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Profiling of Differentially Expressed Genes in *LRRC4* Overexpressed Glioblastoma Cells by cDNA Array

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Abstract Our previous study has shown that LRRC4 is a novel member of the leucine-rich repeat (LRR) superfamily and has the potential to suppress brain tumor growth. In order to further analyze the functions of *LRRC4* on the maintenance of normal function and suppression of tumorigenesis in the central nervous system, we investigated alterations in gene expression related to neurobiology by the Atlas array in two inducible dual-stable *LRRC4*-overexpressing cell lines. Seventeen of 588 genes spotted on the Atlas membrane showed altered expression levels in *LRRC4* transfected U251MG Tet-on cells, which are involved in cell proliferation and cell cycle progression, tumor invasion and metastasis, and neurotransmitter synthesis and release. In addition, cell invasion assay results showed that LRRC4 can inhibit the U251MG cell migration. These studies represent the first cDNA array analysis of the effects of LRRC4 on the involvement of different neurobiological genes in U251MG glioblastoma cells and provide new insights into the function of LRRC4 in glioma.

Key words differential expression; leucine-rich repeat; *LRRC4*; glioma; cDNA microarray

The leucine-rich repeat (LRR) superfamily is composed of a very heterogeneous group of proteins containing leucine-rich motifs, thought to be involved in highly specific protein-protein interactions or cell adhesion. Many LRR proteins are involved in the differentiation and development of normal nervous tissues [1,2]. *LRRC4* (GenBank accession number AF196976) was recently identified and characterized as a novel member of this family, which displayed significant downregulation in primary brain tumor biopsies [3], and could inhibit tumorigenesis and cell proliferation of U251MG glioblastoma cells [4,5]. Its predicted protein shares high homology with nervous system-expressed LRR proteins such as NGL-1 [6,7] and LRRN6A [7], which suggests that *LRRC4* is a novel gene of relevance in the molecular and cellular neurobiology of vertebrates, and may play an important role in the maintenance of normal function and the inhibition of tumorigenesis in the nervous system. However, the molecular mechanism by which LRRC4 suppresses glioma tumorigenesis and cell proliferation has not been fully explained. Gliomas are the most common primary brain tumors, which occur at any age, but especially in young to middle-aged people, and are comparatively more common in men [8]. Thus, it is critical to systemically examine the molecular changes related to neurobiology and to illuminate the *LRRC4* mechanism involved in glioma tumorigenesis.

The microarray technique first reported in 1995 by Schena *et al*. [9] allows simultaneous parallel expression analysis of thousands of genes. Information provided by cDNA microarray analysis might be useful for tumor classification, elucidation of the key factor in tumors, and identification of genes that might be applied to diagnostic purposes or as therapeutic targets [10–12]. The Atlas human cDNA expression system provides a convenient

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and quick method for profiling the expression of many hundreds of genes at the same time.

In order to gain insights into the mechanism by which LRRC4 acts on glioma and to further unveil the function of LRRC4, this study represents the first cDNA array analysis of the effects of LRRC4 on the neurobiological genes differentially expresssed in U251MG glioblastoma cells.

Materials and Methods

Cell culture

U251MG Tet-on-*LRRC4* cell lines (P27, P28) were constructed by our own laboratory [13]. U251MG Tet-on-*LRRC4* cells were cultured in RPMI 1640 (Gibco BRL, Grand Island, USA) containing 10% doxycycline-free fetal bovine serum (BD Biosciences Clontech, Palo Alto, USA) at 37 ºC in an incubator (Thermo Forma Scientific, Philadelphia, USA) with 5% CO_2.

Atlas human neurobiology array

Atlas human neurobiology array 7736-1 was purchased from BD Biosciences Clontech. The membrane contained 10 ng of each gene-specific cDNA from 588 known genes and 9 housekeeping genes. Several plasmid and bacteriophage DNAs and blank spots were also included as negative and blank controls to confirm hybridization specificity. The 588 known genes spotted on the Atlas membrane consisted of cDNAs for cell-cycle control proteins, neurotrophic factor receptors, neurotransmitter-associated proteins, DNA transcription factors, extracellular cell signaling and communication proteins, and stress response proteins. A complete list of the genes with their array positions and GenBank accession numbers is available at http://www.clontech.com.

RNA extraction

Total RNA was extracted from the cell by the standard Trizol method (Invitrogen, Carlsbad, USA). The RNA sample was digested with DNase I (10 U/μg) to remove DNA contamination which might lead to false positives during hybridization. After digestion, DNase I was removed from the sample by phenol-chloroform extraction, followed by ethanol precipitation. The RNA sample was stored at –70 ºC till use. The quantity and quality of the purified total RNA was estimated in a UV spectrophotometer.

cDNA probe synthesis

cDNA was synthesized using a coding DNA sequence (CDS) primer mix (Atlas human neurobiology CDS primer mix 7736-CDS; BD Biosciences Clontech). [α-^{32}P]2′-deoxyadenosine 5′-triphosphate was included in the cDNA synthesis reaction to facilitate probe labeling. The labeled cDNA was column-purified using the Atlas nucleospin extraction kit. The purified labeled probes were stored at –20 ºC till use.

Hybridization

The labeled cDNA probes were hybridized to the microarray nylon membrane (Atlas human neurobiology array 7736-1) according to the manufacturer's protocol. ExpressHyb solution was used for hybridization, with sheared salmon testes DNA as the blocking agent. Along with the probe, Cot-1 DNA was added to block hybridization to repetitive DNA, which might be present in the array. Hybridization was carried out at 68 ºC for 20 h. Following hybridization, the membrane was washed three times in washing solution I [2×standard saline citrate (SSC), 1% sodium dodecyl sulfate (SDS)], and once in washing solution II (0.1×SSC, 0.5% SDS). All the washings were carried out at 68 ºC for 30 min. The membrane was finally rinsed in 2×SSC, wrapped in a Saran wrap and exposed to a phosphor imager screen. The membrane was exposed overnight at –70 ºC. Two independent experiments were performed.

Image analysis

The resultant microarray spots were normalized by a two step normalization process in order to control the background and have uniform signal intensity. Background normalization was done by checking the signal intensities of negative controls; normalization for uniform signal intensity was evaluated against known "housekeeping genes" in the expression array that have a known and stable binding efficiency. Expression uniformity among the housekeeping genes was observed in all hybridization experiments. The qualitative scores of differential expression assigned to each transcript measurement were according to the following system: the fold increase (+) or decrease (–) in the range of (+/–) 0–0.5 were considered as No Change (NC); (+/–) 0.6–2.0 as Marginally Increased (MI) or Marginally Decreased (MD); and (+/–) 2.0 and above as Increased (I) or Decreased (D).

Western blot analysis

Cells were collected by centrifugation at 12,000 *g* for

10 min, then the pellet was resuspended in lysis buffer (1% Nonidet P-40, 40 mM Tris hydrochloride, pH 8.0, 150 mM NaCl) at 4 ºC for 30 min. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, USA) examined with a microplate reader (Elx800; Bio-Tek Instruments Inc., Winooski, USA) at 570 nm. Cell lysates were added to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer with complete protease inhibitors (Roche Applied Science, Indianapolis, USA), separated by SDS-PAGE and transferred to polyvinylidene fluoride (Amersham Biosciences, Piscataway, USA). The blots were incubated with goat anti-RAP1GAP (V-19, sc-10331) and anti-CD44 (N-18, sc-7051) (Santa Cruz Biotechnology, Santa Cruz, USA), rabbit anti-ephrin-B_3 (H-170, sc-20724) and anti-Rab5A (S-19, sc-309) (Santa Cruz Biotechnology), and mouse anti-β-actin (Sigma-Aldrich, St. Louis, USA) antibody, followed by a horseradish peroxidase conjugated anti-goat, anti-rabbit or anti-mouse antibody (Santa Cruz Biotechnology), developed using Supersignal chemiluminescence reagents (Pierce), and exposed to X-ray film.

In vitro cell invasion assay

The invasion assay of tumor cells was performed using a Transwell cell culture chamber (Corning Costar No. 3422; Cambridge, USA). Polyvinylpyrrolidone-free polycarbonate filters with 8 μm pore size were precoated with 1 μg/40 μl of Matrigel (BD Biosciences Clontech, Palo Alto, USA) containing fibronectin (FN) on the lower surface, then 2 μg/10 μl of Matrigel containing FN was applied to the upper surface of the filters. After the filters were dried at room temperature, they were washed gently with phosphate-buffered saline. The U251MG Tet-on-*LRRC4* cells induced with or without doxycycline were removed from the culture flask with 0.1% EDTA and suspended in RPMI 1640 with 0.1% bovine serum albumin at a concentration of 2×10^6 cells/ml. Cell suspension (100 μl) was added to the upper compartment of the chamber and incubated for 20 h at 37 ºC in air atmosphere containing 5% CO_2. After the cells on the upper side of the filters were gently wiped off, the filters were fixed in methanol, stained with hematoxylin and eosin, and mounted on glass slides. The cells that had migrated to the lower side of the filters were counted under a light microscope. The numbers of cells in five defined high power fields (magnification, 200×) were counted, and the average was determined.

Results

Identification of differentially expressed genes by *LRRC4* regulation

To identify changes in gene expression related to neurobiology, the Atlas human cDNA array membranes were hybridized with cDNA derived from U251MG Tet-on-*LRRC4* cell lines (P27, P28) in the absence (Dox–) or presence (Dox+) of doxycycline (2 μg/ml) (**Fig. 1**). No signals were visible in the blank spots and negative control spots, indicating that hybridization was highly specific. Following normalization of the hybridization levels with the housekeeping gene *GAPDH* and the β-actin gene, pairwise comparison was conducted using AtlasImage software (BD Biosciences Clontech). There were 17 genes

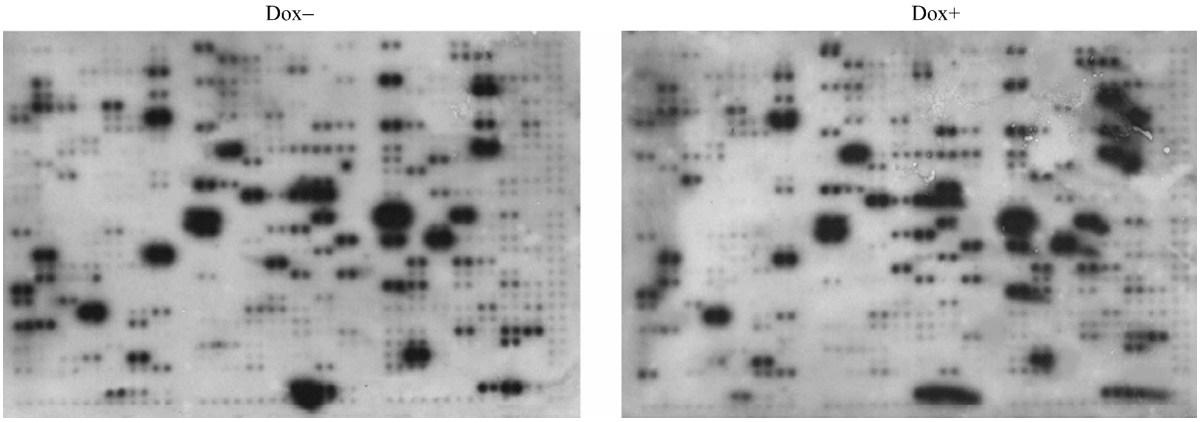

Fig. 1 Parallel analysis of gene expression profiles in U251MG Tet-on-*LRRC4* cells before or after *LRRC4* overexpression
Dox–, without doxycycline; Dox+, with doxycycline.

Table 1 Upregulated genes in *LRRC4*-transfected U251MG Tet-on cells

GenBank No.	Protein	Classification	Induction fold (Dox+ vs. Dox–)
L18983	Protein tyrosine phosphatase N	Protein phosphatases	11.3
U66406	Ephrin-B_3	Intracellular tranducers/Effectors/Modulators	4.4
M64788	Rap1 GTPase activating protein 1 (RAP1GAP)	GTP/GDP exchangers/G-protein GTPase activity modulators	5.3
M81829	Somatostatin receptor 1	Hormone receptors/G protein coupled	3.2
D16826	Somatostatin receptor 4	G protein coupled/Hormone receptors	2.1
X53655	Neurotrophin-3 (NT-3)	Neuropeptides	2.8

Dox–, without doxycycline; Dox+, with doxycycline. GDP, guanosine diphosphate; GTP, guanosine triphosphate.

Table 2 Downregulated genes in *LRRC4*-transfected U251MG Tet-on cells

GenBank No.	Protein	Classification	Induction fold (Dox– vs. Dox+)
D00017	Annexin A2	Other cytoskeleton or motility proteins/Exocytosis	–2.9
M28209	Ras-related protein Rab1A	Other trafficking or targeting proteins/G-proteins	–5.1
X94703	Ras-related protein Rab28	Other trafficking or targeting proteins/G-proteins	–5.9
M28210	Ras-related protein Rab3A	G-proteins/General trafficking	–4.0
M28214	Ras-related protein Rab3B	G-proteins/General trafficking	–3.5
M28215	Ras-related protein Rab5A	G-proteins/General trafficking	–12.1
U18420	Ras-related protein Rab5C	General trafficking	–3.1
D28538	Glutamate receptor metabotropic 5	G protein coupled/Neurotransmitter receptors	–5.0
M59040	CD44	Cell-cell adhesion receptors	–3.9
D50477	Matrix metalloproteinase 16 (MMP16)	Metalloproteinases	–6.1
M92381	Thymosin β-10	Growth factors, cytokines and chemokines	–4.9

Dox–, without doxycycline; Dox+, with doxycycline.

altered in terms of their expression levels, of which 6 were upregulated (**Table 1**) and 11 were downregulated (**Table 2**) following overexpression of *LRRC4* in U251MG cells (**Fig. 2**). Interestingly, genes involved in cell proliferation inhibition and cell cycle arrest, such as the Rap1 GTPase activating protein 1 (RAP1GAP) gene, the ephrin-B_3 gene, the somatostatin receptor genes, the protein tyrosine phosphatase N (PTPN) gene and the neurotrophin-3 (NT-3) gene, were upregulated. Conversely, the genes involved in tumor invasion and metastasis, and neurotransmitter synthesis and release, including *CD44*, *MMP16*, the thymosin β-10 (TB-10) gene, the annexin A2 and Rab protein genes, the glutamate receptor metabotropic 5 (mGlu5) gene, were downregulated.

Confirmation of differential expression

To confirm and validate the results obtained by cDNA array, we analyzed the expression of selected differentially expressed genes by conventional molecular methods. Four genes were measured by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis to verify the accuracy and the universality of the hybridization data. The RT-PCR (data not shown) and Western blot results were consistent with the hybridization data in each of the genes measured (**Fig. 3**). With the induction of doxycycline (2 μg/ml), it presented an increase in the expression levels of RAP1GAP and ephrin-B_3, and a reduction in those of Rab5A and CD44 following *LRRC4* overexpression.

LRRC4-mediated tumor cell invasion suppression

Because the *LRRC4* overexpression resulted in the downregulation of genes involved in tumor invasion and metastasis, such as CD44, MMP16, thymosin β-10 and annexin A2 genes, we next examined whether LRRC4 might affect the migration of U251MG cells. We used a

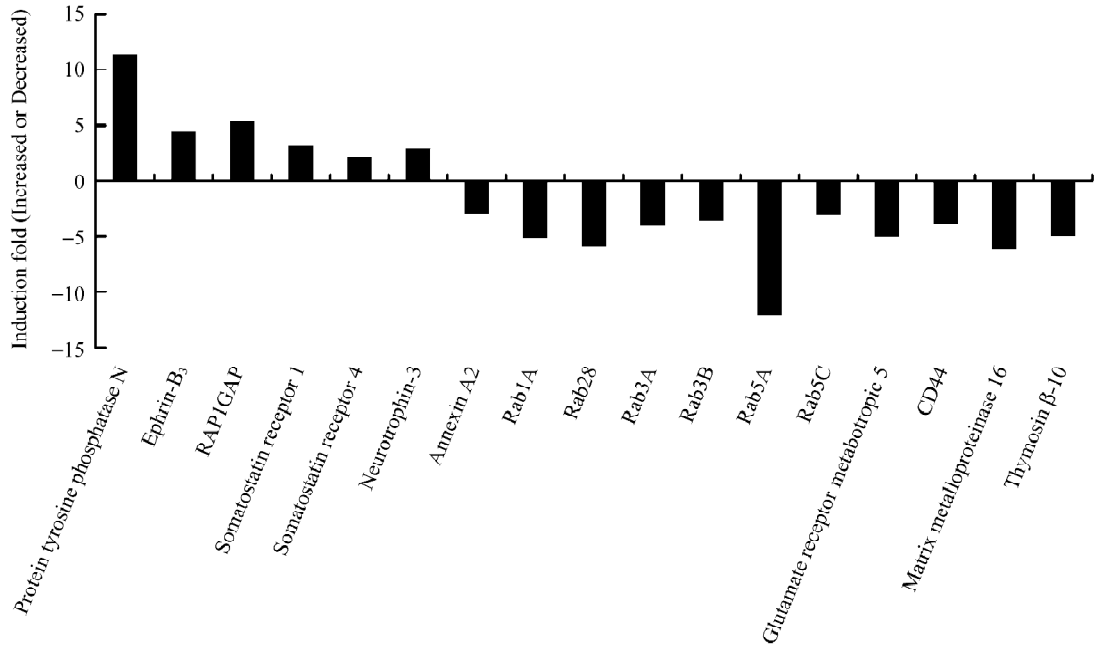

Fig. 2 Upregulated and downregulated transcripts of expression in neurobiology genes in U251MG Tet-on-*LRRC4* cells before or after induction with doxycycline

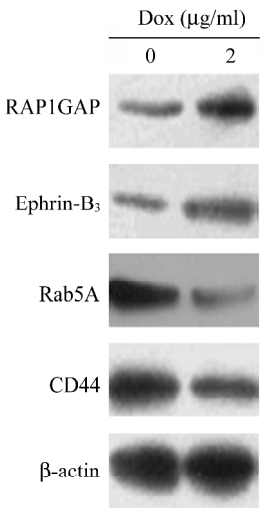

Fig. 3 Western blot analysis of RAP1GAP, ephrin-B_3, Rab5A and CD44 before or after *LRRC4* overexpression

β-actin was also tested as an internal control. Dox, doxycycline.

Transwell chamber in which the upper and lower wells were separated by a filter coated with Matrigel containing FN. As shown in **Fig. 4**, U251MG cells that had not been treated with doxycycline migrated efficiently ($P<0.05$, *t*-test); this migration was almost completely blocked following *LRRC4* overexpression after the addition of doxycycline, indicating that *LRRC4* overexpression may suppress U251MG cell invasion.

Discussion

Cell proliferation, differentiation, apoptosis, migration and interaction are controlled by tightly regulated programs of differential gene expression. Disturbances in the gene expression profiles occur in both tumor initiation and progression [14].

In this study, we concentrated on the differentially expressed genes that might be involved in LRRC4 suppressing glioma occurrence and progression in two inducible U251MG Tet-on-*LRRC4* cell lines using an Atlas human cDNA array.

We presented evidence that overexpression of *LRRC4* can elevate the expression levels of certain cell cycle progression regulators, such as RAP1GAP, ephrin-B_3, somatostatin receptors, PTPN and NT-3, by cDNA array analysis. RAP1GAP is a specific inactivator regulator of Rap1 which is a small GTPase involved in the regulation of cell proliferation, differentiation and morphology [15]. Alterations in the Rap1 signaling pathway are important in the development of human gliomas [16,17]. It was

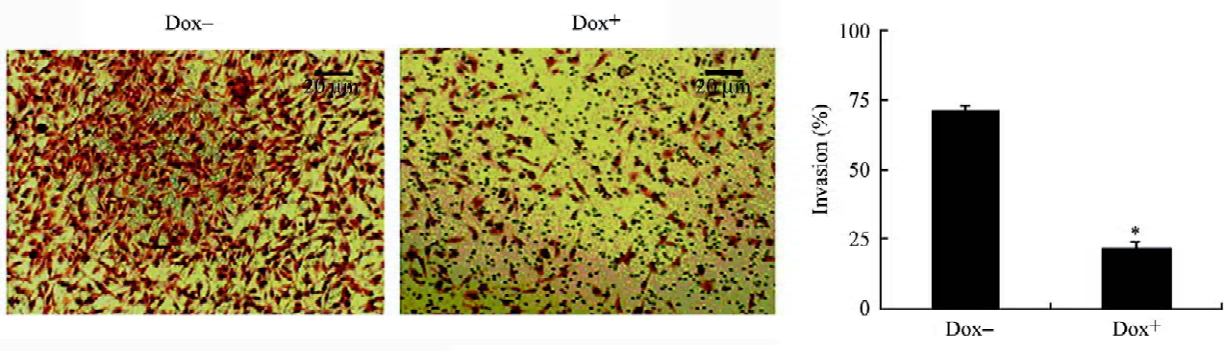

Fig. 4 Inhibition of cell invasion by *LRRC4* overexpression in U251MG cells by cell invasion assay

The cell count and invasion capability of all of the U251MG Tet-on-*LRRC4* positive clones (P27 and P28) with doxycycline were less than the same clones without doxycycline ($P<0.05$, *t*-test). These results were represented as mean±SD ($n=3$). Dox–, without doxycycline; Dox+, with doxycycline.

demonstrated that the majority of sporadically occurring astrocytomas display either loss of tuberin (RAP1GAP) or overexpression of Rap1B [18]. Also, ephrin-B_3, a membrane-bound ligand for the EphB receptor family, plays a critical role in cell cycle arrest by upregulating the expression of *p27* and downregulating the expression of *p19*, *PCNA* and *Stant2* [19]. Similar to RAP1GAP and ephrin-B_3, somatostatin receptor expression is a favorable prognostic factor in human neuroblastoma [20,21]. The Sst2 somatostatin receptor can inhibit cell proliferation through Ras-, Rap1-, and B-Raf-dependent ERK2 activation [22]. It was identified that there is a correlation between the expression of dep-1/PTPeta and the somatostatin antiproliferative effects: the expression and activation of dep-1/PTPeta is required for somatostatin inhibition of glioma proliferation [23]. In addition, the elevated expression of the NT-3 receptor TrkC by childhood medulloblastomas is associated with a favorable clinical outcome of inhibiting tumor growth through the promotion of apoptosis [24]. Our previous study verified that *LRRC4* mediates a delay of the cell cycle, possibly through upregulating the expressions of p21waf1/cip1 and p27kip1, and downregulating the expressions of CDK2, pRb, EGFR, PCNA and the ERK1/2 phosphorylation state [5,7]. This evidence suggested that LRRC4 may have an effect on suppressing cell cycle progression by impacting on Raf/Rap/Ras pathways.

Among the genes differentially expressed, we also focused our attention on the diminution of the expression of cell adhesion molecules including CD44, MMP16, TB-10 and annexin A2 involved in tumor invasion and metastasis. CD44 and MMP are key factors in the migration and invasion of deadly tumors. Glioma invasion *in vitro* is also mediated by CD44-hyaluronan interaction [25–27] and MT1-MMP/CD44/caveolin interaction [28], which could represent a potential target for anticancer therapies. Thymosin β-10 has an identified presence in a number of human tumor cell lines derived from the nervous system [29] and plays a critical role in the regulation of the anchorage-independent growth and assembly of actin filaments [30,31]. Annexin A2, a calcium and phospholipid binding protein and a substrate for protein-tyrosine kinases, is highly expressed in glioblastoma multiforme [32], and is a likely second messenger in the mitogenic pathways known to be important for the growth of these tumors [33]. Increased levels of annexin II have been observed in various cancer cells and tissues, and have been proposed as a marker of malignancy *in vivo* [34]. In addition, the annexin II tetramer can serve as a binding protein for procathepsin B and can cause tumor cell invasion and metastasis [35]. These findings indicated that LRRC4 might act as a receptor for a certain trophic factor or for an adhesion molecule participating in the maintenance of normal brain function and the inhibition of tumorigenesis, like the other LRR superfamily members. Furthermore, cell invasion assay verified *LRRC4* overexpression could markedly suppress the migration and invasion capabilities of U251MG cells. These findings imply that LRRC4 may inhibit the glioma tumor cell invasion and metastasis through regulating the expression of the above-mentioned invasion-related molecules.

The cDNA array analysis also revealed a panel of neurobiological molecules associated with neurotransmitter synthesis and release, which can be downregulated by *LRRC4* overexpression. These kinds of molecules included a set of Rab proteins and mGlu5. Rab proteins are members of the superfamily of monomeric GTPase, which belongs to the Ras superfamily of small GTPase. Rab proteins have emerged as central regulators of vesicle budding, motility and fusion [36,37]. Most are expressed

ubiquitously, such as Rab1A, Rab1B [38–40] and Rab5A [41,42], but Rab3 showed restricted tissue distributions and appeared to play specialized roles in regulated secretion or protein sorting in nerve terminals or endocrine cells [43–46]. Glutamate is an important nutritional amino acid involved in a number of biochemical pathways and is the main excitatory amino acid transmitter in the mammalian central nervous system. Glutamate excitotoxicity has been proposed to be the final common pathway in a number of nervous system diseases [47–49]. Glioma cells were shown to be impaired in their ability to remove glutamate from the extracellular space. Moreover, the tumor may actively induce neuronal death and allow tumor cells to grow by releasing glutamate at concentrations that can induce widespread neurotoxicity [50]. The alterations of these molecules indicated LRRC4 may protect nervous system normal function and suppress glioma tumorigenesis by preventing the synthesis and release of toxic neurotransmitters.

In conclusion, we have identified functionally-related groups of genes differentially expressed in two dual-stable cell lines overexpressing *LRRC4* derived from glioblastoma using a 588-gene cDNA microarray. The observations made in the present study reveal that LRRC4 possesses at least three characteristics that impact on the maintenance of normal function and the inhibition of glioma tumorigenesis in the nervous system. These studies represent the first cDNA array analysis of the effects of LRRC4 on the involvement of different neurobiological genes in U251MG glioblastoma cells and provide new insights into the function of LRRC4 in glioma. Such investigations should be performed in further studies to elucidate the possible relationship between LRRC4-regulated genes and LRRC4's precise role in glioma.

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