Identification of driver genes and key pathways of prolactinoma predicts the therapeutic effect of genipin

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Abstract. The purpose of the present study was to identify the potential targets and markers for diagnosis, therapy and prognosis in patients with prolactinoma at the molecular level and to determine the therapeutic effects of genipin in prolactinoma. The gene expression profiles of GSE2175, GSE26966 and GSE36314 were obtained from the Gene Expression Omnibus database. The differentially expressed genes (DEGs) were identified after comparing between gene expression profiles of the prolactinoma tissues and normal tissues. Then, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis and protein-protein interaction (PPI) network analysis were conducted. In addition, *in vitro*, scratch assay, colony-forming assay, Cell Counting Kit 8 (CCK8) assay and flow cytometry were performed to verify the functional effects of genipin. An aggregate of 12,695, 3,847 and 5,310 DEGs were identified from GSE2175, GSE26966 and GSE36314, respectively. The results of GO and KEGG analysis showed that the DEGs significant and important for prolactinoma were mostly involved with 'spindle pole' and 'oocyte meiosis'. A total of 20 genes were selected as hub genes with high degrees after PPI network analysis, including mitogen-activated protein kinase 1 (MAPK1), MYC, early growth response 1 (EGR1), Bcl2 and calmodulin 1 (CALM1). CCK8 assay, colony-forming assay and scratch assay were performed to verify the anti-prolactinoma effect of genipin. The results of flow cytometry showed that apoptosis was increased by genipin. MAPK1, MYC, EGR1, Bcl2 and CALM1 were screened as main hub genes. Genipin upregulated the expression level of EGR1 and p21 (downstream mediator of EGR1) and EGR1, inhibited the proliferation and migration of prolactinoma cells. Genipin is a promising drug for treatment of patients with prolactinoma.

Introduction

Prolactinoma is one of the most common central nervous system tumors, which is derived from the hormone secreting epithelial cells in the anterior of the pituitary gland (1). According to the 2016 World Health Organization classification of tumors, prolactinomas are grade I/II (2), which are considered to be benign tumors. In addition, prolactinoma is one of the functional pituitary tumors, which are classified into adrenocorticotropic hormone-secreting pituitary adenoma, growth hormone-secreting pituitary adenoma and prolactinoma (3).

Recently, imaging has become important in the diagnosis of prolactinoma. As the most important tool for imaging diagnosis of prolactinoma, MRI can clearly display the size, shape and position of prolactinoma, as well as its interaction with surrounding structures. Medicinal therapy has become the first choice treatment for prolactinoma, with surgery coming second and other treatments, such as gene therapy, molecular therapeutics, chemotherapeutics, radiotherapy and physiotherapy, used as adjunctive therapy. The dopamine agonists (DAs) are the best medicinal treatment of prolactinoma and can reduce the secretion of prolactin: 70-90% of patients with microadenomas have normalized prolactin levels after DAs treatment, with menstruation

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resuming, lactation ceasing, fertility restored and shrinkage of the tumor (4). However, its side effects and adverse reactions cannot be ignored: ≤4% of properly regulated patients with prolactinoma can develop acromegaly according to a previous study (5). A recent study noted that cabergoline, one type of DA, increases the risk of valvular heart disease. In addition, there are also some studies indicating that cabergoline is associated with pituitary apoplexy after initiation of cabergoline therapy (6,7). Selective resection of prolactinoma is another common treatment method that results in the symptoms relief after surgery in the majority of patients. Although prolactinomas can be removed by a frontal or a butterfly pathway, it is often difficult to completely excise and postoperative serum prolactin levels are difficult to recover. A previous study reported that 40-80% surgeries will result in temporary improvement and half of them will relapse (8). Thus, a novel treatment strategy with improved efficacy or fewer side effects is needed.

Recently, research has focused increasingly on the initiation, progression and metastasis of prolactinoma using bioinformatics methods and microarray technology, aiming to reveal the genetic alteration and molecular mechanisms in prolactinoma (9-11). For instance, Zhang *et al* (12) showed that upregulated solute carrier family 2, facilitated glucose transporter member 11 and chromogranin B (CHGB) may be important in prolactinoma progression. Faraoni *et al* (13) also suggested that the transforming growth factor β1 system may be involved in prolactinoma progression. However, the number of studies on the explicit molecular mechanisms is small, and there is a limited and shallow understanding of prolactinoma. Therefore, further study of the molecular mechanisms of prolactinoma is urged, particularly the identification of differentially expressed genes (DEGs) and key pathways. In the present study, early growth response 1 (EGR1) was found to have a significant role in the occurrence and progression of prolactinoma by complete bioinformatics analysis, while genipin was reported as an effective targeted drug for EGR1 as it could upregulate the expression level of EGR1 and p21 (downstream mediator of EGR1), and was applied to inhibit gastric cancer in a previous report (14). Therefore, it is hypothesized that genipin may also be able to treat prolactinoma by targeting EGR1.

Bioinformatics is a technology that comprehensively applies biology, computer science and information technology to analyze numerous complex biological data. Bioinformatics combined with microarray has been broadly used to identify genetic alterations during tumorigenesis (15). In the present study, three mRNA microarray datasets were selected to identify DEGs between prolactinoma tissues and normal pituitary tissues. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were subsequently conducted. Then, a Cell Counting Kit 8 (CCK8) assay, colony-forming assay, scratch assay and flow cytometry were performed to verify the anti-prolactinoma effect of genipin. In brief, the present study aimed to provide data to determine the occurrence and progression mechanism of the prolactinoma and identify precise, promising targets for the treatment of patients with prolactinoma as well as validation of the anti-prolactinoma effect of genipin. The framework of the present study is outlined in Fig. 1.

Materials and methods

Microarray data. Three human gene expression profiles (GSE2175, GSE26966 and GSE36314) (16-18) were obtained from the Gene Expression Omnibus (GEO; ncbi.nlm.nih.gov/geo/) database in September 2017. GSE2175 contained one normal pituitary sample and one prolactinoma sample. GSE26966 consisted of nine normal pituitary samples and 14 human prolactinoma samples. GSE36314 included three normal pituitary samples and four prolactinoma samples.

Identification of DEGs. The analysis of three files was performed in September 2017, using the GeneSpring GX software (version 11.5; Agilent Technologies, Inc.), which provides powerful, accessible tools for intuitive data analysis and visualization, and this software has been extensively applied in bioinformatics analysis (19). Through the analysis of the three sequences, the DEGs between prolactinoma samples and normal samples were identified. Hierarchical clustering analysis and principal component analysis were applied to determine the probe quality control in GeneSpring. Probes with intensity values below the 20th percentile were filtered out using the 'filter probesets by expression' option. Classical t-test was used to identify DEGs, with a two-fold cutoff and P<0.01 applied to determine statistical significance, the samples size was increased to reduce false positives and false negatives. Then, a Venn plot was created using DEGs of the three datasets (bioinformatics.psb.ugent.be/webtools/Venn; version 2.1).

GO and pathway enrichment analysis of DEGs. The Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8) is an online program that provides a comprehensive set of functional annotation tools for researchers to understand biological meaning behind plenty of genes (20). GO analysis is a method that analyzes gene function, cell component and biological process. KEGG is a base for gene function analysis and genomic information link. The two were conducted online based on DAVID, and the annotation tool applied. Gene Set Enrichment Analysis (GSEA; broadinstitute.org/gsea/index.jsp) was performed (also in September, 2017) to determine which sets of genes exhibited statistical significance.

Protein-protein interaction (PPI) network construction and module selection. A PPI network of DEGs was constructed through the Search Tool for the Retrieval of Interacting Genes (STRING) database. In the present study, Molecular Complex Detection was performed based on Cytoscape (version 3.4.0) software to reveal modules of the PPI network (21). GO and KEGG pathway enrichment analysis were performed for genes in the modules. The Molecular Complex Detection (MCODE) application was applied to screen modules of PPI network in Cytoscape with degree cutoff=2, node score cutoff=0.2.

Cell lines. A rat pituitary tumor cell line (GH3) was obtained from the American Type Culture Collection and mouse pituitary tumor cell line (GT1-1) from BeNa Culture Collection. The cell lines were cultured in DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (Gibco; Thermo
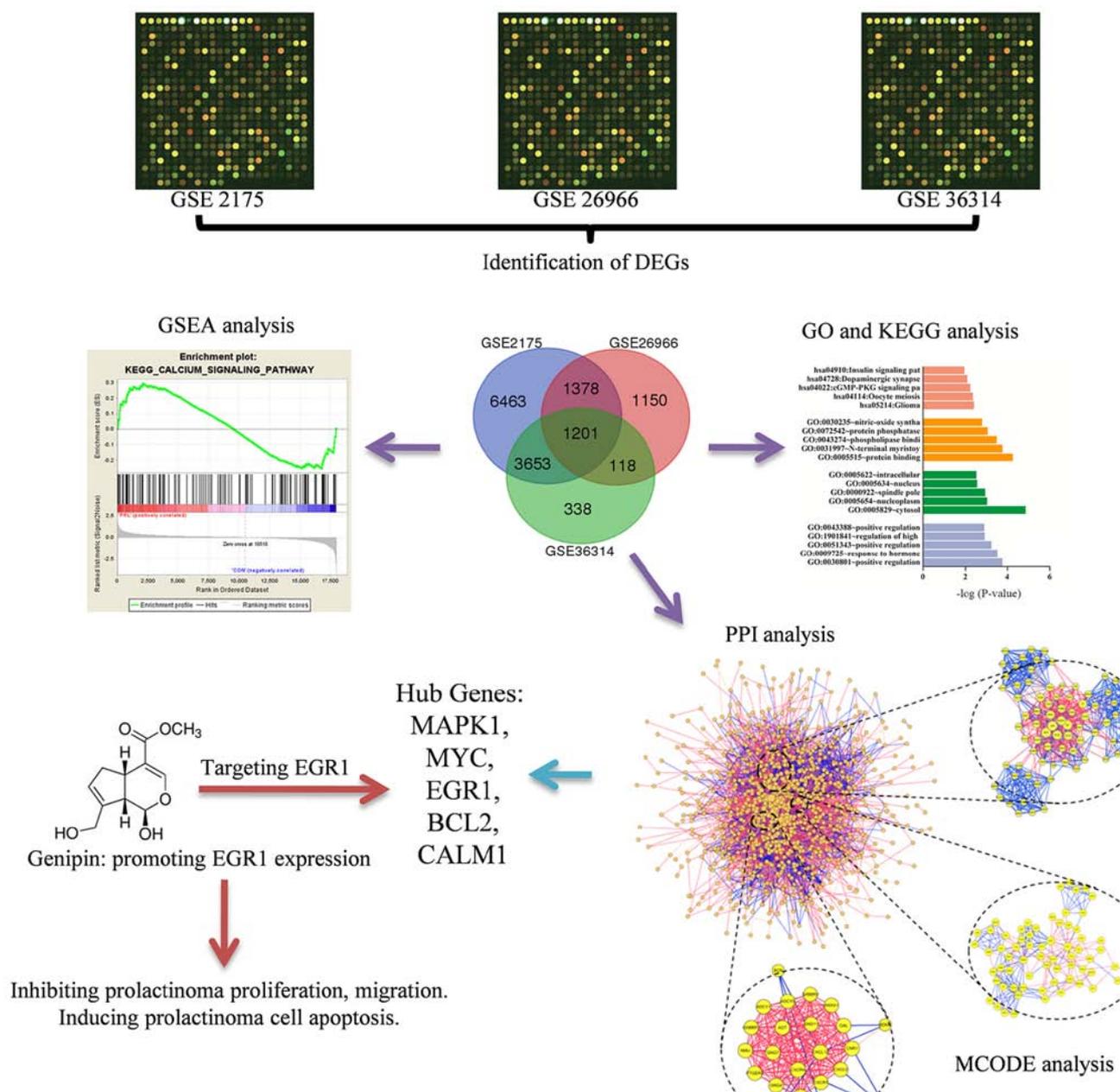
Figure 1. Study framework. The top three images were selected to represent information of tissue datasets from the Gene Expression Omnibus database. DEG, differentially expressed gene; GSEA, gene set enrichment analysis; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; EGR, early growth response 1; MAPK, mitogen-activated protein kinase; CALM, calmodulin 1; PPI, protein-protein interaction.

Fisher Scientific, Inc.). An atmosphere of 5% CO_2 and 95% air at 37°C was maintained for the cultivation of the cell lines. Genipin was purchased from Apexbio, Inc.

CCK8 assay. A total of 2,000 cells were seeded in 96-well plates and cultured in DMEM including 10% FBS for 6 days. Then, 10 μl CCK-8 was added into each well and the plate was incubated at 37°C for 2 h. The viability of cells was determined at 24 h. The cell viability was determined by measuring absorbance at 450 nm on an ELX800 UV universal microplate reader (BioTek Instruments Inc.). The experiments were repeated three times.

Colony-forming assay. The prolactinoma cells were cultured in Petri dishes with the density of 50 cells/cm². They were cultivated for 24 h *in vitro* and then treated with different doses of genipin (0, 0.5 and 2.0 μmol/l). Colonies were counted and described according to Franken *et al* after 10 days *in vitro* growth (22). Then, colonies were rinsed with PBS, fixed in 4% paraformaldehyde at 4°C for 30 min, stained with 5% crystal violet for 10 min at room temperature and rinsed twice with water. This experiment was repeated three times.

In vitro scratch assay. The GT1-1 cells were cultured to confluence on 24-well Permanox plates. A 10 μl pipette tip across each well was used to create a consistent cell-free area which the loose cells were washed out gently using DMEM. Afterwards, the cells were exposed to different doses of genipin (0, 0.5 and 2.0 μmol/l). After the scratch and at 12, 24 h, images of the scraped area were captured using phase

contrast microscopy. The remaining wounded area and the scratch width at six different points per image were measured. This experiment was repeated three times.

Flow cytometry. Prolactinoma cells in the log growth phase were seeded into 6-well plates with a density of $2x10^5$ cells/well and the cells were treated with different doses of genipin (0, 0.5 and 2.0 μmol/l). Following culture for 48 h, the cells were harvested using accutase detachment solution (Sigma-Aldrich; Merck KGaA) and Annexin-V-FITC/propidium labeling was conducted using Annexin-V-FITC/PI apoptosis detection kit (KGI Biotechnology. Ltd.) according to the manufacturer's protocol. The flow cytometer was used to detect the stained cells and the results analyzed with the FACSDiva version 6.2 (BD Biosciences). This experiment was repeated three times.

Statistical analysis. All statistical data were entered into SPSS version 18.0 (SPSS Inc.) for analysis. One-way analysis of variance was performed to analyze quantitative data and Tamhane's T2 was used to conduct post hoc testing. Data are presented as mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of DEGs. An aggregate of 12,695 DEGs were identified from GSE2175, of which 8,314 were upregulated and 4,381 were downregulated. A total of 3,847 DEGs were identified in GSE26966, among which 1,766 were upregulated and 2,081 were downregulated. A total of 5,310 DEGs were picked up from GSE36314, which 2,246 genes were upregulated and 3,064 genes were downregulated. Venn plot demonstrated that there were 1,201 common DEGs among the three datasets (Fig. 2A and Table I). Among them, 570 mutual upregulated genes and 631 mutual downregulated genes were identified. Heat maps of the DEGs in GSE26966 and GSE36314 that perform the most important role are presented in Fig. 2B and C.

Functional and pathway enrichment analysis. To further investigate the function of identified DEGs, the mutual downregulated and upregulated DEGs were entered into DAVID for GO and KEGG pathways analysis. The GO analysis and KEGG analysis of DEGs are presented in Table II. For biological process, upregulated DEGs were significantly enriched in 'positive regulation cyclic nucleotide metabolic process', 'response to hormone' and 'positive regulation of cyclic-nucleotide phosphodiesterase activity' terms, while downregulated DEGs were enriched in 'peptidyl-tyrosine phosphorylation', 'signal transduction' and 'positive regulation of cell cycle'. For cell component, the upregulated genes were mainly enriched in the 'associated cytosol', 'nucleoplasm' and 'spindle pole', and the downregulated DEGs enriched in 'cytoplasm', 'receptor complex' and 'integral component of plasma membrane' terms. For molecular function, the upregulated DEGs enriched in 'protein binding', 'N-terminal myristoylation domain binding' and 'phospholipase binding' terms; the downregulated DEGs tended to enrich in 'protein binding', 'protein tyrosine kinase activity' and 'transmembrane receptor protein tyrosine kinase activity' terms. Table II also presents the most significant enriched pathways of the mutual

upregulated DEGs and downregulated DEGs according to KEGG analysis. The upregulated DEGs were enriched in the terms 'glioma', 'oocyte meiosis' and 'cGMP-PKG signaling pathway', while the downregulated DEGs were mostly involved in pathways including 'cancer', 'focal adhesion' and 'PI3K-Akt signaling pathway' (Fig. 2D and E). In addition, the results of GSEA analysis indicated that the expression profiles of prolactinomas were mainly enriched in 'calcium signaling pathway' and 'Parkinson's disease' (Fig. 2F and G).

Module screening from the PPI network. The PPI network of DEGs consisted of 1,142 nodes and 7,278 edges. Degrees >72 were set as the cut-off criterion. A total of 20 genes were selected as hub genes: MAPK1, JUN, MYC, FOS, EGR1, Bcl2, IL6, CALM1, IL8, STAT3, ESR1, RPS27A, ACACB, CD44, INSR, ERBB2, PPP2CA, CDK2, PTPRC and SMAD3 (Table III). Among those genes, the node degree of Mitogen-activated protein kinase 1 (MAPK1) was the highest, at 183. The top three significant modules were selected (Fig. 3). Functional annotation of the modules genes is shown in Table IV. The genes of module 1 were associated with cellular structure, including 'endoplasmic reticulum lumen', 'extracellular matrix organization' and 'collagen trimer'. Genes of module 2 were associated with 'cancer', 'GABAergic synapse' and 'morphine addiction'. Genes of module 3 associated with 'type I interferon signaling pathway', 'nucleoplasm' and 'PI3K-Akt signaling pathway'.

Genipin reduces proliferation of prolactinoma cells. CCK8 assay was performed to evaluate the effects of genipin on cell survival. The results revealed that the viability of cells in cell lines GH3 and GT1-1 were significantly decreased by genipin, as in Fig. 4A. For further confirmation, a colony-forming assay was conducted to define the effects of genipin in prolactinoma cells. As demonstrated in Fig. 4B and C, there were fewer and smaller clonogenicities in the genipin group compared with the control group, and the percentage of clone formation in the control was significantly higher than the drug groups (0.5 and 2 μmol/l; $P<0.05$), and the percentage of clone formation was found to be significantly different between the two different drug dose groups.

Genipin induces migration of prolactinoma cells. A scratch assay was performed to verify the effect of genipin on invasion and migration of prolactinoma cells, with the widths of scratched areas recorded 0, 12 and 24 h following the scratch. As demonstrated in Fig. 4D and E, the widths of scratched were clearly smaller after 24 h in the control group, but only slightly decreased in genipin group, while after 24 h, it was found that the wounds in control group were also clearly smaller compared with the genipin group. With time, the wound widths decreased in the control and the genipin groups, and the control group showed a significant decrease compared with the genipin group.

Genipin induces apoptosis of prolactinoma cells. Flow cytometry was performed to clarify the mechanism of genipin inhibition in prolactinoma cells and the prolactinoma cells were measured following culture with different doses of genipin for 48 h. Fig. 5A and B demonstrate that the percentage of GH3

Table I. Venn plot analysis results of DEGs among the three datasets.

Name	Total DEGs	Elements
GSE2175, GSE26966 and GSE36314	1,201	CREB3L1, SMARCD3, RALYL, BBOX1, CXCR4…
GSE2175 and GSE26966	1,378	MSRB1, ELMO2, PKNOX2, CLK4, IRAK1…
GSE2175 and GSE36314	3,653	MMP2, SAMD4A, GPR98, KCNG1, DECR1…
GSE26966 and GSE36314	118	TMSB10, KCNA6, NEFH, ID1, CGA…
GSE2175	6,463	AACS, RPS11, PNMA1, HAUS2, A4GNT…
GSE26966	1,150	CLMP, CD99P1, LOC100287896, TMC4, LINC01279…
GSE36314	338	RPS18, ZG16, SERPINF1, NUDT1, LOC101930303 /// TRIM16 /// TRIM16L

DEG, differentially expressed gene. /// genes sharing one genetic locus.
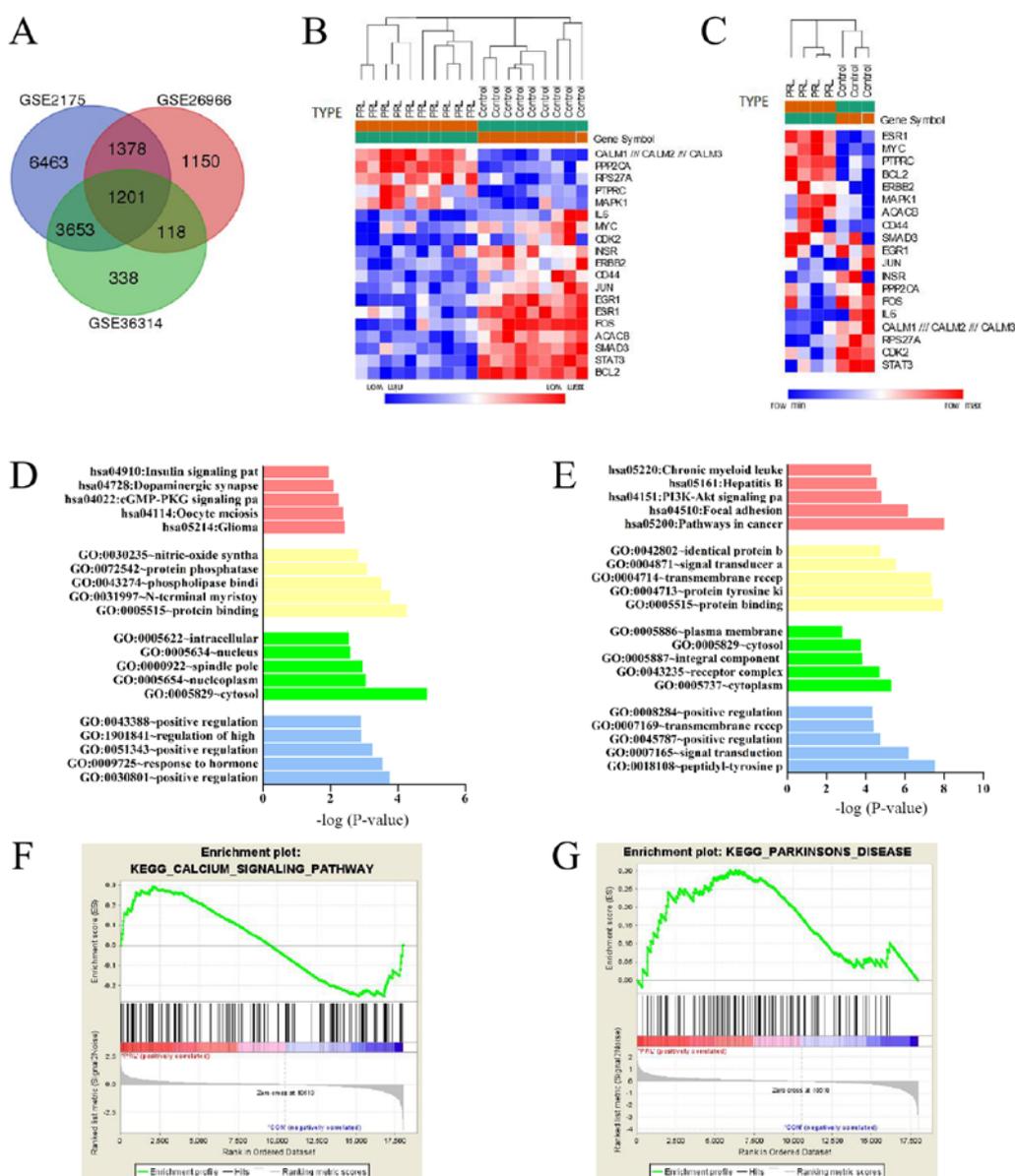
Figure 2. (A) Venn plot of DEGs among the three datasets. (B) DEGs expression heat map of GSE26966. (C) DEGs expression heat map of GSE36314; red represents upregulated genes and blue downregulated genes). (D) Functional and pathway enrichment analysis of upregulated genes among the three datasets. (E) Functional (GO) and pathway (KEGG) enrichment analysis of downregulated genes among the three datasets. The results of GO include biological processes, cell component, and molecular function, marked with different colors, as well as results of KEGG. Gene set enrichment analysis results among the three datasets for (F) 'calcium signaling pathway' and (G) 'Parkinson's disease'. The raw data were downloaded from Gene Expression Omnibus. DEG, differentially expressed gene; PRL, prolactinoma; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table II. Functional and pathway enrichment analysis of upregulated and downregulated genes among three datasets (the primitive data downloaded from Gene Expression Omnibus database).

A, Upregulated

Category	Term	Count	%	P-value
GOTERM_BP_DIRECT	GO:0030801 positive regulation of cyclic nucleotide metabolic process	3	0.012881618	1.72×10^{-4}
GOTERM_BP_DIRECT	GO:0009725 response to hormone	5	0.021469363	2.81×10^{-4}
GOTERM_BP_DIRECT	GO:0051343 positive regulation of cyclic-nucleotide phosphodiesterase activity	3	0.012881618	5.68×10^{-4}
GOTERM_BP_DIRECT	GO:1901841 regulation of high voltage-gated calcium channel activity	3	0.012881618	1.18×10^{-3}
GOTERM_BP_DIRECT	GO:0043388 positive regulation of DNA binding	4	0.017175491	1.23×10^{-3}
GOTERM_CC_DIRECT	GO:0005829 cytosol	45	0.193224269	1.38×10^{-5}
GOTERM_CC_DIRECT	GO:0005654 nucleoplasm	35	0.150285543	8.99×10^{-4}
GOTERM_CC_DIRECT	GO:0000922 spindle pole	6	0.025763236	1.10×10^{-3}
GOTERM_CC_DIRECT	GO:0005634 nucleus	55	0.236162995	2.63×10^{-3}
GOTERM_CC_DIRECT	GO:0005622 intracellular	20	0.085877453	2.86×10^{-3}
GOTERM_MF_DIRECT	GO:0005515 protein binding	89	0.382154665	5.46×10^{-5}
GOTERM_MF_DIRECT	GO:0031997 N-terminal myristoylation domain binding	3	0.012881618	1.68×10^{-4}
GOTERM_MF_DIRECT	GO:0043274 phospholipase binding	4	0.017175491	3.12×10^{-4}
GOTERM_MF_DIRECT	GO:0072542 protein phosphatase activator activity	3	0.012881618	8.26×10^{-4}
GOTERM_MF_DIRECT	GO:0030235 nitric-oxide synthase regulator activity	3	0.012881618	1.53×10^{-3}
KEGG_PATHWAY	hsa05214: Glioma	5	0.021469363	3.70×10^{-3}
KEGG_PATHWAY	hsa04114: Oocyte meiosis	6	0.025763236	4.22×10^{-3}
KEGG_PATHWAY	hsa04022: cGMP-PKG signaling pathway	7	0.030057109	5.62×10^{-3}
KEGG_PATHWAY	hsa04728: Dopaminergic synapse	6	0.025763236	8.28×10^{-3}
KEGG_PATHWAY	hsa04910: Insulin signaling pathway	6	0.025763236	1.23×10^{-2}

B, Downregulated

Category	Term	Count	%	P-value
GOTERM_BP_DIRECT	GO:0018108 peptidyl-tyrosine phosphorylation	13	0.050286245	2.95×10^{-8}
GOTERM_BP_DIRECT	GO:0007165 signal transduction	31	0.119913353	6.22×10^{-7}
GOTERM_BP_DIRECT	GO:0045787 positive regulation of cell cycle	6	0.023209036	1.74×10^{-5}
GOTERM_BP_DIRECT	GO:0007169 transmembrane receptor protein tyrosine kinase signaling pathway	8	0.030945381	3.96×10^{-5}
GOTERM_BP_DIRECT	GO:0008284 positive regulation of cell proliferation	16	0.061890763	4.64×10^{-5}
GOTERM_CC_DIRECT	GO:0005737 cytoplasm	75	0.290112951	4.95×10^{-6}
GOTERM_CC_DIRECT	GO:0043235 receptor complex	9	0.034813554	1.96×10^{-5}
GOTERM_CC_DIRECT	GO:0005887 integral component of plasma membrane	28	0.108308835	1.56×10^{-4}
GOTERM_CC_DIRECT	GO:0005829 cytosol	50	0.193408634	1.74×10^{-4}
GOTERM_CC_DIRECT	GO:0005886 plasma membrane	55	0.212749497	1.53×10^{-3}
GOTERM_MF_DIRECT	GO:0005515 protein binding	116	0.44870803	1.13×10^{-8}
GOTERM_MF_DIRECT	GO:0004713~protein tyrosine kinase activity	12	0.046418072	3.50×10^{-8}
GOTERM_MF_DIRECT	GO:0004714 transmembrane receptor protein tyrosine kinase activity	8	0.030945381	4.77×10^{-8}
GOTERM_MF_DIRECT	GO:0004871 signal transducer activity	12	0.046418072	2.91×10^{-6}
GOTERM_MF_DIRECT	GO:0042802 identical protein binding	21	0.081231626	1.76×10^{-5}
KEGG_PATHWAY	hsa05200: Pathways in cancer	23	0.088967972	9.94×10^{-9}
KEGG_PATHWAY	hsa04510: Focal adhesion	15	0.05802259	6.84×10^{-7}
KEGG_PATHWAY	hsa04151: PI3K-Akt signaling pathway	17	0.065758935	1.57×10^{-5}
KEGG_PATHWAY	hsa05161: Hepatitis B	11	0.042549899	2.67×10^{-5}
KEGG_PATHWAY	hsa05220: Chronic myeloid leukemia	8	0.030945381	5.28×10^{-5}

GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table III. Hub genes among the three datasets.

Gene symbol	Degree	Betweenness
MAPK1	183	0.08796778
JUN	163	0.05703413
MYC	147	0.06479138
FOS	123	0.02616212
EGR1	120	0.0140681
Bcl2	119	0.0274578
IL6	117	0.04994624
CALM1	116	0.06850938
IL8	110	0.02885198
STAT3	108	0.02679268
ESR1	103	0.03289612
RPS27A	94	0.04228441
ACACB	89	0.04358763
CD44	88	0.02006192
INSR	84	0.01708239
ERBB2	81	0.01628601
PPP2CA	77	0.02323539
CDK2	73	0.02347513
PTPRC	73	0.01304584
SMAD3	72	0.02005951

cells that were defined as non-apoptotic, necrotic, late apoptosis or early apoptosis were 96.0, 0.26, 0.39 and 3.31%, respectively, in the control group, 21.8, 1.39, 69.6 and 7.25% in the low dose group, and 1.72, 2.02, 88.4 and 7.81% in the high dose group; while the percentage of non-apoptotic, necrotic, late apoptosis or early apoptosis GT1-1 cells were 62.9, 9.92, 15.0 and 12.2% in the control group, 0.94, 0.23, 49.7 and 49.1% in the low dose group, and 0.69, 0.02, 42.1 and 57.2% in the high dose group, respectively. Fig. 5C and D demonstrates that normal (non-apoptotic) cells were dominant in the control group; in the low and dose groups, the percentage of late and early apoptosis cells was increased.

Discussion

Prolactinoma is the most common pituitary tumor with a secretory function. Prolactinoma monoclonal adenomas account for ~15% of primary intracranial neoplasms. Prolactinoma is usually benign, but secretes excess hormones, which may lead to distinct endocrine syndromes, and tumor growth or compressive symptoms (23). Despite the common application treatment with DAs and selective resection of prolactinoma, their side effects and adverse reactions are difficult to ignore. Recently, studies focusing on prolactinoma have been performed (12,13), but the mechanism of prolactinoma occurrence remains poorly understood and further study is required. Therefore, promising therapy targets for prolactinoma and data to illustrate the progression mechanism of prolactinoma are provided in the present study. In addition, genipin is reported as a drug promoting EGR1 expression (14), which was identified as hub gene in the present study. CCK8 assay,

colony-forming assay, scratch assay and flow cytometry were performed to verify the anti-prolactinoma effects of genipin.

The present study extracted data from GSE2175, GSE26966 and GSE36314 datasets, and identified 12,695, 3,847 and 5,310 DEGs, respectively. A total of 1,201 mutual DEGs were screened among the three datasets, with 570 mutual upregulated DEGs and 631 mutual downregulated DEGs. These DEGs may have an important role during the pathogenesis of prolactinoma and could be applied as diagnostic or prognosis markers and treatment targets in the future.

GO and KEGG pathway enrichment analysis were performed in order to gain further insight into the molecular mechanism of the prolactinoma occurrence. The upregulated DEGs were mostly associated with 'spindle pole', 'oocyte meiosis' and 'nucleus', which are associated with development of tumors. The downregulated genes were mainly associated with 'signal transduction', 'cytoplasm' and 'receptor complex', which meant the decline of normal cell functions. In addition, it was noted that 'peptidyl-tyrosine phosphorylation gene' decreased, which may be connected with the activation of proto-oncogenes or the inactivation of the tumor suppressor genes. According to the results of KEGG analysis, the upregulated DEGs were mainly enriched in 'cGMP-PKG signaling pathway', 'glioma' and 'oocyte meiosis', while the downregulated DEGs were associated with 'focal adhesion' and 'PI3K-Akt signaling pathway'. A number of studies have demonstrated that disruption of the PKG signaling pathway leads to various pathological changes in the heart, including vascular and ventricular dysfunction, fibrosis and hypertrophy (24,25). The cGMP-PKG signaling pathway has also been reported as having a close association with colon cancer and has been considered as a therapeutic strategy for colon cancer (26). It has been hypothesized that blocking the PKG signaling pathway might be an effective solution for patients with prolactinoma (27). Previous studies have shown that the PI3K-Akt signaling pathway performs a crucial role in the growth, proliferation, metabolism, survival and angiogenesis of cancer cells (28,29). For instance, Wu *et al* (30) identified that the PI3K-Akt signaling pathway effectively ameliorated gastric tumor development by delaying growth, inducing apoptosis, and inhibiting metastasis and angiogenesis. The present study hypothesized that reduced function of PI3K-Akt signaling pathway may be associated with prolactinoma metastasis and invasion. The GSEA analysis suggested that prolactinoma has a close association with Parkinson's disease and the calcium signaling pathway, which supports the results of GO and KEGG analysis. This indicates that there may be a link between the progression mechanism of prolactinoma and Parkinson's disease, but this requires further investigation.

In order to obtain the hub genes among the identified DEGs, the 1,021 DEGs were analyzed using PPI network according to the STRING database. A total of 24 genes were selected with degrees >72; EGR1, MAPK1, MYC, Bcl2 and calmodulin 1 (CALM1). EGR1, a primary response gene that encodes a zinc finger DNA-binding protein, is reported to be critical in cancer metastasis and tumor invasion (31). This gene was found to be regulated by gonadotropin-releasing hormone (GnRH) to regulate the expression of luteinizing hormone β polypeptide, one of the GnRH-responsive genes, and was revealed to be associated with prolactinoma in a previous study (31). Previous
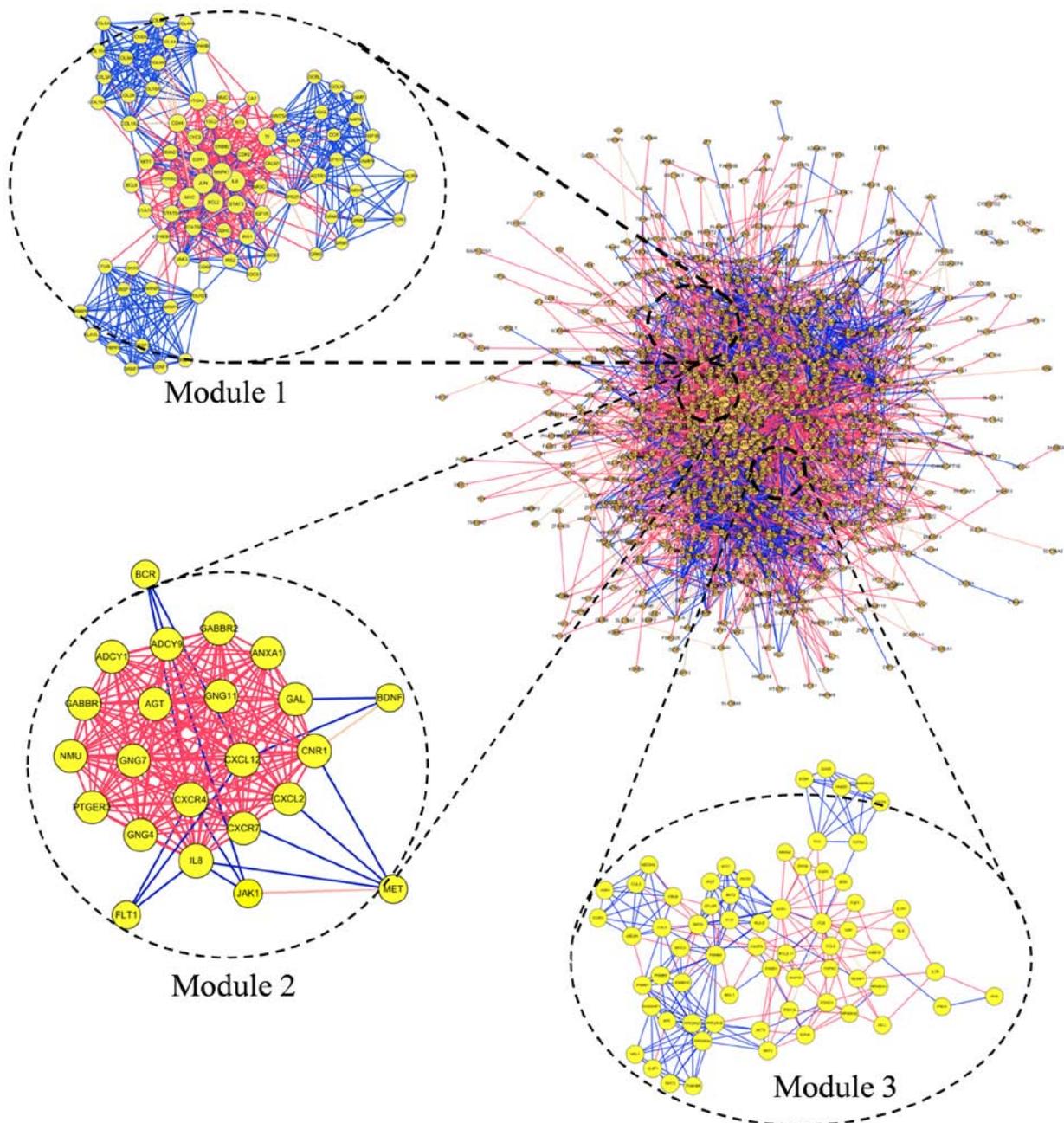
Figure 3. Top three modules from the protein-protein interaction network.

studies have suggested that genipin inhibits the proliferation of various cancer cells by promoting EGR1 function, such as gastric, colon and breast cancer (32,33). Ko *et al* (14) demonstrated that genipin can induce apoptosis in the AGS human gastric cancer cell via a p53-independent EGR1/p21 signaling pathway. Thus, it was hypothesized that genipin might have the same effect on prolactinoma. MAPK1 interacts with a series of important signaling components and phosphorylation events, which are vital in the process of neoplasia. Extracellular signals are transmitted by MAPK1, which adjusts cell growth, differentiation, proliferation, apoptosis and migration (34). For instance, microRNA-585 can suppress tumor proliferation and migration in gastric cancer by directly targeting MAPK1 (35). In addition, a previous study demonstrated that fulvestrant was effective in suppressing prolactinoma cells by inhibiting

the estrogen receptor and MAPK pathway (36). This implies that MAPK1 is a potential treatment target for prolactinoma and the role of MAPK1 in prolactinoma requires further study. MYC, a transcription factor that is overexpressed in cancers, is a proto-oncogen, which is extensively expressed in Burkitt lymphoma (37). MYC was one of hub genes identified in the present study and exhibited abnormal expression compared with normal tissue and prolactinoma tissue; it served an important role in the progression of prolactinoma. Kim *et al* (38) demonstrated that MYC is involved in preventing immune cells from attacking non-small cell lung cancer cells. A previous study noted that translocations of MYC contributed to lymphoma, especially involving Bcl2, and indicated a poor prognosis (39). High serum LDH levels are often associated with malignant tumor, which may be related

Table IV. Functional and pathway enrichment analysis of the modules genes.

A, Module 1

Term	Count	P-value	False discovery rate	Genes
GO:0005788 endoplasmic reticulum lumen (CC)	8	4.18×10^{-7}	4.93×10^{-4}	P4HB, COL9A2, COL2A1, COL16A1, COL11A1, COL5A2, COL4A6, COL4A5
GO:0030198 extracellular matrix organization (BP)	8	8.27×10^{-7}	1.23×10^{-3}	COL9A2, CD44, COL2A1, COL16A1, COL11A1, COL5A2, COL4A6, COL4A5
GO:0005581 collagen trimer (CC)	6	3.06×10^{-6}	3.61×10^{-3}	COL9A2, COL2A1, COL11A1, COL5A2, COL4A6, COL4A5

B, Module 2

Term	Count	P-value	False discovery rate	Genes
hsa05200: Pathways in cancer (KEGG)	11	4.30×10^{-9}	4.68×10^{-6}	ADCY1, PTGER3, BCR, ADCY9, CXCR4, MET, JAK1, GNG11, GNG4, CXCL12, GNG7
hsa04727: GABAergic synapse (KEGG)	7	3.22×10^{-8}	3.50×10^{-5}	ADCY1, ADCY9, GABBR1, GNG11, GABBR2, GNG4, GNG7
hsa05032: Morphine addiction (KEGG)	7	4.87×10^{-8}	5.29×10^{-5}	ADCY1, ADCY9, GABBR1, GNG11, GABBR2, GNG4, GNG7

C, Module 3

Term	Count	P-value	False discovery rate	Genes
GO:0060337 type I interferon signaling pathway (BP)	7	4.29×10^{-9}	5.96×10^{-6}	EGR1, IFI27, IFIT1, ISG15, BST2, PSMB8, ISG20
GO:0005654 nucleoplasm (CC)	18	5.93×10^{-6}	6.46×10^{-3}	PRKCA, ITGB3BP, IER2, EGR1, PPP2R5C, FOXO1, SMC3, PSMB8, PSMB9, ISG20, CUL2, RPS6KA1, ISG15, CASP8, MAPK9, FGF1, AKT3, AKT2
hsa04151: PI3K-Akt signaling pathway (KEGG)	9	9.02×10^{-6}	1.05×10^{-2}	GH2, PRKCA, PRLR, PPP2R5A, PPP2R5C, FGF1, IL7R, AKT3, AKT2

GO, gene ontology; CC, cellular component; BP, biological process; KEGG, Kyoto Encyclopedia of Genes and Genomes.

to prolactinoma and play an important role in the diagnosis of prolactinoma (40). Bcl2, the most important inhibitor of apoptosis in the Bcl family, is associated with the development and progression of the majority of cancers. It inhibits cell apoptosis and resists other forms of cell death leading to an increase in the number of cells, which has a positive effect on the growth of tumors (41). A previous study noted that the expression level of Bcl2 could be changed apparently by fulvestrant during medical treatment (42). Geng *et al* (43) also demonstrated that bromocriptine can induce autophagy-dependent cell death in pituitary adenomas by decreasing Bcl2. Thus, it was hypothesized that Bcl2 may be another driver gene in prolactinoma, drug targeting Bcl2 may inhibit prolactinoma. CALM1 serves a vital role in regulating a series of cellular functions through interaction with multiple target proteins (44). Calmodulin and interconnected calmodulin-regulated systems are involved in tumor growth, tumor-associated angiogenesis and metastasis (45), which is consistent with the GSEA results of the current study, which indicated that the expression profiles of prolactinomas were enriched in 'calcium signaling pathway'.

These findings suggest that CALM1 may be a potential therapeutic target for prolactinoma. In addition, the present study revealed that the genes JUN, FOS, Bcl2, STAT3, RPS27A, ACACB, INSR, ERBB2, PPP2CA, CDK2 and PTPRC are also associated with prolactinoma; these genes are potential diagnosis biomarkers, treatment targets and prognosis markers for patients with prolactinoma.

The functional analysis and enrichment of modules genes were also investigated in the present study, those modules were the most important gene clusters associated with prolactinoma, and may have vital roles in the occurrence and development mechanism of prolactinoma. The genes in module 1 were mainly associated with 'endoplasmic reticulum lumen', 'extracellular matrix organization' and 'collagen trimer', which were mainly associated with cellular structure. The endoplasmic reticulum (ER) is a major site for protein synthesis, lipid production and calcium storage in eukaryotic cells. When the endoplasmic reticulum is filled with unfolded proteins, misfolded proteins or changes in calcium concentration, ER stress (ERS) can be induced, leading to
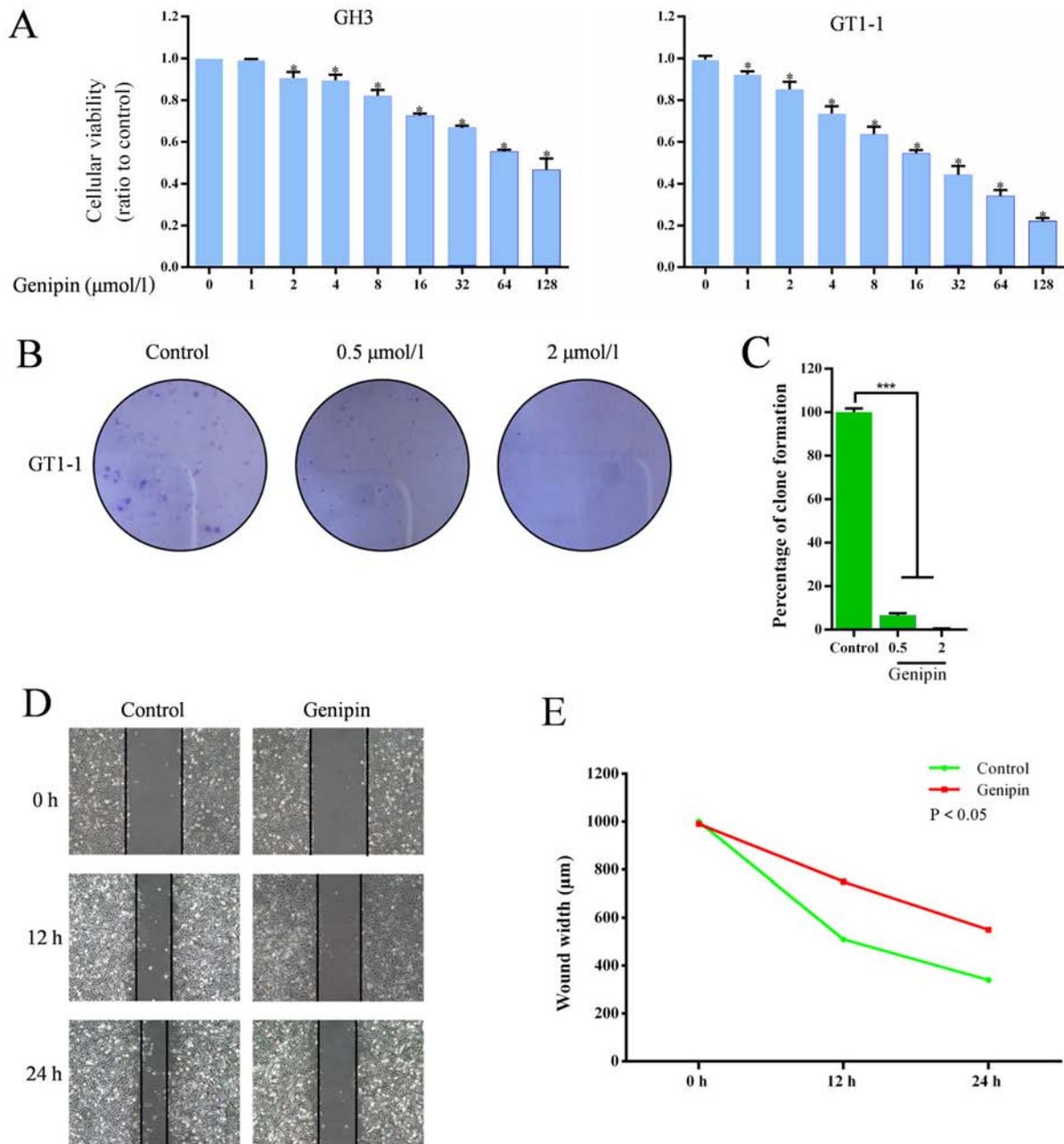
Figure 4. Anti-prolactinoma effect of Genipin (A) Cellular viability of prolactinoma cells treated with genipin. (B) Clonogenicities in Petri dishes with different dose of genipin in GT1-1 cell line. (C) Percentage of clone formation in GT1-1 cell line. (D) Scratch assay in control and genipin groups in GT1-1 cell line. (E) Wound width in control and genipin groups in GT1-1 cell line. *P<0.05 and ***P<0.01 vs. Control.

apoptosis. A number of previous studies have demonstrated that ERS is associated with progression and proliferation of hepatic carcinoma and ovarian cancer (46,47). The genes of module 2 and 3 were associated with 'GABAergic synapse', 'morphine addiction', 'type I interferon signaling pathway' and 'PI3K-Akt signaling pathway'. 'GABAergic synapses' regulate the activity of excitatory neurons in different brain regions. The release of neurotransmitter γ-aminobutyric acid is caused by the activation of inhibitory neurons (48). Therefore, there is a phenomenon in which excitatory neurons and inhibitory neurons co-activate during normal brain activity. The disruption of the balance between excitability and inhibition of neurons occurs in neurodegenerative and

psychiatric disorders, including epilepsy, schizophrenia and Parkinson's disease (49,50). Moreover, the enriched KEGG pathways included 'morphine addiction', so it is hypothesized that taking drugs may increase the risk of prolactinoma.

The anti-prolactinoma effects of genipin were evaluated in present study with CCK8 assay, colony-forming assay and scratch assay *in vitro*. In CCK8 assay, the cellular viability (ratio to control) in cell lines GH3 and GT1-1 decreased with increasing drug doses of genipin. In colony-forming assay, the number and size of clonogenicities in genipin group were clearly fewer than the control group and the percentage of clone formation was significantly different in groups with different genipin doses. Those results verified that the proliferation of
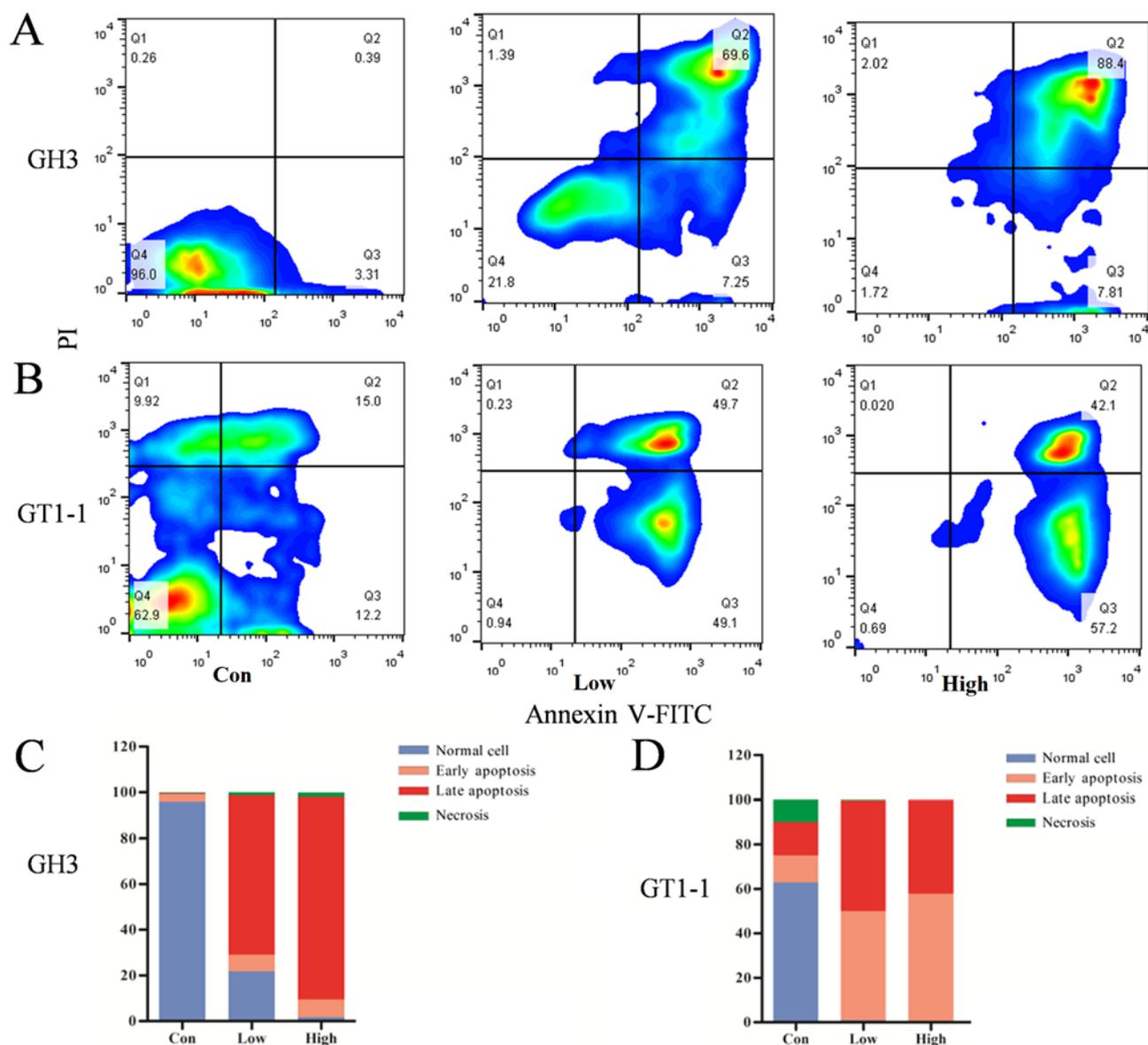
Figure 5. (A) The distribution of cells in apoptosis with different doses of genipin in GH3 cell lines. (B) The distribution of cells in apoptosis with different doses of genipin in GT1-1 cell lines. The percentage of apoptosis cell in (C) GH3 cell lines and (D) GT1-1 cell lines treated with different doses of genipin. Normal cells were dominant in the control group; in the low and dose groups, the percentage of late and early apoptosis cells was increased (upper right quadrant is early apoptosis, upper left quadrant is late apoptosis, lower left quadrant is normal and lower right quadrant is necrosis). Con, control, Low 0.5 μmol/l and High 2.0 μmol/l doses of genipin.

prolactinoma cells was reduced by genipin and the effects were dose-dependent. In scratch assays, the wound widths in the control group decreased over time and were smaller than the genipin group after 24 h; this suggested that genipin reduced the migration of prolactinoma cells. Flow cytometry was performed to further investigate the anti-prolactinoma effect of genipin in prolactinoma cells, which were treated with different dose of genipin for 48 h. The results demonstrated that the percentage of the apoptosis cells increased with the genipin dose, implying that the anti-prolactinoma effects of genipin were caused by inducing apoptosis and that this effect is dose-dependent. The absence of animal experiments was a limitation of this study, due to the lack of an ideal prolactinoma animal model; the effects of genipin in animals requires further investigation, as do the details of the mechanisms of prolactinoma.

In summary, an aggregate of 12,695, 3,847 and 5,310 DEGs were identified from datasets GSE2175, GSE26966 and GSE36314 respectively. GO and KEGG analysis showed that the enriched functions and pathways in upregulated genes were mainly related to 'spindle pole', 'oocyte meiosis' and 'nucleus'; while the downregulated genes were associated with 'signal transduction', 'cytoplasm' and 'receptor complex'. MAPK1, MYC, Bcl2, CALM1 and EGR1 were identified as main hub genes. Genes JUN, FOS, Bcl2, STAT3, RPS27A, ACACB, INSR, ERBB2, PPP2CA, CDK2 and PTPRC were revealed to be associated with prolactinoma for the first time, to the best of our knowledge. The proliferation and invasion of prolactinoma cells could be reduced by genipin via inducing apoptosis of prolactinoma cells. The anti-prolactinoma effect of genipin is dose-dependent. Genipin is a promising and potential drug for patients with prolactinoma.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SZ, BW, XW and DS were major contributors in writing the manuscript, downloading datasets and conducting bio-information analysis. DL, SJ and XL performed the analysis of the results. JG, YZ and XZ performed cell experiments and contributed to figures and tables. RJ and YC supervised the study, and contributed to the experiments and data analysis.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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