Identification of up-regulated genes in malignant glioma with subtraction hybridization

Yong-Jae Cho

Department of Medicine
The Graduate School, Yonsei University

Identification of up-regulated genes in malignant glioma with subtraction hybridization

Directed by professor Kyu-Sung Lee

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This certifies that the Doctoral Dissertation of
Yong-Jae Cho is approved

_____
Thesis Supervisor: Professor Kyu-Sung Lee

_____
Professor Jin-Yang Joo

_____
Professor Dong-Seok Kim

_____
Professor Tai-Soon Yong

_____
Professor Hyeyoung Kim

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Yonsei University
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I dedicate the dissertation to my loving family.

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Abstract

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Identification of the genes that are differentially expressed between brain tumor tissue and normal brain tissue is very important for understanding the molecular basis of these nervous tumors and for defining possible targets for therapeutic intervention. A variety of genetic alterations in human glioblastomas comprise signal transduction and cell cycle arrest control of cellular processes. This investigation is intended to obtain differentially expressed genes related to human malignant glioma using Subtractive hybridization. Subtractive hybridization is potentially faster methods for identifying differentially expressed genes associated with a particular disease state. Using the technique of subtraction, we isolated a novel gene that is over-expressed in glioblastoma as compared to normal brain tissue. Glioblastoma was used as tester and brain normal was used as driver. We identified the 7 novel genes by BLAST of the digested 130 clones. Seven genes were not homologous to any of the known genes in the Genbank™ database. Using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), the mRNA expression levels for

these 7 genes were markedly higher in human glioblastomas tissue than in normal brain tissue. In order to learn more about the expression profile of these genes, RT-PCR was performed using various commercially available normal or human carcinoma cell lines. In human carcinoma cell line expression, two different expression patterns were also identified. Some of these novel genes strongly expressed in glioblastoma tissue sample and human glioma cell line compared to normal brain tissue sample without the over-expression in other human cancer cell line. Theses cloned novel genes may play a role in brain tumorigenesis. To determine whether this novel gene was associated with cell cycle regulation, a serum stimulation study was used for its examination. The time-course expression of this novel gene indicated a significant increase for G1-phase arrest. These genes were time dependent activation during time course of serum stimulation. Expression and activation of these genes might be important and specific development roles of glioblastoma. Further studies including full DNA sequencing of theses 7 novel genes, verification of oncogene, cancer protein, and glioblastoma induction to animal model are needed.

Key words: malignant glioma, genes, gene cloning, subtraction hybridization

Identification of up-regulated genes in malignant glioma with subtraction hybridization

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I. INTRODUCTION

Cancer cells are characterized by unconstrained cell growth, ability to invade surrounding normal tissues, and to metastasize to distant organs. The development of cancer is a complex and multi-step process in which a series of progressive changes culminates in deregulation of all proliferation in nervous cells. These events lead to expression changes of numerous genes, accompanied by different histological or clinical classification of this abnormal cell growth. The formation and progression of tumors develop from single altered cells that begin to proliferate abnormally. Additional mutations lead to the selection of cells with progressively increasing capacities for proliferation, survival, invasion, and metastasis. Radiation and many chemical carcinogens are causes of cancer by damaging DNA and inducing mutations[3]. The developments of novel biological therapies for primary brain tumors have been continue to be intensively research. Alterations of gene expression and several genetic aberrations have been shown to occur during malignant growth, transformation, and progression of glioblastoma [20]. The glioblastoma is the most common tumor among primary brain tumor, and has approximately 60 percent of intracranial tumor [2]. Their distinct ability the normal surrounding tissue makes them difficult to control and nearly impossible to completely remove surgically, thus accounting for the

extraordinarily high lethality associated with malignant gliomas [16]. A variety of genetic alterations in human gliomas comprise signal transduction and cell cycle arrest control of cellular processes. The first, important growth factor signaling pathways of epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) are frequently altered in human glioblastoma as a result of amplification or over-expression mechanisms. The second, mutations with disruption of cell cycle arrest pathways revealed in human gliomas. Mutations of important regulators of cell cycle progression are amplification of CDK4, CDK6, CYCLIN D1and MDM2, and deletions of RB, deletions INK4A-ARF, deletions or mutations of p53 [4].

Current methods for comparing global gene-expression profile changes in different tissues or different pathological specimens include mRNA differential display(Liang and pardee, 1992), serial analysis of gene expression (SAGE)(Velculescu et al., 1998), cDNA microarray(Chee et al., 1996), large-scale cDNA sequencing, expressed sequence tag (EST) database comparison, two-dimensional gel electrophoresis of cellular proteins, subtractive library construction, representational difference analysis (RDA) and suppression subtractive hybridization (SSH)(Diatchenko et al., 1996) [1,9, 17, 19]. Whereas mRNA differential display is the most complicated and labor-intensive, both SAGE and cDNA array are limited for identifying unknown genes because they formerly require known genetic information[20, 22, 25]. The identification of differentially expressed genes has been reported microarray technology. The chip technology is highly efficient, but also requires formerly require known genetic information and requires expensive equipment for making the analysis. Thus, chip technology about glioblastoma study is not yet widely available for the searching of unknown genes [18, 28, 30].

Suppression subtractive hybridization (SSH) is an efficient and versatile PCR-based method of identifying rare, tumor-specific transcripts[20, 25, 28].

Since development of glioblastoma is a complex process involving a large number of genes, we believe it is important to identify additional new genes, which might be involved in the formation of this tumor. To this end, we employed the suppression subtractive hybridization (SSH) technique, which was originally designed to identify genomic differences between two complex genomes. SSH was later adapted to enable the isolation of gene transcripts which are differentially expressed in one sample relative to another, allowing the detection of low-abundance transcripts in a sample.

In this study, using the technique of suppression subtraction hybridization (SSH) we isolated a novel gene that is over-expressed in glioblastoma as compared to normal non-neoplastic brain tissue. SSH is a powerful technique that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the others[5, 10,12,21,23]. Also, it is potentially faster methods for identifying differentially expressed genes associated with a particular disease state. We involve hybridization of cDNA from glioblastoma tissue (tester) to excess of mRNA (cDNA) from normal brain tissue (driver) and then separation of the unhybridized fraction (target) from hybridized common sequences[13]. In this report, novel genes involved in glioblastoma were identified by SSH and characterized by RT-PCR and sequenced. Confirmation of the result obtained from SSH screen was performed by RT-PCR of non-neoplastic brain tissue, human glioma cell lines and other human cell line. To detect a change of mRNA expression of cells in different phase during the time course, serum stimulation in Normal WI-38 Fibroblasts was done.

II. MATERIALS AND METHODS

1. Tissue Materials

Through June 2004 to September 2005, glioblastoma patients consented to

use of their tissues. This study investigated tissues of 15 patients in whom craniotomy and surgical removal was performed due to cerebral glioblastoma. Tumor and normal brain tissues (normal brain tissues; N = 15; tumor tissues; N=15) were collected during the neurosurgical operations. The patient materials consisted altogether of 15 patients – 5 females and 10 males, aged 49 to 70 years. Mean age at diagnosis was 55.2 years ± 6.9 (SD). All patients underwent craniotomy and the study was done on diagnostic material received for frozen section and histology. The histological diagnosis was made by two neuropathologists according to World Health Organization (WHO) classification of the astrocytic tumors. Among 15 pairs of tissues (normal brain tissue and glioblastoma tissue), 10 pairs was used to identification of new glioblastoma specific genes and 5 pairs was used to know expression of identified new glioblastoma specific genes.

2. RNA extraction and SMART™ PCR cDNA Synthesis

Total RNA of the normal brain tissue and glioblastoma tissue was prepared using the Trizol reagent according to manufacturer's instructions (Clontech, Mountain View, CA, USA). The concentration and quality of each RNA sample was measured for the synthesis of high quality cDNA by determined spectrophotometry of a 260/280 ratio. About 1 μg of total RNA samples were treated with DNase I at 37°C for 15 min, at 65°C for 10 min, to get rid of genomic DNA contamination. The RNA was dissolved in DEPC-treated water, semi-quantitative spectrophotometrically and total RNA analyzed on 1.2% agarose gel. SMART cDNA synthesis used total RNA 1μg and was reverse-transcribed in 10μl mixture with PowerScript™ Reverse transcriptase (BD Biosciences Clontech, Mountain View, CA, USA) using 12μM of modified oligo dT primer (CDS primer II A; 5´-AAGCAGTGGTATCAACGCAGGGTACTT-3´) and SMART™ II A primer

(5´-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3´). When RT reaches the 5´ end of the mRNA, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3´ end of the cDNA. The BD SMART™ II A Oligonucleotide, which has an oligo (G) sequence at its 3´ end base-pairs with the deoxycytidine stretch creating an extended template. RT then switches templates and continues replicating to the end of the oligonucleotide. The resulting full-length, single-stranded (ss) cDNA contains the complete 5´ end to the mRNA, as well as sequences that are complementary to the BD SMART Oligonucleotide. The first-strand of cDNA was diluted to a final volume of 40μl with 1×TE buffer (10mM Tris-HCl, pH 8.0, 1nM EDTA). One μl of the diluted cDNA was used to generate the cDNA by long distance PCR with BD Advantage 2 polymerase mix (BD Biosciences Clontech, USA), using PCR primer II A (5´-AAGCAGTGGTATCAACGCAGAGT-3´) following the manufacture's instructions. The PCR-amplified second-strand cDNA was purified using column chromatography and then was digested with 10units/$\mu\ell$ RsaI in a final volume of 50$\mu\ell$ at 37°C for 3 hr to obtain blunt-ended fragments. The final concentration of digested second strand cDNA was 300μg/$\mu\ell$.

3. PCR-based Suppression Subtraction Hybridization (SSH)

SSH was performed with the PCR-Select cDNA Subtraction Kit according to the manufacturer's protocol. Driver ds cDNA was synthesized from 1μg each of total RNA, using SMART™ PCR cDNA Synthesis Kit user Manual (Clontech, Mountain View, CA, USA). First- and second- strand cDNA synthesis and blunt-ending of DNA ends by T4 DNA polymerase were carried out according to the manufacturer's protocol. The second double-stranded cDNA was digested with 10units/ul *Rsa*I in a final volume of 50μl at 37°C for 3hr. After extraction and precipitation of digested second strand cDNAs, the

pellet was dissolved in 7μl of sterile H_2O when precipitate was washed in 80% ethanol and residual ethanol was evaporated after the supernatant was removed. The final concentration of driver was ≈ 300ng/μl. *Rsa*I digested ds tester cDNA was prepared as described above for the driver. Digested tester cDNA (1μl) was diluted in 5μl of H_2O. The diluted tester cDNA (2μl) was then ligated to 2μl of adaptor land adaptor 2R (10μlM) in separate ligation reactions in a total volume of 10μl at 16℃ overnight, using 400units/μl of T4 DNA ligase in the buffer supplied from the manufacturer. After ligation, 1μl of 20×EDTA/glycogen was added and the samples were heated at 72°C for 5min to inactivate the ligase and stored at -20°C.

cDNA hybridization was used with tester1-1 and tester1-2 that were respectively mixed with adaptor1 and adaptor 2R. 1.5μl of tester1-1 with adaptor1, and tester1-2 with adaptor 2R was respectively hybridized with 1.5μl digested first stranded driver cDNA of brain tumor in 1μl 4×hybridization buffer solution at 68°C for 8hr. Tester1-2 hybridization sample was drawn into the pipette tip. Afterwards, 1μl denatured mixture from 1μl digested second stranded driver cDNA, 2μl H_2O, 1ul 4×hybridization buffer solution at 98°C was drawn into pipette tip with a slight air space below the droplet of the above tester1-2 hybridization sample. Sequentially, the entire mixture of pipette tip was transferred to a tube containing the above tester1-1 hybridization sample overnight at 68°C. The final hybridization was then diluted in 200μl of dilution buffer (20mM HEPES / 50mM NaCl / 0.2mM EDTA), heated at 68°C for 7min and stored at -20°C. For each subtraction, we performed two PCR amplifications. The primary PCR was conducted in 25μl. It contained 1μl of diluted, subtracted cDNA, 1μl of PCR primer 1 (10μlM), and 23μl of PCR master mixture prepared using the 50×Advantage cDNA polymerase PCR Kit (Clontech, Mountain View, CA, USA). PCR was performed with the following parameters: 75°C for 5min; 30cycle at 94°C for 25sec, 94°C for 10sec, 64°C

for 30sec, 72°C for 1.5min. 3μl of primary PCR mixture was diluted in 27μl of water for providing the nested-PCR reaction for 11cycle (94°C for 10sec, 68°C for 30sec, 72°C for 1.5min) with the same reagent except the two nested primer1, 2R.

4. Transformation and Sequencing of the subtracted cDNA

The subtracted cDNAs obtained after secondary PCR were cloned with a T/A Cloning Kit (Promega, Madison, WI, USA). The cDNA were ligated into T/A vectors by incubating 3μl of the secondary PCR amplification and 1μl of the vector (50ng/μl) with 3Weiss units of T4 DNA ligase at 4°C overnight. Then, 5μl of ligated product was transformed into 50μl of competent JM109 cells for heat shock. Competent JM109 cells have transformed by ligated product was grown on LB medium agar/ampicillin/IPTG/X-Gal plates at 37°C overnight. White colonies generally contained inserts obtained following transformation of competent cells.

White colonies were placed into LB medium and shaken overnight at 37°C overnight. The plasmid DNA with inserted fragments was extracted using SV minipreps (Promega, Madison, WI, USA) according to the manufacture's protocol. The extracted DNA was digested with *Eco*RI restriction enzyme and product was analyzed on 1.2% agarose gel. DNA sequencing was performed at Biotechnology Center Macrogen (Seoul, Korea).

Nucleic acid homology searches were performed using the NCBI BLAST program (http://www.ncbi.nih.gov/BLAST).

5. RT-PCR in glioblastoma sample and normal brain tissue

For expression of isolated seven genes' mRNA levels from glioblastoma tumor tissues and normal brain tissue, RT-PCR using previous primers was performed.

mRNA of glioblastoma tissue sample and normal brain tissue which were previously mentioned to 5 pairs of samples was reverse transcribed to cDNA using Superscript™ First-Strand Synthesis (Invitrogen, Carlsbad, CA, USA) as previously described. Reverse transcription-polymerase chain reaction was performed on samples with 1㎍ total RNA. The reaction mixture was supplemented with 1 ㎕ of SuperScript II reverse transcritase(50 units), 1㎕ oligo dT(0.5㎍/㎕), 2㎕ 10X RT buffer, 2㎕ 0.1M DTT, 1㎕ 10mM dNTP. The reverse transcription was carried out for 50 min at 42°C. Semi-quantitative RT-PCR was used to assess mRNA amounts of Clones in glioblastoma tissue sample and normal brain tissue. PCR amplification was performed with gene specific primer and 18srRNA was used as the reference gene using the TaKaRa Ex Taq™. Gene specific primers used in this study were designed from the known sequences of the isolated genes shown in Table 1. Amplification conditions were denatured at 94°C for 5 min, followed by 35cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. A final extension at 72°C for 10 min and then run for 30 min at 100V on 1% agarose/EtBr gel with ethidium bromide staining. Densitometric analyses were performed including standardization from the control (18srRNA) band.

6. RT-PCR in human glioma cell lines

RT-PCR analyses was performed for genes of the SSH experiments in five human glioma cell lines (SF188, SF539, SF126, U87, U251) (SNU Cancer Research Center, Seoul, Korea).

After 1μg total RNA using Superscript™ First-Strand Synthesis, RT-PCR was performed on normal brain tissue and human glioma cell line (Invitrogen,

Carlsbad, California, USA). The reaction mixture was supplemented with 1μl oligo dT (0.5μg/ μl), 2μl 10×RT buffer, 2μl 0.1M DTT, 1μl 10mM dNTP mix and water to a final volume of 19μl. After 2min of incubation at 42°C, 1μl of SuperScript™ (50units) was added to the mixture and incubations continued as mentioned above. The reverse transcription was carried out for 50min at 42°C. Semi-quantitative RT-PCR was used to assess mRNA amount in tissues, based on the relative expression of 8 mRNAs: “Clones 7, 10, 15, 25, 28, 43, and 47” and 18srRNA. To normalize mRNA amounts of these clones between tissue samples, 18srRNA was used as the reference gene. Primers used were listed in Table 1. PCR was performed using the TaKaRa Ex Taq™ under the following PCR conditions: first 3min at 94°C , then 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C for 35cycle, 10min at 72°C.

After PCR, each of the PCR products were electrophoretically separated on 1.2% agarose gel and stained with ethidium bromide. Densitometric analyses were performed including standardization from the control (18srRNA) band.

7. RT-PCR in other human cell lines

RT-PCR was performed for genes of the SSH experiments in other human cell lines. Other cell lines used were lung (WI-38), colon **(**CCD-18Co), prostate (RWPE-1), SV40-immortalized cell line (WI-38 VA13), lung carcinoma (NCI-H596), colon carcinoma (KM1214) and prostate carcinoma

(DU145). These cell lines were purchased from ATCC, Manassas, VA, USA and Nikken Cell line bank, Japan. It was done with 1μg total RNA using Superscript™ First-Strand Synthesis (Invitrogen, Carlsbad, California, USA). The reaction mixture was supplemented with 1μl oligo dT (0.5μg/ μl), 2μl 10×RT buffer, 2μl 0.1M DTT, 1μl 10mM dNTP mix and water to a final volume of 19μl. After 2min of incubation at 42°C, 1μl of SuperScript™ (50units) was added to the mixture and incubations continued as mentioned above. The reverse transcription was carried out for 50min at 42°C. Semi-quantitative RT-PCR was used to assess mRNA amount in tissues, based on the relative expression of 8 mRNAs: “Clones 7, 10, 15, 25, 28, 43, and 47” and 18srRNA. To normalize mRNA amounts of these clones between tissue samples, 18srRNA was used as the reference gene. Primers used were listed in Table 1. PCR was performed using the TaKaRa Ex Taq™ under the following PCR conditions: first 3min at 94°C , then 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C for 35cycle, 10min at 72°C.

The product of the PCR amplification was analyzed on 1.2% agarose gel. Densitometric analyses were performed including standardization from the control (18srRNA) band.

Table 1. The primer sequences of genes of interest.

Clones	Primer sequences	Size (bp)
Clone 7	Sense: 5'-ATACCTCACATGCTCTCCAC-3' Anti-sense: 5'-ATTTTGTAAAGGGCTTCCTC-3'	161bp
Clone 10	Sense: 5'-AGTTCAATTTTGTGGGATTG-3' Anti-sense: 5'-AAGGGGATTTCTTAAGGTTG-3'	153bp
Clone 15	Sense: 5'-CCATTTTATTTCTGACCCCT-3' Anti-sense: 5'-AGTCATTCCTGACCTGAGTG-3'	206bp
Clone 25	Sense: 5'-ACAGAGCCAGTTGTGTTTTT-3' Anti-sense: 5'-GGTTCTACGGTGAATCTCAA-3'	217bp
Clone 28	Sense: 5'-CCTAGGAGACATCAAACAGC-3' Anti-sense: 5'-GGGAGTGCCTTATAATGATG-3'	208bp
Clone 43	Sense: 5'-GGTTCCTCCTACAGGGTATT-3' Anti-sense: 5'-GTCTTACAGGTCAGCGATTC-3'	288bp
Clone 47	Sense: 5'-CCACAGGTATCTCCCACTAA-3' Anti-sense: 5'-CTACCTTCCCTTCCTCATCT-3'	222bp
18srRNA	Sense: 5'-TACCTACCTGGTTGATCCTG-3' Anti-sense: 5'-GGGTTGGTTTTGATCTGATA-3'	255bp

8. Serum Stimulation

The WI-38 (8PDL) was routinely maintained in Eagle's minimal essential medium (E-MEM) supplemented with 10% fetal bovine serum, essential amino acids, and non-essential amino acids. Cells were seeded at a density of 2×106 cells in a 60-mm dish. The cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. WI-38 were cultured at a density of 60% cells, the cells were washed with PBS, and finally, medium supplemented with 1% FBS was added. Again, after 48h, cells were washed with PBS and complemented with fresh 10% FBS medium. Cells were then harvested at various times for 0, 0.5, 1, 2, 4, 6, and 16hr after the 10% FBS treatment. To identify of expression, we were analyzed "clones 7, 10, 15, 25, 28, 43, and 47"

mRNA levels by RT-PCR. PCR was performed using the TaKaRa Ex Taq™ under the following PCR conditions: first 3min at 94°C , then 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C for 35cycle, 10min at 72°C. After PCR, each of the PCR products were electrophoretically separated on 1.2% agarose gel and stained with ethidium bromide.

III. **RESULTS**

1. Identification of novel gene over-expressed in glioblastoma using SSH

Using the suppression subtractive hybridization (SSH) of the human glioblastoma tissue compared to normal brain tissue, we have obtained subtracted individual clones. To determine the efficiency of the SSH, the both subtracted cDNA and unsubtracted cDNA have been amplified by PCR with GAPDH primers for different cycles: 18, 23, 28, and 33 cycle. The Expression of GAPDH was observed in unsubtracted cDNA after 18cycle and in subtracted cDNA after 33cycle. In this result, the abundance of GAPDH decreased significantly after subtraction. It was important to confirm that individual clones indeed represent differentially expressed genes. We compared tumor specific genes between glioblastoma tissue and brain normal tissue, glioblastoma was used as tester and brain normal was used as driver. The subtracted nested PCR products, from the SSH, have been cloned in T/A vectors and plated on ampicillin agar plates, X-gal and IPTG. With this experimental design, 130 plasmid clones were obtained (Fig. 1). We evaluated the differential expression of genes in each of hybridizing tester and driver cDNAs to digested 130 clones with EcoRI restriction enzyme. The digested cDNA clones were searched for sequence homologies in the GenBank DNA database by BLAST. DNA sequencing results of the digested 130 clones

detected high homology to known genes in the public database of the 14 kinds (Table 2). We identified the 21 new kinds of genes by BLAST of the digested 130 clones, not homologous to any of the known genes in the Genbank database (Table 3). In these 21 kinds of new genes, we selected 7 genes which were highly expressed in glioblastoma compared to normal brain tissue by RT-PCR (Fig. 2). In this study, we considered these 7 genes as up-regulated novel glioblastoma genes.

Fig. 1. Various Plasmid clones by digestion with EcoRI restriction enzyme (SSH libraries).

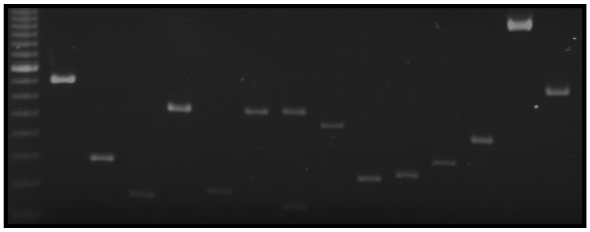


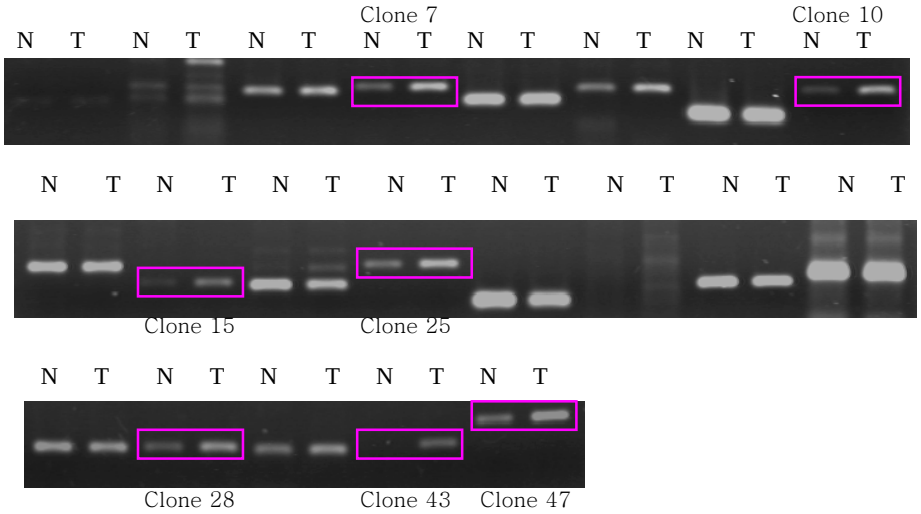
Table 2. Identification of known genes from SSH libraries.

GeneBank accession number	Matching gene
AY034480	S100 calcium-binding protein A6 gene
U28386	hSRP1 alpha (Karyopherin alpha 2)
BC000260	Aldo-keto reductase family 1
AF267864	DC42 mRNA
AF346971	Mitochondrion
NM_000980	Ribosomal protein L18a (RPL18A), mRNA
BC056870	Voltage dependent anion channel 3
U94747	WD repeat protein HAN11 mRNA
X64644	C6.1B mRNA
AF276948	HMG box containing protein (HBP2)
AY335768	Tumor rejection antigen 1gp96 (TRA1)
AF328729	CTCL tumor antigen
X98294	DNA double-strand break repair
AY327035	Ixodes ricinus cytochrome oxidase subunit 1mRNA

Table 3. Unknown 21 clones by BLAST program

Clone	Matching gene
10 , 69	Homo sapiens chromosome 16 clone
13	Homo sapiens
14 , 40	Homo sapiens genomic DNA, chromosome 11
15 ,16,42	Human DNA sequence from clone RP11-20F24
20	Homo sapiens cDNA clone IMAGE:3842446
25	Homo sapiens clone RP11-340F1 from 7p14-15
28	Homo sapiens chromosome 16 clone RP11-132F7
32	Human DNA sequence from clone RP11-454L1
34	Homo sapiens chromosome 17, clone RP11-580I16
37	Sinorhizobium meliloti 1021 complete chromosome
41	Homo sapiens chromosome 5 clone RP11-265K23
43	Homo sapiens 12 BAC RP11-15I13
47	Human chromosome 14 DNA
50	Homo sapiens, clone MGC:13410
54	Homo sapiens, clone IMAGE:3951562
57	Human DNA sequence from clone RP11
58	Human DNA sequence from clone RP11
60	Homo sapiens, clone MGC:22874
9,65,67	Homo sapiens, clone MGC:13410
68	Homo sapiens chromosome 15
7	Homo sapiens BAC clone RP11

Fig. 2. RT-PCR analysis for differential expression of 21 clones between glioblastoma (tester) and normal brain tissue (driver). Differential expressions of seven genes were confirmed. N: normal brain tissue, T: glioblastoma

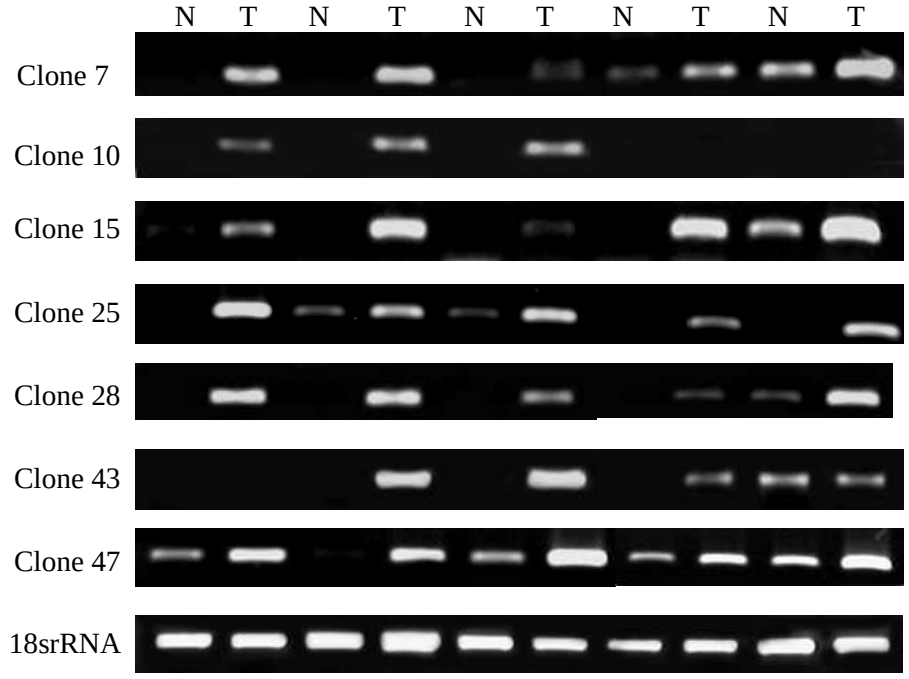


2. Different expression of genes in glioblastoma tissues and normal brain tissue

To be considered up-regulated in glioblastomas, the tested genes had to show a threefold greater expression in tumor samples than in normal brain tissue by semi-quantitative RT-PCR. To examine whether this novel genes specifically expressed in glioblastoma tissues, the expression patterns of transcription were examined by RT-PCR analysis for 5 pairs of glioblastoma tissues and normal brain tissues. Using gene specific primers, RT-PCR was performed on mRNA of 5-paired samples with 18srRNA as control gene. The mRNA of seven clones was determined to be highly express in mRNA of tumor tissues but only weak or no expression in most of the normal tissues (Fig. 3). We showed that the mRNA levels of seven clones were significantly high-expressed in glioblastoma tissue. In this study, we showed that the seven genes were significantly up-regulated in glioblastoma tissues compared with

normal brain tissues.

Fig. 3. Expression of seven genes' mRNA levels from glioblastoma tissues by RT-PCR. The expressions of mRNA of these clones were significantly higher than brain normal tissues. N: normal brain tissues; T: glioblastoma tissues; 18srRNA: control.

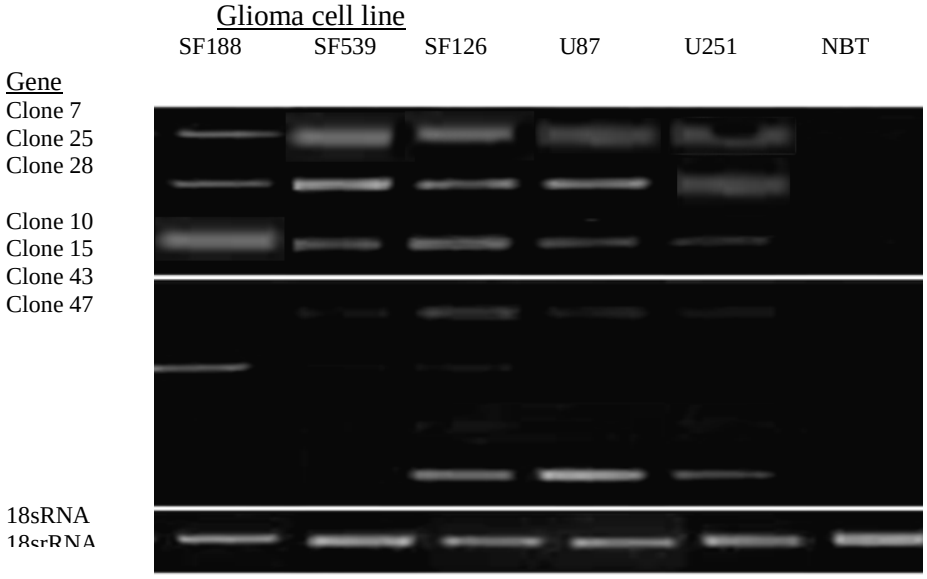


3. Expression of genes in human glioma cell lines

For further target study, semi-quantitative RT-PCR analysis was performed for verification of differential expression of genes in glioma cell lines. Brain tissues and glioma cell lines were used for validation by semi-quantitative RT-PCR with housekeeping gene 18srRNAas control[24]. In five glioma cell lines (SF188, SF539, SF126, U87, U251), we compared the mRNA of genes expression. RT-PCR products of genes were analyzed on 1.2% agarose gel (Fig. 4). All 7 genes were over-expressed than normal non-neoplastic brain tissue. But the expression profiles in the array of cell lines could be categorized two groups: (1) genes that were over-expressed by all the cell line

(clone 7, 25, 28) and (2) genes that showed variable expression across the cell lines without any distinct pattern (clone 10, 15, 43, 47).

Fig. 4. Expressions of seven genes' mRNA from glioma line by RT-PCR. The expressions of mRNA of seven clones were significantly higher than brain normal tissues. In glioma cell lines, two different type of the gene expression profile were observed. One type was genes that were over-expressed by all the cell line (clone 7, 25, and 28) and the other was genes that showed variable expression across the cell lines without any distinct pattern (clone 10, 15, 43, and 47). 18srRNA (control) demonstrates equal expression across the panel. NBT: normal brain tissue

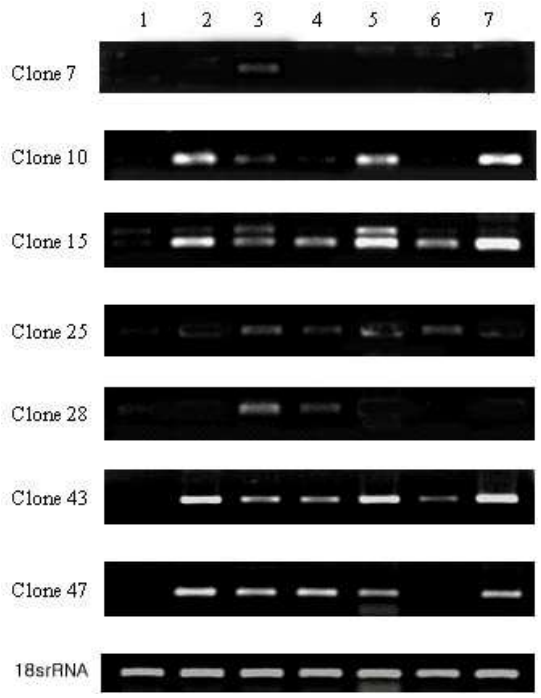


4. Expression of gene in other human cell lines

To examine whether this novel genes specifically expressed, the expression patterns of transcription were examined by RT-PCR analysis for other various human cell lines. Using gene specific primers, RT-PCR was performed on mRNA of 5-paired samples with 18srRNA as control gene. The mRNA of clone 10, 15, 43, and 47 was determined to be over-expressed in human lung cancer, prostate cancer cell line than each human non-cancer cell lines as shown in Fig. 5. We showed that the mRNA levels of four clones were

significantly high-expressed in the SV40-immortalized lung cell line, lung carcinoma cell line, and prostate carcinoma cell line than each normal cell lines. In this study, we showed that the four genes were significantly up-regulated in various other cancer cell lines compared with each normal cell lines. But expression of clone 7, 25, 28 in various human cell lines was not identified.

Fig. 5. Expressions of seven genes' mRNA from various human cell line by RT-PCR. The expressions of mRNA of clone 10, 15, 43, 47 in cancer cell lines were significantly higher than each normal cell lines. In cancer cell lines, clone 10, 15, 43, 47 strongly expressed in the SV40-immortalized (WI-38 VA13) cell line, lung carcinoma cell line (NCI-H596), and prostate adenocarcinoma cell line (DU 145). However, it was similar detection in colon and colon carcinoma (KM 1214). The expression of clone 7, 25, 28 was not expressed or weak-expressed in various human cell lines.
Lane 1, normal lung (WI-38); Lane 2, SV40-immortalzed (WI-38 VA13); Lane 3, Lung carcinoma (NCI-H596); Lane 4, Normal colon (CCD-18CO); Lane 5, Colon carcinoma (KM 1214); Lane 6, Normal prostate (RWPE-1); Lane 7, Prostate carcinoma cell line (DU 145); and control (18srRNA).

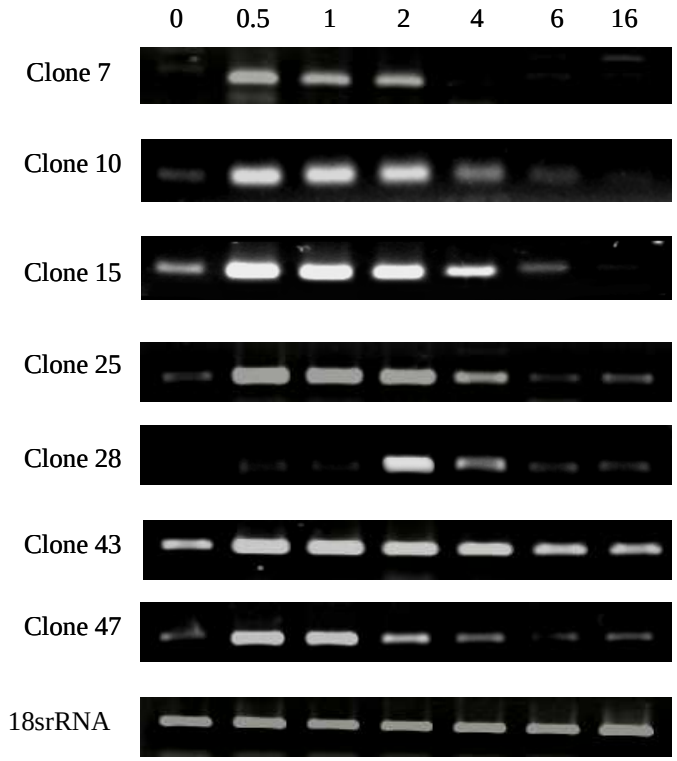


5. Serum stimulation in Normal WI-38 Fibroblasts

To determine whether the association in serum stimulation conditions, normal WI-38 fibroblast cell was cultured at a density of 60% cells, and than treated medium containing 0.1% FBS for 48hr. During 48hr of treated, WI-38 cells achieved an induction of G1-phage arrest of the cell cycle.[1, 3]

As shown in Fig. 5, the time course experiment showed that WI-38 cell stared in serum-free medium was not detected mRNA expression for 0 hr. At 0.5 ~ 4 hr after 10% FBS treatment, expression of each clone was significantly increased; afterwards gradually decreases in WI-38 cell of 10% FBS treatment for 6 hr. At 16 hr after 10% FBS treatment, seven clones were not expression by PCR. (Fig.6.) In this result, we identified that seven clones of serum stimulation conditions showed a time dependent activation.

Fig. 6. WI-38 fibroblast cell time course of seven genes. The expressions of seven genes were significantly increased from 0.5 and 1hr after 10% FBS treatment. At 2hr after the treatment, levels of seven genes' mRNA resulted in a drastic decrease.



IV. DISCUSSION

Identification of genes that are differentially expressed between cancer and nonmalignant cells of the same phenotype may lead to a greater understanding of which oncogenic processes and signaling pathways are pertinent in cellular transformation leading to cancer. Human glioblastoma, as with many human cancers, is characterized by genetic and phenotypic heterogeneity despite a fairly uniform appearance by light microscopic findings. As different genomic, transcriptomic, and proteomic profiles may give rise to a specific tumor type, it is becoming increasingly important to map these profiles since each may be associated with responses to different treatment regimens. As which, our study was designed to identify genes with differential expression between glioblastomas and cells of non-neoplastic brain.

The identification of differentially expressed genes has provided several methods, such as, RNA fingerprinting[29], differential display polymerase chain reaction (DD-PCR)[14], subtractive hybridization[7], subtractive suppression hybridization PCR (SSH)[5,10,12,21], serial analysis of gene expression (SAGE)[27], expressed sequenced tags (EST) analysis[26], and cDNA library array technology[15,18].

To achieve our goal, we have used the SSH that is ideally suited for the identification of new novel genes and known genes. When we used the tester (glioblastoma tissue) and driver (normal brain tissue) DNA fragments, the drive cDNA would have eliminated of the common sequences between the tester and driver cDNA samples during the first and second hybridization step. The PCR amplification of the SSH technique isolated clones of new genes up-regulated from glioblastoma tissue[5, 8]. In this study, we isolated known genes and novel genes that are over-expressed in glioblastoma tissue as compared to brain normal tissue.

Over 130 libraries were screened after SSH, from which 21 differentially regulated clones were identified. Their plasmids were then purified and cDNA

sequenced. The percentage of up-regulated clones was therefore approximately 16%. Of the 21 clones identified, 6 were multiple hits and we selected 7 genes for further evaluation. (Table 1) The seven genes (clone 7, 10, 15, 25, 28, 43, 47) were subjected to further analysis by RT-PCR. We performed to determine the mRNA level of seven novel genes in glioblastoma tissue and brain normal tissues by RT-PCR. In most glioblastoma samples, the expressions of mRNA of seven novel genes of glioblastoma tissue were significantly higher than brain normal tissues.(Figure 3) The lack of expression of all of these in the non-neoplastic brain is further evidence of efficacy of the SSH technique. The uniform expression of 18srRNA control indicates equal template loading of the RNA from the tumor specimen.

The expression profile of the 7 genes in five human glioma cell lines (SF188, SF539, SF126, U87, U251) were categorized into two groups: One type was genes that were over-expressed by all the glioma cell line (clone 7, 25, 28) and the other was genes that showed weak or variable expression across the cell lines without any distinct pattern (clone 10, 15, 43, 47).

In various human cell lines, most of 7 genes were highly expressed in cancer cell line (SV40-immortalized cell line, lung carcinoma cell line, colon cancer cell line and prostate cancer cell line) than matched normal cell line(normal lung cell line, colon cell line and prostate cell).

Two expression patterns were also shown like human glioma cell line expression in human cell lines. Expression of Clone 7, 25, 28 was weak or not in other human cancer cell lines, but that of clone 10,15,43,47 was mostly strong expressed. The later genes were very strongly expressed in the SV40-immortalized cell line, lung carcinoma cell line, and prostate adenocarcinoma cell line compared to lung and prostate normal cell lines.

Interestingly, in the first category, clone 7, 25, 28, although not expressed in the other cancer cell lines, were expressed in the glioma cell line suggesting that these genes may be specific to the glioblastoma. In the later category,

clone 10,15,43,47, were also expressed throughout the panels of human glioma cell line and human other cancer cell line and could represent genes that are important in a proliferative state regardless of tumor phenotype.

Next we tested to detect a change of mRNA expression of cells in different phase during the time course. WI-38 cells were achieved by starving in serum-free medium for 48h and cells were in Go phase at the end of this time period[6]. This study were arrested cell for 48hr by 1% FBS. Such arrested cells then enter a quiescent stage of the cell cycle called Go, in which they can remain for long periods of time without proliferating[3]. Using semi-quantitative RT-PCR, we confirmed the mRNA expressions of seven genes were up-regulation for 0.5~1hr of WI-38 cell differentiation. These genes had time dependent activation during time course of serum stimulation.

V. CONCLUSION

Identification of differentially expressed genes presents in glioblastoma but not in normal brain tissue is important not only to better understand the molecular basis of these cancers, but also to generate diagnostic DNA chips, which may be useful in future therapeutic intervention. In this study, we cloned seven novel genes in glioblastomas. The heterogeneity of glioblastomas was revealed by testing with multiple genes that were derived from our subtractive suppression hybridization screen. The levels of seven novel genes' mRNA were significantly up-regulated in human glioblastoma tissues compared to the normal brain tissues. The mRNA expressions of new genes were up-regulation for 0.5~1hr of WI-38 cell cycle differentiation. These genes had time dependent activation during time course of serum stimulation. Some of these novel genes strongly expressed in glioblastoma tissue and human glioma cell line compared to normal cell line without the over-expression in other human cancer cell line. Theses 3 cloned novel genes

may play a role in the tumorigenesis of glioblastoma. Also, the activation of these genes may be necessary for the development of cancer. Further studies including the full sequencing of theses genes are needed to fully characterize the functional significances and roles of these genes in glioblastoma tumorigenesis and progression, as well as their potential relevance for diagnosis and prognosis assessment.

REFERENCES

1. Chook YM, Blobel G. Karyopherins and nuclear import. Curr Opin Struct Biol 2001: 11; 703-715.
2. Christensen HC, Kosteljanetz M, Johansen C. Incidences of gliomas and meningiomas in Denmark, 1943 to 1997. Neurosurgery 2003:52; 1327-1333.
3. Cooper GM, Hausman RE: The Cell. A Molecular Approach, ed 3. Washington, DC: ASM press 2004: 631-635.
4. Dai C, Holland EC. Astrocyte differentiation states and gliomas formation. Cancer J 2003:9; 72-81.
5. Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, et al. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 1996: 93; 6025-6030.
6. Dong Y, Zhang H, Hawthorn L, Ganther HE, Ip C. Delineation of the molecular basis for selenium-induced growth arrest in human prostate cancer cells by oligonucleotide array. Cancer Res 2003:63; 52-59.
7. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993:75;817-825.

8. Han FC, Gong M, Ng HC, and Ho B. Identification of H. pylori strain specific DNA sequences between two clinical isolates from NUD and gastric ulcer by SSH. World J Gastroenterology 2003:9; 1747-1751
9. Hartmann TB, Thiele D, Dummer R, Schadendorf D, Eichmuller S. SEREX identification of new tumor-associated antigens in cutaneous T-cell lymphoma. Br J Dermatol 2004:150; 252-258.
10. Hufton SE, Moerkerk PT, Brandwijk R, de Bruine AP, Arends JW, Hoogenboom HR. A profile of differentially expressed genes in primary colorectal cancer using suppression subtractive hybridization. FEBS Lett 1999:463; 77-82.
11. Ingman M, Kaessmann H, Paabo S, Gyllensten U. Mitochondrial genome variation and the origin of modern humans. Nature 2000:408; 708-713.
12. Ji W, Wright MB, Cai L, Flament A, Lindpaintner K. Efficacy of SSH PCR in isolating differentially expressed genes. BMC Genomics 2002:3; 12.
13. Kim M, Kim S, Kim S, Kim BD. Isolation of cDNA clones differentially accumulated in the placenta of pungent pepper by suppression subtractive hybridization. Mol Cells 2001:11; 213-219.
14. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 1992:257; 967-971.
15. Miyasaka Y, Enomoto N, Nagayama K, Izumi N, Marumo F, Watanabe M, et al. Analysis of differentially expressed genes in human hepatocellular carcinoma using suppression subtractive hybridization. Br J Cancer 2001:85; 228-234.
16. Nutt CL, Zerillo CA, and Kelly GM, Hockfield S. Brain enriched hyaluronan binding (BEHAB)/brevican increases aggressiveness of

CNS-1 gliomas in Lewis rats. Cancer Res 2001:61; 7056-7059.

17. Palackal NT, Lee SH, Harvey RG, Blair IA, Penning TM. Activation of polycyclic aromatic hydrocarbon trans-dihydrodiol proximate carcinogens by human aldo-keto reductase (AKR1C) enzymes and their functional overexpression in human lung carcinoma (A549) cells. J Biol Chem 2002:277; 24799-24808.
18. Porkka KP, Visakorpi T. Detection of differentially expressed genes in prostate cancer by combining suppression subtractive hybridization and cDNA library array. J Pathol 2001:193; 73-79.
19. Rehman I, Cross SS, Azzouzi AR, Catto JW, Deloulme JC, et al : S100A6 (Calcyclin) is a prostate basal cell marker absent in prostate cancer and its precursors. Br J Cancer 2004:91; 739-744.
20. Sallinen SL, Sallinen PK, Haapasalo HK, Helin HJ, Helen PT, Schraml P, et al. Identification of differentially expressed genes in human gliomas by DNA microarray and tissue chip techniques. Cancer Res 2000:60; 6617-6622.
21. Shridhar V, Sen A, Chien J, Staub J, Avula R, Kovats S, et al. Identification of underexpressed genes in early- and late-stage primary ovarian tumors by suppression subtraction hybridization. Cancer Res 2002:62; 262-270.
22. Smith JM, Bowles J, Wilson M, Koopman P. HMG box transcription factor gene Hbp1 is expressed in germ cells of the developing mouse testis. Dev Dyn 2004:230; 366-370.
23. Swearingen ML, Sun D, Bourner M, Weinstein EJ. Detection of differentially expressed HES-6 gene in metastatic colon carcinoma by combination of suppression subtractive hybridization and cDNA library array. Cancer Lett 2003:198; 229-239.
24. Tanaka T, Yamakawa N, Usui M. Level of glucocorticoid receptor mRNA expression in iris tissue and postoperative ocular

inflammation in patients with uveitis. Graefes Arch Clin Exp Ophthalmol 2003:241;81-84.

25. Van Nocker S, Ludwig P. The WD-repeat protein superfamily in Arabidopsis: conservation and divergence in structure and function. BMC Genomics 2003:4; 50.
26. Vasmatzis G, Essand M, Brinkmann U, Lee B, and Pastan I. Discovery of three genes specifically expressed in human prostate by expressed sequence tag database analysis. Proc Natl Acad Sci U S A 1998:95: 300-304.
27. Velculescu VE, Zhang L, Zhou W, Vogelstein J, Basrai MA, Bassett DE Jr, et al. Characterization of the yeast transcriptome. Cell 1997:88; 243-251.
28. Wandrey M, Trevaskis B, Brewin N, Udvardi MK. Molecular and cell biology of a family of voltage-dependent anion channel porins in Lotus japonicus. Plant Physiol 2004:134; 182-193.
29. Welsh J, Chada K, Dalal SS, Cheng R, Ralph D, McClelland M. Arbitrarily primed PCR fingerprinting of RNA. Nucleic Acids Res 1992:20; 4965-4970.
30. Zeindl-Eberhart E, Haraida S, Liebmann S, Jungblut PR, Lamer S, Mayer D, et al. Detection and identification of tumor-associated protein variants in human hepatocellular carcinomas. Hepatology 2004:39; 540-549.

국문요약

악성교종에서 subtraction hybridization 을 이용한 이상 발현된 유전자의 동정

< 지도교수 이규성 >

연세대학교 대학원 의학과

뇌에 발생하는 악성신경교종의 치료에 있어서 이상 발현된 종양유전자를 찾는 일은 악성신경교종의 분자생물학적 기전을 이해하는데 기초가 될 뿐만 아니라, 이 질환의 치료에 결정적 도움을 줄 것이다. 악성신경교종에서 관찰되는 유전학적 변이의 다양성은 신호전달의 이상과 세포주기상의 변이로 요약 될 수 있다.

본 실험은 subtraction hybridization을 이용하여 악성신경교종에서 이상 발현된 종양유전자를 찾고 이를 여러 세포 주 (cell line)에서 검증하는 과정으로 진행되었다. subtraction hybridization은 Diatchenko 등에 의해 1996년 도입된 이래, 종양과 같은 특정 질환에서 정상 세포에 비해 비정상적으로 발현된 유전적 정보를 찾아내는 데 주로 이용되어 왔으며, 다른 분자생물학적 방법에 비해 빠르고 강력하다고 알려져 왔다. 우리는 subtraction hybridization을 통해 정상뇌세포보다 이상 발현된 고유한 종양유전자를 찾을 수 있었다. 악성신경교종 조직을 tester로, 같은 환자의 정상 뇌 조직을 driver로 하여 subtraction hybridization을 통해 130개의 종양관련 유전적 정보를 얻었다. 이를 Genbank™ system을 통해 분석하여 7개의 그간에는 확실하게 동정되지 않았던 유전자를 확인하였다.

Semi-quantitative RT-PCR을 통해 다른 샘플에서의 정상뇌조직과 악성신경교종조직에서의 7개 유전자의 mRNA 발현을 비교한 결과, 정상뇌조직에 비해 유의하게 발현이 증가한 소견을 보였다. 또 7개 유전자의 mRNA 발현을 관찰하기 위한 기존의 악성신경교종 세포 주와 폐암, 대장암, 전립선암 세포 주에서 시행한 RT-PCR에서, 7개의 유전자 중 3개의 유전자에서 악성신경교종 세포 주에서는 mRNA 발현이 유의하게 증가되었지만, 다른 악성 종양세포 주에서는 발현되지 않았거나 약하게 발현됨을 관찰할 수 있었다. 나머지 4개의 유전자는 악성신경교종 세포 주에서는 mRNA 발현이 약하게 또는 관찰

되지는 않았지만, 다른 악성 종양세포 주에서는 발현됨을 관찰할 수 있었다.

7개의 클론의 세포주기상의 변화를 관찰 하기 위해 시행한 serum stimulation에서 이들 클론들의 time dependent activation을 관찰할 수 있었다. 본 실험을 통해 동정한 7개의 클론 중 3개가 악성신경교종에서 특이하게 발현되는(glioblastoma-specific tumorigenesis) 유전자와 관련이 있으며, 나머지 4개는 종양의 종류와는 상관없이 종양의 성장(proliferative)에 관여하는 유전자와 관련이 있지 않나 추정할 수 있었다.

향후, 이번에 검출한 7개 유전자의 전체 염기서열분석과 이를 통한 동정된 유전자들의 검증, 관련 종양단백질 실험, 그리고 본 유전자를 통한 종양발생 동물실험이 앞으로 필요할 것으로 생각된다.

핵심 되는 말: 악성신경교종, 유전자, gene cloning, subtraction hybridization