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Laboratory Investigation

Detection of Differentially Expressed Genes in Glioblastoma by Suppression Subtractive Hybridization

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Objective : A variety of genetic alterations in human glioblastoma comprises signal transduction and cell cycle arrest control of cellular processes. Subtractive hybridization is potentially a faster method for identifying differentially expressed genes associated with a particular disease state. Using the technique of subtraction, we isolated novel genes that are overexpressed in glioblastoma tissue as compared to normal brain tissue.

Methods : We evaluated the differential expression of genes in each of hybridizing tester and driver cDNAs to digested 130 clones. After sequencing of 130 clones and homology search, this study performed to determine mRNA expression of the unknown gene, "clone 47", in brain tissue, glioblasoma, and several cancer cell lines by reverse transcription-polymerase chain reaction (RT-PCR). To test the time course for Go-phase arrest, serum stimulation and expression at various times for RT-PCR performed.

Results : We identified 23 novel genes by BLAST of the digested 130 clones. The expressions of "clone 47" mRNA of glioblastoma and several cancer lines were significantly higher than normal brain tissues and several normal cell lines. We confirmed the mRNA expression of "clone 47" was up-regulation for 0.5 ~ 1hr of WI-38 cell differentiation.

Conclusion : The novel gene, "Clone 47" is upregulated in glioblastoma tissue and several cancer cell lines. This gene is time dependent activation during time course of serum stimulation. This result suggests that "clone 47" play a role in brain tumorigenesis and the activation of this "clone 47" may be necessary for the development of cancer.

KEY WORDS : Genes · Glioblastoma · Tumorigenesis · Suppression subtractive hybridization.

Introduction

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[4]. The developments of novel biological therapies for primary brain tumors have been continued to be intensively researched. Alterations of gene expression and several genetic aberrations have been shown to occur during malignant growth, transformation, and progression of glioblastoma[1,21]. The glioblastoma is the most common tumor among primary brain tumor, and has approximately 60 percent of intracranial tumors[3]. Their distinct ability to invade 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[17]. A variety of genetic alterations in human glioblastomas comprises 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

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glioblastomas as a result of amplification or overexpression mechanisms. The second, mutations with disruption of cell cycle arrest pathways revealed in human glioblastomas. 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[5].

The identification of differentially expressed genes has been reported microarray technology. cDNA microarrays for molecular profiling of human tumors has been used recently for defining differentially expressed genes in glioblastoma[21]. The chip technology is highly efficient, but requires expensive equipment for making the microarrays. Thus, chip technology about glioblastoma study is not yet widely available[19].

In this study, using the technique of suppression subtraction hybridization(SSH) we isolated a novel gene that is overexpressed in glioblastoma as compared to normal 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 other[6,11,13,22,24]. Also, it is a potentially faster method 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 un-hybridized fraction (target) from hybridized common sequences[14]. In this report, novel genes involved in glioblastoma were identified by SSH and characterized by RT-PCR and sequenced.

Materials and Methods

Tissue materials

Through June 2002 to September 2003, glioblastoma patients consented to use of their tissues. Tumor and normal brain tissues (normal brain tissues; N = 12; tumor tissues; N=12) were collected. The patient materials consisted altogether of 12patients - 4females and 8males, aged 49 to 70years. Mean age at diagnosis was 55.2years $\pm$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.

RNA extraction and SMART(tm) PCR cDNA Synthesis

Total RNAs of the normal brain tissue and glioblastoma tissue were prepared using the Trizol reagent according to manufacturer's instructions (Nalgene, USA). The RNA was dissolved in DEPC-treated water, quantitated spectrophotometrically, and total RNA analyzed on 1.2% agarose gel. SMART cDNA synthesis used total RNA 1μg and was reverse-transcribed in 10$\mu\ell$ mixture with PowerScript™ Reverse transcriptase (BD Biosciences Clontech, 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(tm) 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 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 40$\mu\ell$ with 1$\times$TE buffer (10mM Tris-HCl, pH 8.0, 1nM EDTA). One $\mu\ell$ 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.

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 each of 1μg total RNA, using SMART™ PCR cDNA Synthesis Kit user Manual (BD Biosciences Clontech, 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 *RsaI* in a final volume of 50$\mu\ell$ at 37°C for 3hr. After extraction and precipitation of digested second strand cDNAs, the pellet was dissolved in 7$\mu\ell$ 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 $\approx$ 300ng/$\mu\ell$. *RsaI* digested ds tester cDNA was prepared as described above for the driver. Digested tester cDNA (1$\mu\ell$) was diluted in 5$\mu\ell$ of H_2O. The diluted tester cDNA (2$\mu\ell$) was then ligated to 2$\mu\ell$ of adaptor land adaptor 2R (10$\mu\ell$M) in separate ligation reactions in a total volume of 10$\mu\ell$ at 16°C overnight, using 400units/$\mu\ell$ of T4 DNA ligase in the buffer supplied from the manufacturer. After ligation, 1$\mu\ell$ of 20$\times$ 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. 1.5㎕ of tester1-1 with adaptor1, and tester1-2 with adaptor 2R was respectively hybridized with 1.5㎕ digested first stranded driver cDNA of brain tumor in 1㎕ 4×hybridization buffer solution at 68°C for 8hr. Tester1-2 hybridization sample was drawn into the pipette tip. Afterwards, 1㎕ denatured mixture from 1㎕ digested second stranded driver cDNA, 2㎕ 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 200㎕ 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 25㎕. It contained 1㎕ of diluted, subtracted cDNA, 1㎕ of PCR primer 1(10㎕M), and 23㎕ of PCR master mixture prepared using the 50(Advantage cDNA polymerase PCR Kit (BD Biosciences Clontech, 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. 3 ㎕ of primary PCR mixture was diluted in 27㎕ 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.

Cloning and analysis of the subtracted cDNA

The subtracted cDNAs obtained after secondary PCR were cloned with a T/A Cloning Kit (Promega, USA). The cDNA were ligated into T/A vectors by incubating 3㎕ of the secondary PCR amplification and 1㎕ of the vector (50ng/㎕) with 3Weiss units of T4 DNA ligase at 4°C overnight. Then, 5㎕ of ligated product was transformed into 50㎕ of competent JM109 cells for heat shock. Competent JM109 cells transformed by ligated product were 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. The plasmid DNA with inserted fragments was extracted using SV minipreps (Promega, USA) according to the manufacture's protocol. The extracted DNA was digested with EcoRI restriction enzyme and product was analyzed on 1.2% agarose gel. DNA sequencing was performed at Biotechnology Center Macrogen (Korea). Nucleic acid homology searches were performed using the BLAST program.

Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription-polymerase chain reaction(RT-PCR) was performed on normal brain and glioblastoma with 1㎕ total RNA using Superscript™ First-Strand Synthesis (Invitrogen, USA). Other cells 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). The reaction mixture was supplemented with 1㎕ oligo dT(0.5㎍/㎕), 2㎕ 10×RT buffer, 2㎕ 0.1M DTT, 1㎕ 10mM dNTP mix and water to a final volume of 19㎕. After 2min of incubation at 42°C, 1㎕ 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 of "clone 47" in tissues, based on the relative expression of 2 mRNAs : "Clone 47" and 18srRNA. To normalize mRNA amounts of "clone 47" between tissues sample, 18srRNA was used as the reference gene. Primers used were "clone 47" sense, 5′ -CCACAGGT ATCTCCCACTAA-3′ , and anti-sense, 5′ -CTACCTTCCC TTCCTCATCT-3′ , leading to a 222-bp PCR product, 18srRNA sense, 5′ -TACCTACCTGGTTGATCCTG-3′ , and anti-sense, 5′ -GGGTTGGTTTTGATCTGATA-3′ , leading to a 255bp PCR product. PCR was performed using the TaKaRa Ex Taq™ under the following PCR conditions: first 3min at 94°C , then 30sec at 94°C, 30sec at 55°C, 30sec at 72°C for 35cycle, 10min at 72°C. The product of the PCR amplification was analyzed on 1.2% agarose gel.

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, non-essential amino acids. Cells were seeded at a density of 2×10^6 cells in a 60-mm dish. The cultures were maintained at 37°C in a humidified 5% CO_2 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 analyzed "clone 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 30sec at 94°C, 30sec at 55°C, 30sec at 72°C for 35cycle, 10min at 72°C. The product of the PCR amplification was analyzed on 1.2% agarose gel.

Results

Identification of genes overexpressed in glioblastoma using subtraction hybridization

The two populations of mRNAs were used to find tumor specific genes. We identified specific genes in glioblastoma tissue, comparing with normal brain tissue. Glioblastoma was used as tester and normal brain 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. We evaluated the differential expression of genes in each of hybridizing tester and driver cDNAs to digested 130 clones with *Eco*RI restriction enzyme. The digested cDNA clones searched for sequence high homologies in the GenBank DNA database by BLAST. DNA sequencing result identified known genes of the 14 kinds (Table 1). We identified the 23 novel genes by BLAST of the digested 130 clones. In this study, we selected the one cDNA of highly expressed novel genes in glioblastoma tissue.

Expression of "clone 47" mRNA in brain tumor tissues and cancer cell lines by RT-PCR

For further target study, semi-quantitative RT-PCR analysis performed for verification of differential expression of "clone 47" in sample. Brain tissues and carcinoma cell lines were used for validation by semi-quantitative RT-PCR with housekeeping gene 18srRNA[25] as control. We compared the mRNA expression between glioblastoma tissue with 1μg of total RNA prepared from normal brain tissue and glioblastoma tissue. RT-PCR products of "clone 47" were analyzed on 1.2% agarose gel (Fig. 1). The expressions of mRNA of "clone 47" of glioblastoma tumors were significantly higher than normal brain tissues. In cancer cell lines, "clone 47" very strongly expressed in the SV40-immortalized (WI-38 VA13) cell line, lung carcinoma cell line (NCI-H596), and prostate carcinoma cell line (DU 145). However, it was similar detection in colon and colon carcinoma (KM 1214) (Fig. 2). Several of these cell lines showed differentially expressed "clone 47" from tumor than normal cell line, such as lung (WI-38), colon (CCD-18Co), and prostate (RWPE-1).

Table 1. Identification of known tumor specific genes from suppression subtraction hybridization libraries

GeneBank accession no.	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

Serum stimulation in normal WI-38 fibroblasts

To test the time course for G_0-phase arrest, WI-38 fibroblast

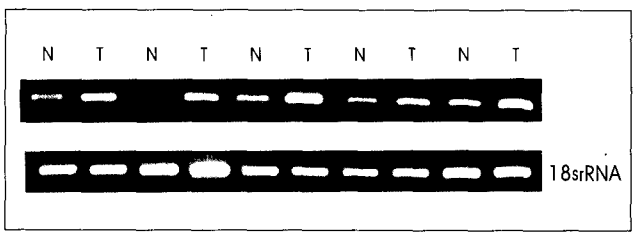

Fig. 1. Expression of brain "clone 47" mRNA from human glioblastoma tissues by RT-PCR. The expressions of mRNA of "clone 47" of glioblastoma are significantly higher than normal brain tissues. N : normal brain tissues; T : glioblastoma tissues; 18srRNA : control.

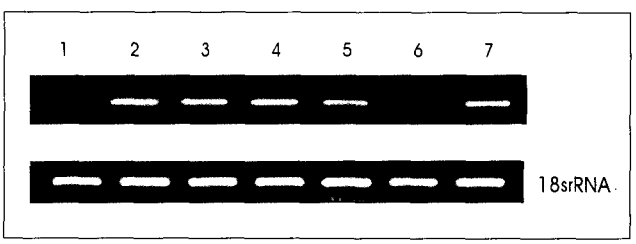

Fig. 2. Expression of "clone 47" gene mRNA from human cell line by RT-PCR. The expressions of mRNA of "clone 47" of glioblastoma are significantly higher than brain normal tissues. In cancer cell lines, "clone 47" very strongly expressing in the SV40-immortalized (WI-38 VA13) cell line, lung carcinoma cell line (NCI-H596), and prostate carcinoma cell line (DU 145). However, it is similar detection in colon and colon carcinoma (KM 1214). Lane 1, normal lung (WI-38); lane 2, SV40-immortalized (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).

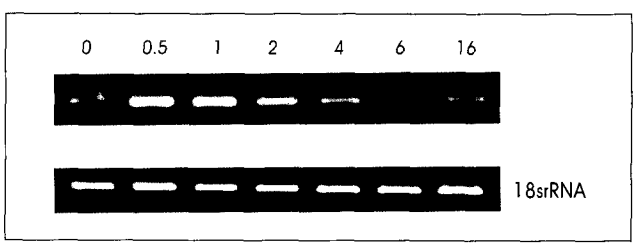

Fig. 3. WI-38 fibroblast cell time course of "clone 47". The expression of "clone 47" is significantly increased from 0.5 and 1hr after 10% FBS treatment. At 2hr after the treatment, the level of "clone 47" mRNA shows a drastic decrease.

was cultured at a density of 60% cells. We treated normal WI-38 fibroblast cells with 1% FBS and then stimulated by the addition of fresh medium containing FBS 10%, after 48hr. 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 analyzed "clone 47" mRNA levels by semi-quantitative RT-PCR on 1.2% agarose gel (Fig. 3). The time course experiment showed that the expression of "clone 47" was significantly increased from 0.5 and 1hr after 10% FBS treatment. At 2hr after the treatment, the level of "clone 47" mRNA showed a drastic decrease. We found that "clone 47" of serum stimulation conditions during the time course showed a time dependent activation.

Discussion

The identification of differentially expressed genes has provided several methods, such as, RNA fingerprinting[30], differential display polymerase chain reaction(DD-PCR)[15], subtractive hybridization[8], subtractive suppression hybridization PCR(SSH)[6,11,13,22], serial analysis of gene expression(SAGE)[28], expressed sequenced tags(EST) analysis[27], and cDNA library array technology[16,19].

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[6,9]. In this study, we isolated novel genes and known genes that is overexpressed in glioblastoma tissue as compared to brain normal tissue. DNA sequencing result identified known genes of the 14 kinds. Karyopherin appeared to have some substrate specificity and provided valuable insights into understanding the molecular mechanism of nuclear import, substrate release by GTPase and interactions with the nuclear pore complex[2]. Cutaneous T-cell lymphoma(CTCL) is a clonal lymphoproliferative disorder of mainly CD4+ T cells, with primary manifestation in the skin[10]. S100A6 (Calcyclin) is a calcium-binding protein that has been implicated in a variety of biological functions as well as tumorigenesis[20]. Mitochondrial DNA (mtDNA) has been a ability tool in our understanding of human evolution, apparent lack of recombination, owing to characteristics such as high copy number, high substitution rate and maternal mode of inheritance[12]. Human multiple tissue expression array analysis showed that aldo-keto reductase (AKR1C) was highly expressed in the human lung carcinoma cell line[18] and human aldose reductase-like protein-1 (hARLP-1) was the most prominent tumor-associated AKR (aldo-keto reductase) member detected in HCC (human hepatocellular carcinomas)[31]. HMG box containing protein 1(HBP1) is a high mobility group domain transcriptional repressor that regulates proliferation in differentiated tissues[23]. WD repeats (WDRs) are known to serve as platforms for the assembly of protein complexes or mediators of transient interplay among other proteins[26]. Voltage-dependent anion channels(VDACs) are generally considered as the main pathway for metabolite transport across the mitochondrial outer membrane[29].

This study performed to determine the mRNA level of "clone 47" gene in glioblastoma tissue and normal brain tissue by RT-PCR. The expressions of mRNA of "clone 47" novel genes of glioblastoma tissue were significantly higher than normal brain tissues. In cancer cell lines, "clone 47" very strongly expressed in the SV40-immortalized (WI-38 VA13) cell line, lung carcinoma cell line, and prostate carcinoma cell line. "Clone 47" is clearly more frequently and more strongly expressed in carcinoma cell line compared to lung and prostate. The SV40-immortalized WI-38 VA13 cell line have overcome crisis, but do not yet show any signs of malignant transformation. These cancer cell lines and glioblastoma tissue showed differential expression. These findings suggest that "clone 47" play a role in brain tumorigenesis. The activation of this "clone 47" may be necessary for the development of cancer.

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[7]. Cells were arrested for 48hr by 1% FBS. Such arrested cells then entered a quiescent stage of the cell cycle called Go, in which they can remain for long periods of time without proliferating[4]. Using semi-quantitative RT-PCR, we confirmed the mRNA expression of "clone 47" gene was up-regulated for 0.5~1hr. This gene showed time dependent activation during time course of serum stimulation.

Conclusion

In conclusion, the level of "clone 47" mRNA is significantly up-regulated in human glioblastoma tissues compared to the normal brain tissues. The "Clone 47" strongly expressed in carcinoma cell line and SV40-immortalized (WI-38 VA13) cell line compared to normal cell line. This result suggests that "clone 47" play a role in brain tumorigenesis and the activation of this mRNA may be necessary for the development of cancer.

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