Identification of microRNAs associated with the aggressiveness of prolactin pituitary tumors using bioinformatic analysis

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Abstract. Aggressive prolactin pituitary tumors, which exhibit aggressive behaviors and resistance to conventional treatments, are a huge challenge for neurosurgeons. Many studies have investigated the roles of microRNAs (miRNAs) in pituitary tumorigenesis, invasion and metastasis, but few have explored aggressiveness-associated miRNAs in aggressive pituitary tumors. Differentially expressed miRNAs (DEMs) between aggressive and nonaggressive prolactin pituitary tumors were screened using the GSE46294 miRNA expression profile downloaded from the GEO database. The potential target genes of the top three most highly upregulated and downregulated DEMs were predicted by miRTarBase, and potential functional annotation and pathway enrichment analysis were performed using the DAVID database. Protein-protein interaction (PPI) and miRNA-hub gene interaction networks were constructed by Cytoscape software. A total of 43 DEMs were identified, including 19 upregulated and 24 downregulated miRNAs, between aggressive and nonaggressive prolactin pituitary tumors. One hundred and seventy and 680 target genes were predicted for the top three most highly upregulated and downregulated miRNAs, respectively, and these genes were involved in functional enrichment pathways, such as regulation of transcription from RNA polymerase II promoter, DNA-templated transcription, Wnt signaling pathway, protein binding, and transcription factor activity (sequence-specific DNA binding). In the PPI network, the top 10 genes with the highest degree of connectivity of the upregulated and downregulated DEMs were selected as hub genes. By constructing an miRNA-hub gene network, it was found that most hub genes were potentially modulated by hsa-miR-489 and hsa-miR-520b. Targeting hsa-miR-489 and hsa-miR-520b may provide new clues for the diagnosis and treatment of aggressive prolactin pituitary tumors.

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Abbreviations: miRNAs, microRNAs; DEMs, differentially expressed miRNAs; PPI, protein-protein interaction; TMZ, temozolomide; mRNA, messenger RNA; DE-miRNAs, differentially expressed miRNAs; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GEO, Gene Expression Omnibus; DAVID, Database for Annotation, Visualization and Integrated Discovery; MCODE, Molecular Complex Detection; BiNGO, Biological Networks Gene Oncology tool; BP, biological process; CC, cellular component; MF, molecular function; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; TKI, tyrosine kinase inhibitor

Key words: aggressive pituitary tumor, pituitary carcinoma, prolactinoma, microRNA, bioinformatic analysis

Introduction

Pituitary tumors represent approximately 10-15% of intracranial tumors, of which prolactin-secreting pituitary adenomas (prolactinoma) are the most common subtypes, accounting for 30-40% of pituitary tumors (1,2). Most of these tumors are noninvasive, show slow growth and are easily treated by surgery or medical treatment, including cabergoline and dopamine agonists. However, a small subset, accounting for 2.5-10% of pituitary adenomas, are defined as aggressive pituitary tumors and can exhibit aggressive behaviors, resistance to conventional treatments and/or temozolomide (TMZ), and multiple recurrences despite standard therapies combining surgical, medical and radiotherapy treatment approaches (3,4). Early identification of aggressive pituitary tumors is challenging but is of major clinical importance as these tumors are associated with increased morbidity and mortality (5). Numerous studies have been performed to explore potential predictive and prognostic biomarkers for the molecular pathogenesis underlying the aggressive behavior and malignant transformation of pituitary tumors, yet research results remain fairly unreliable and controversial (4,6,7).

MicroRNAs (miRNAs/miRs) are a large family of short endogenous noncoding RNAs, approximately 21-25 nucleotides

in length, that can directly bind to the 3'-untranslated region of messenger RNA (mRNA), thereby leading to suppression of protein translation or mRNA degradation (8,9). Subsequently, miRNAs can negatively regulate the expression of target genes involved in proliferation, apoptosis, cell cycle differentiation, invasion and metabolism (9). Aberrant expression of miRNAs contributes to tumorigenesis, invasion and metastasis by derepressing or silencing key regulatory proteins in various types of tumors, including pituitary adenomas (10-12). Many studies have investigated the roles of miRNAs in pituitary tumorigenesis, dysfunction, neurodegeneration and metastasis by comparing tumoral to normal pituitary tissues (13-16). However, currently, there are few studies that have explored aggressiveness-associated miRNAs in 'aggressive' pituitary tumors, especially aggressive prolactinoma, one of the most common subtypes of pituitary adenomas, based on large-scale human tissue datasets.

In recent years, microarray technology and bioinformatic analysis have been widely used to help us discover novel clues to identify reliable and functional miRNAs. In the present study, differentially expressed miRNAs (DEMs, DE-miRNAs) between aggressive and nonaggressive prolactin pituitary tumors were screened using the GSE46294 miRNA expression profile (17). The potential target genes of the top three most highly upregulated and downregulated DE-miRNAs were predicted by miRTarBase. Subsequently, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and protein-protein interaction (PPI) network analyses were performed to help us understand the molecular mechanisms underlying the aggressiveness of pituitary tumors. Finally, 20 hub genes were identified, and an miRNA-hub gene network was constructed by Cytoscape software. In conclusion, our study aimed to explore the aggressiveness-associated miRNAs in aggressive prolactin pituitary tumors and their potential molecular mechanisms based on bioinformatic analysis and to provide candidate biomarkers for early diagnosis and individualized treatment of aggressive prolactin pituitary tumors.

Materials and methods

Microarray data. The Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) is a public functional genomics data repository of high-throughput gene expression data, chips and microarrays (18). After extensive data screening in the GEO database, only the GSE46294 dataset was selected as it compared the miRNA expression of aggressive and nonaggressive prolactin pituitary tumors (17). GSE46294, based on the GPL13264 platform (Agilent-021827 Human miRNA Microarray), contained four aggressive prolactin pituitary tumor samples and eight nonaggressive prolactin pituitary tumor samples.

Data processing. GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) is an interactive web tool that can compare different groups of samples from the GEO series to identify DEMs across experimental conditions (19). The DEMs between aggressive and nonaggressive prolactin pituitary tumor samples were screened using GEO2R. Adjusted P-values (adj. P) were applied to correct the false-positive results by using the default

Benjamini-Hochberg false discovery rate method. Adj. P<0.01 and |fold change (FC)| >2 were considered the cut-off values for identifying DEMs. A DEM hierarchical clustering heat map was constructed using MeV (Multiple Experiment Viewer, http://mev.tm4.org/), which is a cloud-based application supporting the analysis, visualization, and stratification of large genomic data, particularly RNASeq and microarray data. The potential target genes of the top three most highly upregulated and downregulated DE-miRNAs were predicted by miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php/), which is a database for experimentally validated miRNA-target interactions (20).

Functional and pathway enrichment analyses. The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.ncifcrf.gov/) is an online tool for gene functional classification, which is an essential foundation for high-throughput gene analysis to understand the biological significance of genes (21). DAVID was introduced to perform functional annotation and pathway enrichment analysis, including GO (Gene Ontology) enrichment and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis, for the predicted target genes of 6 selected DEMs (22,23). A P-value <0.05 was considered statistically significant.

PPI network construction and module analysis. The target genes obtained from the upregulated and downregulated DEMs were first mapped to the STRING database (http://string-db.org) to assess functional associations among these target genes, with a combined score >0.4 defined as significant (24). Then, PPI networks were constructed using Cytoscape, which is a biological graph visualization software for integrated models of biologic molecular interaction networks (25). The Molecular Complex Detection (MCODE) plugin of Cytoscape was used to identify the most significant module in the PPI networks (26). The criteria for selection were as follows: Degree cut-off=2, node score cut-off=0.2, maximum depth=100 and k-core=2. Moreover, GO and KEGG enrichment analyses were performed using DAVID for genes in the modules.

Hub gene analysis and miRNA-hub gene network construction. Hub genes were selected by considering the high degree of connectivity in the PPI networks analyzed by the cytohubba plugin of Cytoscape. The top 10 genes with the highest degree of connectivity were selected as the hub genes of the upregulated and downregulated DEMs, respectively. Subsequently, GO and KEGG enrichment analyses were performed for the selected 20 hub genes. The biological process analysis of hub genes was performed and visualized using the Biological Networks Gene Oncology tool (BiNGO) plugin of Cytoscape (27). The latest information of functional roles of hub genes was downloaded from GeneCards in Nov. 2018 (https://www.genecards.org/). Subsequently, an miRNA-hub gene network was constructed by Cytoscape.

Results

Identification of DEMs and their target genes. Following analysis of the GSE46294 dataset using GEO2R, a total

Table I. Top 10 upregulated and downregulated DEMs between aggressive and nonaggressive prolactin pituitary tumors.

miRNAs (DEMs)	P-value	t	B	logFC
Upregulated				
hsa-miR-489	0.00677	3.25	-4.58	7.07
hsa-let-7d*	0.02591	2.53	-4.58	6.09
hsa-miR-138-1*	0.02569	2.54	-4.58	5.26
hsa-miR-886-3p	0.00191	3.94	-4.58	4.36
hsa-miR-576-5p	0.04773	2.2	-4.59	3.83
hsa-miR-135b	0.01671	2.77	-4.58	3.72
hsa-miR-137	0.03877	2.32	-4.59	3.29
hsa-miR-886-3p	0.00235	3.82	-4.58	3.2
hsa-miR-551b	0.02074	2.66	-4.58	3.04
hsa-miR-296-3p	0.04524	2.23	-4.59	3.02
Downregulated				
hsa-miR-520b	0.00732	-3.21	-4.58	-6.36
hsa-miR-875-5p	0.04037	-2.29	-4.59	-5.66
hsa-miR-671-3p	0.01453	-2.85	-4.58	-5.49
hsa-miR-372	0.00348	-3.61	-4.58	-5.49
hsa-miR-586	0.02631	-2.53	-4.58	-5.44
hsa-miR-367*	0.02421	-2.57	-4.58	-4.84
hsa-miR-302b	0.01052	-3.02	-4.58	-4.49
hsa-miR-187	0.0322	-2.42	-4.59	-4.35
hsa-miR-193b*	0.02207	-2.62	-4.58	-4.31
hsa-miR-452*	0.00322	-3.65	-4.58	-4.17

miRNA names with '*' are also mature miRNAs as annotated in miRBase (http://www.mirbase.org). For example, hsa-let-7d* is hsa-let-7d-3p; hsa-miR-138-1* is hsa-miR-138-1-3p; hsa-miR-367* is hsa-miR-367-5p; hsa-miR-193b* is hsa-miR-193b-5p; hsa-miR-452* is hsa-miR-452-3p. DEMs, differentially expressed miRNAs; hsa, *Homo sapiens*.

of 43 DEMs were identified, including 19 upregulated and 24 downregulated miRNAs between aggressive and nonaggressive prolactin pituitary tumors. For better visualization, the top 10 most highly upregulated miRNAs and the top 10 most highly downregulated miRNAs are presented in Table I, and the hierarchical clustering heat map of the DEMs is presented in Fig. S1. According to their FC values, hsa-miR-489, hsa-let-7d* and hsa-miR-138-1* were the top 3 most highly upregulated miRNAs, and hsa-miR-520b, hsa-miR-875-5p and hsa-miR-671-3p were the top 3 most highly downregulated miRNAs (Table I). One hundred seventy potential target genes were predicted for the top 3 most highly upregulated miRNAs and 680 potential target genes were predicted for the top 3 most highly downregulated miRNAs by miRTarBase.

Functional and pathway enrichment analyses. GO analysis, including biological process (BP), cellular component (CC) and molecular function (MF), was performed on the potential target genes of top 3 most highly upregulated miRNAs (Table II) and the top 3 most highly downregulated miRNAs

(Table III). GO functional annotation analysis showed that in the BP category, the target genes of the top 3 most highly upregulated miRNAs were significantly enriched in DNA-templated transcription, signal transduction, and positive regulation of transcription from RNA polymerase II promoter (Fig. 1A), while the target genes of the top 3 most highly downregulated miRNAs were enriched in DNA-templated transcription, DNA-templated regulation of transcription, and regulation of transcription from RNA polymerase II promoter (Fig. 1B). In the CC category, the target genes of the top three most highly upregulated miRNAs were significantly enriched in cytoplasm, nucleus and cytosol (Fig. 2A), while the target genes of the top three most highly downregulated miRNAs were enriched in nucleus, nucleoplasm and cytosol (Fig. 2B). In the MF category, the target genes of the top 3 most highly upregulated miRNAs were significantly enriched in protein binding, transcription factor activity, sequence-specific DNA binding, transcriptional activator activity, and RNA polymerase II core promoter proximal region sequence-specific binding (Fig. 3A), while the target genes of the top 3 most highly downregulated miRNAs were enriched in protein binding, DNA binding and transcription factor activity, and sequence-specific DNA binding (Fig. 3B). In addition, KEGG pathway analysis revealed that the target genes of the top 3 most highly upregulated miRNAs were mainly enriched in the Wnt signaling pathway, cGMP-PKG signaling pathway and renal cell carcinoma (Fig. 4A), while the target genes of the top three most highly downregulated miRNAs were mainly enriched in pathways in cancer, proteoglycans in cancer, measles and influenza A (Fig. 4B) (Tables II and III).

PPI network construction and module analysis. The PPI networks of the target genes of the top 3 most highly upregulated and downregulated DEMs were constructed (Fig. 5), and the most significant module was obtained using the MCODE plugin of Cytoscape. The genes in the most significant module of the upregulated DEMs were *SF1, SNRPD3* and *SNRPA1*, while the genes in the most significant module of the downregulated DEMs were *RNF34, RNF19B, ASB16, FBXL7, UBE2V2, RBBP6, KBTBD6, WSB1, KLHL21, CUL3, TCEB1, UBOX5* and *RNF115*. Functional analyses of the genes involved in the module of the downregulated DEMs were performed using DAVID, showing that genes in this module were mainly enriched in protein K48-linked ubiquitination (BP), polar microtubule (CC), ubiquitin-protein transferase activity (MF), and ubiquitin-mediated proteolysis(KEGG).

Hub gene analysis and miRNA-hub gene network construction. For the upregulated miRNAs, the hub genes included *RHOB, PTPN11, SNAI2, UBE2D1, SF1, PDPN, NUP43, YY1, HIF1A* and *SNRPD3*. For the downregulated miRNAs, the hub genes were *EGFR, CTNNB1, ESR1, CDKN1A, CCND1, CYCS, DNAJC10, IL8, CUL3* and *IGF1R*. The abbreviations, full names and functions of these 20 hub genes are shown in Table IV. Among these genes, *EGFR* (epidermal growth factor receptor) demonstrated the highest node degrees, which suggested that *EGFR* may be a key target associated with prolactin pituitary tumor

Table II. Functional and pathway enrichment analysis for target genes of the top 3 upregulated miRNAs.

Category	Term	Pathway description	Count	P-value
Upregulated miRNAs				
GO BP	GO:0060412	Ventricular septum morphogenesis	3	0.020464503
GO BP	GO:0007286	Spermatid development	4	0.021020749
GO BP	GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	12	0.021742388
GO BP	GO:0006351	Transcription, DNA-templated	24	0.022393279
GO BP	GO:0030154	Cell differentiation	9	0.025194909
GO BP	GO:0097411	Hypoxia-inducible factor-1α signaling pathway	2	0.030146509
GO BP	GO:0030177	Positive regulation of Wnt signaling pathway	3	0.030678983
GO BP	GO:0007165	Signal transduction	16	0.030948235
GO BP	GO:0030336	Negative regulation of cell migration	4	0.036066871
GO BP	GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	14	0.03646379
GO CC	GO:0005737	Cytoplasm	52	0.0134897
GO CC	GO:0031519	PcG protein complex	3	0.016939042
GO CC	GO:0005634	Nucleus	52	0.026624876
GO CC	GO:0005794	Golgi apparatus	13	0.026655792
GO CC	GO:0005654	Nucleoplasm	29	0.053523267
GO CC	GO:0031526	Brush border membrane	3	0.054869988
GO CC	GO:0000139	Golgi membrane	9	0.072820488
GO CC	GO:0044798	Nuclear transcription factor complex	2	0.078554642
GO CC	GO:0005829	Cytosol	32	0.094144731
GO MF	GO:0005515	Protein binding	83	0.007060503
GO MF	GO:0050693	LBD domain binding	2	0.030452531
GO MF	GO:0003700	Transcription factor activity, sequence-specific DNA binding	14	0.034027538
GO MF	GO:0001077	Transcriptional activator activity, RNA polymerase II core sequence-specific binding	6	0.035934263
GO MF	GO:0030620	U2 snRNA binding	2	0.045331887
GO MF	GO:0008517	Folic acid transporter activity	2	0.052686367
GO MF	GO:0001078	Transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding	4	0.054345955
GO MF	GO:0004726	Non-membrane spanning protein tyrosine phosphatase activity	2	0.059984623
GO MF	GO:0003714	Transcription corepressor activity	5	0.071931973
GO MF	GO:0004871	Signal transducer activity	5	0.07295342
KEGG	hsa04310	Wnt signaling pathway	5	0.006641183
KEGG	hsa04022	cGMP-PKG signaling pathway	5	0.01255563
KEGG	hsa05211	Renal cell carcinoma	3	0.049309583

In the event there were more than five terms enriched in this category, the top 5 terms were selected per P-value. GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; Count, numbers of enriched genes in each term; hsa, *Homo sapiens*.

aggressiveness. Biological process analysis of the hub genes is shown in Fig. 6A. Functional and pathway enrichment analyses for the hub genes of the top 3 upregulated and downregulated miRNAs are presented in Tables V and VI. As shown in Fig. 6, KEGG analysis showed that the hub genes of the upregulated miRNAs were mainly enriched in renal cell carcinoma and proteoglycans in cancer (Fig. 6B, Table V), while the hub genes of the downregulated miRNAs were mainly enriched in proteoglycans in cancer, prostate cancer and pathways in cancer (Fig. 6C, Table VI).

Table III. Functional and pathway enrichment analysis for target genes of the top 3 downregulated miRNAs.

Category	Term	Description	Count	P-value
Downregulated miRNAs				
GO BP	GO:0046777	Protein autophosphorylation	15	0.000170888
GO BP	GO:0006355	Regulation of transcription, DNA-templated	59	0.001639464
GO BP	GO:0006357	Regulation of transcription from RNA polymerase II promoter	23	0.002882721
GO BP	GO:0016567	Protein ubiquitination	20	0.002898481
GO BP	GO:0006351	Transcription, DNA-templated	71	0.003602574
GO BP	GO:0006123	Mitochondrial electron transport, cytochrome c to oxygen	4	0.014446477
GO BP	GO:0042119	Neutrophil activation	3	0.017126856
GO BP	GO:0007223	Wnt signaling pathway, calcium modulating pathway	5	0.018278676
GO BP	GO:0008654	Phospholipid biosynthetic process	5	0.019902891
GO BP	GO:0048468	Cell development	5	0.019902891
GO CC	GO:0005654	Nucleoplasm	112	6.68468E-07
GO CC	GO:0005634	Nucleus	170	0.001202665
GO CC	GO:0017053	Transcriptional repressor complex	7	0.002719148
GO CC	GO:0005758	Mitochondrial intermembrane space	8	0.002811554
GO CC	GO:0005813	Centrosome	22	0.003323275
GO CC	GO:0005739	Mitochondrion	50	0.006368195
GO CC	GO:0031463	Cul3-RING ubiquitin ligase complex	7	0.007248149
GO CC	GO:0005829	Cytosol	106	0.009417134
GO CC	GO:0015629	Actin cytoskeleton	13	0.010602362
GO CC	GO:0005741	Mitochondrial outer membrane	10	0.014535489
GO MF	GO:0005515	Protein binding	269	9.14069E-05
GO MF	GO:0003677	DNA binding	62	0.004456722
GO MF	GO:0004842	Ubiquitin-protein transferase activity	18	0.005935513
GO MF	GO:0003700	Transcription factor activity, sequence-specific DNA binding	39	0.006954496
GO MF	GO:0004672	Protein kinase activity	18	0.013435998
GO MF	GO:0004879	RNA polymerase II transcription factor activity, ligand-activated sequence-specific DNA binding	5	0.013877869
GO MF	GO:0003707	Steroid hormone receptor activity	6	0.015112205
GO MF	GO:0043565	Sequence-specific DNA binding	23	0.017535243
GO MF	GO:0031625	Ubiquitin protein ligase binding	15	0.018757483
GO MF	GO:0004674	Protein serine/threonine kinase activity	18	0.020173241
KEGG	hsa05162	Measles	10	0.005987506
KEGG	hsa05215	Prostate cancer	8	0.006312095
KEGG	hsa05200	Pathways in cancer	19	0.009215433
KEGG	hsa05205	Proteoglycans in cancer	12	0.011467731
KEGG	hsa05219	Bladder cancer	5	0.018691786
KEGG	hsa04962	Vasopressin-regulated water reabsorption	5	0.023655555
KEGG	hsa04919	Thyroid hormone signaling pathway	8	0.023895596
KEGG	hsa05164	Iinfluenza A	10	0.030429321
KEGG	hsa05218	Melanoma	6	0.032052212
KEGG	hsa04390	Hippo signaling pathway	9	0.035379929

If there were more than five terms enriched in this category, the top 5 terms were selected per the P-value. GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; Count, numbers of enriched genes in each term; hsa, *Homo sapiens*.
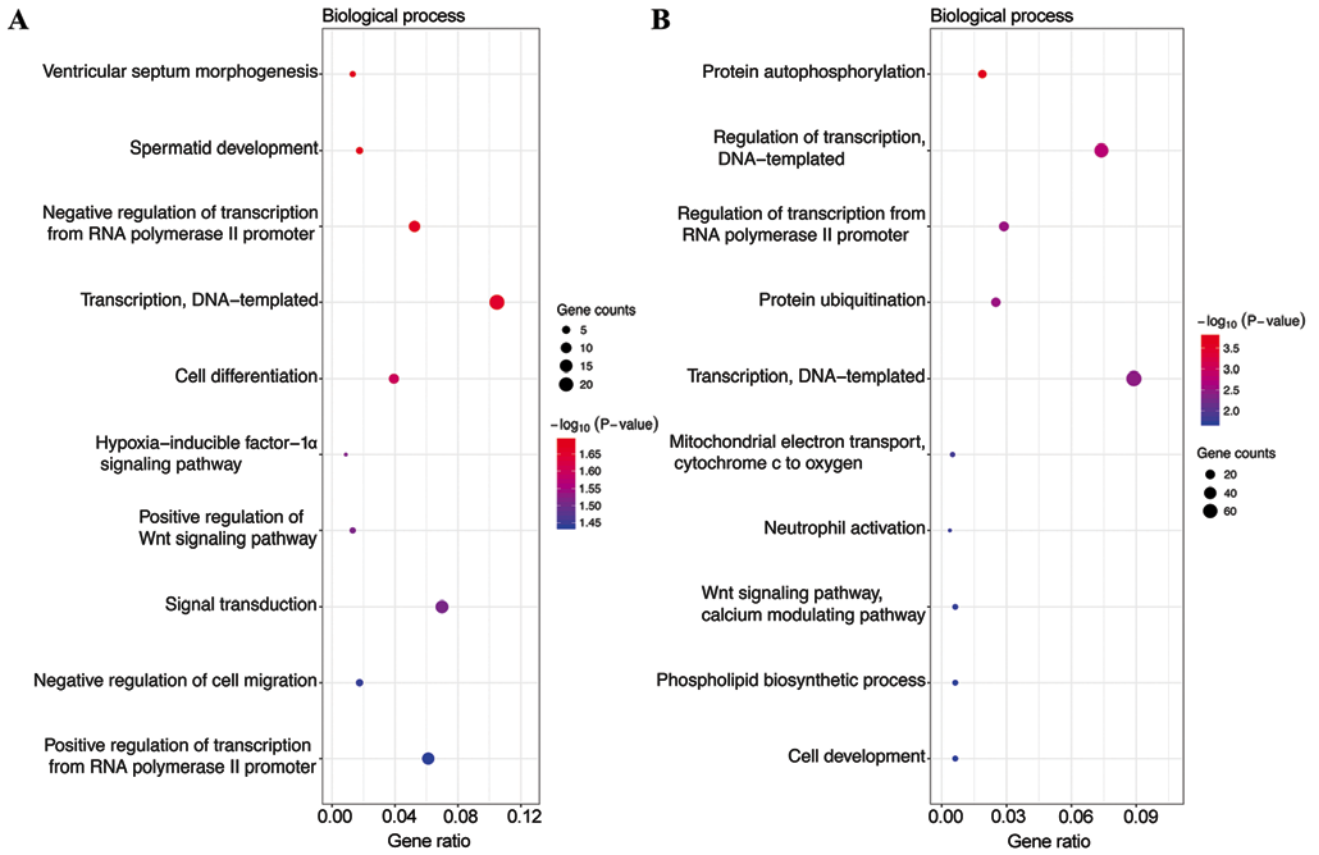
Figure 1. Gene Ontology (GO) functions for the target genes of the top 3 most highly upregulated miRNAs and the top 3 most highly downregulated miRNAs. (A) Enriched biological processes of the upregulated miRNAs; (B) enriched biological processes of the downregulated miRNAs.
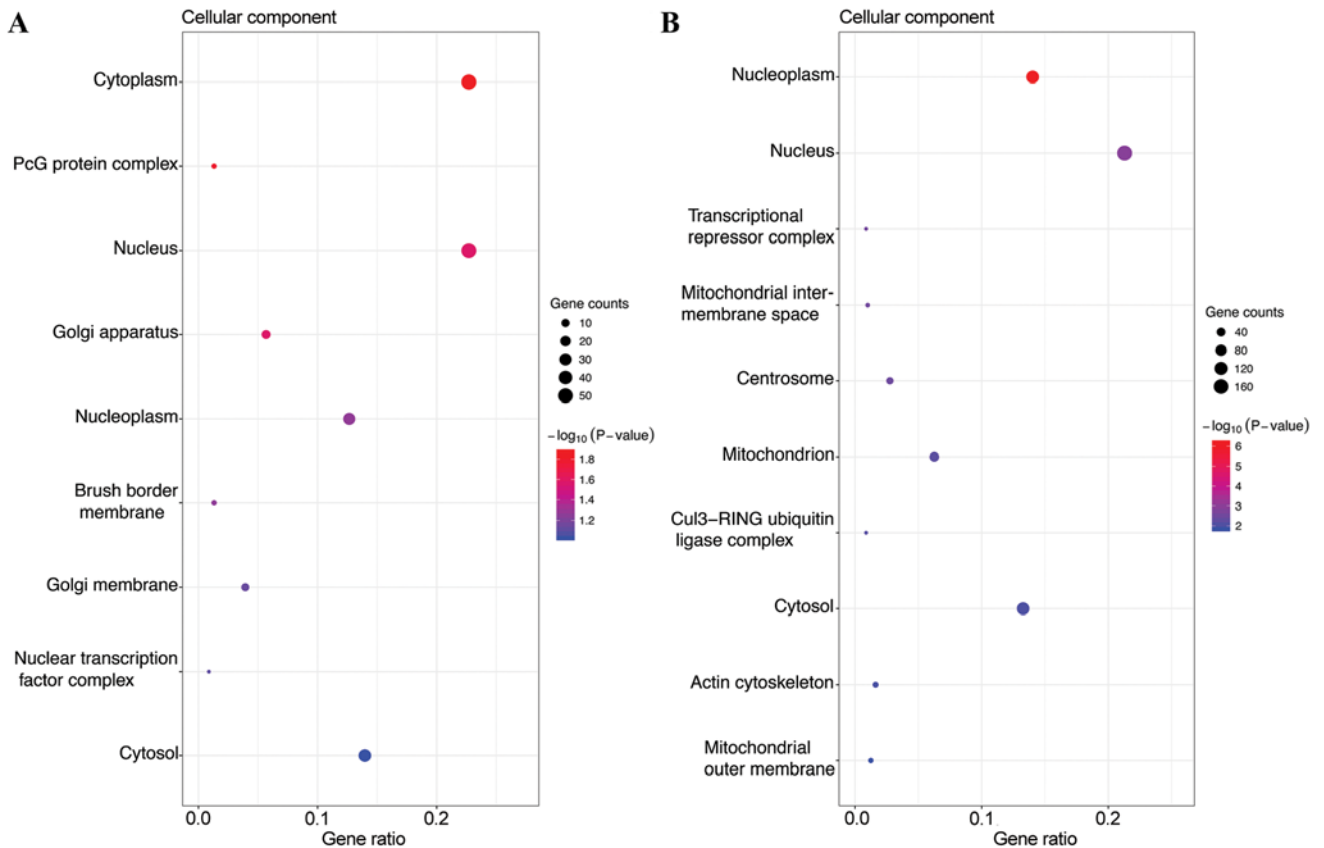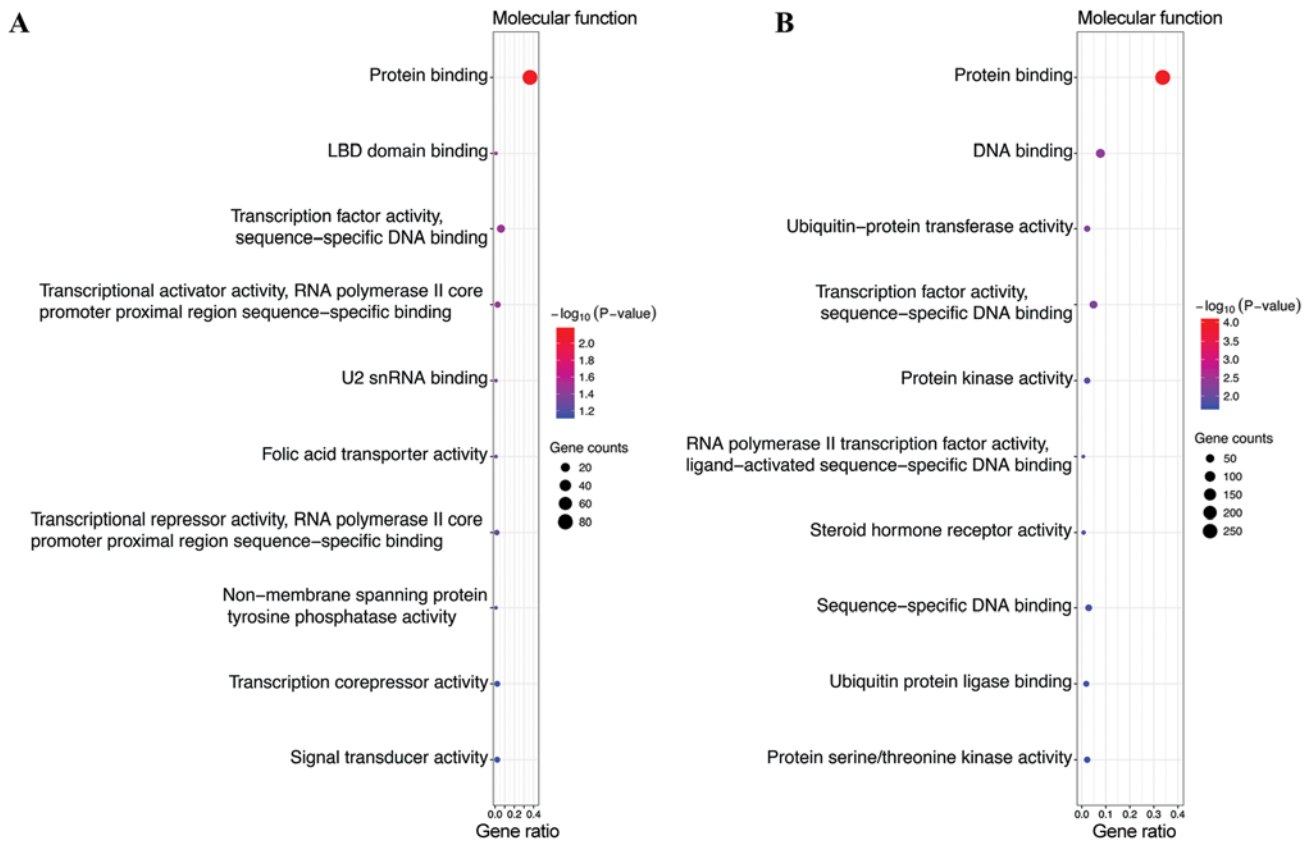

Figure 2. Gene Ontology (GO) functions for the target genes of the top 3 most highly upregulated miRNAs and the top 3 most highly downregulated miRNAs. (A) Enriched cellular components of the upregulated miRNAs; (B) enriched cellular components of the downregulated miRNAs.

Figure 3. Gene Ontology (GO) functions for the target genes of the top 3 most highly upregulated miRNAs and the top 3 most highly downregulated miRNAs. (A) Enriched molecular functions of the upregulated miRNAs; (B) enriched molecular functions of the downregulated miRNAs.
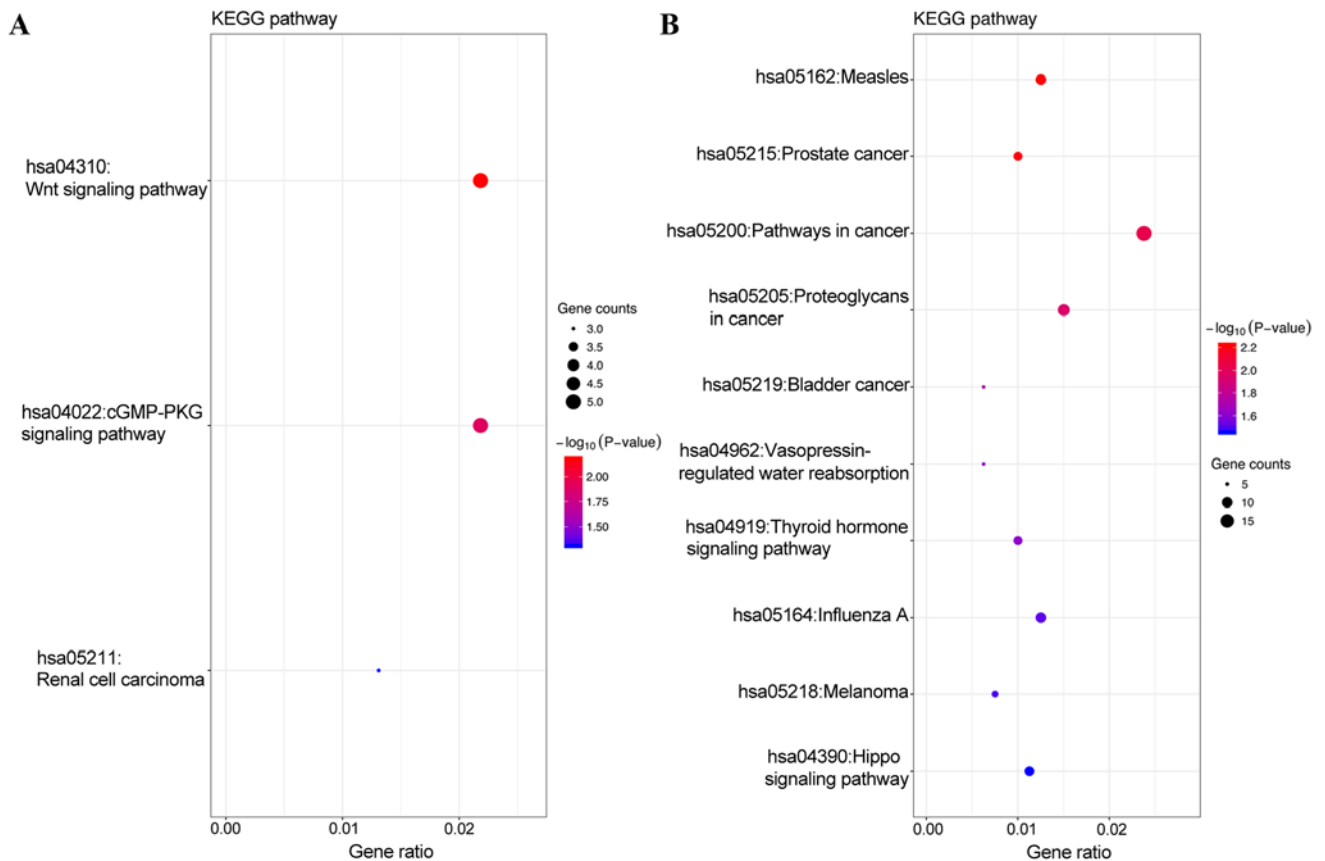

Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for the target genes of the top 3 most highly upregulated miRNAs and the top 3 most highly downregulated miRNAs. (A) Enriched KEGG pathways of the upregulated miRNAs; (B) enriched KEGG pathways of the downregulated miRNAs.
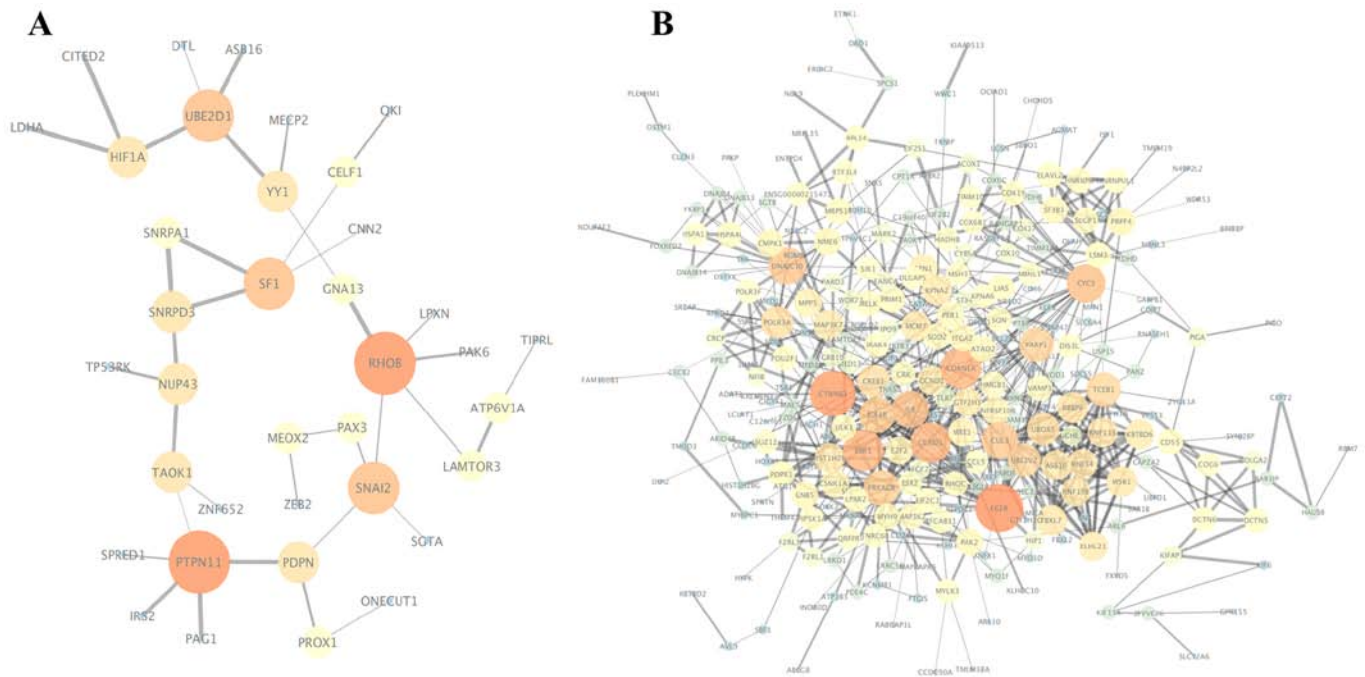
Figure 5. (A) Protein-protein interaction (PPI) network of the target genes of the top 3 most highly upregulated differentially expressed miRNAs (DEMs). (B) PPI network of the target genes of the top 3 most highly downregulated DEMs. Node size indicates the connectivity degree, and larger circles indicate a higher degree. Edge size indicates the combined scores between genes, which represent the confidence of protein interactions. The color gradually increases from dark (blue) to bright (red), representing the gradually increase in the number of interacting genes.

Subsequently, miRNA-hub gene networks were constructed by Cytoscape (Fig. 7). As shown in Fig. 7A, hsa-miR-489, the most highly upregulated DEM, potentially could target 9 (*RHOB, PTPN11, SNAI2, UBE2D1, SF1, PDPN, NUP43, YY1* and *HIF1A*) of 10 hub genes. Five hub genes and 2 hub genes potentially were regulated by upregulated hsa-miR-138-1-3p and hsa-let-7d*, respectively. Additionally, according to Fig. 7B, hsa-miR-520b, the most highly downregulated DEM, potentially could also target 9 (*EGFR, ESR1, CDKN1A, CCND1, CYCS, DNAJC10, IL8, CUL3* and *IGF1R*) of 10 hub genes. Three hub genes and 1 hub gene potentially were regulated by downregulated hsa-miR-875-5p and hsa-miR-671-3p, respectively. The results suggested that hsa-miR-489 and hsa-miR-520b may be the most important regulators of prolactin pituitary tumor aggressiveness.

Discussion

Prolactin-secreting pituitary adenoma is the most common (30-40%) subtype of pituitary tumors and commonly presents with headache, visual disturbances, amenorrhea, galactorrhea, infertility and hyposexuality (1,2). Most prolactinomas are noninvasive and easily treated by surgery, radiotherapy or medical treatment, including cabergoline and dopamine agonists, which are highly effective drugs for prolactinoma. However, aggressive prolactin pituitary tumors, with unknown incidence, are entities whose pathological behaviors lie between those of benign pituitary adenomas and malignant pituitary carcinomas. They display a rather distinct aggressive behavior with marked invasion of nearby anatomical structures, a tendency for resistance to conventional treatments and/or TMZ, and early postoperative recurrences (3,4). Extensive research

has been performed to explore potential biomarkers for early diagnosis and treatment of aggressive pituitary tumors. The Raf/MEK/ERK, PI3K/Akt/mTOR, and VEGFR pathways were found to be upregulated in pituitary tumors, suggesting that these pathways may be utilized to control pituitary tumor growth and progression (28-32). However, most targeted therapies based on the above pathways have been administered to patients with aggressive pituitary tumors without success (32-34). Therefore, further research is needed to discover aggressiveness-associated biomarkers with diagnostic and therapeutic value for aggressive prolactin pituitary tumors.

miRNAs are a group of small, endogenous noncoding RNAs that can repress protein expression by cleaving mRNA or inhibiting translation (8,9). Mostly, miRNAs are recognized as having a significant role in the negative regulation of target gene expression, which contributes to tumorigenesis, invasion and metastasis in various types of tumors (10-12). Recent studies have shown that aberrant miRNA expression is involved in tumorigenesis and tumor development of pituitary adenomas, especially prolactin pituitary tumors (13-16). D'Angelo *et al* (35) found that miR-603, miR-34b, miR-548c-3p, miR-326, miR-570 and miR-432 were downregulated in prolactinomas, which can affect the G1-S transition process. Mussnich *et al* (36) found that miR-15, miR-26a, miR-196a-2, miR-16, Let-7a and miR-410 were downregulated in prolactinomas, which can negatively regulate pituitary cell proliferation. Roche *et al* (17) demonstrated that miR-183 was downregulated in aggressive prolactin tumors and inhibited tumor cell proliferation by directly targeting KIAA0101, which is involved in cell cycle activation and the inhibition of p53-p21-mediated cell cycle arrest. However, few studies, except for one reported by Roche *et al* (17) in 2015, have been

Table IV. Functional roles of the hub genes of the top 3 upregulated/downregulated miRNAs identified in the PPI interaction.

Gene symbol	Degree	Full name	Function
Upregulated miRNAs			
RHOB	16	Ras homolog family member B	Protein coding gene. Among its related pathways are ERK signaling and focal adhesion. GO annotations related to this gene include GTP binding and GDP binding.
PTPN11	15	Protein tyrosine phosphatase, non-receptor type 11	Protein coding gene. Among its related pathways are immune response Fcε RI pathway and EGF/EGFR signaling pathway. GO annotations related to this gene include protein domain-specific binding and protein tyrosine phosphatase activity.
SNAI2	15	Snail family transcriptional repressor 2	Protein coding gene. Among its related pathways are ERK signaling and adherens junction. GO annotations related to this gene include sequence-specific DNA binding and tran scriptional repressor activity, RNA polymerase II proximal promoter sequence-specific DNA binding.
UBE2D1	14	Ubiquitin conjugating enzyme E2 D1	Protein coding gene. Among its related pathways are gene expression and cell cycle, mitotic. GO annotations related to this gene include ligase activity and acid-amino acid ligase activity.
SF1	14	Splicing factor 1	Protein Coding gene. Among its related pathways are Oct4 in mammalian ESC pluripotency and mRNA splicing-major pathway. GO annotations related to this gene include nucleic acid binding and RNA binding.
PDPN	14	Podoplanin	Protein coding gene. Among its related pathways are cytoskel etal signaling and response to elevated platelet cytosolic Ca^{2+}. GO annotations related to this gene include amino acid trans membrane transporter activity and folic acid transmembrane transporter activity.
NUP43	13	Nucleoporin 43	Protein coding gene. Among its related pathways are cell cycle, mitotic and transport of the SLBP independent mature mRNA.
YY1	13	YY1 transcription factor	Protein coding gene. Among its related pathways are gene expression and translational control. GO annotations related to this gene include DNA binding transcription factor activity and transcription coactivator activity.
HIF1A	11	Hypoxia inducible factor 1 subunit α	Protein coding gene. Among its related pathways are ERK signaling and central carbon metabolism in cancer. GO anno tations related to this gene include DNA binding transcription factor activity and protein heterodimerization activity.
SNRPD3	11	Small nuclear ribonu cleoprotein D3 polypeptide	Protein coding gene. Among its related pathways are mRNA splicing-major pathway and processing of capped intronless pre-mRNA. GO annotations related to this gene include histone pre-mRNA DCP binding.
Downregulated miRNAs			
EGFR	33	Epidermal growth factor receptor	Protein coding gene. Among its related pathways are ERK signaling and gene expression. GO annotations related to this gene include identical protein binding and protein kinase activity.
CTNNB1	31	Catenin β1	Protein coding gene. Among its related pathways are ERK signaling and focal adhesion. GO annotations related to this gene include DNA binding transcription factor activity and binding.
ESR1	25	Estrogen receptor 1	Estrogen resistance and myocardial infarction. Among its related pathways are gene expression and integrated breast cancer pathway. GO annotations related to this gene include DNA binding transcription factor activity and identical protein binding.

Table IV. Continued.

Gene symbol	Degree	Full name	Function
CDKN1A	25	Cyclin dependent kinase inhibitor 1A	Protein coding gene. Among its related pathways are gene expression and Akt signaling. GO annotations related to this gene include ubiquitin protein ligase binding and cyclin binding.
CCND1	24	Cyclin D1	Protein coding gene. Diseases associated with CCND1 include myeloma, multiple and Von Hippel-Lindau syndrome. Among its related pathways are ERK signaling and focal adhesion. GO annotations related to this gene include protein kinase activity and enzyme binding.
CYCS	23	Cytochrome *c*, somatic	Protein coding gene. Diseases associated with CYCS include thrombocytopenia 4 and autosomal thrombocytopenia with normal platelets. Among its related pathways are gene expression and activation of caspases through apoptosome-mediated cleavage. GO annotations related to this gene include iron ion binding and electron transfer activity.
DNAJC10	21	DNAJ heat shock protein family (Hsp40) member C10	Protein coding gene. Among its related pathways are protein processing in endoplasmic reticulum. GO annotations related to this gene include chaperone binding and protein disulfide oxidoreductase activity.
IL8	21	C-X-C motif chemokine ligand 8	Protein coding gene. Among its related pathways are Akt signaling and rheumatoid arthritis. GO annotations related to this gene include chemokine activity and interleukin-8 receptor binding.
CUL3	20	Cullin 3	Protein Coding gene. Among its related pathways are RET signaling and Class I MHC mediated antigen processing and presentation. GO annotations related to this gene include protein homodimerization activity and ubiquitin-protein transferase activity.
IGF1R	19	Insulin like growth factor 1 receptor	Protein coding gene. Among its related pathways are ERK signaling and mTOR pathway. GO annotations related to this gene include identical protein binding and protein kinase activity.

PPI, protein-protein interaction; GO, Gene Ontology. Online database GeneCards (https://www.genecards.org).

performed to explore aggressiveness-associated miRNAs in aggressive prolactin pituitary tumors based on large-scale human tissue datasets. Additionally, based on the GSE46294 dataset, our study obtained different DEMs compared with those reported by Roche *et al*. The reasons may be due to different softwares or different algorithms when analyzing differentially expressed genes or RNAs, and due to the small sample size of the GSE46294 dataset (37).

In the present study, some aggressiveness-associated miRNAs were screened by performing a differential expression analysis based on an miRNA expression profile downloaded from the GEO database. The potential target genes of the top 3 most highly upregulated and most highly downregulated DEMs were collectively enriched for regulation of transcription from RNA polymerase II promoter, DNA-templated transcription, Wnt signaling pathway, protein binding, and transcription factor activity (sequence-specific DNA binding). Moreover, by constructing PPI networks, we identified the top 10 hub genes with the highest degree of connectivity

with the top 3 most highly upregulated and downregulated DEMs. Hub genes of the upregulated DEMs were mainly enriched for proteoglycans in cancer, while hub genes of the downregulated DEMs were mainly enriched for proteoglycans in cancer, pathways in cancer, FoxO signaling pathway, and focal adhesion. Those pathways were all reported by previous studies to be associated with tumor growth, progression invasion and metastasis of various tumors (38-40). In our study, proteoglycan in cancer is the enriched pathway shared by both upregulated and downregulated DEMs. However, there is little research on proteoglycan in tumorigenesis, invasiveness and progression of pituitary tumors. Matano *et al* reported that endocan, a novel soluble dermatan sulfate proteoglycan, can function as a new invasion and angiogenesis marker of pituitary adenomas (40). More studies are needed to further research the functions of proteoglycan in pituitary adenomas, especially aggressive tumors.

Among the 20 hub genes, *EGFR* demonstrated the highest node degrees, suggesting that *EGFR* was a key target associated
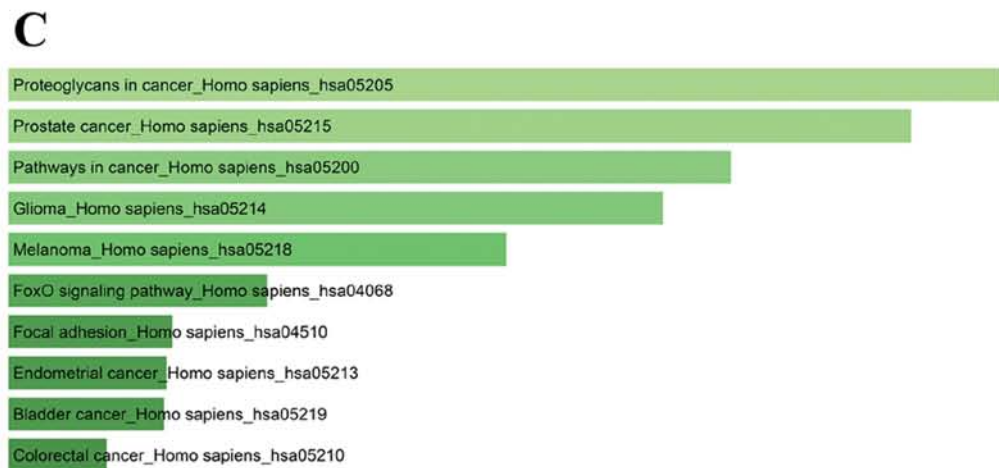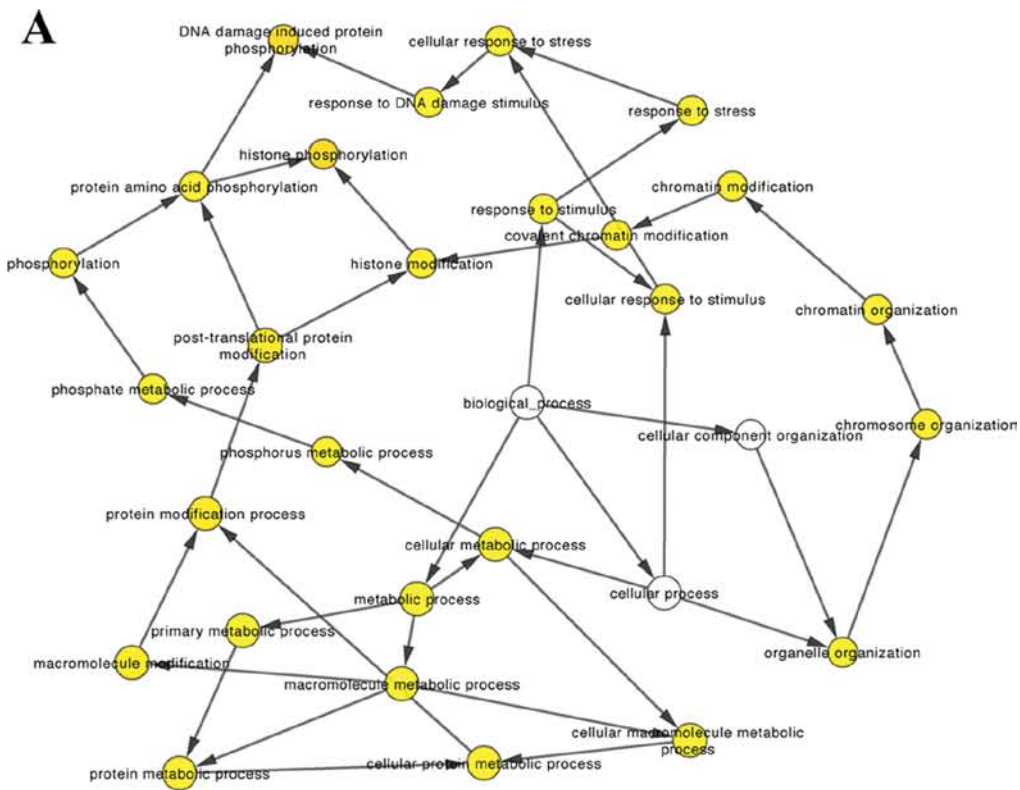
Figure 6. (A) The biological process analysis of hub genes. Node color depth refers to the corrected ontology P-values. Node size indicates the number of genes involved in the ontologies. P<0.01 was considered statistically significant. (B) Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for the hub genes of the top 3 most highly upregulated miRNAs. (C) Enriched KEGG pathways for the hub genes of the top 3 most highly downregulated miRNAs.

Table V. Functional and pathway enrichment analysis for the hub genes of the top 3 upregulated miRNAs.

Category	Term	Pathway description	Genes
Upregulated miRNAs			
GO BP	GO:0032364	Oxygen homeostasis	*HIF1A*
GO BP	GO:0032909	Regulation of transforming growth factor β2 production	*HIF1A*
GO BP	GO:0033483	Gas homeostasis	*HIF1A*
GO BP	GO:0032642	Regulation of chemokine production	*SNAI2, HIF1A*
GO BP	GO:0046885	Regulation of hormone biosynthetic process	*HIF1A*
GO BP	GO:0043619	Regulation of transcription from RNA polymerase II promoter in response to oxidative stress	*HIF1A*
GO BP	GO:0070099	Regulation of chemokine-mediated signaling pathway	*HIF1A*
GO BP	GO:0032352	Positive regulation of hormone metabolic process	*HIF1A*
GO BP	GO:0010839	Negative regulation of keratinocyte proliferation	*SNAI2*
GO BP	GO:0071364	Cellular response to epidermal growth factor stimulus	*SNAI2, PTPN11*
GO CC	GO:0031528	Microvillus membrane	*PDPN*
GO CC	GO:0000243	Commitment complex	*SNRPD3*
GO CC	GO:0005683	U7 snRNP	*SNRPD3*
GO CC	GO:0005687	U4 snRNP	*SNRPD3*
GO CC	GO:0034709	Methylosome	*SNRPD3*
GO CC	GO:0031527	Filopodium membrane	*PDPN*
GO CC	GO:0071437	Invadopodium	*PDPN*
GO CC	GO:0031011	Ino80 complex	*YY1*
GO CC	GO:0005685	U1 snRNP	*SNRPD3*
GO CC	GO:0031258	Lamellipodium membrane	*PDPN*
GO MF	GO:0000400	Four-way junction DNA binding	*YY1*
GO MF	GO:0001227	Transcriptional repressor activity, RNA polymerase II transcription regulatory region sequence-specific binding	*YY1, SNAI2*
GO MF	GO:0019956	Chemokine binding	*PDPN*
GO MF	GO:0043565	Sequence-specific DNA binding	*YY1, SNAI2, HIF1A*
GO MF	GO:0061631	Ubiquitin conjugating enzyme activity	*UBE2D1*
GO MF	GO:0000217	DNA secondary structure binding	*YY1*
GO MF	GO:0061650	Ubiquitin-like protein conjugating enzyme activity	*UBE2D1*
GO MF	GO:0005158	Insulin receptor binding	*PTPN11*
GO MF	GO:0035326	Enhancer binding	*YY1*
GO MF	GO:0001078	Transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding	*YY1, SNAI2*
KEGG	hsa05211	Renal cell carcinoma	*PTPN11, HIF1A*
KEGG	hsa05205	Proteoglycans in cancer	*PTPN11, HIF1A*
KEGG	hsa04150	mTOR signaling pathway	*HIF1A*
KEGG	hsa05120	Epithelial cell signaling in *Helicobacter pylori* infection	*PTPN11*
KEGG	hsa05230	Central carbon metabolism in cancer	*HIF1A*
KEGG	hsa05220	Chronic myeloid leukemia	*PTPN11*
KEGG	hsa04920	Adipocytokine signaling pathway	*PTPN11*
KEGG	hsa04520	Adherens junction	*SNAI2*
KEGG	hsa05231	Choline metabolism in cancer	*HIF1A*
KEGG	hsa04066	HIF-1 signaling pathway	*HIF1A*

GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; hsa, *Homo sapiens*.

with the aggressiveness of prolactin pituitary tumors, which is consistent with previous studies (4,41). *EGFR* encodes a transmembrane glycoprotein that is located on the cell surface and binds to epidermal growth factor (EGF). Binding

Table VI. Functional and pathway enrichment analysis for the hub genes of top 3 downregulated miRNAs.

Category	Term	Pathway description	Genes
Downregulated miRNAs			
GO BP	GO:0070141	Response to UV-A	CCND1, EGFR
GO BP	GO:0097193	Intrinsic apoptotic signaling pathway	CDKN1A, CUL3, DNAJC10, CYCS
GO BP	GO:0032355	Response to estradiol	CTNNB1, ESR1, EGFR
GO BP	GO:1903798	Regulation of production of miRNAs involved in gene silencing by miRNA	ESR1, EGFR
GO BP	GO:0033674	Positive regulation of kinase activity	CDKN1A, EGFR, IGF1R
GO BP	GO:0001934	Positive regulation of protein phosphorylation	CDKN1A, CCND1, EGFR, IGF1R
GO BP	GO:0045737	Positive regulation of cyclin-dependent protein serine/threonine kinase activity	CCND1, EGFR
GO BP	GO:0045740	Positive regulation of DNA replication	EGFR, IGF1R
GO BP	GO:0006367	Transcription initiation from RNA polymerase II promoter	CDKN1A, CCND1, ESR1
GO BP	GO:0034333	Adherens junction assembly	CTNNB1
GO CC	GO:0030128	Clathrin coat of endocytic vesicle	EGFR
GO CC	GO:0030122	AP-2 adaptor complex	EGFR
GO CC	GO:0030131	Clathrin adaptor complex	EGFR
GO CC	GO:1990907	β-catenin-TCF complex	CTNNB1
GO CC	GO:0005719	Nuclear euchromatin	CTNNB1
GO CC	GO:0000791	Euchromatin	CTNNB1
GO CC	GO:0035327	Transcriptionally active chromatin	ESR1
GO CC	GO:0000790	Nuclear chromatin	CTNNB1, ESR1
GO CC	GO:0005758	Mitochondrial intermembrane space	CYCS
GO CC	GO:0016342	Catenin complex	CTNNB1
GO MF	GO:0097472	Cyclin-dependent protein kinase activity	CDKN1A, CCND1
GO MF	GO:0019900	Kinase binding	CDKN1A, CCND1, CTNNB1, ESR1
GO MF	GO:0004693	Cyclin-dependent protein serine/threonine kinase activity	CDKN1A, CCND1
GO MF	GO:0004709	MAP kinase kinase kinase activity	EGFR, IGF1R
GO MF	GO:0001223	Transcription coactivator binding	ESR1
GO MF	GO:0044389	Ubiquitin-like protein ligase binding	CDKN1A, CUL3, EGFR
GO MF	GO:0019901	Protein kinase binding	CDKN1A, CCND1, ESR1, EGFR, IGF1R
GO MF	GO:0030331	Estrogen receptor binding	CTNNB1, ESR1
GO MF	GO:0016671	Oxidoreductase activity, acting on a sulfur group of donors, disulfide as acceptor	DNAJC10
GO MF	GO:0046934	Phosphatidylinositol-4,5-bisphosphate 3-kinase activity	ESR1, EGFR
KEGG	hsa05205	Proteoglycans in cancer	CDKN1A, CCND1, ESR1, CTNNB1, EGFR, IGF1R
KEGG	hsa05215	Prostate cancer	CDKN1A, CCND1, CTNNB1, EGFR, IGF1R
KEGG	hsa05200	Pathways in cancer	CDKN1A, CCND1, CTNNB1, CYCS, EGFR, IGF1R
KEGG	hsa05214	Glioma	CDKN1A, CCND1, EGFR, IGF1R
KEGG	hsa05218	Melanoma	CDKN1A, CCND1, EGFR, IGF1R
KEGG	hsa04068	FoxO signaling pathway	CDKN1A, CCND1, EGFR, IGF1R
KEGG	hsa04510	Focal adhesion	CCND1, CTNNB1, EGFR, IGF1R

Table VI. Continued.

Category	Term	Pathway description	Genes
KEGG	hsa05213	Endometrial cancer	*CCND1, CTNNB1, EGFR*
KEGG	hsa05219	Bladder cancer	*CDKN1A, CCND1, EGFR*
KEGG	hsa05210	Colorectal cancer	*CCND1, CYCS, CTNNB1*

GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; hsa, *Homo sapiens*.
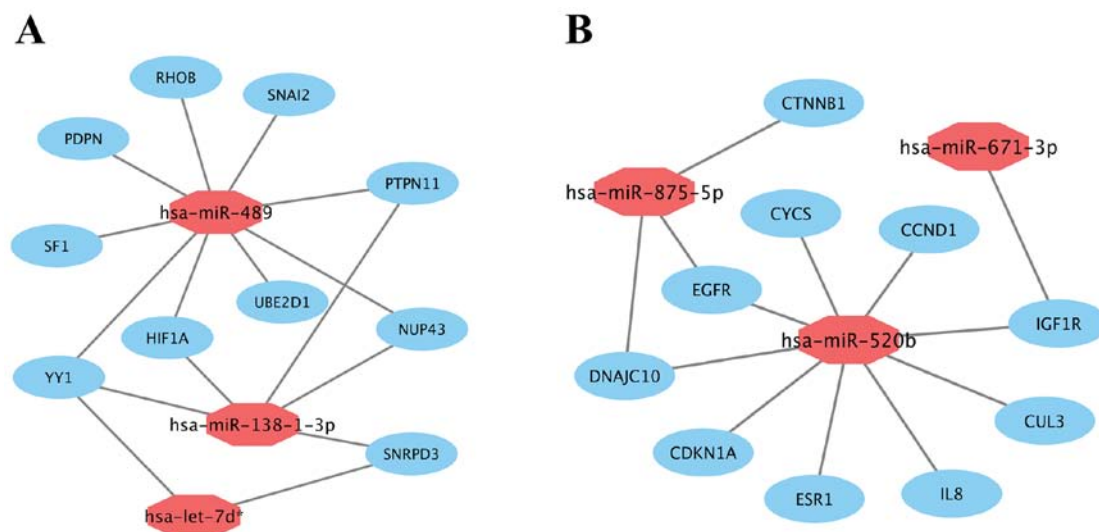

Figure 7. miRNA-hub gene network (A) for the top 3 most highly upregulated miRNAs and their hub genes; (B) for the top 3 most highly downregulated miRNAs and their hub genes.

of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation, leading to cell proliferation. *EGFR* involvement in the tumorigenesis and invasion of pituitary tumors, especially aggressive prolactinomas, has been reported by previous studies, and mutations in this gene can be utilized as potential targets in the treatment of aggressive prolactinomas. As reported in the literature, tyrosine kinase inhibitors (TKIs), such as lapatanib, sunitinib and erlotinib, have been trialed as first- or second-line treatments based on the VEGFR pathway, but most of them have failed (4,29-32,34). In addition, in the present study, we found that *EGFR* may be negatively modulated by hsa-miR-520b using the miRTarBase database; furthermore, hsa-miR-520b can be regulated by *EGFR* due to its association with the biological process regulation of production of miRNAs involved in gene silencing by miRNA (30-32). This interesting finding may allow the use of this potential pathway for the diagnosis or treatment of aggressive prolactinomas in the future.

Subsequently, by constructing an miRNA-hub gene network, we found that most hub genes were potentially modulated by hsa-miR-489 and hsa-miR-520b, suggesting that these miRNAs may be the most important regulators of prolactin pituitary tumor aggressiveness. Recent studies demonstrated that hsa-miR-489 acts as a tumor suppressor in hepatocellular carcinoma (42), gastric cancer (43), breast cancer (44), glioma (45), hypopharyngeal squamous cell carcinoma (46),

bladder cancer (47) and colorectal cancer (48). Downregulation of miR-489 was reported to be associated with the tumorigenesis, invasion, and metastasis of various tumors, suggesting an important role for hsa-miR-489 in predicting prognosis and acting as a drug target. However, the roles of hsa-miR-489 in pituitary tumors, especially aggressive prolactinomas, have not been previously studied. Additionally, hsa-miR-520b was reported to have a suppressive effect on tumor cell proliferation, migration, invasion and epithelial-to-mesenchymal transition (EMT) in colorectal cancer (49), glioblastoma (50), hepatoma (51), head-neck cancer (52), breast cancer (53), lung cancer (54) and gastric cancer (55). Expression of hsa-miR-520b is lower in tumor tissues than in normal tissues, significantly promoting the proliferation, migration, and invasion of tumor cells. Unlike other tumors, Liang *et al* (56) reported that hsa-miR-520b was upregulated in nonfunctioning and gonadotropin-secreting pituitary adenomas relative to normal pituitaries, which indicated that miR-520b functions as a tumor inducer in benign pituitary adenoma (56). However, whether hsa-miR-520b acts as a promoter or suppressor in aggressive prolactin pituitary tumors has not been previously studied. According to our study, we speculate that upregulation of hsa-miR-489 suppresses aggressiveness and progression, while downregulation of hsa-miR-520b promotes the aggressiveness and progression of aggressive prolactinomas. Such ambivalent miRNA expression might be one of the reasons

that aggressive prolactin pituitary tumors lie on the spectrum between 'benign' pituitary adenomas and 'malignant' pituitary carcinomas. It will be extremely meaningful to authenticate the functions of hsa-miR-489 and hsa-miR-520b and elucidate the mechanisms by which they regulate aggressive behaviors, resistance to treatments and early recurrence in aggressive prolactin pituitary tumors.

There are some limitations of the present study. First, the sample size of GSE46294 is rather small (only 12 samples), which may cause some bias when identifying the differentially expressed miRNAs. Second, the expression of the differentially expressed miRNAs was not validated by RT-qPCR analysis with our clinical pituitary samples. Further studies are needed to experimentally verify the results of this study.

In conclusion, we successfully identified one key target gene, *EGFR*, and two crucial miRNAs, hsa-miR-489 and hsa-miR-520b, associated with aggressiveness based on bioinformatic analysis. These findings may provide potential candidate biomarkers for the early diagnosis and individualized treatment of aggressive prolactin pituitary tumors. However, further research is needed to experimentally verify the results of this study.

Availability of data and materials

The GSE46294 datasets analyzed during the present study are available in the GEO repository (http://www.ncbi.nlm. nih.gov/geo/). The potential target genes of DEMs were predicted by miRTarBase (http://mirtarbase.mbc.nctu.edu. tw/). The DAVID database (http://david.ncifcrf.gov/) was used to perform functional annotation and pathway enrichment analysis for genes. The STRING database (http://string-db. org) was used to assess functional associations among genes.

Authors' contributions

All authors conceived and designed the study. LG, XG and CF performed data curation and analysis. KD and WL analyzed and interpreted the results. ZW and BX drafted and reviewed the manuscript. All authors read and approved the final manuscript.

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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