Use of SMARTTM-Generated cDNA for Gene Expression Studies in Multiple Human Tumors

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ABSTRACT

We demonstrate here that SMARTTM PCR-amplified cDNAs arrayed on a nylon membrane are suitable for high-throughput tissue expression profiling when starting biological materials are limited. We show that SMART cDNA accurately reflects gene expression patterns found in total RNA by comparing the expression level of several target genes in SMART PCR-amplified cDNAs and their corresponding total RNAs. We also arrayed cDNAs from 68 matched tumor and normal samples on a nylon membrane to determine whether SMART PCR-amplified cDNA could be used for detecting differentially expressed genes in these tissues. These arrays containing normalized tumor and normal cDNAs were hybridized with probes for glutathione peroxidase and gelsolin. The hybridization results revealed cancer-related and patient-specific gene expression differences between tumor and normal tissues for these genes. These studies show that SMART PCR-amplified cDNAs maintain the complexity of the original mRNA population and are thus suitable for high-throughput studies to compare the relative abundance of target genes and to detect differentially expressed genes in a wide variety of tissues simultaneously.

INTRODUCTION

Identifying differentially expressed genes is a common starting point in understanding the molecular mechanisms underlying biological processes including differentiation, development, and tumorigenesis. Techniques currently used to identify differentially expressed genes include (i) subtractive

hybridization (11,18,28), (ii) differential display (20,22), (iii) serial analysis of gene expression (SAGE) (31), and (iv) cDNA microarrays (10,25). These techniques usually require verification steps to prove differential expression of identified genes. Two different approaches are often used for verification: RT-PCR and northern blotting. RT-PCR is a sensitive method and commonly used for detecting differentially expressed genes; however, it can be laborious when many genes and samples are analyzed simultaneously. Northern blot analysis of differential gene expression is also time consuming and typically requires 10 µg total RNA or 1–5 µg poly(A)⁺ RNA.

Acquiring sufficient RNA when working with clinical samples such as biopsies, microdissected tumors, and laser-captured cells is very difficult. The use of such tissues is becoming more common in clinical research, and methods for amplifying small amounts of RNA while maintaining the original mRNA representation are extremely valuable.

Generation and subsequent amplification of cDNA is a potential method for overcoming the obstacle of limited tissue samples. However, amplification of cDNA requires the presence of primer binding sites at both cDNA ends. These primer binding sites can be attached by (i) homopolymer tailing on the 3' end of the first-strand cDNA (2,5,12), (ii) single-stranded anchor ligation to the 5' end of mRNA (21,27) or to the 3' end of the first-strand cDNA (3), and (iii) double-stranded adaptor ligation to the 5' end of double-stranded cDNA (15). Each of these approaches requires several inefficient and complex manipulations.

SMARTTM PCR cDNA synthesis provides an elegant method for amplifying cDNA samples. SMART cDNA technology combines, in one step, first-strand cDNA synthesis and attachment of anchor sequences at the 5' and 3' ends of single-stranded cDNA. This is achieved by utilizing two intrinsic properties of Moloney murine leukemia virus (MMLV) reverse transcriptase: (i) the ability to add nontemplated nucleotides

(predominantly dC) to the 3' end of the first-strand cDNA and (ii) the ability to switch templates (6,7,19). In SMART cDNA synthesis, the SMART oligonucleotide, which has an oligo (G) sequence at its 3' end, anneals to the dC-rich cDNA tail and serves as an extended template for reverse transcriptase. When reverse transcriptase reaches the 5' end of the mRNA, the enzyme switches templates and continues replicating to the end of the SMART oligonucleotide. The resulting first-strand cDNA contains the complete 5' end of the mRNA as well as sequences that are complementary to the SMART oligonucleotide. The SMART anchor sequence then serves as a universal PCR primer binding site for amplifying the entire cDNA population.

SMART PCR-generated cDNA has been successfully used for cDNA library construction (6) and for the PCR-based suppression subtractive hybridization (11). SMART cDNA can also be used as a substitute for northern blot analysis (6,13, 14). This approach termed as "virtual northern" is very useful for researchers who wish to analyze transcript size and expression patterns but lack sufficient RNA for northern blots. SMART-generated cDNA can also be used instead of RNA as a hybridization probe for cDNA and oligonucleotide-based microarrays when starting material is limited (17,24,32).

We report that SMART PCR-generated cDNA retains the original mRNA message profile and, therefore, is a suitable material for high-throughput tissue expression profiling in multiple tissues when arrayed on a nylon membrane.

MATERIALS AND METHODS

The following RNA sources were used for SMART cDNA synthesis: human total RNA from liver, placenta, whole brain, kidney, lung, spleen, testis, fetal brain, thymus, pancreas, prostate, and HeLa cells. Total RNA from cancer cell lines: human (Burkitt's) lymphoma (Daudi), human (chronic myelogenous) leukemia (K-562), human (promyelocytic) leukemia (HL-60), human melanoma (G361), human lung carcinoma (A549), human (lymphoblastic) leukemia (MOLT-4), human colorectal adenocarcinoma (SW480), and human (Burkitt's) lymphoma (Raji) (all from ATCC, Manassas, VA, USA). Matched human tumor and normal total RNA samples from the same patients: breast, kidney, lung, uterus, cervix, ovary, prostate, stomach, colon, rectum, and small intestine (all from Clontech Laboratories, Palo Alto, CA, USA).

SMART PCR cDNA Synthesis

Total RNAs were reverse-transcribed using the SMART PCR cDNA Synthesis Kit (Clontech Laboratories) according to the user manual, with some modifications. Briefly, 1 µg total RNA was mixed with 1 µL 10 µM oligo(dT) (CDS primer), 1 µL 10 µM SMART II Oligonucleotide, and deionized water for a total volume of 5 µL. For annealing, this mixture was heated at 72°C for 2 min, cooled on ice for 2 min, and spun briefly. The reaction was followed by the addition of 2 µL 5× Reaction Buffer (250 mM Tris-HCl, pH 8.3, 30 mM MgCl₂, and 375 mM KCl), 1 µL 20 mM dithiothreitol (DTT), 1 µL 10 mM dNTP (10 mM each dATP, dCTP, dGTP, and dTTP) (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 0.5 µL 100 U/µL PowerScript™ reverse transcriptase (Clontech Laboratories), and 0.5 µL 28 U/µL ANTI-RNase™ (Ambion, Austin, TX, USA). Samples were incubated at 42°C for 1 h in an air incubator, followed by inactivation of reverse

transcriptase at 72°C for 7 min. Then, 40 µL TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) were added to each sample, which was subsequently stored at -20°C. To determine the number of PCR cycles necessary for optimal amplification of cDNA, 1 µL from each first-strand cDNA reaction mixture was combined with 10 µL 10× Advantage™ Polymerase Buffer (Clontech Laboratories) [40 mM Tricine-KOH, pH 9.2, 15 mM KOAc, 3.5 mM Mg(OAc)₂], 1 µL PCR primer (5'-AAGCAGTGGTAACAACGCAGAGT-3'), 2 µL 10 mM dNTP (10 mM each dATP, dCTP, dGTP, and dTTP), and 1 µL Advantage cDNA Polymerase Mix (Clontech Laboratories). Samples were then amplified in a PTC 200 thermal cycler (MJ Research, Watertown, MA, USA) using the following program: 1 cycle at 95°C for 1 min, then 15 cycles at 95°C for 15 s, 65°C for 30 s, and 68°C for 6 min. After 15 cycles, 15 µL of the reaction mixture was transferred to a fresh 0.5-mL tube and subjected to three additional cycles, while the remaining 85 µL of the PCR mixture was kept at 4°C. After three additional cycles, 5 µL reaction mixture was aliquoted for gel analysis, while the remaining 10 µL of the reaction mixture was subjected to another three cycles for a total of 21 cycles. To determine the optimal number of cycles, 5 µL of each of the reaction mixture (i.e., 15-, 18-, and 21-cycle PCR products) were analyzed on a 1.1% agarose gel in Tris-acetate EDTA (TAE) buffer. Based on results of the agarose gel analysis, samples were subjected to additional cycles as necessary.

RT-PCR

We performed the RT-PCR of specific gene fragments using total RNAs of matched tumor and normal samples using the Advantage RT-for-PCR Kit (Clontech Laboratories). Briefly, 1 µg total RNA with 1 µL 20 µM oligo(dT)₁₈ primers was heated at 70°C for 2 min, then quenched rapidly on ice. The RT reaction was followed by adding 4 µL 5× Reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 1 µL dNTP mixture (10 mM each dATP, dGTP, dCTP, and dTTP), 20 U recombinant RNase inhibitor, 200 U MMLV reverse transcriptase in a 20-µL reaction volume. Samples were incubated at 42°C for 1 h in an air incubator, followed by inactivation of reverse transcriptase at 95°C for 5 min. Then, 80 µL nuclease-free water was added to a final volume of 100 µL. We used 5 µL diluted cDNA for each 50-µL reaction. PCR was performed using Advantage cDNA Polymerase Mix according to the following program: 1 cycle at 94°C for 4 min, then 20 cycles at 94°C for 30 s, 68°C for 1.5 min, and 1 cycle at 68°C for 5 min. After 20 PCR cycles, 5 µL of the PCRs were analyzed on a 2% TAE agarose gel, and based on the gel results, additional cycles were performed.

Normalization of matched tumor and normal cDNA samples for RT-PCR was performed by PCR using primers for ubiquitin (UBI) (5'-AGTCCACCCTGCACCTGGTTCTCCG-3' and 5'-CCTCAAGCGCAGGACCAAGTGCAGAG-3'). Normalized amounts of cDNA were then used to perform PCR with primers for glutathione peroxidase (GP) (5'-CATCTGACCGCTCTTCTGG-3' and 5'-TGGGGCCTTGAGTGATAGGA-3'), and gelsolin (GSN) (5'-TTTTTGCAGGGCTATT-TTTGGA-3' and 5'-GCTTTGAGCCTCCCTCCTTT-3').

Preparation of cDNA Array Membranes

cDNAs were purified using the NucleoTrap® PCR Purifi-

cation Kit (Clontech Laboratories) and eluted in 55 μ L TE buffer. One microgram of total RNA yields up to 10 μ g purified SMART cDNA.

Prenormalized cDNA arrays were prepared by spotting 50 ng cDNA on Nytran® Nylon membranes (Schleicher & Schuell, Keene, NH, USA) in a 96- or 384-well format using a Biomek® 2000 laboratory automation workstation (Beckman Coulter, Fullerton, CA, USA). Each pair of matched tumor and normal cDNA was then normalized independently as follows. Three arrayed membranes were probed separately with the following housekeeping genes: UBI, ribosomal protein S9 (RPS9), and 23 kDa highly basic protein (23 kDa) (1,8,9,23). The hybridization signals were quantified by phosphorimaging. The percentage (P) that each cDNA sample in a pair contributed to the sum of the signals of both members of the pair was calculated for all three housekeeping genes. A normalization factor (N_F) was calculated for each matched pair by dividing the sum of the percentages of signal intensity for normal cDNA dots ($P_{N-UBI} + P_{N-23kD} + P_{N-RPS9}$) by the sum of the percentages of signal intensity for matching tumor cDNA dots ($P_{T-UBI} + P_{T-23kD} + P_{T-RPS9}$). The calculated normalization factor was then used to raise or decrease the amount of either normal or tumor cDNA to achieve a comparable value for both dots (see References 8 and 9).

Normalized amounts of cDNAs were then spotted on nylon membranes along with nine cancer cell line cDNAs (50 ng/spot); negative controls (50 ng/spot) including yeast total RNA (NC-1), yeast tRNA (NC-2), *E. coli* DNA (NC-3), poly r(A) (NC-4), human C₀t-1 DNA (NC-5), and human genomic DNA (NC-6); and UBI cDNA (10 ng/spot) as a positive control.

Hybridization of cDNA Arrays

cDNA probes were labeled using DECAprime™ II DNA labeling kit (Ambion) and [α -³²P]dATP (Amersham Pharmacia Biotech) according to the user manual. cDNA membranes were prehybridized and then hybridized overnight at 68°C with radiolabeled cDNA probes in a hybridization bottle containing ExpressHyb™ Hybridization Solution (Clontech Laboratories), 100 μ g/mL sheared salmon sperm DNA, and 1–2 $\times 10^6$ dpm/mL labeled probe. cDNA membranes were washed three times with 2 $\times$ standard saline citrate (SSC), 1% SDS for 30 min, once with 0.2 $\times$ SSC, 0.5% SDS for 30 min, then rinsed in 2 $\times$ SSC, and exposed to Biomax® MS X-ray film with an intensifying screen (Eastman Kodak, Rochester, NY, USA). Signal intensities were calculated for individual spots using a Storm™ 860 PhosphorImager® (Amersham Pharmacia Biotech).

RESULTS

To determine whether SMART PCR-generated cDNA accurately represents the original mRNA population, we first amplified cDNAs using SMART technology from five human total RNA samples purified from liver, placenta, brain, kidney, and HeLa cells. Fifty nanograms of the purified cDNA were spotted on nylon membranes alongside 1 μ g each corresponding total RNA. Arrayed membranes were hybridized with labeled cDNA probes for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Figure 1 A) and vascular endothelial growth factor (VEGF) (Figure 1B). Signal intensities on the membranes were then quantified by phosphorimaging. Both experiments reveal a strong correlation between the rel-

ative signal intensities of total RNA and corresponding SMART cDNA in all five different tissues (Figure 1, bottom rows). The results show that 50 ng SMART cDNA produce the same gene expression profile across the samples as 1 μ g of the original total RNA samples, while yielding a higher sensitivity of detection.

To evaluate whether SMART cDNA yields signals that are both specific and quantitative, we performed the following

Figure 1. Array of SMART cDNAs and original total RNA samples probed with G3PDH (A) and VEGF (B). Samples (50 ng) of cDNAs and 1 μ g corresponding total RNAs from five different tissues were spotted on nylon membranes. Membranes were hybridized with radiolabeled probes for G3PDH (A) and VEGF (B). Hybridization signals were detected by phosphorimaging. Due to the different hybridization characteristics of cDNA and RNA, hybridization signal values for both G3PDH and VEGF were quantified and represented as a percentage of the total signal from all five spots for either cDNA or RNA (bottom rows). 1, liver; 2, placenta; 3, brain; 4, kidney; and 5, HeLa cells.

experiment. We synthesized SMART cDNA from total RNA from the following 12 human tissues: spleen, placenta, kidney, lung, adult liver, testis, whole brain, fetal liver, thymus, pancreas, prostate, and HeLa cells. Purified cDNA (50, 30, and 10 ng each) were arrayed on a nylon membrane. The membrane was hybridized with a cDNA probe for the liver-specific gene, tyrosine aminotransferase (TAT) (29). Figure 2 shows that TAT specifically hybridizes with only cDNAs synthesized from adult liver and fetal liver (panel A). Figure 2 also demonstrates a near linear correlation between each hybridization signal intensity and the spotted amount of cDNA (panel B). Additionally, these experiments show that SMART cDNAs generate much stronger hybridization signals than corresponding total RNAs providing higher sensitivity for detecting the expression of low-abundance RNA transcripts.

Our next goal was to show that arrays of SMART PCR-generated cDNA from multiple matched tumor and normal tissues could be used for high-throughput tissue expression profiling. We generated SMART cDNAs from 68 matched tumor and normal total RNAs from the following 11 tissues: breast, kidney, lung, uterus, cervix, ovary, prostate, stomach, colon, rectum, and small intestine. Each tumor SMART cDNA was then independently normalized to its matched normal cDNA to ensure valid comparisons. For normalization, 50 ng each matched cDNA were spotted on the membrane, and the three arrayed membranes were separately hybridized with the following housekeeping genes: UBI, RPS9, and 23 kDa highly basic protein. Based on the relative intensities of hybridization signals for these three genes, we adjusted the amount of each cDNA for spotting on nylon membranes (see Materials and Methods).

Next, we printed the normalized amount of each cDNA on

a nylon membrane along with cancer cell line cDNAs, negative, and positive controls. These normalized membranes were then used to perform differential gene expression studies in normal and cancer tissues using probes for GP and GSN. The expression of GP was predominantly detected in normal kidney samples with down-regulation in kidney tumors (Figure 3B). The hybridization also indicates down-regulation of GP expression in breast, colon, and lung tumors. No GP expression was detected in cancer cell lines. Figure 3B shows the hybridization patterns for GSN. GSN expression was down-regulated in breast tumors as well as prostate and colon tumors. RT-PCR was used to confirm the hybridization results for several of the kidney and uterus samples (Figure 3). The hybridization of these arrayed membranes with GP and GSN showed that SMART cDNAs could be used to detect differentially expressed genes between cancer and normal tissues.

DISCUSSION

Generation of PCR-amplified cDNAs with a high representation of mRNA transcripts is critical for gene expression profiling and for other molecular biology applications. To avoid biased amplification of cDNAs, we restricted PCR amplification to an exponential phase, which is necessary to generate highly representative cDNA (26). In our experience, the optimal number of PCR cycles tends to vary with different templates, amounts of starting material, and type of thermal cycler. Each cDNA synthesis reaction should be monitored on an agarose gel to avoid overcycling of PCR products. In our experiments, the optimal number of cycles is typically one cycle less than is needed to reach plateau.

To determine how accurately SMART PCR-generated cDNA reflects the original mRNA population, we compared the expression level of G3PDH and VEGF in SMART cDNA and corresponding total RNA from five different tissues. SMART cDNAs for this experiment were prepared with the optimized number of PCR cycles. We then quantified the hybridization intensity of each spot and compared cDNA with corresponding total RNA. The results show that 50 ng SMART cDNA preserves the relative abundance of both G3PDH and VEGF that was observed in 1 μ g of the original total RNA (Figure 1). Furthermore, a comparison of hybridization signal intensities of 50 ng/dot of cDNA and 1 μ g/dot of total RNA showed that SMART cDNA yields a 5–10-fold higher sensitivity of detection than the corresponding total RNA.

The specificity of PCR amplification for an entire cDNA population was also considered. Nonspecific amplification can easily be determined when a single gene is amplified, but the issue is more complex for an entire cDNA population. To evaluate the specificity of SMART PCR amplification, we arrayed cDNAs from 12 different tissues on a nylon membrane and hybridized with a tissue-specific gene. Only liver and fetal liver cDNA specifically hybridized with the probe for the liver-specific gene, TAT. Here, we spotted different amounts of cDNAs: 10, 30, and 50 ng (Figure 2A). Phosphorimager quantitation of the hybridization signals generated by the various amounts of cDNAs on a membrane revealed a linear correlation between intensity and cDNA quantity, indicating that the cDNA amounts applied to the membrane did not reach a saturation level (Figure 2B). This experiment also showed the higher sensitivity of cDNA in comparison with 150 ng/dot of corresponding poly(A)⁺ RNA.

Figure 2. Array of different amounts of SMART cDNA hybridized with a TAT probe. (A) cDNAs were prepared from 12 different human total RNA samples. Ten, 30, and 50 ng each purified cDNA were arrayed on a nylon membrane along with 150 ng corresponding poly(A)⁺ RNA. The membrane was hybridized with a radiolabeled probe for TAT and visualized by phosphorimaging. (B) The linear correlation between the intensity of each hybridization signal and the amount of spotted cDNAs for adult liver and fetal liver. 1, spleen; 2, placenta; 3, kidney; 4, lung; 5, adult liver; 6, testis; 7, whole brain; 8, fetal liver; 9, HeLa cells; 10, thymus; 11, pancreas; and 12, prostate.

We also investigated the possibility of using SMART PCR-generated cDNA arrayed on a nylon membrane to detect differentially expressed genes in multiple matched tumor and normal samples from different patients. In addition, we evaluated its potential use for quick confirmation of differentially expressed genes, which are identified by other methods such as microarrays, differential display, and subtractive hybridization.

SMART cDNAs synthesized from total RNAs from normal and tumor samples were normalized against each other using three housekeeping genes (1,23,30). Normalization, based on the relative abundance of housekeeping genes, is necessary for comparative studies but is particularly challenging in cancer and normal tissues because of significant changes in gene expressions in cancer cells (16). We have chosen three housekeeping genes, UBI, RPS9, and 23 kDa, which show a relatively moderate variation in expression between cancer and normal tissues (unpublished data).

Normalized arrays were then hybridized with cDNA probes for genes that have been shown to be involved in cancer. Down-regulation of glutathione peroxidase was previously detected in lung tumors by subtractive hybridization (unpublished data). In fact, our cDNA array reveals the down-regulation of GP in lung tumors of three independent patients. In addition, our hybridization data reveal the down-regulation of GP in every one of kidney, breast, and colon tumors. GSN expression has been shown to be down-regulated in human breast ductal carcinoma by immunohistochemistry using a monoclonal antibody (4). Our matched tumor/normal expression array confirms the down-regulation of GSN in breast tumors and reveals its down-regulation in prostate and colon tumors. RT-PCR results for some of the kidney and uterus cDNA samples confirmed the hybridization results for all three genes (Figure 3).

We conclude that the SMART PCR-generated cDNA

Figure 3. Arrays of normalized matched tumor/normal cDNAs probed for GP and GSN. cDNAs were normalized as described in Materials and Methods. The normalized membranes were then hybridized with radiolabeled probes for GP (A), and GSN (B). Three cDNA pairs from kidney (A2, A4, and A5) and three cDNA pairs for uterus (C2, C4, and C6) (outlined boxes) were used in RT-PCR to confirm the differential expression of GP and GSN (A and B). Signals were visualized by phosphorimaging. M, 1-kb DNA ladder size markers; N, cDNA from normal tissue; T, cDNA from tumor tissue; CC, cancer cell line cDNAs (F1-9, HeLa cells, Daudi, K-562, HL-60, G361, A549, MOLT-4, SW480, and Raji, left to right); UBI, positive control cDNA for UBI (10 ng/spot) on the right top and bottom corners of the membrane. Six negative controls are included on the right of the membrane (NC-1–NC-6; see Materials and Methods). The matched tumor and normal cDNAs are from: kidney (15 pairs, A1–14 and C9), breast (9 pairs, B1–9), prostate (3 pairs, B11–13), uterus (7 pairs, C1–7), ovary (3 pairs, C10–12), cervix (1 pair, C14), colon (11 pairs, D1–11), lung (3 pairs, D13–15), stomach (8 pairs, E1–8), rectum (7 pairs, E10–16), and small intestine (1 pair, E18).

could be beneficial to investigators who only have access to small amounts of tissue (*i*) for quick detection of differentially expressed genes between tumor and normal tissues, (*ii*) to confirm differentially expressed genes identified by other techniques, and (*iii*) to screen for differentially expressed genes in a large number of independent samples.

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