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Combination of PCR Subtraction and cDNA Microarray for Differential Gene Expression Profiling

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ABSTRACT

PCR subtraction hybridization has been used effectively to enrich and single out differentially expressed genes. However, identification of these genes by means of cloning and sequencing individual cDNAs is a tedious and lengthy process. In this report, an attempt has been made to combine the use of PCR select cDNA subtraction hybridization and cDNA microarrays to identify differentially expressed genes using a nonradioactive chemiluminescent detection method. mRNA from human prolactin (hPRL) or human prolactin antagonist (hPRL-G129R) treated and non-treated breast cancer cells was isolated, and cDNAs were synthesized and used for the PCR subtraction to enrich the differentially expressed genes in the treated cells. The PCR-amplified and sub-

tracted cDNA pools were purified and labeled using the digoxigenin method. Labeled cDNAs were hybridized to a human apoptosis cDNA microarray membrane and identified by chemiluminescence. The results suggest that the strategy of combining all three methods will allow for a more efficient, nonradioactive way of identifying differentially expressed genes in target cells.

INTRODUCTION

Our previous studies have shown that hPRL demonstrates a stimulatory effect on human breast cancer cell proliferation (1). We have also reported that an hPRL mutant with a single amino acid substitution mutation at position 129 (hPRL-G129R) acts as an hPRL receptor antagonist on human breast cancer cells (1). Further investigation of hPRL-G129R demonstrated that its inhibitory effects on breast cancer cells are through the induction of apoptosis (1). However, the exact mechanism of hPRL-G129R-induced apoptosis is still awaiting further investigation.

To study the physiological mechanisms of different cell types and of cells under different conditions, PCR subtraction hybridization has been used widely over the years (4-6). This technique gives a representation of differentially expressed genes from one group of cells as compared with another. The theory behind the technique is very simple. It first uses mRNA from two populations of cells and converts them into cDNA. The cDNA from cells that contain differentially expressed genes is referred to as the "tester", and the reference cDNA is referred to as the "driver". Both tester and driver cDNAs are first digested using a 4 base-cutter restriction enzyme to create shorter blunt-ended molecules. The ends of the tester cDNAs are modified by ligating adaptors that will serve as PCR primers. The tester cDNAs are then hybridized with driver cDNAs, which have no adaptors on their ends. Suppression PCR, using the adaptors as primers, is then performed to allow exponential amplification of the differentially expressed genes.

Identifying these genes by the use of conventional methods such as cloning, sequencing, and northern blot analysis is a tedious and expensive process (6,9).

Recently, the vast emergence of the cDNA microarray techniques greatly expands the ability of researchers to identify previously cloned sequences in pools of cDNAs. This technique has proved to be an essential tool when trying to identify which genes are responding to a certain condition (6,9). One of the drawbacks of using directly isolated mRNA/cDNA as probes in screening commercial membranes is a high signal-to-noise ratio (2). In this study, we combine the use of suppression subtractive hybridization to enrich differentially expressed cDNAs upon treatment with hPRL and hPRL-G129R. We will then identify the differentially expressed cDNAs by the use of microarray technology. Also, nucleotide hybridization detection without the use of radioisotopes is of interest to many researchers (3,7,10,11). Therefore, we employed the use of a nonradioactive chemiluminescent system,

digoxigenin (DIG), to label the cDNAs as an alternative way of identifying genes on the microarray membrane.

MATERIALS AND METHODS

Cell Lines and Growth Conditions

The T-47D human breast cancer cell line was obtained from ATCC (Manassas, VA, USA). Cells were maintained in phenol red-free RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) (both from Invitrogen, Carlsbad, CA, USA) and grown at 37°C in an atmosphere containing 5% CO₂. Before treatment with hPRL or hPRL-G129R, cells were split into three groups (10 T75 flasks were used for each group) and depleted with RPMI 1640 supplemented with 10% Charcoal/Dextran-treated FBS (CSS) for six days until cells reached 80%

confluency. After depletion, approximately 10⁸ cells from each group were treated with either 500 ng/mL hPRL (Tester A) (hPRL was kindly supplied by Dr. A.F. Parlow, National Hormone and Pituitary Program, NIH, USA) in RPMI 1640 supplemented with 1% CSS or 500 ng/mL of hPRL-G129R (Tester B) (hPRL-G129R was produced in our laboratory; Reference 8) or cultured with 1% CSS alone as the untreated control (Driver). Cells were treated for 48 h, and then mRNA was isolated in the following section.

PCR cDNA Subtraction Hybridization

mRNA isolation was performed using the Micro-Fast Track™ 2.0 kit from Invitrogen according to the manufacturer's instructions. RNA yield was determined by measuring absorbance at 260 nm. Subtractive hybridization was per-

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formed using the PCR Select™ cDNA Subtraction Kit from Clontech Laboratories (Palo Alto, CA, USA). Briefly, 10 µg mRNA were used for synthesizing Tester A, Tester B, and Driver cDNAs. Restriction enzyme digests of the cDNAs, adaptor ligation of the testers, and two rounds of hybridization between tester and driver were carried out following the manufacturer's instructions. After the hybridizations were complete, primary PCR was used to amplify the products. Conformation of the subtractions was performed on the primary PCR products using the primers for the housekeeping gene, G3PDH, supplied in the kit.

Generation of enriched cDNAs, which will be labeled and used as probes for screening the microarray, were prepared with a secondary PCR. Four 50-µL reactions were performed and pooled. cDNAs were purified using QIAquick™ PCR Purification Kit (Qiagen, Valencia, CA, USA). DNA yield was determined by measuring the absorbance at 260 nm.

Labeling of cDNA Probes

Purified cDNAs from the PCR subtraction hybridization were randomly primed and labeled with DIG-dUTP, alkali-labile DNA Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany). Three micrograms of cDNA were labeled according to the manufacturer's protocol and incubated for more than 20 h. An overnight incubation was performed, as previous results have demonstrated, to ensure an efficient yield of newly synthesized DIG-labeled DNA.

cDNA Microarray

Atlas™ Human Apoptosis Arrays from Clontech Laboratories containing all currently known apoptosis related genes on a nylon membrane were pre-hybridized with DIG Easy Hyb® solution (Roche Molecular Biochemicals) at 37°C for 2 h in a hybridization incubator with gentle rotation. Three micrograms of DIG-labeled probes were purified and resuspended in 20 µL distilled water. The probes were boiled for 10 min and quickly chilled on ice for ≥ 5 min. After pre-hybridization, 20 µL DIG-labeled probe were added to

the microarray membrane in which 5 mL fresh DIG Easy Hyb had been added. Membranes were hybridized overnight at 68°C in a hybridization incubator with gentle rotation. The following day, membranes were washed at 38°C twice (5 min/wash) in 2× standard saline citrate (SSC), 1% SDS, and twice at 68°C (15 min/wash) in 0.1× SSC, 0.5% SDS.

Chemiluminescent Detection

Hybridized Atlas membranes were developed using the DIG Luminescent Detection Kit (Roche Molecular Biochemicals) according to the manufacturer's specifications. CSPD® (Roche Molecular Biochemicals) was used as the chemiluminescent substrate. After 5 min of incubation with CSPD, membranes were wrapped in plastic wrap, placed in an autoradiography cassette, and incubated at 37°C for 15 min. The membrane was then exposed to Kodak® Biomax™-MR film (Eastman Kodak, Rochester, NY, USA) at room temperature for various amounts of time to obtain an optimal exposure.

RESULTS AND DISCUSSION

In this report, we successfully combined three proven effective commercially available methods to profile genes in breast cancer cells in response to various treatments using nonradioactive techniques.

Figure 1. Efficiency test of the PCR select subtraction hybridization. The housekeeping gene G3PDH (approximately 400-bp fragment) amplified for 12 cycles with primers provided from PCR Select cDNA Subtraction Hybridization kit on a 1% agarose/ethidium bromide gel. Lane 1 is λ DNA/*Hind*III-digested molecular weight marker. Lanes 2 and 4 are subtracted Testers A and B, respectively, and lanes 3 and 5 are the unsubtracted tester controls.

When performing the mRNA isolation, we have found that it is important to start with at least 10^8 cells to obtain sufficient mRNA for completing the experiment. We have found that a minimum of 10 µg mRNA should be used (instead of 2 µg as recommended) to produce the optimal amount of cDNAs for the remainder of the experiment. Once the primary and secondary PCRs are completed, an efficiency test must be performed to verify the subtraction efficiency. As shown in Figure 1, after two separate PCR runs were performed, the G3PDH was greatly reduced in the subtracted samples as compared to the unsubtracted.

To obtain enough cDNAs to be used for labeling and probing of the microarray, we recommend pooling multiple secondary PCRs together when making the cDNA probes. Figure 2 shows the results of the cDNA enrichment reactions. The cDNA shown on the gel represents 1/10 of the purified pool of cDNAs obtained from the final PCR product (keep in mind that housekeeping genes such as G3PDH could not be amplified after subtraction; see Figure 1). A total of 3 µg amplified final PCR-produced cDNAs was used for labeling with DIG to increase the amount of genes to be labeled properly.

To search for any apoptosis-related genes present in the cDNA pools after

Figure 2. Analysis of enriched cDNAs from PCR select subtraction hybridization. After the secondary PCR, a sample of the purified hPRL-specific cDNAs (lane 2) and hPRL-G129R-specific cDNAs (lane 3) was run on a 1% agarose/ethidium bromide gel. Lane 1 is λ DNA/*Hind*III-digested molecular weight marker.

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Figure 3. Microarray hybridized with enriched subtracted cDNAs. Human apoptosis cDNA microarrays were hybridized with DIG-labeled, hPRL-specific cDNAs (A) or hPRL-G129R-specific cDNAs (B). A section of the hPRL-specific array (a) or hPRL-G129R-specific array (b) was enlarged for comparison purposes. We have identified that cDNAs represented by 10F in the hPRL-treated cancer cells and various others in the hPRL-G129R-treated cells are of special interest.

hPRL or hPRL-G129R treatments, an Atlas human apoptosis cDNA microarray containing 205 apoptosis related genes was used. Figure 3 shows the results after hybridization with the DIG-labeled cDNAs. There are distinct differences found with the treatment of either hPRL or hPRL-G129R. Many apoptosis-related genes are being expressed in the hPRL-G129R-treated cells, as would be expected since previous experiments showed it to induce apoptosis. Various caspases are shown in Figure 3, lane 11. There is no presence of these genes in the hPRL-treated cDNAs, as shown in Figure 3.

These results prove that, by combining the use of PCR select cDNA subtraction hybridization, cDNA microarrays, and chemiluminescent detection, one is able to detect and identify differentially expressed genes. This technique is valuable in that it can be performed with any desired cell lines, with any treatment of the researcher's choice, and probed using any variation of cDNA microarray

membranes. These methods together will allow researchers the ability to target and study specific differentially expressed genes in a cost-efficient and environmentally friendly manner.

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