

Direct comparison of microarray gene expression profiles between non-amplification and a modified cDNA amplification procedure applicable for needle biopsy tissues

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Abstract

Global gene expression profiling by cDNA microarray analysis has been used to discover the biomarkers for early diagnosis of various cancers, subclassing cancer type, and prediction of patient's treatment outcome. The information provided by gene expression profiling may contribute to the design of molecular mechanism-based strategies for cancer prevention and/or treatment. However, the standard procedure for cDNA microarray analysis requires 5 µg of good quality total RNA as starting material for each target preparation reaction. Thus, there is a limit for needle biopsy samples, laser capture microdissected tissues, or flow-sorted cells to successfully utilize the microarray technology. In order to profile the gene expression of needle biopsy tissue, we have modified the standard protocol by carrying out cDNA amplification after cDNA synthesis. We compared percentage present calls, absent calls, reproducibility, and concordance in needle biopsy samples processed by standard microarray protocol (cDNA non-amplification method) and our modified protocol (cDNA amplification method). The results showed that cDNA amplification method provided high reproducibility, representation, and concordance with the standard cDNA non-amplification method. We have successfully analyzed the gene expression profiles of needle biopsy tissues using the modified method without significantly changing the expression profiles. These results suggest that the global gene expression profiles of small biopsy samples can be achieved by our modified method to facilitate the analysis of gene expression profiles for clinical application. © 2003 International Society for Preventive Oncology. Published by Elsevier Ltd. All rights reserved.

Keywords: Gene expression; Microarray; Biopsy tissue

1. Introduction

There has been a growing interest generated in the subject of microarray, which makes it possible to analyze a large number of gene expressions simultaneously and rapidly [1]. cDNA microarray provides a window of opportunity for determining the molecular characteristics of various cancers and the molecular effects of anti-cancer agents on cancer cells. This will contribute to the design of molecular mechanism-based strategies for cancer prevention and/or treatment. The gene expression profiles of various cancers have been analyzed by cDNA microarray [2–8]. The alterations of gene expression profiles by several anti-cancer agents have also been reported [9–12].

The high-throughput gene chips (Human Genome U95 and U133 Arrays from Affymetrix), which contain ~12,000 and 23,000 known genes, respectively, are more widely used for profiling gene expression. However, the standard procedure for expression analysis on the Affymetrix arrays requires approximately 5 µg of total RNA as starting material for each target preparation reaction. Thus, there is a limit for needle biopsy samples, laser capture microdissected tissues, or flow-sorted cells to utilize the chips. There are few commercial kits available for small sample microarray; however, no study regarding needle biopsy microarray has been reported that analyzed the data systematically. In order to profile the gene expression of needle biopsy tissues, we have modified the standard protocol by carrying out cDNA amplification after cDNA synthesis, and have successfully analyzed the gene expression profiles of needle biopsy tissues using a modified protocol without significantly changing the expression profiles. Here, we report the comparisons of gene

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expression profiles obtained by standard non-amplification and the modified cDNA amplification methods.

2. Materials and methods

The flowchart for standard method (cDNA non-amplification methods for more than 5 μg of RNA sample) and modified method (cDNA amplification methods for small sample) is presented in Fig. 1.

2.1. Total RNA preparation

The fresh biopsy samples were submerged in RNAlater RNA stabilization reagent (QIAGEN, Valencia, CA) and stored at -70°C until proceeding RNA extraction. Two needle biopsy samples from human mammary tissues were obtained prior to and 24 h after the initiation of doxorubicin-based chemotherapy using 16 gauge biopsy needle from one patient (Harper University Hospital, Wayne State University School of Medicine, Detroit, MI). The samples were labeled as pre or post sample accordingly. PC3 human prostate cancer cells (ATCC, Manassas, VA) were cultured

in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C . The cells were treated with 50 μM genistein or 0.5 mM Na_2CO_3 (vehicle control) for 6 h before RNA extraction. Total RNA from biopsy samples or cultured cells was isolated by Trizol (Invitrogen, Carlsbad, CA) and purified by RNeasy Mini Kit and RNase-free DNase Set (QIAGEN, Valencia, CA) according to the manufacturer's protocols. The extracted RNA was quantified by spectrophotometer, and the quality of the total RNA was analyzed by spectrophotometer and agarose gel electrophoresis to check for degradation or contamination.

2.2. cDNA synthesis and amplification by cDNA amplification methods for small sample

cDNA for each biopsy or cell culture sample was synthesized from 1 μg of total RNA and, then, amplified by using SMARTTM PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) with modification. T7-(dT)₂₄ primer, SMARTTM Oligonucleotide, and PowerScriptTM reverse transcriptase were used for first-strand cDNA synthesis. When RT reaches the 5' end of the mRNA, the enzyme's terminal transferase activity added a few additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA. SMARTTM Oligonucleotide, which has an oligo(G) sequence at its 3' end, base-paired with the deoxycytidine stretch, creating an extended template. Reverse transcriptase then switches templates and continues replicating up to the end of the oligonucleotide. The resulting full-length, single-stranded cDNA contained the complete 5' end of the mRNA, as well as sequences that are complementary to the SMARTTM Oligonucleotide (Fig. 2).

The first-strand reaction mixture was used as template for PCR amplification. The T7-(dT)₂₄ primer and 5' PCR Primer IIA (included in the kit), which has 23 base sequence same as the sequence of SMARTTM Oligonucleotide were served as PCR primers. The first-strand cDNA was amplified in PCR thermal cycler PE480 (Applied Biosystems, Foster City, CA) using following condition: 95°C 1 min, then 30 cycle of 95°C 15 s, 65°C 30 s, 68°C 6 min. The amplified double-stranded cDNA was purified with phenol/chloroform extraction and precipitated with ethanol, and resuspended in 12 μl RNase-free water. The purified cDNA were subjected to in vitro transcription as mentioned below.

2.3. cDNA synthesis by cDNA non-amplification method for more than 5 μg of RNA sample

If the total RNA obtained from biopsy sample or cells is more than 5 μg , the standard protocol from Affymetrix was used for cDNA synthesis. cDNA for each biopsy or cell sample was synthesized by using Superscript cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to the

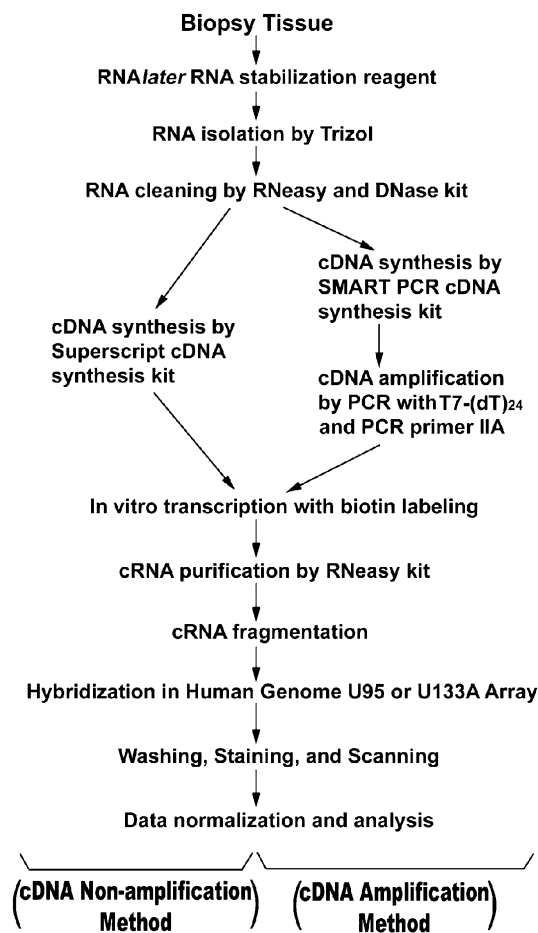


Fig. 1. Flowchart for standard method (cDNA non-amplification method) and modified method (cDNA amplification method).

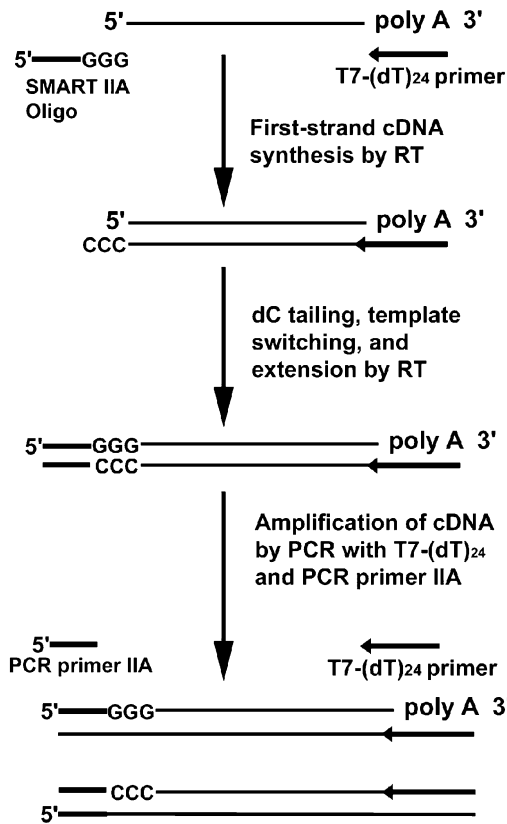


Fig. 2. Flowchart for cDNA synthesis and amplification.

manufacturer's protocol with modification. T7-(dT)₂₄ primer was used in place of the oligo(dT) provided in the kit.

2.4. *In vitro* transcription, array hybridization and scanning

After cDNA synthesis from large scale sample or cDNA synthesis and amplification from small sample, the biotin-labeled cRNA was transcribed *in vitro* (IVT) from cDNA by using BioArray HighYield RNA Transcript Labeling Kit (ENZO Biochem, New York, NY), and purified by RNeasy Mini Kit. The purified cRNA was fragmented by incubation in fragmentation buffer (200 mM Tris-acetate pH 8.1, 500 mM KOAc, 150 mM MgOAc) at 95 °C for 35 min and chilled on ice. The fragmented labeled cRNA was then applied to Human Genome U95 or U133A Array (Affymetrix, Santa Clara, CA), and hybridized to the probes in the array. After washing and staining, the arrays were scanned by using HP GeneArray™ Scanner (Hewlett-Packard, Palo Alto, CA).

2.5. Microarray data normalization and analysis

The gene expression levels of samples were normalized and analyzed by using Microarray Suite, MicroDB™, and Data Mining Tool software (Affymetrix, Santa Clara, CA). The signal value of the post or treatment array was multiplied

by normalization factor to make its mean intensity equivalent to the mean intensity of the pre or control array using Microarray Suite software according to manufacturer's protocol. The absolute call (representing transcript signal; present: transcript is present; marginal: transcript signal is marginal; absent: transcript is undetected) and average difference (representing relative level of transcript expression) of gene expressions in a sample, and the absolute call difference (change of expression; D: decrease of expression; I: increase of expression), fold change (change fold of expression), average difference (difference of transcript expression level between samples) of gene expressions between two or several samples were analyzed using above software. Average-linkage hierarchical clustering of the data was applied by using Cluster [13] and the results were displayed by using TreeView [13]. Correlation statistical analysis for the data obtained from the two methods was accessed by using Pearson Product Moment Correlation Coefficient.

3. Results

3.1. Comparison of absolute call from two methods

To evaluate whether the two methods produced similar data with respect to sensitivity and specificity, the microarray data from either the cDNA non-amplification method for more than 5 µg of total RNA or cDNA amplification method for 1 µg of the same sample RNA were analyzed by using Microarray Suite software. The percentages of probe sets in each experiment called present or absent are shown in Fig. 3. The cDNA amplification samples resulted in 42.83–54.55% present calls and 43.92–55.52% absent calls, comparable to 46.42–54.18% present and 44.81–52.01% absent calls that was routinely obtained from 5 µg of total RNA without cDNA amplification. There were only maximum 2.87% difference in present calls and 3.53% difference in absent calls between two methods using same sample, suggesting that two methods have similar sensitivity and specificity.

3.2. Reproducibility of two methods

To evaluate the reproducibility of the two methods and concordance of detectable transcripts detected by the two different methods, we analyzed the probe sets detected as present using one method and absent using another method. Firstly, we analyzed the present and absent probe sets in two duplicated PC3 control cell samples processed by using same cDNA non-amplification method. We found that there was a total of 11.39% discordant calls between these two experiments using same method (Fig. 4). Then, we compared the discordant in same samples between two methods using RNA extracted from breast biopsies. As demonstrated in Fig. 4, 13.04–13.23% discordant calls were found between two methods in biopsy samples. A similar percentage of discordant calls were also observed in PC3 control cells

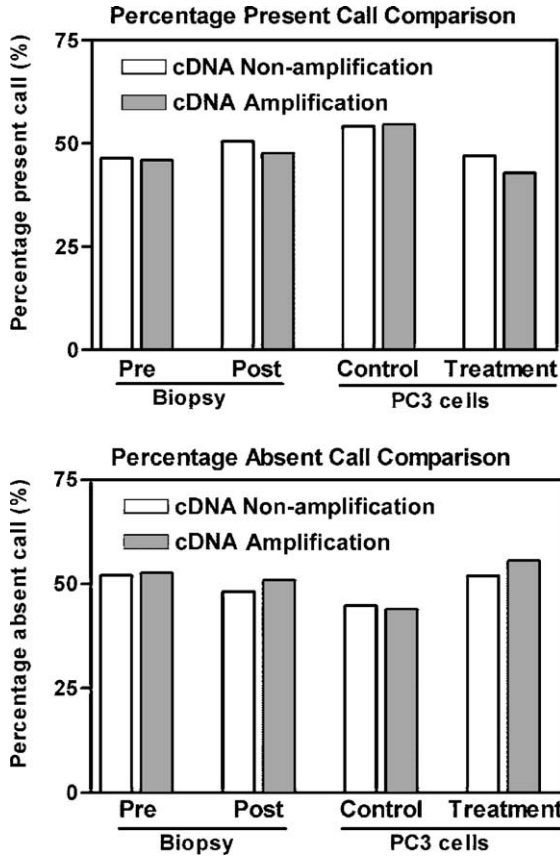


Fig. 3. Percentage present and absent call comparison between two methods. (Pre: biopsy sample before treatment; post: biopsy sample after 24 h of treatment.)

and genistein treated cells using two different methods. These results suggest that the cDNA amplification method performed as good as the standard cDNA non-amplification method with similar reproducibility.

In order to demonstrate whether the cDNA amplification method selectively amplifies only certain transcripts,

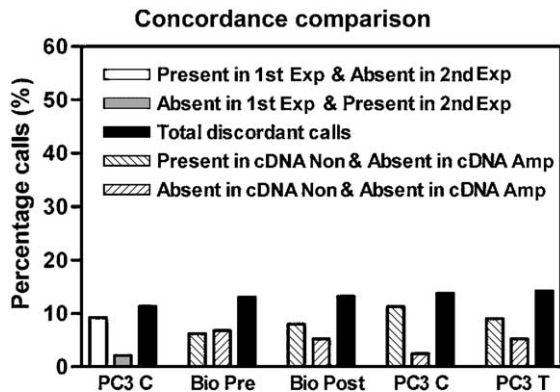


Fig. 4. Concordance comparison between two methods. (Bio pre: biopsy sample before treatment; bio post: biopsy sample after 24 h of treatment; PC3 C: PC3 control cells; PC3 T: PC3 cells treated with genistein; exp: experiment; cDNA non: cDNA non-amplification; cDNA amp: cDNA amplification.)

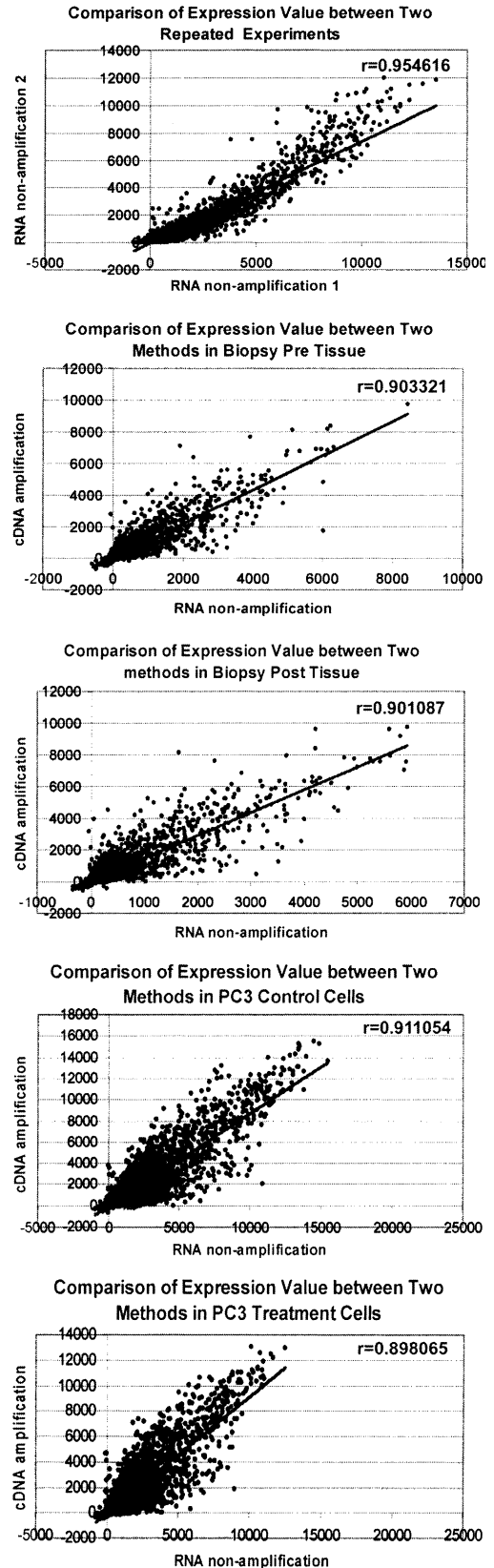


Fig. 5. Expression value comparison between the two methods.

we further analyzed the Absolute Calls of probe sets in same PC3 control cells processed by two different methods. If the cDNA amplification method amplifies only a subset of transcripts effectively, a much higher percentage of probe sets, which were called present by cDNA non-amplification method, may be called absent by the cDNA amplification method since some transcripts may have dropped off. We found that this type of discordant calls remained at around 11%, compare to 9.23% when using the same method (Fig. 4). In contrast, if the cDNA amplification method amplifies transcripts nonspecifically, an increase in the percentage of probe sets called absent by the cDNA non-amplification method, but present by the cDNA amplification method will be apparent. In the present study, the probe sets called absent by the cDNA non-amplification method, but present by the cDNA amplification method also remained

constant at low percentage (2.45%), similar to the percentage found using the same method (2.16%) (Fig. 4), suggesting that two methods have similar sensitivity and specificity.

3.3. Statistical analysis of results obtained from two methods

In order to further analyze the reproducibility of two methods, the average difference values, which represents the degree of expression, of whole set of probe obtained from two methods or two experiments using same method were assessed by the Pearson product moment correlation coefficient. The r value for two duplicated samples processed using same cDNA non-amplification method was 0.954 (Fig. 5). An r of 0.898–0.911 was obtained when comparing average difference values in same sample processed

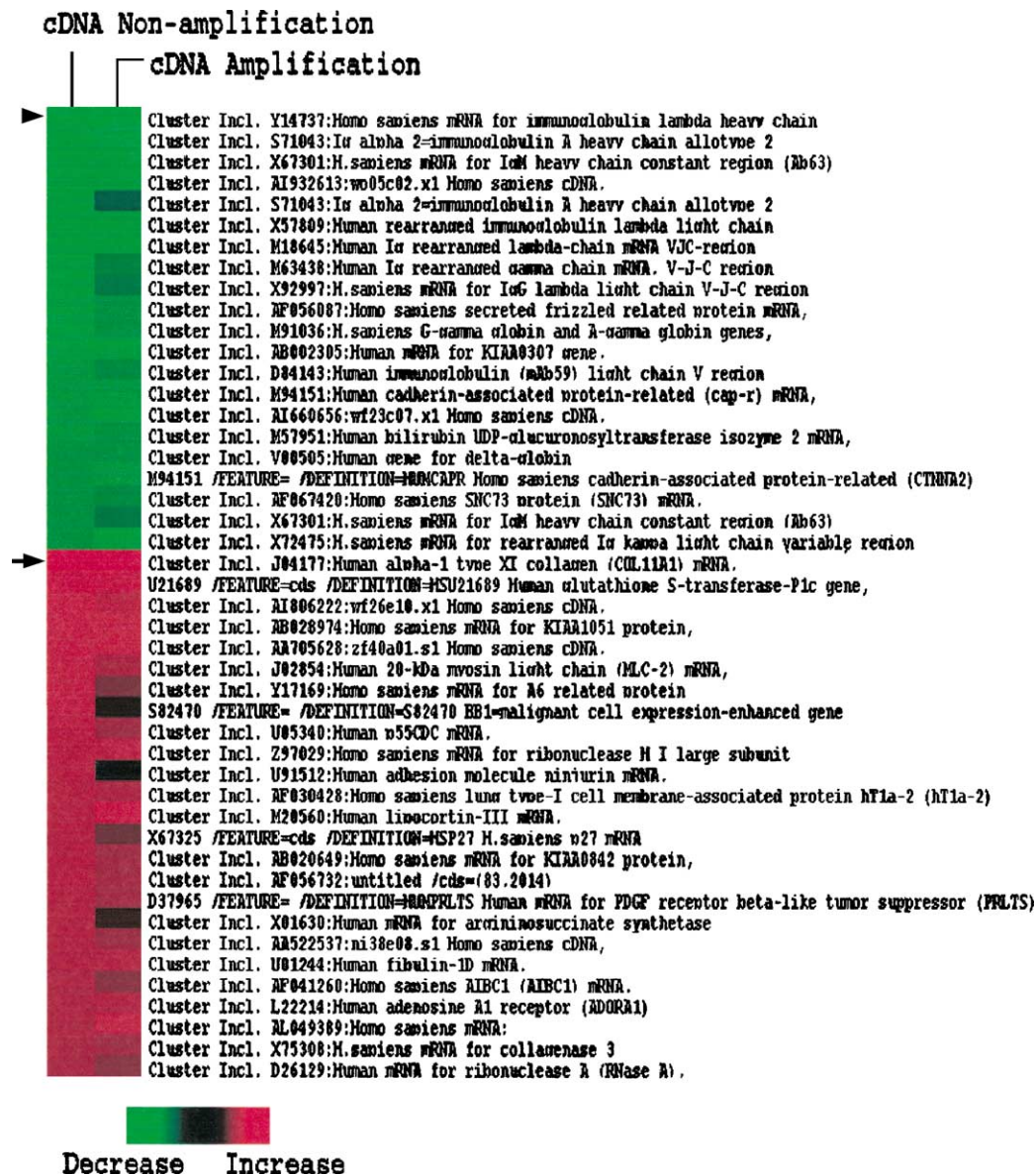


Fig. 6. Cluster map of selected genes showing alternations in mRNA expression (▶: maximum up; →: maximum down).

Table 1
The most altered genes after treatment in biopsy tissue processed by the two methods

Methods	Absolute call	Change	Fold change	Gene
Maximum up				
cDNA non-amplification	P	I	68.1	J04177 human alpha-1 type XI collagen
cDNA amplification	P	I	53.9	J04177 human alpha-1 type XI collagen
Maximum down				
cDNA non-amplification	P	D	−84.9	Y14737 human immunoglobulin lambda heavy chain
cDNA amplification	P	D	−175.4	Y14737 human immunoglobulin lambda heavy chain

P: present; I: increase; D: decrease.

using the two different methods, similar to the samples processed with the cDNA non-amplification method. This indicates a good concordance between the two methods.

3.4. Comparison of the altered expressions level from two methods

To further evaluate the concordance of alternation of gene expression detected by the microarray with and without cDNA amplification, we analyzed the increase and decrease in gene expression of post versus pre biopsy samples processed by two different methods. We found that the genes, which showed most altered expression after treatment in biopsy samples detected by two methods were same (Table 1, Fig. 6). The gene expression profiles of needle biopsy tissues achieved from standard and modified methods showed similar patterns, although the fold change in the expression level was not exactly same between these two different methods (Fig. 6). Similar results were observed in the samples from cultured cells with or without treatment as detected by two methods, suggesting a good concordance between the two methods.

4. Discussion

Early diagnosis of cancer will provide an opportunity for curative treatment. However, the biomarkers for early diagnosis of cancer remain little known. Thus, it is important to better understand the molecular abnormalities in patients with cancers and to find the molecular abnormalities which identify the individuals at high risk of tumor. The molecular abnormalities in patient with cancer also provide information for evaluation of therapeutic efficacy and predicting patient outcome [14,15]. Early testing of therapeutic response in patient and successful prediction of patient outcome will help to devise therapeutic strategies which will ultimately lead to improved therapy for cancer patient. However, the utility of prognostic factors discovered years ago is limited because they mainly measure tumor differentiation or tumor stage, not molecular biological properties that likely control tumor behavior. Recently, global gene expression profiling by cDNA microarray analysis has been used to discover the biomarkers for early diagnosis of various cancers, sub-

classing cancer type, and prediction of patient's treatment outcome [2,5,6,8,14–16]. The alternations of gene expression profiles by several anti-cancer agents have also been reported [9–11]. The information provided by gene expression profiling may contribute to the development of novel mechanism-based therapy against cancers. To achieve gene expression profiling, it is essential to get biopsy samples from individuals or patients. Smaller biopsy tissue is better for patients; however, the standard cDNA microarray requires more than 5 µg of good quality RNA, which is rarely obtainable from small samples. Thus, our modified microarray method for small biopsy tissues could be very useful for clinical application because our data showed high concordance between the standard cDNA non-amplification and modified cDNA amplification methods.

In the present study, we compared percentage present call, absent call, reproducibility, and concordance in same sample processed by standard microarray protocol (cDNA non-amplification method) and our modified protocol (cDNA amplification method). The results showed that cDNA amplification method provided high reproducibility, representation, and concordance with standard cDNA non-amplification method. It is important to note that the genes, which showed most altered expression in biopsy tissues detected by the two different methods, remained the same. The gene expression profiles of needle biopsy tissues obtained by standard and modified methods showed similar patterns, although the fold change in the expression level varied between these two different methods. These results support the future use of the modified cDNA amplification method for microarray expression analysis for needle biopsy tissues.

The difference in the procedure between cDNA non-amplification method and cDNA amplification method is the addition of cDNA amplification step after cDNA synthesis. This enhances the sensitivity of the modified method and ensures enough cDNA for next step of the protocol when using small biopsy samples. However, it is important to note that the signal intensities obtained from the two different methods cannot be directly compared since distinct levels of cDNA are generated with or without cDNA amplification. Although we found that the data obtained from the two different methods were highly reproducible, we recommend comparing two small biopsy samples using

the same cDNA amplification method to detect alternations of gene expression between these two samples.

In summary, we obtained the global gene expression profiles of needle biopsy samples using a modified method without significantly affecting the gene expression profiles obtained by standard method. Analysis of the differential gene expression profiles of needle biopsy tissues will be beneficial for the early diagnosis, prediction of treatment outcome, and perhaps devising therapeutic strategies against cancers.

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