Journal of Surgical Oncology 1998;67:234–241

Application of the Differential Hybridization of Atlas™ Human Expression Arrays Technique in the Identification of Differentially Expressed Genes in Human Glioblastoma Multiforme Tumor Tissue

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Background and Objectives: Several molecular biology techniques are utilized to study changes in gene expression during the genesis of human tumors. Our objective was to identify genes that showed altered expression between normal brain tissue (NBT) and glioblastoma multiforme tumor tissue (GMTT).

Methods: The technique of differential hybridization of two Atlas™ Human cDNA expression array was used. In this technique, dCTP^{32}-labeled complimentary DNA from NBT and GMTT was hybridized to two identical human cDNA expression array membranes containing 588 known genes.

Results: Autoradiographic analysis showed that of the 588 genes analyzed, 52 are overexpressed in GMTT and 57 in NBT. A gene-specific semiquantitative reverse transcription polymerase chain reaction (RT-PCR) method was used to confirm the expression pattern of seven known genes. RT-PCR results demonstrate that the expression pattern of a majority of genes agreed with the expression pattern observed on expression array. The known tumor suppressor genes retinoblastoma (RB) and p53 showed loss of expression in GMTT compared with NBT.

Conclusions: We conclude that the differential hybridization technique of Atlas™ Human cDNA expression array can be a useful method in identifying genes that are differentially expressed either in NBT or GMTT.

J. Surg. Oncol. 1998;67:234–241.

KEY WORDS: brain; polymerase chain reaction; expression arrays; tumor markers; brain tumors

INTRODUCTION

Brain tumors are among the leading cause of death among young children and adults. A survey by the American Cancer Society documented that 13,300 people died of brain tumors in the United States in 1996 [1]. It is estimated that over 17,600 new brain tumor

Grant sponsors: Deke Slayton Foundation, Northwest Hospital, and Wilkins Charitable Foundation, Seattle WA.

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Accepted 16 December 1997

cases will be identified in 1997 [2]. Glioblastomas and astrocytomas are the most common brain tumors that affect adults [3].

Glioblastoma multiforme is a high-grade astrocytoma that grows rapidly and contains malignant cells [3]. Several genetic changes at the chromosomal and gene expression level are well documented for astrocytomas and glioblastoma multiforme [4]. For example, the loss of tumor suppressor genes at chromosome 10, mutations in p53, or overexpression of epidermal growth factor receptor are some of the major events involved in glioblastoma multiforme [4]. However, the exact series of events that leads to the initiation or progression of glioblastoma are not known.

Overexpression of oncogenes and loss or mutation of tumor suppressor genes are key events in the neoplastic transformation of normal cells [5]. Thus, identification and characterization of changes in the gene expression profile during the process of transformation does not only lead to a better understanding of this process, but also aids in designing cancer therapy protocols. Previously, several molecular biology techniques of differential library hybridization, suppression subtractive hybridization (SSH), subtractive library construction, direct differential cDNA sequencing, and differential display polymerase chain reaction (PCR) have been utilized to isolate and characterize differentially expressed genes [6,7]. All these techniques have several advantages and disadvantages. The major advantages for these techniques include: 1) analysis of a subset of genes; 2) comparison of more than two biological conditions; and 3) high efficiency. The major disadvantages are: 1) the presence of a large number of false-positive clones; and 2) the extensive time required to clone and characterize isolated genes. The techniques of differential library hybridization and subtractive library construction have proved to be useful when two different biological conditions are compared [8]. The technique of differential display PCR, on the other hand, can be utilized in identifying genes with altered expression in several different biological situations [9]. Previously, we utilized the technique of differential display PCR to isolate potential tumor suppressor and tumor-associated genes in human brain tumor tissues [10]. The technique of human cDNA arrays has been previously utilized to study the expression pattern of genes in normal and tumor tissues [11,12]. Here we report the utilization of a new technique of differential hybridization of Atlas™ Human cDNA expression array to study the differences in gene expression between NBT and GMTT.

MATERIALS AND METHODS

Human Tissues and Cell Lines

Brain and non-brain tumor and normal tissues were procured from the tissue bank maintained by Pacific Northwest Cancer Foundation Tissue Bank, Northwest Hospital, Seattle, WA.

Human cDNA Expression Array

Two Atlas™ Human cDNA expression array membranes were purchased from Clontech (Palo Alto, CA). Each membrane contained the cDNAs from 588 known genes and 9 housekeeping genes. As shown in Figure 1, the known genes are divided into six categories: A) oncogenes, tumor suppressor genes, and cell cycle regulator genes; B) stress response, ion channel and transport, and intracellular signal transduction modulators and effector genes; C) apoptosis-related, DNA synthesis, repair, and recombination genes; D) transcription factors and DNA binding proteins; E) receptors, cell surface antigens, and cell adhesion genes; and F) cell-cell communication genes. Each of these genes were PCR amplified using gene-specific primers to generate 200–500 base pair products. One hundred nanograms of each PCR product was then spotted in duplicate on to a positively charged membrane.

Differential Hybridization of Atlas™ Human cDNA Expression Arrays

Total RNA was isolated from human GMTT using the RNAzol solution from Gibco/BRL(Gaithersburg, MD). Total RNA from NBT was purchased from Clontech (Palo Alto, CA). Ten micrograms of total RNA for each tissue sample was treated with 5 μl of DNaseI (2 units/μl) (Amersham, Arlington heights, IL) for 30 minutes at 37°C. The first-strand cDNA synthesis was carried out as described previously [10]. Briefly, 20 μg of total RNA from NBT and GMTT was treated with 5 μl of DNase I (2 units/μl) at 37°C for 30 minutes. RNA was then precipitated at −80°C for 30 minutes using 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volume of 100% ethanol. After drying, the RNA pellet was resuspended in 6.5 μl sterile water and 1 μl each of oligo dT, random hexamer, and CDS primer (0.02 μM; Clontech, Palo Alto,CA). RNA and primer mix was incubated at 80°C for 3 minutes. After placing the reaction mix on ice, 4 μl of 5× reaction buffer, 2 μl dNTP mix (dCTP, dATP, dGTP, and dTTP, 5 mM each), 100 mM DTT, 2 μl of MMLV reverse transcriptase (50 units/μl) were added and incubated at 42°C for 60 minutes. A 0.5 volume of the first-strand cDNA reaction was labeled with 40 μCi $dATP^{32}$ (3,000 Ci/mmole) using a random prime labeling kit (Amersham). Equal amounts of cDNA (1×10^6 cpm/μl) from GMTT and NBT were then hybridized to two Atlas™ Human cDNA expression arrays in separate bags for 18 hours at 65°C. The expression arrays were washed in wash solution 1 (2× SSC and 1% SDS) for 60 minutes at room temperature. A second wash was performed at 65°C for 40 minutes in wash solution 2 (0.1× SSC and

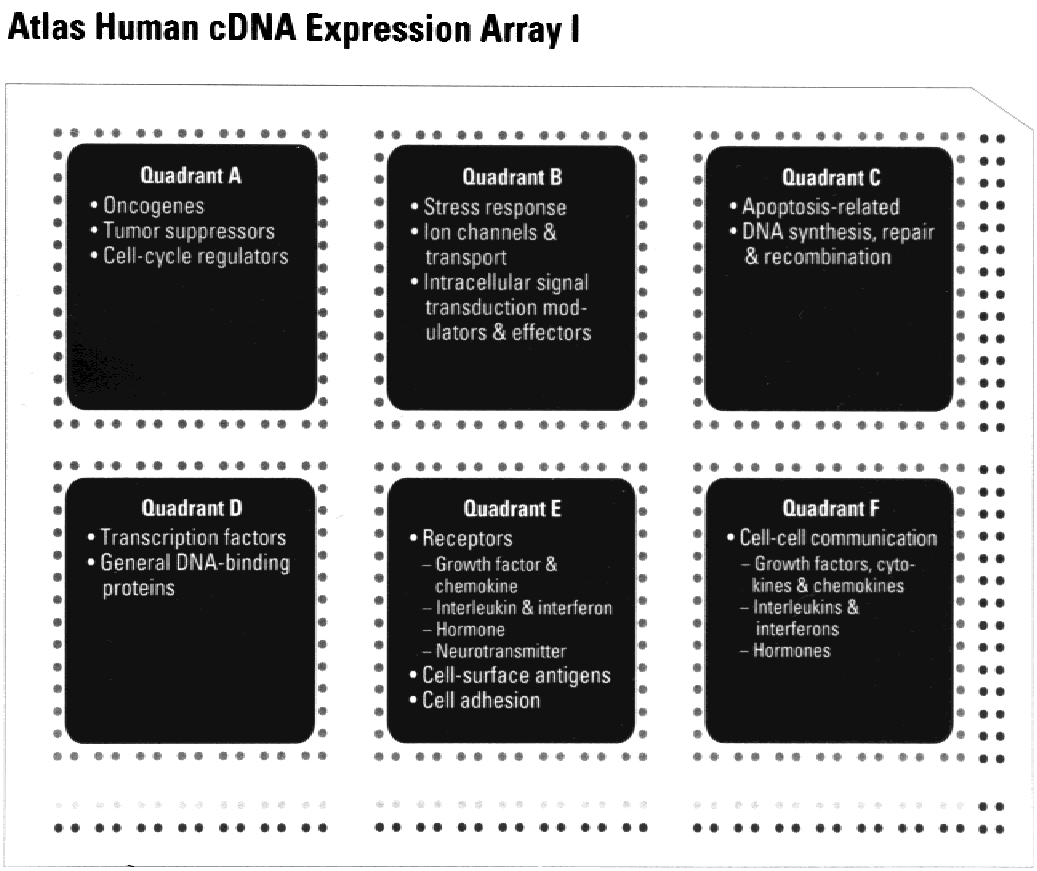


Fig. 1. Schematic of Atlas™ Human cDNA expression array. The membrane is divided into six quadrants (A through F). Each quadrant has 96 genes, 100 ng of each is spotted in duplicate. Black dots at the bottom and in the right hand corner represent DNA samples (negative control).

0.5% SDS) and then exposed to X-ray film at −80°C for 48 hours.

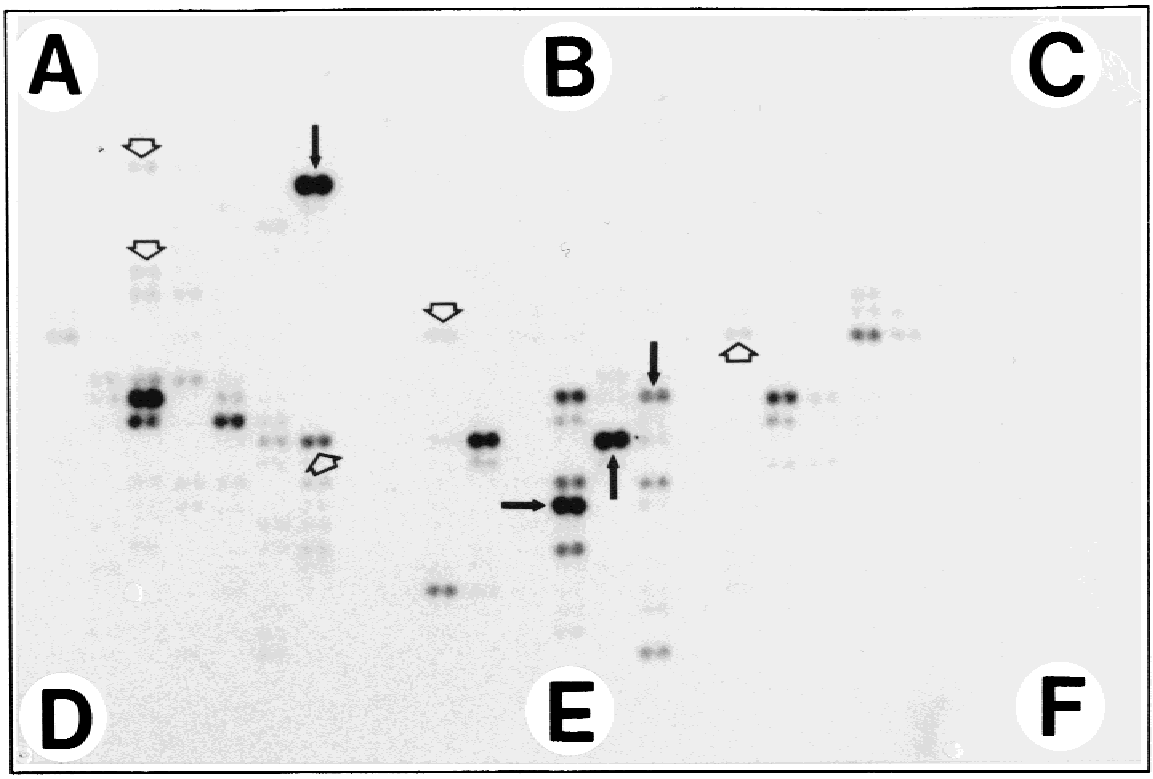
Gene-Specific RT-PCR Analysis

To confirm the differential expression of genes identified on the expression array, we used the technique of gene-specific RT-PCR as described previously [10]. Briefly, 10 μg of total RNA was treated with DNase I (2 units/μl) for 30 minutes at 37°C. RNA was then extracted and precipitated using 3 M sodium acetate, pH 5.2. First-strand cDNA synthesis was carried out using the advantage 1st strand cDNA synthesis kit from Clontech (Palo Alto, CA) using oligo $(dT)_{18}$ and random hexamer primers. Approximately 125 ng of 1st strand cDNA synthesis product was used for carrying out the PCR reactions for 35 cycles using the PCR kit from Qiagen (Valencia, CA). The thermocycler conditions for denaturing, annealing, and extension of the template cDNA was done for 30 seconds each at 94°C, 50°C, and 72°C respectively. The PCR product was then run on a 1.2% agarose gel. DNA was transferred onto a Hybond N^+ magnacharge membrane (Amersham) using standard Southern blotting conditions as described previously [13]. The membrane was prehybridized for 18 hours in a prehybridization buffer containing 50% formamide [13]. Hybridization was done using a 1×10^6 cpm/μl gene-specific probe at 42°C for 18 hours. Gene-specific probes were prepared by multiprime labeling, the internal primer for each gene using the Megaprime labeling kit from Amersham.

Gene-Specific Primers

For gene-specific RT-PCR and labeling of gene-specific probes, we used the following PCR primers and internal primer for eight of the genes tested: RB (5′ggtggcactgtgtacacctctggatt3′, 5′agtgatctgaatggtataaaaaccaa3′, 5′ctatcttccaaatgcaatttg attgactgcccattcaccaaaattatcct3′); DCC (5′tccaacctgtatgcctatgaaagcatg3′, 5′cttccagtttataagatagtccat3′, 5′gccgatttgtccgtctcagctggcgcc cacctgcagaagc3′); HM89 (5′ctctccaaaggaaagcgaggtggacat3′, 5′agactgtacactgtaggtgctgaaatca3′, 5′atctgtttccactgagtctgatcttcaagttttcacccagctaacaca3′); ERBB-2 (5′agttcccagatggctggaaggggtc3′, 5′cccccacacttgcctccccatacaaca3′, cggccctaagggagtgtctaagaacaaaagcgacccattcagagactgt3′); AF-1 (5′tggaagagatcaagccatcggagctgc3′, 5′attaattacattttaagccagcacacca3′, 5′taattgcttgttagcaaaatggatatgacacatctctgatacttttttca3′); GSTT-2 (5′cctcacatctccttagctgacctcgta3′, 5′aggacacaaggcctcagtgtgcatcatt3′, 5′tgagctgggaaacctcacccttgcaccgtcctcag-

NORMAL BRAIN TISSUE



GLIOBLASTOMA MULTIFORME TUMOR TISSUE

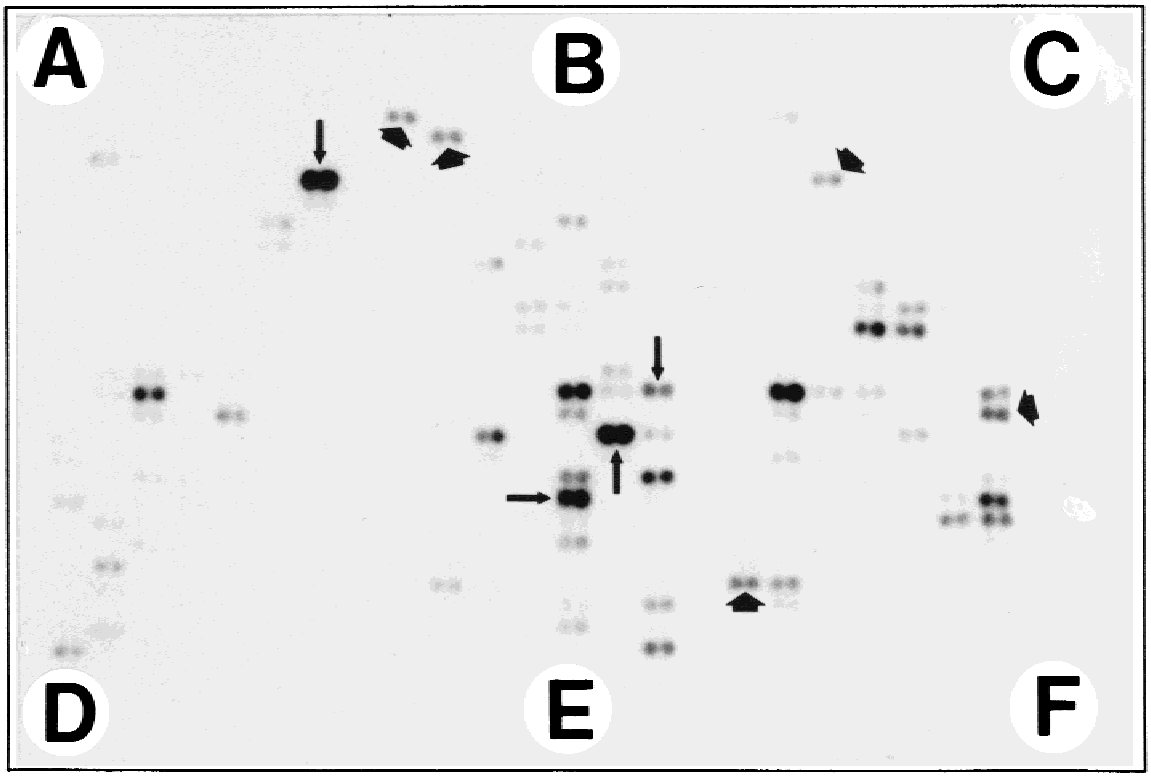


Fig. 2. Expression pattern of genes in normal brain tissue (NBT) and glioblastoma multiforme tumor tissue (GMTT). Differential hybridization of two identical Atlas™ Human cDNA expression arrays was performed as described in Materials and Methods. The top and bottom panels represent the expression array membrane hybridized with cDNA from NBT and GMTT respectively. Thick open arrows indicates genes that are over expressed in NBT, thick black arrow indicates genes over expressed in GMTT. Genes that are expressed at similar levels in both NBT and GMTT are indicated by thin black arrows. Six quadrants in the expression arrays are shown by letters A through F.

TABLE I. Listing of Genes Identified Using the Technique of Differential Hybridization of Atlas™ Human cDNA Expression Arrays

Genebank accession no.	Gene name	Genebank accession no.	Gene name
Genes overexpressed in glioblastoma multiforme tumor tissue			
J03241	TGFB3	L11353	MERLIN
X01060	Transferrin receptor protein	D10924	HM89
L32976	Protein kinase MLK-3	M34356	CREB
U02081	NET1	U05875	AF-1
U09564	Serine kinase	U09607	JAK1
X70326	Macmarcks	L36719	MKK3
M16038	LYN	M64174	JAK1
M65066	cAMP-dependent PK	M84489	ERK2
L34583	Tyrosine phosphatase	D42108	Phospholipase C
M68516	Protein C inhibitor	U04806	Tyrosine kinase ligand
X79389	Glutathione S-transferase	U71364	CAP-3
L20046	ERCC5	L24564	RAD
M97796	ID-2	M28372	CNBP
L14922	DNA binding protein PO-GA	D13316	E4TF1-47
D13318	E4TF1-60	D26120	ZFM1
M30257	VCAP	M62829	ETR103
M62831	ETR101	M33374	SQM1
M34064	N-cadherin	U43522	CAK beta
M76673	FMLP-related protein	K03515	Neuroleukin
M22488	BMP1	M22489	BMP2A
M65199	Endothelin-2	M96956	TDGF-3
X78686	ENA-78	M57627	IL-10
L06801	IL-13	L15344	B cell growth factor
J03634	Inhibin beta	A00914	ACE
J04040	Glucagon	M13981	Inhibin alpha chain
M14200	Acetyl-CoA-binding protein	M31159	Insulin-like growth factor binding protein
Genes overexpressed in normal brain tissue			
Y00285	IGFR-2	M11730	ERBB-2
M54915	PIM-1	X16416	ABL
X76132	DCC	M15400	RB
M15990	YES	X16706	FRA-2
X16707	FRA-1	X51521	EZRIN
X59932	C-SRC kinase	L16464	ETS oncogene
U24166	EB1	U26710	C-CBL
U61262	DCC (neogenin)	M26708	Prothymosin alpha
L26584	CDC 25	L13738	SYK
U18087	HPDE4A6	U33635	NGFR
U39657	MKKK6	U40282	ILK
U43408	TNK1	X07270	HSP 86
X54079	HSP 27	M11717	HSP 70
M3215	TNFR2	M33294	TNFR1
L07414	CD 40 ligand	M32865	Ku antigen
M29971	Methyl transferase	L12579	CCAAT DISP protein
Z30094	BTF	D28468	D binding protein
M34960	TFIID	M62829	TF ETR103
M62831	TF ETR101	M87503	ISGF3 gamma subunit
M96944	PAX-5	U08191	R Kappa B
U12535	NCK	U05040	Fuse binding protein
U08015	NF-AFc	U08853	LCR-F1
U10324	NF90	U14755	LIM-1
U01839	FYA-B^{+}	X60592	CD40L receptor
A09781	INF gamma receptor	M59911	Integrin alpha-3
J03133	SP1	M83221	RELB
M97190	SP2	M97191	SP3
D11117	HOX 4A	D45132	Annexin
U28838	TF IIIB		

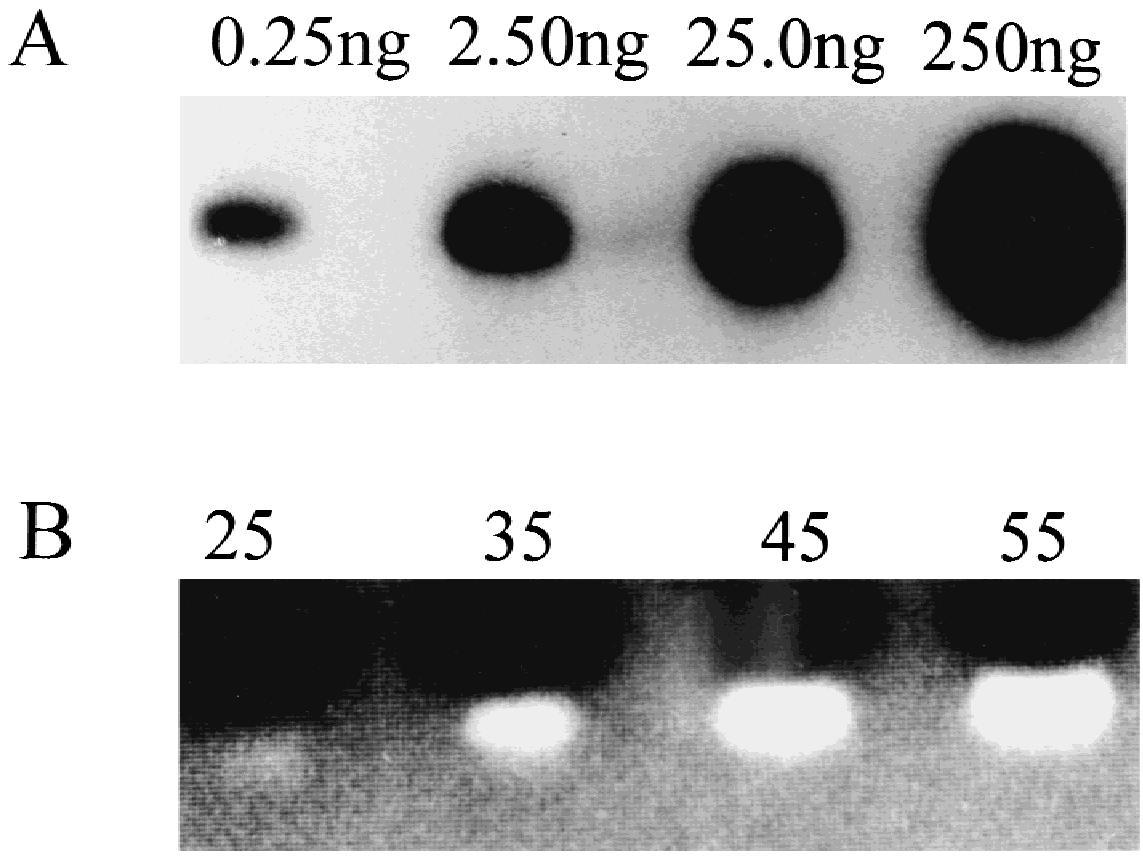


Fig. 3. Demonstration of the linear nature of the reverse transcriptase-polymerase chain reaction (RT-PCR) technique. Panel **A** shows RT-PCR analysis of the HM 89 gene performed using an increasing amount of cDNA (indicated above the autoradiogram). Panel **B** shows the PCR product of HM 89 run on a 1.2% agarose gel stained with ethidium bromide (the number of PCR cycles were 25, 35, 45 and 55).

cagtccacaaagcat3′); LYN (5′taccagcagcagccttagagcacagg3′, 5′agaaatgtggtggagctgacgctgggag3′, 5′aacttggacttgtcctcagcagctggtaatattgctctgcttgacaacat3′); and D1-2 (5′cggagcaatatgaaatgatct3′, 5′gcaaatacagctcctattg3′, 5′taggcctgactggcattgtattagcaaactcatcactaga3′). The gene bank accession numbers for genes tested are: RB (M15400); DCC (X76132); HM89 (D10924); ERBB-2 (M11773); AF-1 (U05875); GSTT-1 (X79389); and LYN (M16038). D1-2 is a mitochondrial cytochrome C oxidase subunit 1 gene sequence (accession number D38112) and we have used it as an internal control for RT-PCR [10]. The world wide web address to access these sequences is http://www.ncbi.nlm.nih.gov/index.html. A complete list of the 588 genes of the Atlas™ Human expression array used here can also be accessed through the world wide web address http://www.clontech.com/clontech/APR97UPD/Atlaslist.html.

RESULTS

Expression Pattern of 588 Known Genes

We used the technique of differential hybridization of Atlas™ Human cDNA expression array to identify differences in the expression pattern of 588 known genes between GMTT and NBT. As shown in Figure 2, several differentially expressed genes were observed. Out of 588 genes spotted onto the Atlas™ Human expression array a total of 143 genes was detected. The remaining 445 genes did not show any signal. Genes detected were divided into two main categories. First, genes that are overexpressed in GMTT compared with NBT (Table I); second, genes that are overexpressed in NBT compared with GMTT. Thirty-four genes were expressed at similar levels in both GMTT and NBT (data not shown). Analysis of the first category indicated 52 known genes that were overexpressed in GMTT compared with NBT. Out of these 52 genes, 1, 15, 6, 8, 5, and 17 fall into categories A–F, respectively, of Figure 1. Conversely, 57 genes were overexpressed in the NBT compared with GMTT. Out of these 57 genes, 19, 9, 4, 16, 6, and 3 fall into categories A–F (Fig. 1). We observed 34 genes that were expressed at similar levels in both NBT and GMTT. Out of these 34 genes 4, 5, 22, and 3 fall into categories A, C, E, and F, respectively (Fig. 1).

Gene-Specific RT-PCR Analysis

We used RT-PCR with different amounts of a known gene (HM89) [14] cDNA and varied the number of cycles in order to confirm its differential expression. As

shown in Figure 3, a linear increase was observed in the product generated. On the basis of this experiment, we used 125 ng of cDNA as starting material and 35 cycles of PCR to test the expression pattern of seven genes that were identified in the expression arrays. As shown in Figure 4, five genes (RB, DCC, HM89, ERBB-2, AF-1) of seven studied showed the same expression pattern by gene-specific RT-PCR as observed using the Atlas ™ human expression arrays technique. The expression pattern of two genes (GSTT-1 and LYN) did not agree with their expression pattern in the human expression array.

DISCUSSION

Several cytogenetic and molecular biology techniques are utilized to identify molecular changes involved in the genesis of glioblastomas [15]. Cytogenetic approaches are successful in identifying brain tumor suppressor genes [15]. Although useful, cytogenetic approaches require extensive chromosomal analysis and sequencing for isolating one gene. Most likely, multiple genetic changes lead to the genesis of human glioblastomas. Thus, it is important to develop molecular biology techniques that detect multiple genetic defects in tumor cells.

Several molecular biology techniques, including subtractive library construction, differential library hybridization, differential library sequencing, and differential display PCR are currently utilized to identify differentially expressed genes under different biological conditions [6,7]. We previously used the technique of differential display PCR to identify differentially expressed genes in human GMTT [10]. The technique of differential display PCR has limitations in the number of genes that can be isolated and characterized [10]. One limitation of DD-PCR is that a large number of false-positive clones are detected. The technique of differential hybridization has been used to isolate differentially expressed genes under different biological conditions. Recently, a modified version of this technique has been designed to increase its sensitivity and efficiency. This technique is referred to as the cDNA expression array technique.

The technique of cDNA expression array is extensively used to study changes in gene expression [11,12]. We utilized this technique in this study and demonstrated that it can be very useful in identifying differentially expressed genes in NBT or GMTT. One unique observation was that a total of 143 out of 588 genes analyzed was detected. This could be due to several reasons. The efficiency of 1st strand cDNA synthesis could have been low due to usage of total RNA. It has been suggested by Clontech that the efficiency of cDNA labeling can be increased if polyA+ RNA is used. Because of the limited amount of tissue available, we utilized total RNA. We are currently refining this technique by increasing the efficiency of cDNA labeling.

Using the technique of gene-specific RT-PCR, we

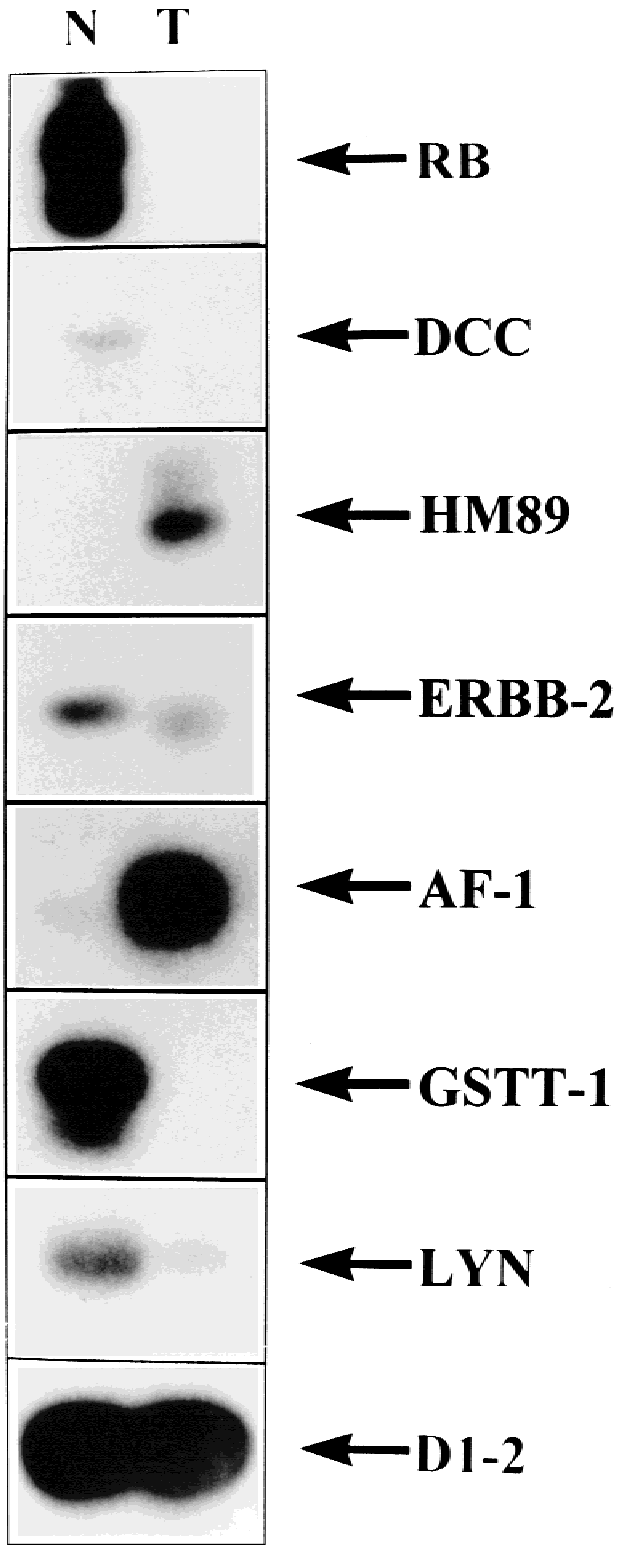


Fig. 4. Gene-specific RT-PCR analysis of seven genes identified using differential hybridization of Atlas™ Human cDNA expression arrays. Gene specific RT-PCR (for RB, DCC, HM89, ERBB-2, AF-1, GSTT-1, LYN and D1-2) was performed as described in Materials and Methods. Lane N represents normal brain tissue and lane T represents glioblastoma multiforme tumor tissue.

demonstrated that five of seven genes analyzed agree in their expression pattern as observed in Atlas™ Human cDNA expression arrays. We observed that the known tumor suppressor genes, RB and DCC, were detected only in NBT and not in GMTT. The loss of RB and DCC has been documented previously in brain tumor [4]. HM89 is a G-protein-coupled receptor that is shown to play an important role in the entry of HIV-1 into CD4+ cells [15]. AF-1 is an accessory factor required for activation of the human gamma interferon receptor. Gene-specific RT-PCR analysis indicated that ERBB-2, a human tyrosine kinase receptor that has extensive homology to epidermal growth factor receptor (EGFR) was detected in NBT but not in GMTT. The expression pattern of GSTT-1 and LYN using gene-specific RT-PCR was the opposite to that observed in Atlas™ Human cDNA expression arrays technique. The reason for this is not known at present. We are currently analyzing the expression pattern of 132 genes that were isolated using this technique.

In total, 96 genes in six categories (A: oncogenes, tumor suppressor genes, cell cycle regulators; B:stress response genes, ion channels transport proteins, intracellular signal transduction modulators/effectors; C: apoptosis related genes, DNA synthesis repair/recombination genes; D: transcription factors/DNA binding proteins, E: cell surface receptors/antigens, cell adhesion molecules; F: cell-cell communication proteins) were analyzed in Atlas™ Human cDNA expression arrays. Our analysis indicates that the majority of genes that were detected in NBT belong to the category of oncogene/tumor suppressor genes and DNA binding proteins. Conversely, gene products involved in the signal transduction and cell surface proteins were overexpressed in GMTT compared with NBT. This clearly indicates that multiple genetic changes and gene products are required for the genesis of human glioblastomas. On the basis of data presented here, we conclude that differential hybridization of Atlas™ Human cDNA expression arrays can be a useful technique to identify differentially expressed genes in human glioblastomas.

ACKNOWLEDGMENTS

We thank Cassie Keener and Sherianne Ricks for their excellent technical contribution to this study.

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