A predicted miR-27a-mediated network identifies a signature of glioma

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Abstract. The dysregulation of physiological microRNA (miRNA) activity has been shown to play an important role in gliomagenesis. In a previous study, using microRNA arrays and glioma tissues found that miR-27a was upregulated, which was also identified in the glioma cell lines and samples by quantitative real-time polymerase chain reaction (qRT-PCR). In this study, in order to explore the potential roles of miR-27a in the progression of glioma, we first utilized text-mining of PubMed abstracts with natural language processing (NLP) to identify 1,168 glioma-related molecules. In addition, miR-27a targets predicted by computational methods were integrated with the results from NLP analysis, followed by Gene Ontology (GO), pathway and network analysis. We identified 33 hub genes by overlap calculation and demonstrated that miR-27a may be involved in the progression of glioma through adherens junction, focal adhesion, the neurotrophin signaling pathway, the MAPK signaling pathway, the transforming growth factor-β (TGF-β) signaling pathway, cytokine-cytokine receptor interactions, the p53 signaling pathway, the apoptotic signaling pathway, as well as others. Our data may provide researchers with a better understanding of the mechanisms of the miR-27a-target network in glioma initiation and progression.

Introduction

Gliomas are the most frequently observed brain tumors, with glioblastoma multiforme (GBM) being the most common and aggressive form in adults (1). Despite the major therapeutic improvements achieved with the combination of neurosurgery, chemotherapy and radiotherapy, the prognosis and survival rate for patients with GBM remains extremely low (2). Therefore, there is an urgent need for innovative and reliable diagnostic or prognostic biomarkers and new therapeutic strategies. Gene signatures that can characterize the heterogeneity of glioma would provide a molecular basis for the pathological or clinical characteristics and the regulatory pathways and networks that are unique to glioma subtypes and would lead to the identification of molecular fingerprints for a more guided treatment plan.

microRNAs (miRNAs) comprise a large group of endogenous non-coding RNAs that can either block mRNA translation or negatively regulate miRNA stability, and therefore play a crucial role in the regulation of gene expression (3). A number of studies have analyzed miRNA expression profiles in glioblstoma and have identified several deregulated miRNAs (4-6). Furthermore, alterations in miR-levels have been implicated in the deregulation of critical players in major cellular pathways, modifying the differentiation, proliferation and survival of tumor cells. For instance, miR-7 and miR-221/222 have been shown to be involved in the activation of the Akt and epidermal growth factor receptor (EGFR) signaling pathways, while miR-34a is a key downstream regulator of p53 (7-10). As cancer-related molecular signatures are definitely not one or two, but rather a series of factors, the comprehensive and systematic analysis of miRNA-target genes is of great importance in glioma treatment and may provide a unique method for the diagnosis and prognosis of glioma, in addition to providing novel targets for the development of therapeutic strategies for glioma.

In the present study, we found that miR-27a is upregulated in human glioma. Furthermore, we systematically analyzed the miR-27a-predicted target genes related to the glioma by a series of data-filtering, including Gene Ontology (GO), pathway and network analyses. Our results provide novel insights into our understanding of the role and mechanism of miR-27a in gliomagenesis and provide a potential therapeutic strategy for glioma treatment in the future.

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Materials and methods

Cell culture and human glioma samples. The human U251, U87, LN229, LN308 and A172 glioblastoma cell lines were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All cells were maintained at 37˚C in a 5% CO2 incubator in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA).

The human glioma samples were obtained from the Department of Neurosurgery, Sir Run Run Shaw Hospital, Medical College, Zhejiang University, Hangzhou, China. Informed consent was obtained from all patients diagnosed with glioblastoma prior to obtaining the specimens. The samples were freshly resected during surgery and immediately frozen in liquid nitrogen for subsequent total RNA extraction.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR). The total RNA was isolated from cultured cells, human glioma specimens, or normal brain tissues (NBTs) using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed in triplicate in the ABI 7500HT fast real-time PCR System (Applied Biosystems) and normalized to U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) endogenous control. Total RNA from NBTs was used as the control. The miR-27a levels were measured with the TaqMan microRNA assay kit in accordance with the manufacturer's instructions (Applied Biosystems).

Natural language processing (NLP) of glioma studies. We conducted a search in PubMed (Medline), in an attempt to cover all studies published with the following keywords: glioma (title). All the sets of genes and proteins associated with the keywords were identified and added to a list, followed by gene mention tagging, using a biomedical named entity recognizer (ABNER; an open source tool for automatically tagging genes, proteins and other entity names in text, http://pages.cs.wisc.edu/~bsettles/abner/) (11) and conjunction resolution. In the present study, the gene symbol in the Entrez gene database of NCBI prevailed as previously described (12,13). A flow chart of the NLP analysis is shown in Fig. 1.

Prediction of miRNA target genes. The analysis of miR-27a predicted targets was determined using three independent softwares (14): PicTar2005(http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi); miRandaV5(http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and TargetScan 5.1(http://www.targetscan.org/) .

GO analysis. GO analysis was conducted with the GSEABase R package (http://www.r-project.org/) statistical Platform (15). miR-27a targets were then classified into three major groups: biological process, cellular component and molecular function.

Pathway analysis. miR-27a targets were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database by using GenMAPP v2.1, and the p-value was calculated for each enriched pathway (16).

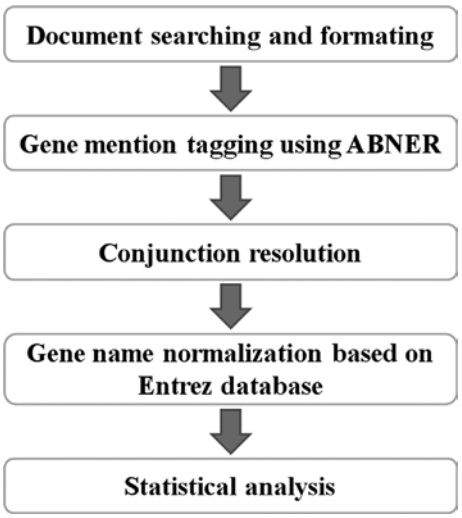

Figure 1. Flow chart of the natural language processing (NLP) analysis of glioma.

Table I. Gene network analysis including three types of interaction: enzyme-enzyme interaction, protein-protein interaction and gene expression interaction.

ECrel	enzyme-enzyme interaction, indicating two enzymes catalyzing successive reaction steps
PPrel	protein-protein interaction, such as binding and modification
GErel	gene expression interaction, indicating correlation between transcription factor and target gene product

Network analysis. We also integrated three different interactions: i) protein interaction, gene regulation and protein modification in the KEGG database; ii) the existing high-throughput experiments, such as the protein-protein interaction confirmed by yeast two-hybrid; iii) the already mentioned interaction between genes. In brief, we downloaded the pathway data from the KEGG database and analyzed the genomic interaction between the genes by using the KEGGSOA (http://www.bioconductor.org/packages/2.4/bioc/html/KEGGSOAP.html) R package (http://www.r-project.org/), including three types of interaction: enzyme-enzyme relation, protein-protein interaction and gene-expression interaction (17) (Table I).

The protein-protein interaction data were downloaded from the MIPS database (http://mips.helmholtz-muenchen.de/proj/ppi/) (18). For the afore-mentioned interaction we used algorithm co-citation in the PubMed abstracts: we analyzed a gene term and all its co-occuring term variants within the sentences in the PubMed abstracts and calculated the frequency of the co-citation gene. Subsequently, we performed a statistical amalysis as described in the NLP analysis. Finally, the network was displayed by using the Medusa software.

Integrative analysis of miR-27a target genes and NLP. The overlap between the miR-27a target genes predicted by bioinformatic tools and the glioma related-genes obtained from

the NLP analysis was calculated. A gene network analysis was subsequently performed.

Statistical analysis. In the analysis of miR-27a expression, all tests were carried out using SPSS Graduate Pack 11.0 statistical software (SPSS, Chicago, IL). Descriptive statistics including the mean ± SE along with one-way ANOVAs were used to determine the significant differences. P<0.05 was considered to indicate a statistically significant difference.

In the NLP analysis, the frequency of each gene occurrence was calculated. The higher the frequency of genes the greater the likelihood of an association between glioma and a certain gene. The total number of references in the PubMed database was recorded as 'N'. The frequency of genes and glioma in the PubMed database was denoted by 'm' and 'n', respectively. The simultaneous occurrence of a gene and the disease was denoted as 'k'. Then by using hypergeometric distribution, we calculated the probability of frequency greater than 'k' co-citation at the completely random conditions. P<0.01 was considered to indicate a statistically significant difference. We used the following formula:

$$p = 1 - \sum_{i=0}^{k-1} p(i \mid n, m, N)$$

$$p(i \mid n, m, N) = \frac{n!(N-n)!m!(N-m)!}{n-i\ !i!\ n-m\ !\ N-n-m+i\ !N!}$$

Results

miR-27a is overexpressed in human glioma. We have previously demonstrated that miR-27a is upregulated in glioblastoma by miRNA arrays (data not shown). To further identify the expression of miR-27a in glioma, we performed TaqMan-based real-time stem-loop RT-PCR analyses using fresh human glioma samples and cell lines. We selected five glioblastoma cell lines, including U251, U87, LN229, LN308 and A172 for testing and used NBT as the normal control. Our data showed that miR-27a was significantly upregulated in all of these glioblastoma cell lines, compared to NBT (Fig. 2A). A similar result was observed in the glioma tissues (Fig. 2B), and all of these samples showed a significant increase (5 to 14 fold) in miR-27a expression. Taken together, our results demonstrated that miR-27a was abnormally overexpressed both in human glioma samples and cell lines.

Integrative analysis of miR-27a target genes and NLP results. After proving the upregulation of miR-27a in glioma, we futher investigated the potential role of miR-27a and its targets in glioma development. We used three computational algorithms: TargetScan4.0, PicTar and miRanda, which are commonly used to predict miRNA targets, and identified 1,884 miR-27a target genes. In addition, the abstracts and titles of 11,150 primary studies were identified for initial reviewing using the search strategies already mentioned and a total of 1,168 glioma-related genes were subsequently identified.

To further explore the potential mechanism of miR-27a, we categorized the integrative results of NLP analysis and miR-27a targets in GO according to biological process, cellular component and molecular function (Table II). Subsequent to the pathway analysis, there were 50 pathways available, out of which eight signaling pathways were significant (p<0.01) (Table III): the adherens junction pathway (NLK, INSR, SNAI1, SMAD2, MET, CDC42, FYN, EGFR, FGFR1, YES1; p=2.25E-06); the focal adhesion pathway (GRB2, PDPK1, XIAP, MET, PDGFRA, PXN, IGF1, CDC42, FYN, RAP1B, EGFR, FN1; p=0.0006989); the neurotrophin signaling pathway (GRB2, IRS1, MAPK14, BAX, CDC42, FASLG, RAP1B, MAPK7, NGFRAP1); the MAPK signaling pathway

Table II. All of the genes obtained from integration of NLP analysis and potential miR-27a targets were categorized in GO, according to biological process, cellular component and molecular function.

Term	Count	p-value
Biological process		
Developmental processes	61	0
Death	28	2.08E-10
Cell cycle and proliferation	28	1.36E-06
Stress response	24	2.58E-05
Cell adhesion	14	0.0007227
Cell organization and biogenesis	29	0.0010944
Protein metabolism	37	0.0016411
Cell-cell signaling	9	0.0021401
Signal transduction	43	0.0126941
RNA metabolism	30	0.0629269
Other biological processes	65	0.0873043
Other metabolic processes	27	0.2618713
Transport	23	0.2850757
DNA metabolism	3	0.6768301
Cellular component		
Nucleus	48	0.0043901
Plasma membrane	33	0.0072076
Cytoskeleton	14	0.0222964
Cytosol	7	0.0245927
Extracellular matrix	6	0.0285326
Other cellular component	64	0.0704202
Non-structural extracellular	18	0.0817679
Other cytoplasmic organelle	5	0.3191817
Mitochondrion	9	0.3869073
Other membranes	59	0.6113681
ER/Golgi	9	0.7024601
Translational apparatus	1	0.8080292
Molecular function		
Kinase activity	21	2.85E-06
Other molecular function	118	0.0003563
Transcription regulatory activity	18	0.0028955
Cytoskeletal activity	9	0.0202878
Signal transduction activity	35	0.1061263
Enzyme regulator activity	7	0.2699902
Nucleic acid binding activity	23	0.3250427
Transporter activity	7	0.7310064

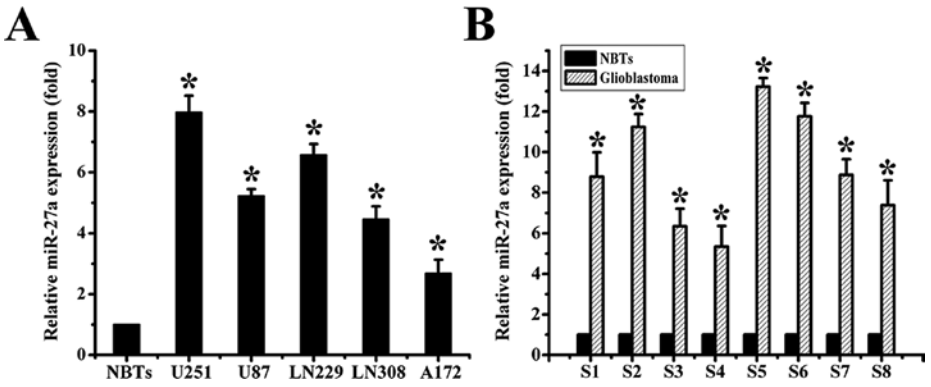

Figure 2. miR-27a is overexpressed in glioma cell lines and samples. (A) miR-27a expression in glioma cells (U251, U87, LN229, LN308 and A172) and normal brain tissue (NBT) by qRT-PCR. (B) qRT-PCR analysis showed that glioblastoma tissues expressed much higher levels of miR-27a compared with the NBT. Data represen the means ± SE of thee replicates; *p<0.05.

Table III. Pathway analysis of the genes obtained from integration of the NLP analysis and potential miR-27a targets[a].

Title	Count	p-value	Genes
Adherens junction	10	2.25E-06	NLK, INSR, SNAI1, SMAD2, MET, CDC42, FYN, EGFR, FGFR1, YES1
Focal adhesion	12	0.0006989	GRB2, PDPK1, XIAP, MET, PDGFRA, PXN, IGF1, CDC42, FYN, RAP1B, EGFR, FN1
Neurotrophin signaling pathway	9	0.0010528	GRB2, IRS1, MAPK14, BAX, CDC42, FASLG, RAP1B, MAPK7, NGFRAP1
MAPK signaling pathway	14	0.0010648	NLK, GRB2, CDC25B, PDGFRA, MAPK14, NF1, CDC42, FASLG, RAP1B, MAPK7, EGFR, MAP2K4, FGFR1, FGF1
TGF-β signaling pathway	7	0.0017426	SMAD2, ID4, SMAD5, SP1, BMPR2, ID3, RPS6KB1
Cytokine-cytokine receptor interaction	13	0.0024092	CXCL2, MET, PF4, IL6R, PDGFRA, IL10, FASLG, CSF3, BMPR2, EGFR, CSF1, IL24, KITLG
p53 signaling pathway	6	0.0024798	PMAIP1, BAX, IGF1, CASP8, CDK6, BBC3
Apoptosis	6	0.008848	XIAP, CFLAR, BAX, FADD, FASLG, CASP8

[a]There were 50 pathways available. Among them, eight signaling pathways were significant (p<0.01).

(NLK, GRB2, CDC25B, PDGFRA, MAPK14, NF1, CDC42, FASLG, RAP1B, MAPK7, EGFR, MAP2K4, FGFR1, FGF1; p=0.0010648); the transforming growth factor-β (TGF-β) signaling pathway (SMAD2, ID4, SMAD5, SP1, BMPR2, ID3, RPS6KB1; p=0.0017426); the cytokine-cytokine receptor interaction (CXCL2, MET, PF4, IL6R, PDGFRA, IL10, FASLG, CSF3, BMPR2, EGFR, CSF1, IL24, KITLG; p=0.0024092); the p53 signaling pathway (PMAIP1, BAX, IGF1, CASP8, CDK6, BBC3; p=0.0024798); and the apoptotic pathway (XIAP, CFLAR, BAX, FADD, FASLG, CASP8; p=0.008848).

The gene network can not only directly reflect the relationship between genes, but also the stability of gene regulatory networks. The highly connected hub genes often play an important role in the stability of the network. Since the hub genes are the core of gene regulation and can affect the majority of genes, a hub gene is generally believed to be of greater importance than another normal gene. In the network analysis of the integrative targets, the connectivity of growth factor receptor-bound protein 2 (GRB2) was also the highest among all 33 hub genes obtained in the miR-27a target network analysis (a total of six genes; z-test, p= 0.0010559): CD28, EGFR, FGFR1, IRS1, MET and PDGFRA (Fig. 3).

The overlap of the miR-27a target genes and the glioma-related genes obtained from NLP analysis was calculated and 44 overlap genes that are not only associated with the development and progression of glioma but are also the potential miR-27a target genes were obtained in this integrative analysis (Fig. 4; Table IV).

Discussion

Gliomas are the most frequently observed brain tumors, with GBM being the most common and aggressive form in adults (1). Unfortunately, the underlying molecular mechanisms resulting in gliomagenesis and local invasion remain obscure and are the major obstruction to finding novel therapeutic strategies.

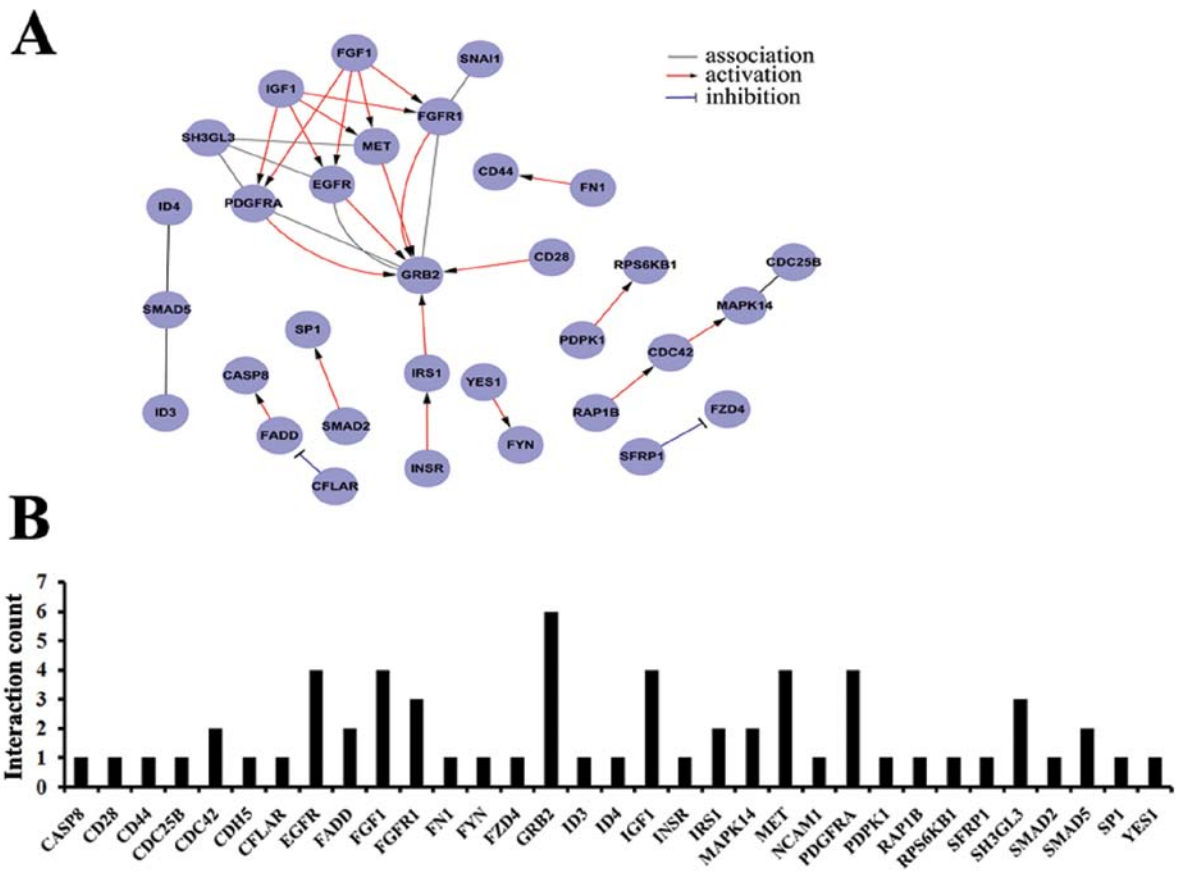

Figure 3. (A) Network analysis of glioma-related genes indentified in the natural language processing (NLP) analysis. The network can reflect the relationship between genes from a situation as a whole. Grey, association; red, activation; blue, inhibition. (B) Connectivity analysis of the glioma-related genes. The connectivity of GRB2 was the highest among the total glioma-related genes (z-test, $p<0.01$).

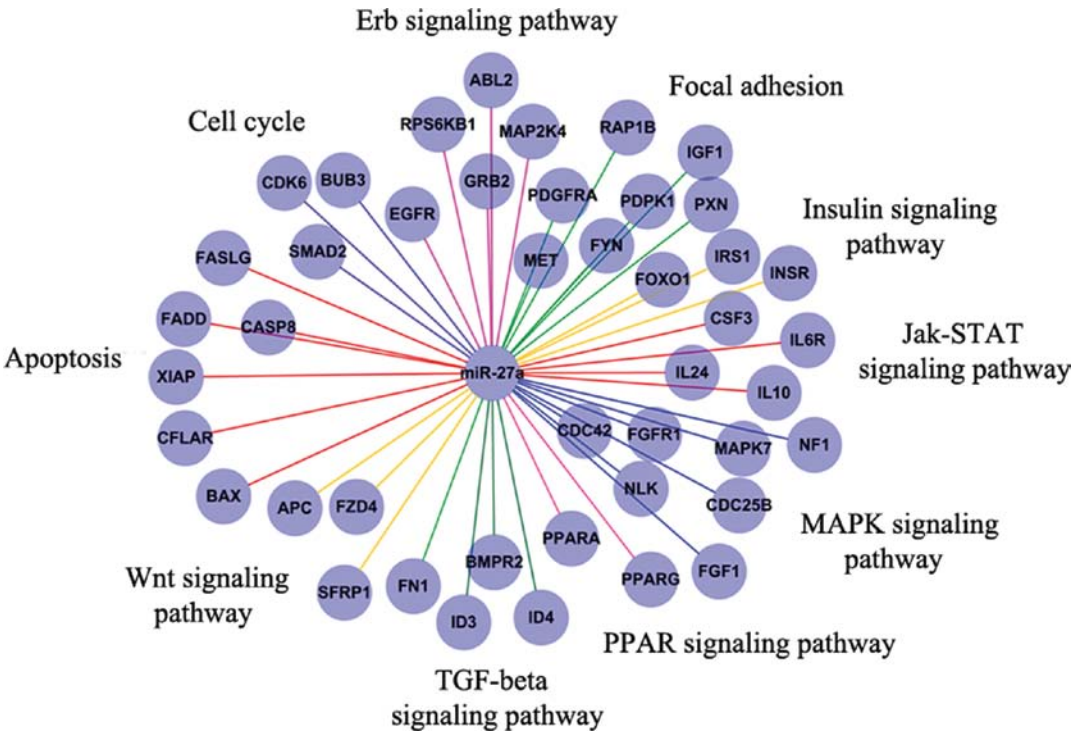

Figure 4. Integrative-analysis of miR-27a target genes and the natural language processing (NLP) results. A total of 44 overlap genes and their functional pathways that are not only associated with the molecular mechanism of glioma but also the potential miR-27a target genes were obtained in this final integrative analysis.

Table IV. Integrative analysis of miR-27a target genes and the NLP results[a].

Targets	PubMed count	p-value	Gene description
FASLG	20	0	Fas ligand (TNF superfamily, member 6)
FADD	8	0	Fas (TNFRSF6)-associated via death domain
CASP8	10	0	Caspase 8, apoptosis-related cysteine peptidase
BAX	13	0	BCL2-associated X protein
NF1	18	0	Neurofibromin 1
IL10	18	0	Interleukin 10
IL24	7	0	Interleukin 24
PDPK1	8	0	3-Phosphoinositide-dependent protein kinase 1
EGFR	227	0	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)
CDK6	6	0	Cyclin-dependent kinase 6
PPARG	16	1.94E-08	Peroxisome proliferator-activated receptor gamma
FGFR1	6	2.23E-06	Fibroblast growth factor receptor 1
PXN	5	9.25E-06	Paxillin
XIAP	5	2.15E-05	X-linked inhibitor of apoptosis
PDGFRA	4	0.0002609	Platelet-derived growth factor receptor, alpha polypeptide
CDC25B	3	0.0002675	Cell division cycle 25 homolog B (S. pombe)
PPARA	4	0.0015537	Peroxisome proliferator-activated receptor alpha
SLTM	1	0.0062219	SAFB-like, transcription modulator
RPS6KB1	2	0.024244	Ribosomal protein S6 kinase,70kDa, polypeptide 1
CFLAR	2	0.038005	CASP8 and FADD-like apoptosis regulator
NLK	1	0.040756	Nemo-like kinase
FZD4	1	0.044739	Frizzled homolog 4 (*Drosophila*)
ID4	1	0.044739	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
BUB3	1	0.054624	Budding uninhibited by benzimidazoles 3 homolog (yeast)
RAP1B	1	0.060506	RAP1B, member of RAS oncogene family
ID3	1	0.07985	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
ABL2	1	0.085576	V-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)
SFRP1	1	0.11919	Secreted frizzled-related protein 1
MAPK7	1	0.12284	Mitogen-activated protein kinase 7
MAP2K4	1	0.15859	Mitogen-activated protein kinase kinase 4
CSF3	1	0.16557	Colony stimulating factor 3 (granulocyte)
BMPR2	1	0.18275	Bone morphogenetic protein receptor, type II (serine/threonine kinase)
FGF1	1	0.20455	Fibroblast growth factor 1 (acidic)
IL6R	1	0.21115	Interleukin 6 receptor
FOXO1	1	0.22093	Forkhead box O1
SMAD2	1	0.32089	SMAD family member 2
CDC42	1	0.40925	Cell division cycle 42 (GTP binding protein, 25kDa)
IRS1	1	0.42502	Insulin receptor substrate 1
FN1	1	0.43215	Fibronectin 1
FYN	1	0.48397	FYN oncogene related to SRC, FGR, YES
INSR	1	0.49774	Insulin receptor
APC	1	0.51621	Adenomatous polyposis coli
GRB2	1	0.58868	Growth factor receptor-bound protein 2
IGF1	1	0.73593	Insulin-like growth factor 1 (somatomedin C)

[a]From the integrative-analysis, 44 overlap genes were obtained that are not only associated with the development and progression of glioma but are also potential miR-27a target genes.

Our approach links putative targets to a function and succeeds not only in identifying multiple targets but also in uncovering an entire network under miRNA control.

miR-27a has been studied by several researcheres. Recently, Zhao *et al* proved that the downregulation of miR-27a inhibits the proliferation of gastric cancer cells *in vitro* and *in vivo*, increases the sensitivity of gastric cancer cells to drugs, and can increase the accumulation or decrease the amount of adriamycin in gastric cancer cells (19). Wang *et al* reported that the upregulation of miR-27a contributes to the malignant transformation of human bronchial epithelial cells induced by the SV40 small T antigen (20). Ma *et al* showed that the inhibition of miR-27a suppressed the growth, colony formation and migration of pancreatic cancer cells (21). Zhang *et al* demonstrated that the downregulation of miR-27a increases the sensitivity of esophageal cancer cells to P-glycoprotein-related and P-glycoprotein-non-related drugs, and can promote ADR-induced apoptosis, accompanied by the increased accumulation and decreased in the amount of ADR (22). However, the precise molecular mechanisms of miR-27a involved in glioma are unclear.

In the present study, we focused on the miR-27a expression and the predicted signature of a miR-27a-mediated network for glioma. We first identified the abnormal overexpression of a miR-27a in glioma by qRT-PCR. To explore the potential mechanisms involved, we carried out NLP analysis, and identified 1,168 genes related to the development and progression of glioma. Given that the biological significance of miRNA deregulation relies on the effect upon their target-protein-coding genes, we analyzed the predicted targets of miR-27a. To date, computational methods have been widely used for the prediction of miRNA target genes. It has been shown that the union of miRNA target genes predicted by three computational algorithms (miRanda, PicTar, and TargetScan) is one of the strategies that demonstrate the highest sensitivity (14). In our study, this strategy predicted a total of 1,884 unique gene symbols targeted by miR-27a. Then the NLP analysis and miR-27a putative targets were integrated and categorized in GO, followed by pathway and network analyses, including the tumor-related genes, CDK6, SMAD2, FASLG, FADD, BAX, CASP8, FOXO1, PPARG and MET. Some of these targets have already been verified by miRNA functional experiments, such as PPARG (23), FOXO1 (24) and FADD (25). A total of 50 pathways were obtained in the miR-27a target pathway analysis and some of them have already been reported to be involved in the development and progression of glioma. Pathways such as the adherens junction (26), the focal adhesion (27), the neurotrophin signaling pathway (28), the MAPK signaling pathway (29), the TGF-β signaling pathway (30), the cytokine-cytokine receptor interaction (31), the p53 signaling pathway (32) and the apoptotic signaling pathway (33) play an important role in the molecular mechanisms of glioma ($p<0.01$). Other pathways, such as the ErbB signaling pathway (34), the insulin signaling pathway (35), the PPAR signaling pathway (36), the Wnt signaling pathway (37), the cell cycle (38) and the Jak-STAT signaling pathway (39) are also involved in glioma progression. Since the highly connected hub genes are the core of gene regulation and affect the majority of genes, it is generally believed that hub genes are of greater importance than other normal genes and often play a crucial role in the stability of the network. We also carried out connectivity analysis in this study, and GRB2 had the highest connectivity (a total of six genes; z-test, $p=0.0010559$), of all the 33 hub genes obtained in the miR-27a target network analysis (Fig. 3). Other hub genes, such as EGFR (40), PDGFRA (32), FADD (41) and MET (42) are also associated with cancer. Cancer-related biomarkers are definitely a group or a series of factors. The joint detection of these 33 hub genes may potentially enable doctors to identify and select high-risk patients for effective adjuvant therapy in order to improve the results of patients with glioma.

A total of 33 hub genes were identified in the final integrative analysis of NLP and miR-27a targets. Our results suggest that miR-27a may plays an important role in the development and progression of glioma through the adherens junction, focal adhesion, neurotrophin signaling, MAPK signaling, TGF-β signaling, cytokine-cytokine receptor interaction, p53 signaling and the apoptotic signaling pathway, as well as others. Our integrative study also suggests that targeting those hub genes and their pathways may be an effective strategy to control cell proliferation in glioma.

In conclusion, the present study provides new insights into the role of miR-27a in human brain tumors. We propose that a systematic analysis of the miR-27a-targets in glioma may be an effective tool for the early diagnosis, prognosis and prediction of the therapeutic response of the individual. Our results may help researchers predict the molecular mechanisms of miR-27a in the development and progression of glioma. The identification of these molecular pathways involved in the malignant biological behavior of glioma may play a prominent role in establishing rational therapeutic approaches.

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