2.0 mm, and that of the top hydrophobic pattern was 4 mm. We immobilized 24mer oligonucleotides on each individual sensor surface, which served as capture probes, as described previously (9). The sequences of the captured probes were complementary to the sequence of each gene in a region specific to that gene where no mutation has been reported.

We examined breast cancer susceptibility genes, i.e., tumor protein p53 (TP53; 1182 bp), heat-shock protein 90 (HSP90; 1632 bp), breast cancer gene 1 (BRCA1; 5592 bp), and Histone H4 (His4; 312 bp), plus a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1008 bp), covering both high-/low-copy number and long/short genes. Hybridization was carried out in a 55 °C water bath in a buffer containing 10 mmol/L Tris-HCl, 1.0 mmol/L EDTA, and 0.10 mol/L NaCl for 30 min. Before the hybridization, nucleic acid samples were denatured at 95 (cDNA) and 70 °C (mRNA) for 10 min and cooled in an ice bath before being added to the sensor array. After hybridization, the sensor array was exposed to 2.5-μL aliquots of 5.0 g/L glucose oxidase-avidin at room temperature for 30 min and then to 2.5-μL aliquots of 5.0 g/L redox polymer, poly(vinylimidazole-co-acrylamide) partially imidazole-complexed with Os(2,2'-bipyridine)2(imidazole) for 10 min. Synthesis of the redox polymer has been described elsewhere (10). The formation of a mixed micro three-dimensional mRNA + glucose oxidase-avidin/redox polymer bilayer allowed elec-
Electrochemical measurements were carried out in a Faraday cage with a Model 660A electrochemical workstation (CH Instruments) equipped with a low-current module. All potentials reported in this work are referred to an Ag/AgCl reference electrode.

Use of cisplatin–biotin conjugates enabled direct labeling of mRNA with biotin moieties in a one-step, nonenzymatic reaction, and specific genes were subsequently detected amperometrically with high sensitivity and specificity (see Fig. 2 in the online Data Supplement). The lowest amount of mRNA needed for successful detection of specific mRNAs was $1.5 \text{ ng}$. Considering that there are an estimated 30,000 genes in this mRNA pool, the detection limit for each specific gene is, on average, in the subfemtogram range, which is in good agreement with the calibration study (see the online Data Supplement).

The relative errors associated with mRNA assays for individual genes were generally $25\%$ in the concentration range of $2.0-300 \text{ fmol/L}$. This allowed us to identify genes from two conditions that differed by less than onefold in expression. Current array technologies can reliably differentiate gene expression differences of more than threefold (11), but in many cases the expression of the most interesting genes differs by less than threefold between different conditions.

To determine our gene expression detection limit, we performed multiple assays on TP53 in extracts containing 50 ng of mRNA. The different amounts of TP53 expression were mimicked by adding various amounts of the mRNA to the test solution. The proposed assay unambiguously detected gene expression differences less than onefold (Fig. 1), and the amperometric response of TP53 was reliably distinguished from that of the original test solution after a mRNA increase of only 0.80-fold. This provides greater accuracy in the identification of differentially expressed genes and decreases the need for excessive replicates. In addition, with a detection limit that is at least 1000-fold lower than those of fluorescence-based assays, the proposed method dramatically decreases the amount of starting mRNA needed from micrograms to nanograms.

mRNAs from eight breast cancer samples and three healthy samples were tested on the sensor array. GAPDH cDNA was used as an internal control. The expression of each selected gene was quantified on the sensor array (10 duplicates for each gene and a total of 14 controls) and was calculated as a ratio relative to GAPDH in the healthy tissues. As can be seen in Table 1, underexpression for BRCA1 and His4 and overexpression for HSP90 were observed in the breast cancer tissues, whereas TP53 showed little difference between the cancer and healthy tissues. This reflects the role of TP53 mutations and not variations in gene expression in the cancer tissues because mutated TP53 gene was also captured by the sensor array. To differentiate the most frequent mutations, capture probes with unique (mutated) sequences must be used. As shown in Table 1, these results are in good agreement with those obtained by conventional RPA as well as the results reported in earlier studies (12–14). Useful clinical information can be drawn only from a statistically valid number of samples in connection with a patient's medical history. Progress in this area is being made in our laboratory in collaboration with hospitals. Nonetheless, the data reported here serve the purpose of proof of principle, confirming the applicability of the sensor array to direct analysis of clinical samples.

In summary, the electrochemical sensor array described

![Fig. 1. Dependence of amperometric responses (six duplicates) on concentration of TP53 mRNA.](image)

**Table 1. Gene expression, as assessed by mRNA quantification, in human breast tissues.***

<table>
<thead>
<tr>
<th>Cancer tissue</th>
<th>TP53</th>
<th>HSP90</th>
<th>BRCA1</th>
<th>His4</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>This method</td>
<td>0.55</td>
<td>1.78</td>
<td>0.14</td>
<td>0.16</td>
<td>0.94</td>
</tr>
<tr>
<td>RPA</td>
<td>0.53</td>
<td>1.84</td>
<td>0.14</td>
<td>0.18</td>
<td>0.97</td>
</tr>
<tr>
<td>Healthy tissue</td>
<td>0.52</td>
<td>0.36</td>
<td>0.23</td>
<td>0.31</td>
<td>0.98</td>
</tr>
<tr>
<td>This method</td>
<td>0.54</td>
<td>0.36</td>
<td>0.24</td>
<td>0.32</td>
<td>0.97</td>
</tr>
<tr>
<td>RPA</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Results are given as ratios of gene-specific mRNA to GAPDH mRNA.

* Mean (SD) of eight breast cancer tissue samples.

* RPA were performed according to a previously described protocol (15).

* Mean (SD) of three healthy tissue samples.
here is capable of directly detecting expression of breast cancer susceptibility genes from mRNA extracts with high sensitivity and specificity without RT-PCR amplification. Gene expression differences less than onefold were successfully detected. The lowest detectable amount of a specific gene was found to be ~800 copies in 1.5 ng of mRNA. Integration of the sensor array into a fully automated microelectromechanical system, from tissue to nucleic acid isolation and quantification, may provide faster, less expensive, and simpler solutions for molecular diagnosis. Development of such an integrated system is being undertaken in our laboratory.

References


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The ABL tyrosine kinase inhibitor imatinib (STI571, Gleevec, Gleevec) has produced dramatic clinical responses in most patients with chronic myeloid leukemia (1–4). However, drug resistance has arisen as a result of increased expression of the BCR-ABL oncogene or the emergence of clones of cells harboring mutations in the ABL portion of the gene that reduce drug binding while retaining aberrant kinase activity (5). Original observations suggested that one specific substitution, T315I, was responsible (6). However, subsequent reports have demonstrated that this is not the case (7–13).

To monitor the emergence of drug resistance in patients treated with imatinib, there is a need to develop a reliable method for the screening of mutations in the BCR-ABL oncogene (5, 7). We report here the use of denaturing HPLC (DHPLC) as a method to screen for mutations in exons 4 and 6 of the ABL gene. Although originally described more than 6 years ago, the widespread application of this technique has only recently become possible with the introduction of commercially available specialized HPLC instrumentation dedicated to the performance of mutation analysis (14). Previous reports have demonstrated that the sensitivity and specificity of the technique are consistently high (15, 16).

Peripheral blood samples were obtained, after receipt of informed consent, from 22 patients with accelerated phase or blast crisis chronic myeloid leukemia. Genomic DNA from control samples was obtained from the WestLakes Research Institute at Cumbria after appropriate ethical consent. Genomic DNA was extracted by a standard phenol–chloroform method. PCR was performed with 50–100 ng of DNA, 2.5 mM MgCl2, 100 μM deoxynucleotide triphosphates, 0.2 μM each of forward and reverse primer, 1.25 U of Taq polymerase (AmpliTaq Gold; Perkin-Elmer), and 1× buffer. Primer pairs were designed with use of Omiga Software (Accelyrs Inc) to flank exons 4 and 6 of the ABL gene, based on criteria recommended by Transgenomic. The primer sequences were as follows: exon 4 forward, 5′-CTG TCT TGG GCT GAA-3′, and exon 4 reverse, 5′-AAA CAC ACT CGG ACT ATG AGA A-3′; exon 6 forward, 5′-GAC TGA GCA GCA GAG TCA GA-3′ and exon 6 reverse 5′-GCC AGC ACT GAG GTT AGA A-3′. PCR conditions were as follows: 10 min of denaturation at 95°C; a touchdown protocol of 20 s at 94°C, 1 min at 66°C (exon 4) or 63°C (exon 6), and 1 min at 72°C (−0.5°C for 14 cycles); and 20 cycles of 20 s at 94°C, 1 min at 59°C (exon 4) or 56°C (exon 6), and 1 min at 72°C, with a final extension for 7 min at 72°C. Heteroduplexes were formed in a thermal cycle by denaturation (95°C for 5 min) and annealing/stabilization (starting at 94°C for 2 min with a −1°C touchdown until 4°C). DHPLC was performed in a Trangenomic WAVE mutation detection apparatus. Samples (10 μL) were eluted from a DNAsep column by a gradient of 250 mL/L acetonitrile in 0.1 mol/L triethylammonium acetate (buffer B) against 0.1 mol/L triethylammonium acetate.