Fabrication of a microarray using a combination of the large circular sense and antisense DNA

KYUNG-OH DOH[1*], YUN-HAN LEE[2*], KIL-HWAN HAN[2*], SEOK-YONG UHM[2], JONG-PIL KIM[2], YUN-UI BAE[1], JEONG-HOH PARK[2], IK-JAE MOON[2] and JONG-GU PARK[2,3,4]

[1]Department of Physiology, Yeungnam University School of Medicine, 317-1 Daemyung-dong, Daegu 705-717; [2]WelGENE Inc., 71B-4L, Hightech Sector 2, Sungseo Industrial Park 3, Dalseogu, Daegu 704-230; [3]Department of Medical Genetic Engineering, and [4]Chronic Disease Research Center and Institute for Medical Science, Keimyung University School of Medicine, Dongsan Medical Center, 194 Dongsan-dong, Joong-gu, Daegu 700-712, Korea

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Abstract. In the present study, single-stranded large circular (LC)-sense molecules were utilized as probes for DNA microarrays and showed stronger binding signals than those of PCR-amplified cDNA probes. A microarray experiment using 284 LC-sense DNA probes found 6 upregulated and 7 downregulated genes in A549 cells as compared to WI38VA13 cells. Repeated experiments showed largely consistent results, and microarray data strongly correlated with data acquired from quantitative real-time RT-PCR. A large array comprising 5,079 LC-sense DNA was prepared, and analysis of the mean differential expression from dye-swap experiments revealed 332 upregulated and 509 downregulated genes in A549 cells compared to WI38VA13 cells. Subsequent functional analysis using an LC-antisense library of overexpressed genes identified 28 genes involved in A549 cell growth. These experiments demonstrated the proper features of LC-sense molecules as probe DNA for microarray and the potential utility of the combination of LC-sense and -antisense libraries for an effective functional validation of genes.

Introduction

Recent developments in DNA microarray technologies have given us the chance to study the relative expression levels of a large number of genes, in a massively parallel mode (1-4). Using array hybridization techniques, the expression of thousands or tens of thousands of genes can be utilized in a variety of applications (5-8). This rapid accumulation of genomic sequence information and expression profiling has created a bottleneck in subsequent target validation for diverse applications including drug discovery. The majority of target gene validation via functional studies has been conducted using a variety of conventional gain-of-function or loss-of-function studies. These loss-of-function studies have been conducted either by gene knockdown using conventional antisense (9,10), decoy (11), ribozymes (12), or more recently small-interfering RNA (siRNA) (13-17).

Although microarrays now play an important role in biomedical sciences, inconsistencies or errors in the microarray data originate from a variety of sources including cross-hybridization, sequencing mistakes, contamination of clones and uneven hybridization conditions across an array (18). The signal intensity of the microarray data is thought to be another important factor for interpretation of experimental results (19). In microarray experiments employing double-stranded DNA as probes, the probe DNA requires denaturation in order to bind a labeled cDNA. Whereas denatured DNAs generate weaker binding signals due to the renaturation of the parental complementary strands, single-stranded DNA generates a stronger binding signal. In fact, single-stranded DNA was previously employed as probe DNA for microarray experiments at the Sanger Institute (Cambridge, UK). The M13 phagemid, a recombinant plasmid vector which is used for the construction of recombinant bacteriophages, can be engineered to generate a large quantity of single-stranded large circular (LC) molecules harboring either the sense or antisense sequence of a gene. Recently, high-throughput functional genomics using LC-antisense molecules has been developed for the identification of genes associated with cancer cell growth (20). The LC-sense DNA of recombinant bacteriophages may result in advantages for higher chances of binding to complementary target cDNA, owing to its considerable length and high degree of sequence fidelity. LC-sense DNA can also be easily generated in a high-

Correspondence to: Dr Jong-Gu Park, Department of Medical Genetic Engineering, Keimyung University School of Medicine, 194 Dongsan-dong, Joong-gu, Daegu 700-712, Korea

Dr Kyung-Oh Doh, Department of Physiology, Yeungnam University School of Medicine, 317-1 Daemyung-dong, Daegu 705-717, Korea

E-mail: jonggu@dsmc.or.kr; kodoh@ynu.ac.kr

*Contributed equally

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throughput and large-scale mode in transformed *E. coli* cultures.

In the present study, the utility of LC-sense molecules instead of LC-antisense was examined for the fabrication of a DNA microarray for the enhanced detection of differential gene expression. The data reproducibility of the LC-sense DNA microarray was further tested by employing quantitative real-time RT-PCR. An LC-antisense library to upregulated genes was next constructed and used in the functional validation of genes associated with the growth of cancer cells.

Materials and methods

Cell culture and RNA preparation. Both the human lung cancer cell line A549 and oncogenic transformants of human lung embryonic fibroblasts WI38VA13 were acquired from the Korean Cell Line Bank and cultured in DMEM and RPMI media, respectively, containing 10% fetal bovine serum (WelGENE, Daugu, Korea). All cultures were maintained in an atmosphere containing 5% CO_2 and harvested for total RNA isolation. RNA was used only in cases in which the OD 260/280 ratio was >1.8.

High-throughput preparation of LC-sense DNA probes in a large quantity. Either pSPORT1 or pT3T7-Pac phagemids, harboring human EST cDNA, were utilized in the production of single-stranded phage genomic DNA harboring the sense cDNA sequences. The recombinant phagemids were transformed into competent *E. coli* cells, XL-1 Blue (Stratagene, La Jolla, CA, USA), which had been infected with the helper bacteriophage, M13K07 (New England Biolabs, Ipswich, MA, USA), in accordance with a previously described method (20,21). All of the LC-sense DNA was adjusted to a uniform concentration of 0.35 g/l.

Microarray preparation, target cDNA labeling and hybridization. The microarrays were prepared via the spotting of the LC-sense probe DNA onto poly-l-lysine glass slides using an Affymetrix 417 Arrayer (Affymetrix, Inc., Santa Clara, CA, USA). In addition, LC-sense molecules to 4 housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase, phospholipase A2, tubulin-α2 and ß-actin) were purified as internal controls, and 3 different vector molecules [pSPORT1, pT3T7-Pac and pBluescript KS(+) phagemids] were purified as negative controls. IntelliGene Human Cancer Chip, ver. 4.0, comprised of the PCR-amplified cDNA probes of 886 cancer-related genes, was purchased from Takara Bio (Kyoto, Japan). Total RNA samples (30 μg) from A549 and WI38VA13 cells were reverse-transcribed with oligo-dT primers in the presence of Cy5-dUTP and Cy3-dUTP, respectively. Hybridization was conducted at 66˚C for 16 h in a humidified chamber. After hybridization, the slides were washed once each in 2X SSC/0.2% SDS for 5 min at 55˚C, 2X SSC/0.2% SDS for 5 min at 65˚C, 2X SSC for 5 min at room temperature, and 0.05X SSC for 5 min at room temperature, then spin-dried in a centrifuge prior to image scanning.

Microarray data acquisition and analysis. The fluorescent target cDNAs were detected by scanning the slides with an Affymetrix 428 Array Scanner (Affymetrix, Inc.) or GenePix 4000B (Molecular Devices, Sunnyvale, CA, USA). The scanned images were then analyzed with ImaGene ver. 4.2 software (BioDiscovery, El Segundo, CA, USA). The signal intensity values were determined by subtracting the background from the median values of the intensity of the spots. The corrected background intensities were then normalized to total signal intensities in order to account for the different input RNA concentrations or labeling efficiency in the individual Cy3 and Cy5 reverse transcriptase reactions. The microarray data (GSE11278) were deposited in Gene Expression Omnibus (GEO).

Quantitative real-time RT-PCR. Total RNA (1 μg) was reverse-transcribed using the random primers provided in the Reverse Transcription System (Promega, Madison, WI, USA). The cDNAs of the target genes were amplified using the Dynamo HS SYBR Green qPCR Kit (Finzymes, Espoo, Finland) and the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). In order to normalize the quantity of total RNA in each reaction, the ß-actin gene was simultaneously amplified.

Transfection of an LC-antisense library into a lung cancer cell line. An LC-antisense library to upregulated genes in the 5K LC-sense DNA microarray experiments was constructed by unidirectional cloning and transfected into A549 cells. The cells ($1x10^3$) were then seeded in each well of 96-well plates in 100 μl of DMEM media supplemented with 10% FBS. The cells were incubated for 12-18 h at 37˚C in a 5% CO_2 incubator. The LC-antisense library (0.1 μg) complexed with 0.3 μg of Enhancer Q and 0.5 μg of WelFect Ex (WelGENE) was added to the cultured cells and incubated for 3 additional days. Equal quantities of control LC-antisense DNA lacking a cDNA insert complexed with transfection reagents were also added to the same number of cells in different 96-well plates and simultaneously assayed. After transfection, microscopic observations and MTT reduction assays were conducted. The percentage of the inhibition of cell growth in each well treated with LC-antisense was calculated via the comparison of the optical density with those of the sham treatments.

Results

Production of LC-sense DNA as microarray probes. The recombinant M13 phagemid was employed in the production of single-stranded LC-sense DNA molecules. We utilized these molecules as probe DNA for the DNA microarray trials. LC-sense molecules were generated as genomic DNA using the F1 replication origin of the phagemid vector. The recombinant phagemids were transformed into competent *E. coli* infected with the helper phage, M13K07. From 100 ml of the culture supernatant, an approximate quantity of 150 μg LC-sense probe DNA was acquired.

Stronger binding signal of LC-sense probe DNA to target cDNAs. In order to compare the signal sensitivity of LC-sense DNA to that of conventional PCR-amplified cDNA in the probe-target interaction, we assessed the signal intensities

obtained under identical hybridization conditions. The differences in the signal intensities between the two types of probes were further studied via serial dilution, using a probe to verify the fold difference of the signal intensities. Both LC-sense and PCR-amplified cDNA probes to the gene of the serine protease inhibitor, Kunitz type 2 (SPINT2) were prepared at concentrations of 0.35 g/l. Whereas the PCR-amplified cDNA probes were spotted at the original concentration, the LC-sense DNA probes were successively diluted from the original concentration by factors of 2-, 5-, 10- and 20-fold (Fig. 1A). The probe samples were then spotted alongside PCR-amplified cDNA probes of ß-actin (positive control) and Lambda A (negative control) genes on poly-l-lysine-coated glass slides and were quality-controlled by examining the uniform spotting in a quantitative manner. When signals from the denatured cDNA probes were compared to those from the LC-sense DNA probes, the median signal intensity generated from the 5-fold dilution of the LC-sense DNA was found to be somewhat higher than those from the PCR-amplified cDNA probes (Fig. 1B). Subsequently, the LC-sense probe DNA to 284 cancer-associated genes was generated to fabricate a DNA microarray. Differential expression profiles between WI38VA13 and A549 cells were evaluated with Cy3-dUTP and Cy5-dUTP labeling of total RNA from respective cells. The LC-sense DNA microarray and cDNA microarray (IntelliGene Cancer DNA Chip, Takara Bio) were hybridized and scanned. Data of the 204 genes shared by the LC-sense DNA microarray and cDNA microarray were selectively scatter-plotted after $\log_2$ transformation (Fig. 1C and D). The scatter plot obtained from the LC-sense DNA microarray showed significantly stronger signals and tighter clustering along the y=x line for most values compared with those from the cDNA microarray. The median signal intensities of Cy3- and Cy5-labeled target cDNAs on the LC-sense DNA microarray were 4.2- and 12.5-fold higher, respectively, than those of the cDNA microarray.

Reproducibility in gene expression profiling with LC-sense probe DNA. In the microarray experiment using LC-sense DNA, 6 of the 284 (~2.1%) genes were upregulated >2-fold in A549 cells. Of these 6 genes, the CatL (22), GPCR19 (23), JAG1 (24), K8 (25) and SPINT2 (26) genes were previously reported as being involved in the progression of lung cancer. Conversely, 7 of the 284 (~2.5%) genes were downregulated >2-fold. Among these 7 genes, the CTGF (27), LAMB2 (28) and tPA (29) genes were previously reported to be downregulated in lung cancer. In addition, the downregulation of the CDH11 gene was detected in astrocytoma cells (30). When the experiments were repeated three more times to determine the reproducibility of the LC-sense DNA, the patterns of upregulation or downregulation of the genes were determined to be largely consistent, with only a minor degree of discordance (Table I).

Validation of LC-sense DNA microarray data with quantitative real-time RT-PCR. The differential expression profiles acquired from the LC-sense DNA microarray platform were then evaluated by real-time RT-PCR for data validation. Thirteen genes were determined to have been either upregulated or downregulated in A549 cells from the

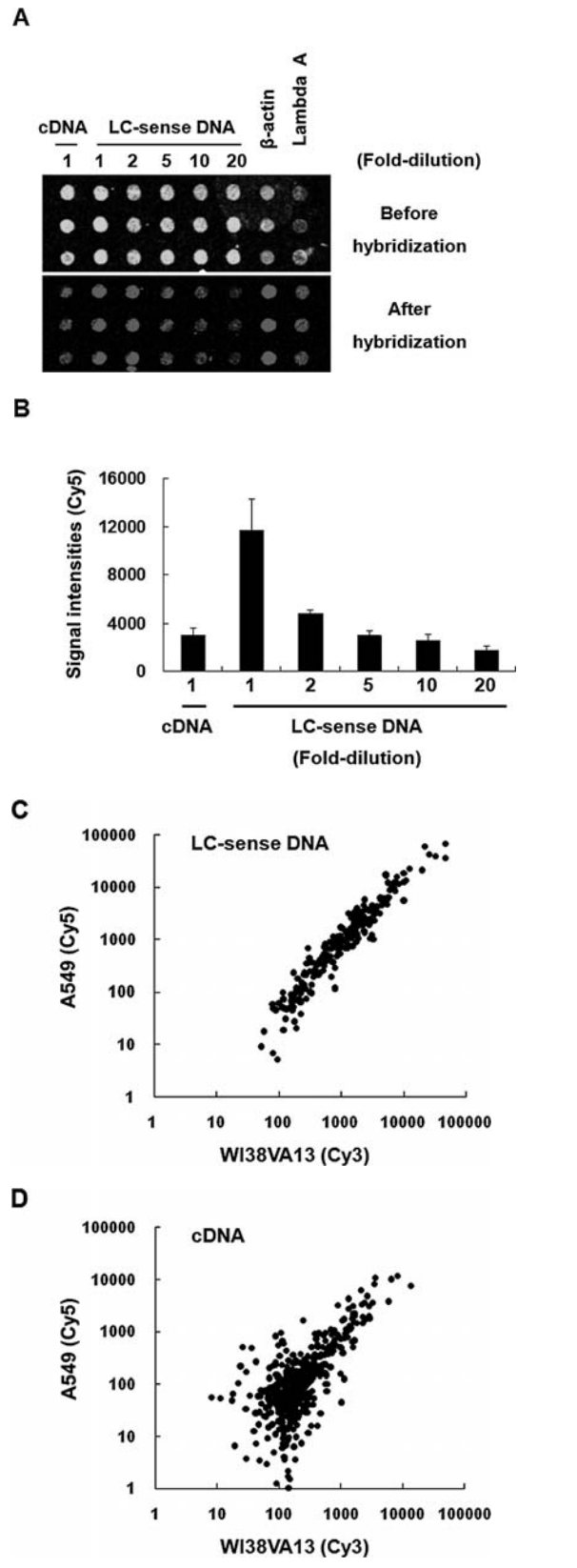

Figure 1. The fold difference in hybridization signals and microarray experiments between the LC-sense DNA and the PCR-amplified cDNA. (A) After confirming the uniform spotting of the probe DNA, the microarray slides were hybridized with Cy5-labeled target cDNAs of A549 cells. (B) The signal intensity of three spots is shown in a bar graph as the mean ± SD. The numbers shown underneath the graph denote the fold dilutions of the probe DNA. (C and D) The labeled target cDNAs from the WI38VA13 (Cy3) and A549 (Cy5) cells were mixed together and hybridized to an LC-sense DNA microarray and cDNA microarray. Each microarray was scanned and scatter-plotted after $\log_2$ transformation.

Table I. Upregulated or downregulated genes in the lung cancer cell line A549.

Gene description	Accession no.	Cy5/Cy3 ratio[a]	SD	k/n[b]
Up-regulated genes in A549				
Cathepsin L (CatL)	AI814383	2.18	0.10	4/4
G protein-coupled receptor 19 (GPCR19)	H07878	2.27	0.27	4/4
Jagged 1 (JAG1)	NM000214	3.24	1.06	3/4
Keratin 8 (K8)	AA598517	5.96	0.38	4/4
Serine protease inhibitor, Kunitz type 2 (tissue factor pathway inhibitor-2, TFPI2)	NM021102	4.15	0.21	4/4
Thioredoxin reductase 1 (TrxR1)	AA464849	2.93	1.12	3/4
Down-regulated genes in A549				
Cadherin 11, type 2, OB-cadherin (osteoblast) (CDH11)	AA136983	0.43	0.07	4/4
Collagen, type VI, α 3 (COL6A3)	R62603	0.36	0.10	4/4
Connective tissue growth factor (CTGF)	AA59874	0.32	0.05	4/4
Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) (CDKN2C)	N72115	0.43	0.08	4/4
Interferon induced transmembrane protein 2 (1-8D) (IFITM2)	AA862371	0.57	0.11	3/4
Laminin, ß 2 (laminin S) (LAMB2)	AA156802	0.48	0.03	4/4
Tissue plasminogen activator (tPA)	AA447797	0.42	0.04	4/4

All genes listed showed at least a 2-fold upregulation or downregulation as compared to the expression level in the WI38VA13 cells on the LC-sense DNA microarray in at least three out of four independent experiments. [a]Each data point represents the average of the repeated experiments. [b]The frequency by which gene expression was upregulated at least 2-fold in the repetitive experiments (k/n) is shown. SD, standard deviation.

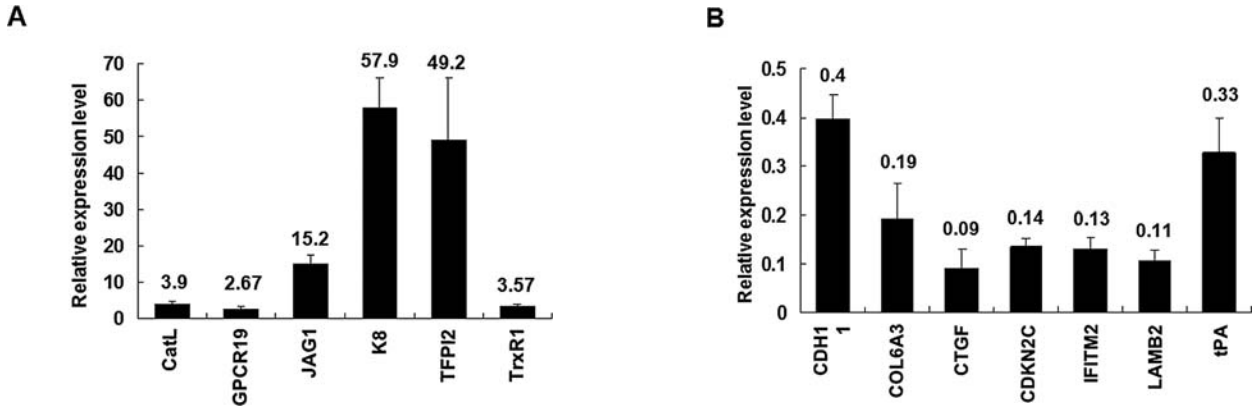

Figure 2. Validation of LC-sense DNA microarray data. The reliability of microarray signals was examined for 6 upregulated genes (A) and 7 downregulated genes (B) by quantitative analysis using real-time RT-PCR in A549 cells. The expression levels were calculated relative to that of ß-actin that had been normalized to 1.0 for WI38VA13 cells. Values of differential expression represent the average and SD (bars) of three independent experiments. Genes with differential expression are shown underneath each figure.

microarray experiments. The expression levels of the genes in A549 cells were quantified relative to those from the WI38VA13 cells. The expression levels of the CatL, GPCR19, JAG1, K8, SPINT2 and TrxR1 genes in A549 cells were higher than those observed in the WI38VA13 cells (Fig. 2A). Meanwhile, the expression levels of the CDH11, COL6A3, CTGF, CDKN2C, IFITM2, LAMB2 and tPA genes in A549 were lower when compared to levels observed in the WI38VA13 cells (Fig. 2B). In contrast, the expression levels of the GLB1 (galactosidase ß 1) and HDAC1 (histone deacetylase 1) genes, which showed almost no changes in the LC-sense DNA microarray experiment, were not significantly altered. Furthermore, fold-changes between the expression levels measured using real-time RT-PCR and those using LC-sense DNA microarray exhibited a correlation coefficiency of R^2=0.9189.

Expression profiling with 5K LC-sense DNA microarray. In order to verify the utility of the system for large scale expression profiling, LC-sense DNA probes to 5,079 human unigene clones were generated and arrayed on glass slides. The 5K LC-sense DNA microarray was then hybridized with a mixture of labeled target cDNAs from A549 and WI38VA13 cells. The microarray was then scanned (Fig. 3A). A dye-

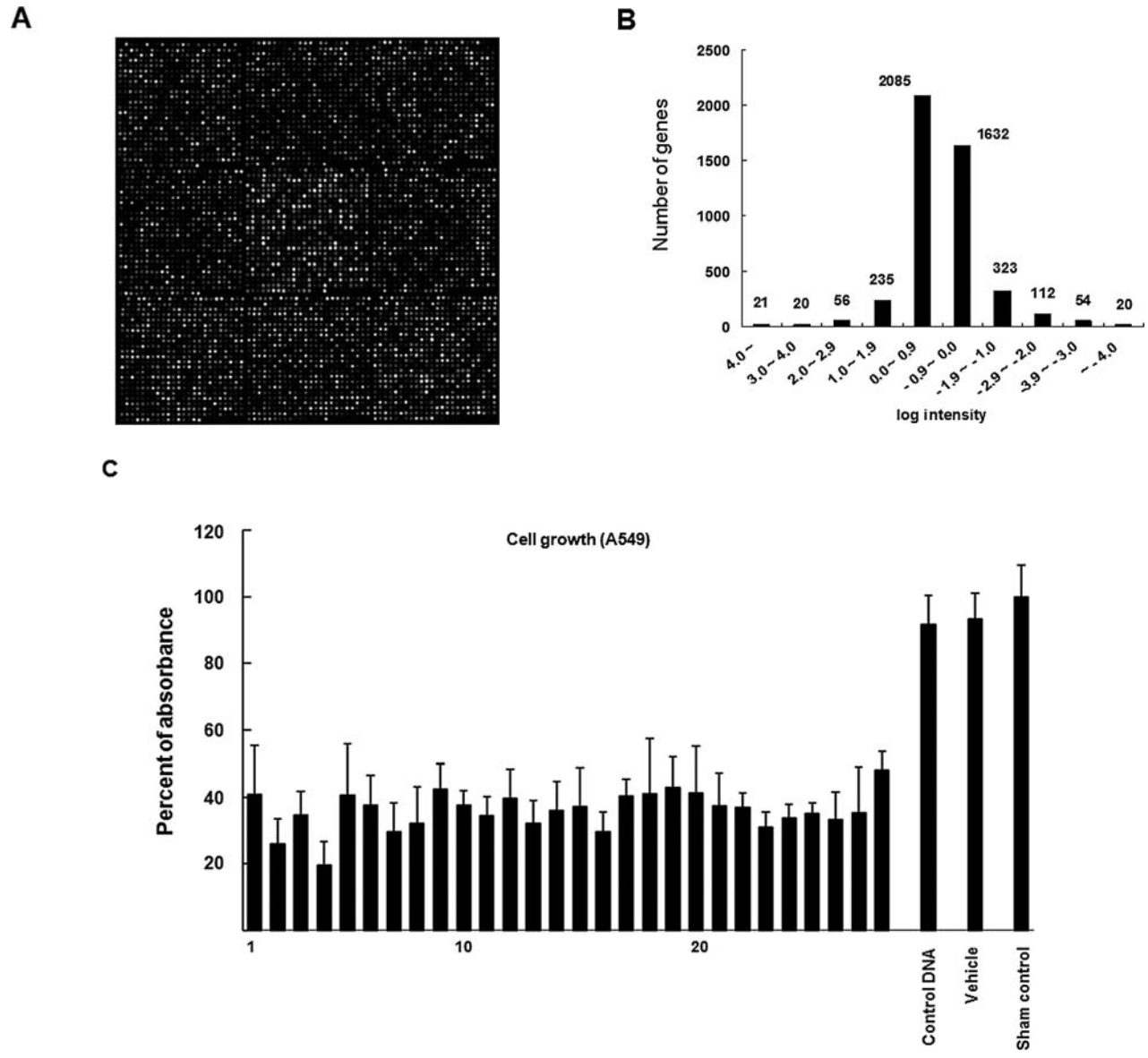

Figure 3. Large-scale expression profiling in A549 cells using a 5K LC-sense DNA microarray. (A) LC-sense DNA probes to 5,079 human unigenes were prepared and arrayed on a glass slide. The microarray slide was preheated and hybridized with a mixture of labeled target cDNAs. The fluorescence image of the microarray was scanned. (B) The number of genes for each expression range from dye-swap microarray experiments is shown on the ordinate of the graph. (C) LC-antisense species of 360 genes were transfected into A549 cells, and the growth rates were assessed by MTT assays in triplicate. Twenty-eight members of the LC-antisenses from the LC-antisense library showed marked inhibition (survival <50%) of growth in A549 cells as compared to the sham control, while these did not significantly inhibit (survival >80%) the growth of WI38VA13 cells. The value of each bar represents the mean ± SD of triplicate experiments.

swap test was conducted for quality control, and showed a correlation coefficiency of R^2=0.9351. A histogram of differential gene expression was also generated (Fig. 3B). Analysis of the mean differential expression levels by log transformation from the dye-swap microarray experiments showed that 332 (6.54%) of 5K were upregulated >2-fold, and 509 (10.02%) out of 5K were downregulated >2-fold, respectively, in A549 cells.

Functional validation of target genes using LC-antisense library. The LC-antisense library to the genes upregulated in A549 cells was constructed and utilized in the identification of genes functionally involved in the growth of lung cancer cells. Three hundred and sixty overexpressed genes selected from preliminary microarray experiments were unidirectionally subcloned into pBluescript SK (-) phagemids. These recombinant phagemids were then independently transformed into competent *E. coli* cells, and LC-antisense molecules were then purified. The transfected cells were quantitatively measured for growth inhibition via MTT assay 3 days after transfection. We tried to select the targets which have strong inhibitory effects on the growth of A549 cells with minimal effects on the growth of WI38VA13 cells. Twenty-eight members (Table II) of 360 antisenses showed marked inhibitory effects (survival <50%) on the growth of A549 (Fig. 3C) compared to WI38VA13 cells, while showing minimal effects (survival

Table II. Twenty-eight LC-antisenses which showed marked inhibition of growth in A549 as compared to WI38VA13 cells.

Representative RNA	Symbol	Description
AA700054	ADFP	Adipose differentiation-related protein
AA774619	ATP5SL	Hypothetical protein FLJ10241
NM_005603.3	ATP8B1	ATPase, class I, type 8B, member 1,
AA026120	BHLHB2	Basic helix-loop-helix domain containing, class B, 2
AA426053	CAPN3	Calpain 3, (p94)
AA430524	CAPZB	Capping protein (actin filament) muscle Z-line, ß
AA490911	CD99L2	CD99 antigen-like 2
AA877213	CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1
AA149095	DUSP1	Dual specificity phosphatase 1
R61289	L3MBTL	L(3)mbt-like (*Drosophila*)
AA625666	LITAF	Lipopolysaccharide-induced TNF factor
AA702487	LY6G5C	Lymphocyte antigen 6 complex, locus G5C
R83837	LYN	V-yes-1 Yamaguchi sarcoma viral-related oncogene homolog
NM_007289.1	MME	Membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10), transcript variant 2b
AA894577	NOL5A	Nucleolar protein 5A (56 kDa with KKE/D repeat)
H61726	NR1H3	Nuclear receptor subfamily 1, group H, member 3
AI739498	PPARA	Peroxisome proliferative activated receptor, α
AA282134	QPCT	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)
NM_006808.2	SEC61B	Sec61 ß subunit (SEC61B)
AA454570	SEMA3F	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F
N47445	SFRP4	Ependymin related protein 1 (zebrafish)
AA402891	SLC29A2	Solute carrier family 29 (nucleoside transporters), member 2
AA490471	SPARCL1	SPARC-like 1 (mast9, hevin)
AA458849	SPINT2	Serine protease inhibitor, Kunitz type, 2
NM_152999.2	STEAP2	Six-transmembrane epithelial antigen of prostate 2
AA455272	TMEM187	Chromosome X open reading frame 12
AI628066	XIAP	Baculoviral IAP repeat-containing 4
BC058011		Hypothetical protein LOC286440

>80%) on the growth of WI38VA13 cells. Cells treated with single-stranded control DNA and the double-stranded DNA-lipid complex showed no significant inhibition of cell growth.

Discussion

The single-stranded LC-sense molecules were utilized as probes for DNA microarrays and showed stronger binding signals than those of the PCR-amplified cDNA probes under identical hybridization conditions. The stronger signals observed with the LC-sense DNA microarray can be attributed to the favorable environment for intermolecular hybridization provided by the single-stranded structural feature of LC-sense DNA, coupled with its considerable length. Repeated microarray experiments using LC-sense probe DNA generated convincing results. Reliability was then confirmed using quantitative real-time RT-PCR, and fold changes obtained from microarray and real-time RT-PCR were well correlated. Large-scale LC-sense DNA microarrays were then prepared, and a dye-swap test was conducted. Result of the dye-swap test also showed good correlation coefficiency. By employing LC-antisense molecules to overexpressed genes in the 5K microarray, 28 genes functionally involved in the growth of lung cancer cells were identified.

Several features of LC-sense probe DNA make it practical for gene expression profiling. First, LC-sense DNA can be generated in a large quantity and at a reasonable cost. LC-sense DNA can be dependably generated from the culture supernatants of competent bacterial cells already harboring helper bacteriophages and transformed with recombinant M13 phagemids (21). Second, the single-stranded nature of LC-sense DNA allows stronger binding to target genes as compared to that of double-stranded cDNA. Third, LC-sense DNA has replication fidelity identical to that of bacterial genomic DNA. Furthermore, target validation can be easily accelerated by subsequent employment of LC-antisense libraries allowing functional analysis of genes.

Massively parallel expression profiling with DNA microarrays has recently emerged as a leading technology in the systematic analysis of cellular physiology (31). DNA micro-

arrays are currently being used in a variety of applications, including gene discovery (32), disease diagnosis (33), drug discovery (34) and toxicological research (35). The intensity of hybridization signals on DNA microarrays is an important factor, as weak signals render the microarray data inconsistent, and limit many of the potential applications of this technology (19). Due to stronger binding signals, LC-sense microarray experiments require smaller amounts of probe DNA for the production of the DNA microarrays, less Cy3 and Cy5 dye in the generation of downstream data, and smaller amounts of RNA samples.

The acquisition of meaningful data from gene expression profiles, such as powerful diagnostic markers or novel targets for the treatment of cancer, is important but difficult. When certain genes are overexpressed in cancer cells, they may have a role in cancer or pathologic cell growth, and may be selected as potential therapeutic targets. Therefore, functional studies on an individual gene employing antisense oligonucleotides (36) or si-RNA (13-15) are necessary for each gene. In this study, we identified 28 candidate genes which inhibited the growth of A549 but not WI38VA13 cells. These 28 genes appear to have functions that are either directly or indirectly associated with the growth of lung cancer cells. Of these genes, several were already known as targets of anticancer therapy or tumor markers. XIAP is a well-known inhibitor of the apoptosis protein family, and RNA interference of XIAP for cancer therapy has been attempted (37). Specific inhibition of Lyn using siRNA significantly decreased tumor growth and also reduced metastases (38), and a recent bioinformatical study revealed that CYP24A1 is a valuable biomarker in lung cancer (39). However, further studies are required which apply these results to clinical settings, since these results were obtained in cell lines.

In conclusion, LC-sense DNA demonstrates suitable properties for microarray experiments. Furthermore, LC-sense and -antisense libraries may be effectively combined for target gene validation in a high-throughput mode.

Acknowledgements

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