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ORIGINAL RESEARCH

Liquiritin potentiate neurite outgrowth induced by nerve growth factor in PC12 cells

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Abstract Neurite outgrowth and neuronal differentiation play a crucial role in the development of the nervous system. Understanding of neurotrophins induced neurite outgrowth was important to develop therapeutic strategy for axon regeneration in neurodegenerative diseases as well as after various nerve injuries. It has been reported that extension of neurite and differentiation of sympathetic neuron-like phenotype was modulated by nerve growth factor (NGF) in PC12 cells. In this study, NGF mediated neurite outgrowth was investigated in PC12 cells after liquiritin exposure. Liquiritin is a kind of flavonoids that is extracted from *Glycyrrhizae radix*, which is frequently used to treat injury or swelling for its life-enhancing properties as well as detoxification in traditional Oriental medicine. The result showed that liquiritin significantly promotes the neurite outgrowth stimulated by NGF in PC12 cells in dose dependant manners whereas the liquiritin alone did not induce neurite outgrowth. Oligo microarray and RT-PCR analysis further clarified that the neurotrophic effect of liquiritin was related to the overexpression of neural related genes such as neurogenin 3, neurofibromatosis 1, notch gene homolog 2, neuromedin U receptor 2 and neurotrophin 5. Thus, liquiritin may be a good candidate for treatment of various neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease.

Keywords Liquiritin · PC12 cells · Neurite · NGF · Microarray

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Introduction

Neurodegenerative disease was caused by changes in the central nervous system including loss of specific nerve cells and/or abnormal cellular accumulation of fibers. Alzheimer's disease (AD) is one of representative neurodegenerative dementias characterized as progressive cognitive impairment. Despite approval of several drugs for AD, the prognosis is poor. The investigative AD therapies are focused on blocking pathogenic β-amyloid protein and rescuing vulnerable neurons from degeneration (Barger and Harmon 1997). Some strategies also tried to prevent the pathogenic effects of apolipoprotein E (Martins et al.

2006) and the microtubule associated protein Tau (Lu et al. 1999). However, in detailed studies about the investigative AD therapies of nerve regeneration have rarely been performed. Based on current situation, development of new candidates for treating neurodegenerative diseases through neurotrophic and neuroprotective mechanism are extremely necessary. Among the numerous candidates of AD therapy, neurotrophic factors are significantly important. Neurotrophic factors, such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and fibroblast growth factor (FGF), play an important role in differentiation, survival and maintenance through modulation of neurite outgrowth in neuron and PC12 cells. Discovery of novel neurodifferentiation compound capable of promoting NGF activity, even at a low level might provide the basis for new treatment neurodegenerative diseases.

The pharmacology and clinical application of traditional Chinese medicine has been well documented for over centuries in China. Some Chinese herbs are effective in promoting good health and vitality, and in curing various diseases. Licorice has a long history of medical application in China due to its wide range pharmacological effects. Studies have shown that licorice extract has neuroprotective effect on the anti-metal induced apoptosis (Hibasami et al. 2005); Isoliquiritigenin was able to improve the impairment of brain reperfusion in mice cognitive model (Chen et al. 2006); Liquiritin can play an antidepressants property through antioxidant function (Brann et al. 2007). Liquiritin is one of the major constituent of *Glycyrrhizae radix* (Wang and Nixon 2001), which comprises flavonoids of 2-phenylchromone as a major constituent with a molecular formula $C_{21}H_{22}O_9$ (Okamura et al. 1999). Liquiritin induces apoptosis in stomach cancer (Hibasami et al. 2005) and promyelotic leukemia cell lines (Chen et al. 2006), and inhibits HIV replication in monocytes (Brann et al. 2007; Takei et al. 2005). We found that liquiritin has induced strong ERβ activity (data not shown) from our estrogen receptor subtype β (ERβ) agonist high-throughput drug screening process (Chen et al. 2006). Brann et al. (2007) reported that estrogen receptor modulators may have multitargeting neuroprotection.

Although numerous studies defined various pharmacological effects of liquiritin and its derivatives, neuroprotection and neurotrophic effects in neuronal cells has not been investigated in detail. Thus, we investigated the effects of liquiritin flavonoids in outgrowth of PC12 cells. We noticed significantly enhanced NGF dependent neurite outgrowth in PC12 cells after liquiritin exposure. Furthermore, we found overexpression of neural related genes such as neurogenin (Nerog) 3, neurofibromatosis (Nf) 1, notch gene homolog (Notch) 2, neuromedin U receptor (Nmur) 2 and neurotrophin (Ntf) 5 after liquiritin exposure.

Materials and methods

Cell lines and materials

PC12 cells, a rat pheochromocytoma cell line was purchased from the Chinese Type Culture Collection Center (China Academy of Medical Science, Beijing). Recombinant NGF was purchased from R&D Systems (Minneapolis, MN). PC12 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GibcoBRL; Gaithersburg, MD) and penicillin (10 U/mL), and streptomycin (10 μg/mL) at 37 °C and 5% CO_2. For the experiment of regeneration of neuritis in PC12 cells, cells were grown in 12-well plate percolate with poly-L Lysine. After serum starvation for 12 h with serum free DMEM, cells were treated with different doses of liquiritin and/or NGF in test medium (DMEM supplemented with 1% FBS, 2% HS) at 37 °C for indicated time.

Cell proliferation and cytotoxic assays

Cell proliferation was assessed by MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] assay. Briefly, at the end of cell culture, MTT was added to the culture (1:10 v/v) and incubated for 4 h at 37 °C. Cells were then solubilized in 50% dimethyl formamide and 10% sodium dodecyl sulfide (pH 4.7). The relative amount of MTT reduction was determined based on the absorbance measured at OD 570 nm using a plate reader (Multiskan MK3, Thermo Labsystems).

The cytotoxicity of liquiritin was determined by the cytotoxicity detection kit (Zhongshengbeikong, Beijing, China) that was based on the detection of

lactate dehydrogenase (LDH) released from dead cells as a result of cytotoxicity. The supernatant from liquiritin treated PC12 cells were collected and then transferred to 96-well plates. Substrate mixture containing tetrazolium salt was added, and then incubated by measuring the absorbance at 440 nm (SABA18, Roma, Italy). At least three independent experiments were performed for each study.

Cell counts and neurite analyses

After treatment, cell counts were performed in random microscopic fields and all counts were performed at least five times. With the software of Axiovison Image System (Carl Zeiss, Germany), neurite outgrowth was evaluated based both on the number of neuritis per cell and on the relative lengths of neuritis. A minimum of 100 clumps per culture were scored per dish and examined by strip counting.

Gene chip analyses

PC12 cells were plated (1×10^6 cells/well) in 6-well plate, cultured and harvested after 24 h incubation with liquiritin (20 μg/mL) in test medium and/or only in test medium. Total RNAs were isolated from PC12 cells using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). cDNA was synthesized and converted into biotin-labeled cRNA with biotin-16-UTP (Roche, Mannheim, Germany) by using a TrueLabeling-AMP Linear RNA amplification kit (SuperArray Bioscience, Frederick, MD). Prior to hybridization, the cRNA probes were purified with the ArrayGrade cRNA cleanup kit (SuperArray Bioscience). The purified cRNA probes were then hybridized to pretreat Oligo GEArray Human Alzheimer's Disease Microarray OHS-057 (SuperArray Bioscience) which covers 200 Alzheimer's disease related genes. Following several washing steps, array spots binding cRNA were detected using alkaline phosphatase-conjugated streptavidin and CDP-Star as a chemiluminescent substrate. Chemiluminescence was detected by exposing the array membranes to X-ray film. The image data were transformed into numerical data using GEArray Expression Analysis Suite software (SuperArray Bioscience). The numerical data were then further evaluated with Microsoft Excel 2007. Data evaluation included background correction (subtraction of minimum value) and median normalization. The data-filtering criteria were: at least one of the spot intensities to be compared had to be more than twice the background intensity, and the spot intensity ratios had to be greater than 3.0 (for overexpression).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent (Molecular Research Center Inc, Cincinnati, OH) according to the manufacturer's instruction. Reverse transcription was carried out using a Moloney murine leukemia virus (M-MLV) and oligo(dT) primer. PCR amplification using primer sets specific for rat Neurog 3, NF1, Ntf 5, Notch 2, Nmur 2 or β-actin was carried out at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and repeated 23 cycles followed by incubation at 72 °C for 7 min. Nucleotide sequences of the specific primers are as follows: Neurog 3 forward, TAG GAA CCT CCA AAG GGT AGA AG; Neurog 3 reverse, AGT CAG TGC CCA AAT GTA GTT GT; NF1 forward, CTG AAA TCG AAG AGA TCT GCC TA; Nf 1 reverse, ACT GTC AAG CAC AAC ATC TAG CA; Ntf 5 forward, CTC AGA ATG CAA GGC TAA ACA GT; Ntf 5 reverse, TTC ACG TAG TCT TTC CTC GTC TC; Notch 2 forward, GGG ACA TGT AAT AAC CTG GTG AA; Notch 2 reverse, GAT GTC CTC CTC ACA GTC CAT AC; Nmur 2 forward, AGA ACA AGG GAC ACC TGT GG; Nmur 2 reverse, AAT CAC CAT GCA CAC CAG AA; β-actin forward, ATC CTG AAA GAC CTC TAT GC; β-actin reverse, AAC GCA GCT CAG TAA CAG TC. The β-actin was used as an internal control to evaluate relative expression of rat neurogenin 3, NF1, Ntf 5 and Notch 2.

Statistical analysis

Statistical analyses were performed using ANOVA followed by the student *t*-test. Data were expressed as mean ± SD and $p < 0.05$ was considered statistically significant.

Results

Effect of liquiritin on viability of PC12 cells

To determine the effect of liquiritin on the proliferation of PC12 cells in the presence of NGF (2 ng/mL), PC12 cells were cultured with various concentrations (0–100 μg/mL) of liquiritin in the presence of NGF (2 ng/mL). NGF (50 ng/mL) was used as a positive control. Cell viability was assessed by observing the amount of MTT reduction. Low dose (5–20 μg/mL) treatment of liquiritin in the presence of NGF (2 ng/mL) did not affect cellular viability of PC12 cells (Fig. 1a). However, high dose (50, 100 μg/mL) treatment of liquiritin in the presence of NGF (2 ng/mL) significantly decreased cellular viability compared to NGF 50 ng/mL alone treatment in the PC12 cells (Fig. 1). More than 30% decrease in cell proliferation was observed with liquiritin 100 μg/mL + NGF 2 ng/mL treatment after 5 days in the PC12 cells (Fig. 1a). Since MTT assays measured the metabolic activities of the cells, the reduction in the cell number could either be a consequence of cell death or the reduction in the cell proliferation. The cytotoxicity test was then performed to examine whether treatment of PC12 cells with liquiritin could induce cells death. LDH assessment showed that at concentration ranging from 0 to 20 μg/mL did not induce neuronal cell death after 3 days incubation. However, at high concentration (50, 100 μg/mL), liquiritin showed weakly cytotoxic effect on the neuronal cells after 3 days treatment.

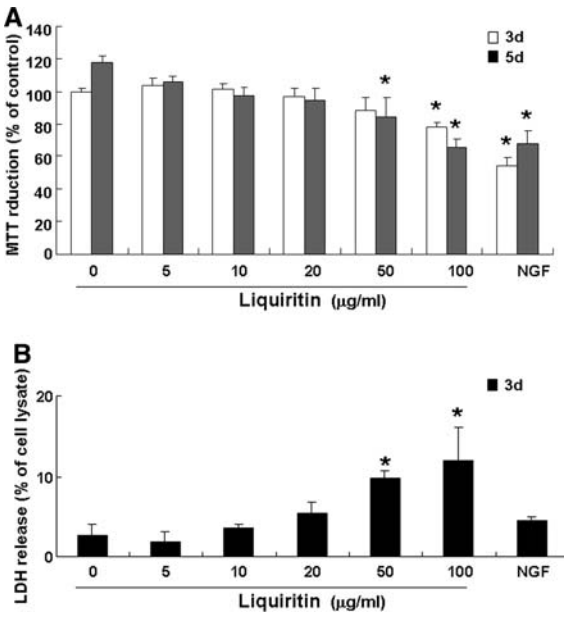

Fig. 1 Effect of liquiritin on the cell survival in PC12 cells. PC12 cells were treated with various concentrations of liquiritin in the presence of NGF (2 ng/mL) for 3 or 5 days. The cell viability was determined by MTT (**a**) or LDH release assay (**b**). The results were expressed as percentage of surviving cells over control cells (**a**) or total LDH release (**b**). The data are the mean ± SD ($n = 3$), and are representative of three or more independent experiments. * $p < 0.05$; significantly different compared to the control treated

Liquiritin enhanced the neuronal differentiation of PC12 cells

In order to investigate the neurotrophic effects of liquiritin, the effects of liquiritin (5–100 mg/mL) on neurite outgrowth in PC12 cells were evaluated (Fig. 2). As shown in Fig. 2, liquiritin enhanced neurite outgrowth in PC12 cell in the presence of low dose NGF (2 ng/mL) after 3 days treatment. Dose–dependent morphological changes were observed in the PC12 cells after liquiritin exposure (Fig. 2a). NFG (2 ng/mL) did not affect neurite outgrowth of PC12 cells. The NGF (50 ng/mL) was used as positive control. Liquiritin alone did not trigger differentiation of PC12 cells. These result demonstrated that liquiritin promote neuronal differentiation through NGF dependent manner in the PC12 cells.

Liquiritin regulates the expression of multiple genes at the mRNA level in PC12 cells

Since numerous molecules were reported to be involved in the neuronal differentiation, we performed Oligo microarray analysis to elucidate the neurotrophic mechanism in the PC12 cells with liquiritin exposure. Out of the 200 genes covered by the Oligo GEArray Alzheimer's Disease Microarray OHS-057, 28 genes were found to be more than twofold up-regulated by liquiritin (20 μg/mL) treatment after 24 h according to the chosen filter criteria (Table 1). These genes comprised a variety of ontological groups, most of which could be ascribed to cellular functions such as cell survival, synaptic formation, and cell signaling.

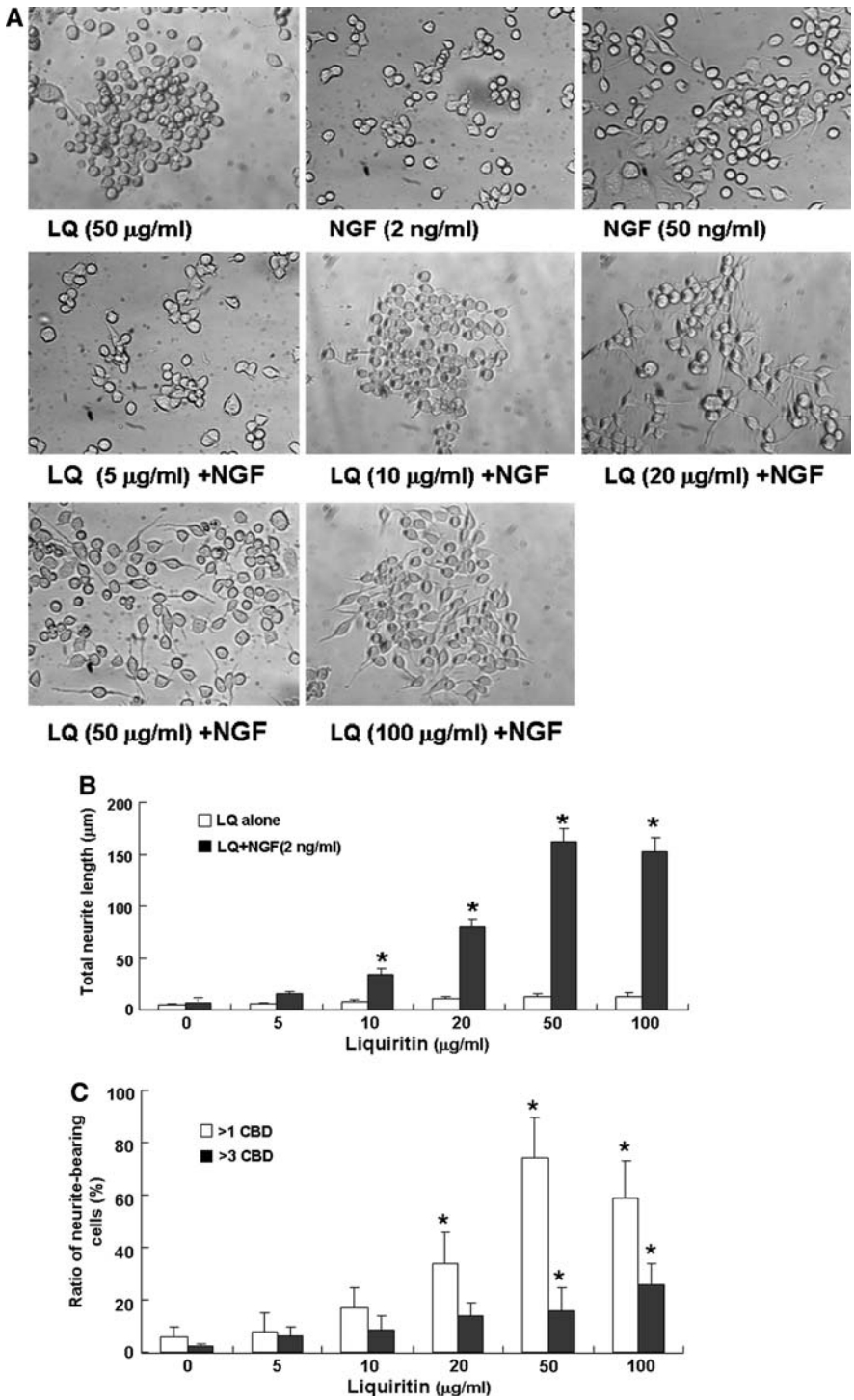

Fig. 2 Liquiritin promotes neurite outgrowth induced through NGF (2 ng/mL) in PC12 cells. **a**, **b** PC12 cells were treated with various concentrations of liquiritin in the presence or absence of NGF (2 ng/mL) for 3 days. NGF 50 ng/mL was used as a positive control. **a** Phase contrast microphotographs (100×). **b** Total cell neurite length or **c** cells with neurite processes longer than the one or three cell body diameter (CBD) were counted, and the percentage of neurite-containing cells was determined. The data are the mean ± SD ($n = 3$), and are representative of three or more independent experiments. More than 100 cells/condition were analyzed in each experiment.* $p < 0.05$; significantly different compared to the control treated. *LQ* Liquiritin

These data indicated a functional role of liquiritin in the cellular response to neuronal damage. Furthermore, 5 of 28 up-regulated genes, such as Neurog 3, Nf 1, Notch 2, Nmur 2 and Ntf 5, were highly suggested to play a role in the neuronal differentiation of PC12 cells after liquiritin exposure (Fig. 3).

Table 1 Overexpressed genes in liquiritin (20 μg/mL) treated PC12 cells analyzed by Oligo GEArray Human Alzheimer's Disease Microarray OHS-057

Gene name	Gene symbol	Fold change
Apba1	Amyloid beta (A4) precursor protein-binding, family A, member 1	2.69
Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1	2.47
Bmp3	Bone morphogenetic protein 3	2.60
Bmp6	Bone morphogenetic protein 6	3.54
Dlx2	Distal-less homeobox 2	4.48
Grin1	Glutamate receptor, ionotropic, *N*-methyl D-aspartate 1	2.86
Hdac5	Histone deacetylase 5	3.65
Hdac7a	Histone deacetylase 7A	7.79
Lhx5	LIM homeobox protein 5	2.72
Ephb2_predicted	Eph receptor B2 (predicted)	5.89
Mtss1_predicted	Metastasis suppressor 1 (predicted)	4.91
Musk	Muscle, skeletal, receptor tyrosine kinase	4.27
Neurog3	Neurogenin 3	13.22
Nf1	Neurofibromatosis 1	2.41
Nmur2	Neuromedin U receptor 2	2.69
Notch2	Notch gene homolog 2 (Drosophila)	5.89
Ntf5	Neurotrophin 5	5.43
Pspn	Persephin	2.44
Pura_predicted	Purine rich element binding protein A (predicted)	4.46
Rfng	Radical fringe gene homolog (Drosophila)	3.36
Runx3	Runt-related transcription factor 3	2.06
Sepp1	Selenoprotein P, plasma, 1	2.85
Serpinf1	Serine (or cysteine) peptidase inhibitor, clade F, member 1	3.12
Shh	Sonic hedgehog homolog (Drosophila)	2.77
Slit2	Slit homolog 2 (Drosophila)	2.19

Confirmation of oligo microarray findings by RT-PCR

We selected Neurog 3, Nf 1, Notch 2, Nmur 2, and Ntf 5 among the up-regulated genes by liquiritin treatment, and observed mRNA expression of Neurog 3, Nf 1, Notch 2, Nmur 2, and Ntf 5 using RT-PCR with rat specific primers. The efficiency of the reaction was adjusted by β-actin amplification. As shown in Fig. 4, the expressions of Neurog 3, Nf 1, Notch 2, Nmur 2, and Ntf 5 were increased by liquiritin (20 μg/mL). The RT-PCR results were consistent with that from the oligo microarray results.

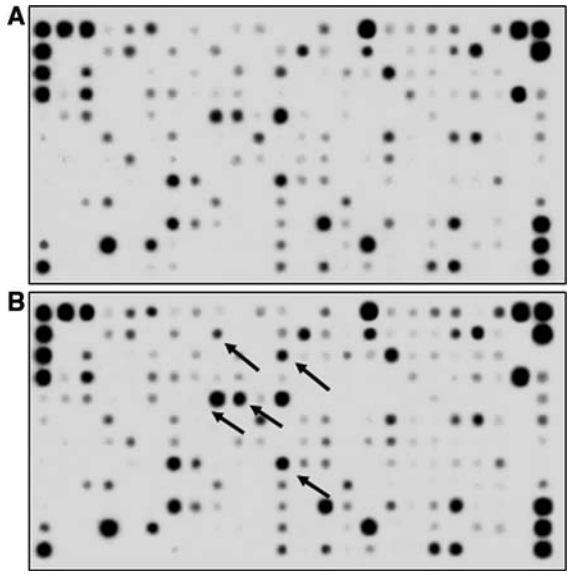

Fig. 3 Differential expression of selected genes on OHS-057 analyzed by oligo SuperArrays specific for human Alzheimer's disease. Labeled RNA extracted from PC12 cells was used to hybridize membrane-based DNA microarrays. The house-keeping genes in four corners were used as quality control in each array. *Arrowed spots* showed mRNA overexpression on OHS-057. A representative experiment out of two is shown in this figure. **a** liquiritin (20 μg/mL), **b** control

Discussion

In this study, we demonstrated that flavonoids liquiritin enhanced neurite outgrowth of PC12 cells with NGF dependent manner. Furthermore, we defined that liquiritin significantly enhanced expression of neuronal related genes such as Neurog 3, Nf 1, Notch 2, Nmur 2 and Ntf 5.

Glycyrrhizae radix (*G. radix*, liquorice) is one of the traditional Chinese medicines (TCM) and is the major constituent in several TCM formulas widely used in the treatment for asthma, coughs, and peptic ulcers. *G. radix* contains flavonoids as major constituents, which include liquiritigenin, isoliquiritigenin, liquiritin, and glycyrrihizin (Kamei et al. 2003).

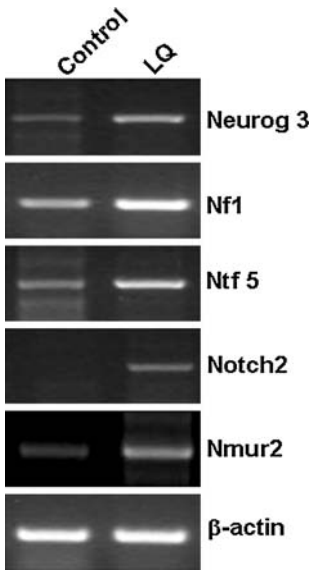

Fig. 4 Confirmation of oligo microarray results of up-regulated genes by RT-PCR. Five genes, Neurog 3, Nf 1, Notch 2, Nmur 2, and Ntf 5 were analyzed by RT-PCR with total RNA from control and liquiritin (20 μg/mL) treated PC12 cells. As an internal control, β-actin was amplified. The data are representative of results obtained from three independent experiments

Previous studies have demonstrated that licorice extract linked to anti-tussive, anti-inflammatory, anti-apoptosis, and anti-psychotic properties. Liquiritin is one of the major constituent of *G. radix* (Wang and Nixon 2001), which comprises flavonoids of 2-phenyl-chromone as a major constituents with a molecular formula $C_{21}H_{22}O_9$ (Okamura et al. 1999). Present results showed that liquritin alone did not stimulate the differentiation of PC12 cells but did so in the presence of a low dose NGF (2 ng/mL) (Fig. 2). PC12 cell, a rat pheochromocytoma cells which has been widely used as a model system for NGF-induced neuronal differentiation (Ishima et al. 2008). NGF binds to the receptor and activates the TrkA in the neuronal cells. Activation of TrkA by NGF stimulates downstream pathway molecules which result in neuronal differentiation and/or promote cell survival. Neuronal differentiation and neurite outgrowth were mediated by activation of the Ras/ERK, PI3K/Akt, and phospholipase-C-gamma 1 (PLC-γ1) signaling pathways (Read et al. 2008). Further studies are needed to elucidate the molecular mechanisms of liquiritin in these pathways.

Our microarray analysis revealed overexpression of 28 genes out of totally 200 genes with liquritin treatment (Table 1; Fig. 4) which include five neuronal related genes, such as Neurog 3, Nf 1, Notch 2, Nmur 2 and Ntf 5. Previous studies demonstrated that these neuronal related genes contribute to various functions: Neurog 3 gene promotes early retinal neurogenesis (Ma et al. 2009); Nf 1 gene, which appears to function as tumor suppressor gene, was found to produces neurofibromin and functions as a Ras-GAP, a negative regulator of Ras and play an important role in the neuronal differentiation of PC12 Cells (Patrakitkomjorn et al. 2008); Ntf 5, which belongs to the neurotrophic factors signaling family, was found to be involved in promoting survival and differentiation of mammalian neurons (Rabacchi et al. 1999); Notch2 gene, a member of the Notch family, is a signaling network which is evolutionarily conserved intercellular signaling pathway to regulate interactions between physically adjacent cells. Previous study demonstrated that notch signaling has a critical role in the maintenance and function of neuronal cells in the adult enteric nervous system (Xu et al. 2008). Taken together, the functions of these neuronal related genes were involved in a number of signaling pathways, such as cell proliferation, cell cycle, apoptosis, anti-inflammatory, antioxidant, nerve regeneration, nerve cell differentiation, migration, neural synapse formation and so on. Based on these results and our data, we believe that liquiritin induce enhancement of cellular differentiation through up-regulation of these neuronal related genes. However, further study was needed to clarify the role of these up-regulated neuronal related genes in the neural synapse formation, nerve regeneration, and nerve cell differentiation after liquiritin treatment.

Further studies are, however, required to evaluate neurotrophic property of the liquiritin in the animal models of neurodegenerative diseases and to understand the precise molecular mechanisms of neurotrophic actions of the liquiritin in vitro as well as in vivo. Nevertheless, our study highly suggested the neurotrophic effects of the liquiritin in particular, promote NGF-induced neurite outgrowth. Future works will focus on clarifying the detailed molecular mechanisms underlying the neurotrophic effects of liquiritin on neuronal cells.

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