

# Global changes of mRNA expression reveals an increased activity of the interferon-induced signal transducer and activator of transcription (STAT) pathway by repression of miR-221/222 in glioblastoma U251 cells

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**Abstract.** MicroRNA (miR)-221 and miR-222 are frequently upregulated in various types of human malignancy including glioblastoma. Previous studies have identified some targets of miR-221 and miR-222, such as p27 and p57. Inter-relationship between miR-221 and miR-222 expression and global mRNA expression remains elusive. Here we knocked down miR-221 and miR-222 expression and found 158 differentially expressed genes with 2-fold changes in U251 glioma cells by microarray analysis. Using the KEGG pathway databases and BioCarta, we found that the IFN- $\alpha$  signaling pathway was the most significant pathway modulated by differentially expressed genes. STAT1 and STAT2 are core proteins in the IFN- $\alpha$  signaling pathway. By Western blotting and immunofluorescence, we found that STAT1 and STAT2 expression and phosphorylation were upregulated in U251 cells with knocked-down miR-221/222. Furthermore, tyrosine phosphorylation of STAT1 and STAT2 was present in the nucleus after repression of miR-221/222 expression in U251 cells. These data indicate for the first time a mechanism involving STAT1/2 upregulation under the transcriptional control of INF- $\alpha$  signaling after knockdown of miR-221/222 cluster in U251 glioma cells.

## Introduction

MiRNAs (miRs) are a class of highly conserved small non-coding single-stranded RNAs, of approximately -22 nt, that

play important regulatory roles at the post-transcriptional level (1). miRNA-mediated regulation of gene expression is considered to be one of the fundamental events at the post-transcriptional level (2-4). miRNAs participate in the determination of cell fate, pattern formation in embryonic development, and in the control of cell proliferation, cell differentiation and cell death (2-4). Recent research suggests the potential involvement of altered miRNAs in the pathogenesis of a wide range of human cancers (2,3). Shortly after the cluster of human miRNAs was identified, miRNA cluster miR-15a-16, which includes miR-15a and miR-16-1, was identified located near 13q14, a region that is characterized by a high frequency of deletions in chronic lymphocytic lymphoma and several solid tumors (5). Another recent study indicated that expressions of miR-15a and miR-16-1 were inversely correlated with the Bcl-2 expression in chronic lymphatic leukemia, and that both of these miRNAs negatively regulate Bcl-2 at the post-transcriptional level; thus potentiating the normal apoptotic response by targeting the anti-apoptotic gene Bcl-2 (6). This finding opens the door to the possibility of designing miRNA-based therapeutics. The best-characterized example of miRNA-based therapeutics is the miR-221/222 cluster. In a mouse subcutaneous tumor model for prostate carcinoma, the anti-miR-221/222 reduced tumor growth by increasing intratumoral p27 amount. The effect is long lasting, as it is detectable as long as 25 days after treatment (7). In general, previous studies have indicated that some miRNAs play an oncogenic role in different human cancers, but relatively little work has been done on the interrelationship between change of miRNAs expression and global changes of mRNA expression in the oncogenesis.

Gene expression analysis based on genome-wide microarray has been largely used to characterize human cancers. Initial microarray investigations support the belief that gliomas in general, and glioblastoma multiforme (GBM) in particular, are molecularly heterogeneous tumors, and that molecular approaches to classification and grading may have distinct diagnostic and prognostic advantages (8-14). This approach

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allowed the identification of genes involved in tumorigenesis (11-14). Furthermore, the discovery of gene expression signatures characteristic of distinctive clinic pathological features suggested that expression profiles could be used for molecular classification and evaluation of clinical prognosis of human gliomas (11-14). Microarray facilitates the comprehensive characterization of miRNA expression profiles in human cancers. Ciafre *et al* used microarray data to compare the global expression levels of 245 miRNAs in glioblastoma tissue to normal brain tissue, and found that the expressions of miR-221/222 were strongly upregulated in glioblastoma cells. They identified nine overexpressed miRNAs in glioblastoma cell lines, which including the miR-221/222 cluster. Thus, the overexpression of miR-221/222 in glioblastoma cells might represent a molecular signature of glioblastoma tissues and cell lines (15). However, the gene expression profile reflecting the genomic changes after interruption of miR-221 and miR-222 expression in gliomas has received little attention.

In the present study, as indicated in Fig. 1, 2'-O-methyl (2'-OMe-) modified antisense oligonucleotides were used to knock down the expression of the miR-221/222 cluster in U251 cells. Global changes in gene expression were evaluated using a human 70-mer oligonucleotide microarray containing 35,000 well-characterized genes. The microarray analysis indicated that 145 genes were upregulated. Pathway analysis of the differentially expressed genes indicated significant changes in the activities of the IFN- $\alpha$  signaling pathway. We found evidence of differential regulation of transcription by eight transcription factors (TFs). We also demonstrated that STAT1 and STAT2 were the core factors affected by knock-down of miR-221/222. A mechanism involving STAT1/2 upregulation under the transcriptional control of INF- $\alpha$  signaling after the knockdown of miR-221/222 cluster in U251 glioma cells was proposed.

## Materials and methods

**Cell culture and transfection.** Human glioma cell line U251, A172 and H4 were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine (Sigma, USA), 100 units of penicillin/ml (Sigma) and 100  $\mu$ g of streptomycin/ml (Sigma). The cells were incubated at 37°C in 5% CO<sub>2</sub> and sub-cultured every 2-3 days. Total RNA was extracted from above-mentioned cells by the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for miRNA profile examination. The miRNA microarray was obtained from CapitalBio Co. (Beijing, China).

The individual oligonucleotide probes were printed in triplicate on chemically-modified glass slides in a 21x21 spot configuration of each subarray for the miRNA microarray. All the oligonucleotide probes were printed in triplicate on one microarray, and each of the four subarrays contained 16 controls [Zip5, Zip13, Zip15, Zip21, Zip23, Zip25, Y2, Y3, U6, New-U2-R, tRNA-R, hsa-let-7a, hsa-let-7b, hsa-let-7c, 50% DMSO (dimethyl sulfoxide) and Hex].

Total RNA (FirstChoice® Total RNA, Ambion, USA) from normal brain was used as a control. The miRNAs were

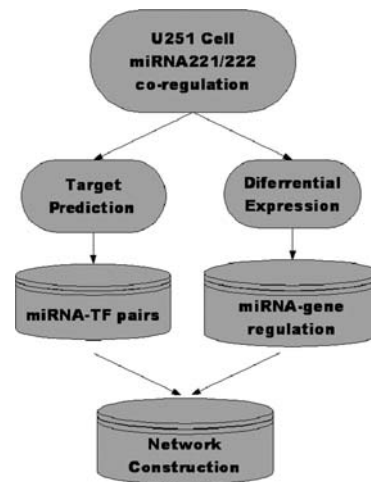


Figure 1. The miR-221 and miR-222 were found upregulated in four glioma cell lines by microarray. The knocked-down miR-221 and miR-222 in U251 cells were analyzed for the mRNA profile of changed genes. miRNA database was searched for the target genes of miR-221 and miR-222. By gene ontology (GO) and the KEGG pathway databases and BioCarta, a network of miR-221/222-TFs-STAT1/2-gene expression was constructed.

enriched from total RNA using a mirVana miRNA isolation kit (Ambion, USA) and labeled with mirVana array labeling kit (Ambion). Labeled miRNAs were used for hybridization on each miRNA microarray, containing 509 probes in triplicate, at 42°C overnight. A double-channel laser scanner (LuxScan 10K/A, CapitalBio) was used to scan the arrays and they were quantified by image analysis software (LuxScan 3.0, CapitalBio).

**Transfection and RNA isolation.** 2'-O-methyl (2'-OMe-) oligonucleotides were chemically synthesized by GenePharma Co., Ltd (Shanghai, China). 2'-O-Methyl oligos were composed entirely of 2'-O-methyl bases and had the following sequences: 2'OMe-miR-221 5'-GUCAACAUCAGUCUGAUAAGCUA-3' (AS-miR-221) and 2'OMe-miR-222 5'-GUCACAUCAGUCUGAUAAGCUA-3' (AS-miR-222). The U251 cells were co-transfected with AS-miR221 and AS-miR-222 in equal amounts at a final concentration of 20  $\mu$ M using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cells were cultured for 48 h until further study. Total RNA was isolated using the TRIzol reagent (Invitrogen) based on the manufacturer's protocol.

**Microarray analysis.** The 35,000-oligonucleotide microarray was constructed by CapitalBio Corp. using the Human Genome Oligo human-V4.0 (Operon), and comprises 35035 5-amino-modified 70-mer probes. In addition to internal controls provided by the Oligo Set vendor, twelve intergenic sequences from yeast were synthesized and used as external controls. After printing, the slides were baked for 1 h at 80°C and stored dry at room temperature until use. Prior to hybridization, the slides were dehydrated over 65°C water for 10 sec and UV cross-linked at 250 mJ/cm<sup>2</sup> energy. The immobilized oligonucleotides were washed off with 0.5% SDS for 10 min at 42°C and SDS was removed by dipping the slides in anhydrous ethanol for 30 sec. An aliquot of 5  $\mu$ g

Table I. miRNAs differentially expressed between human glioma cell lines and normal brain tissues by microarray SAM results (data in this table were normalized to ambion normal brain).

miRNA	Chromosome location	Comparative expression of miRNAs	Potential targets
Up-regulated			
miR-21	17q23.2	7.035±2.409	PTEN, SEPT7
miR-15b	3q25.33	5.433±1.700	CDC25A, EIF2C1
miR-106b	7q22.1	5.105±3.004	IGF2BP1, E2F1
miR-221	Xp11.3	3.496±0.948	TIMP3, LIFR
miR-222	Xp11.3	3.404±1.079	ESR1, PAK1
miR-27a	19p13.12	3.327±0.953	MAP3K7IP3, SLC6A1
miR-23a	19p13.12	3.183±0.365	ITGB8, PTEN
miR-23b	9q23.31	2.383±0.441	SEMA6D, TOP1
Down-regulated			
miR-137	1p21.3	0.116±0.152	EIF3S1, SNF1LK
miR-95	4p16.1	0.114±0.138	NR4A2, OAZ2
miR-132	17p13.3	0.093±0.075	GMFB, PAIP2
miR-128b	2q21.3	0.089±0.067	PDE3A
miR-128a	2q21.3	0.084±0.061	EIF2S2, NF1
miR-342	14q32.2	0.074±0.057	SMG7, SEPT3
miR-127	14q32.31	0.071±0.104	SEPT7, KIF3B
miR-323	14q32.31	0.070±0.033	SEMA6D, IL11
miR-330	19q13.32	0.060±0.068	FGFR1, HOXD8
miR-1	20q13.33	0.056±0.068	IGF1, TGIF2
miR-219	6p21.32	0.037±0.055	NEUROD2
miR-126	9q34.3	0.025±0.009	IRS1
miR-329	14q32.31	0.021±0.011	PAM, TNRC6B
miR-223	Xq12	0.014±0.014	PDPK1, SLC37A3
miR-495	14q32.31	0.010±0.009	CDK6, BDNF
miR-124a	20q13.33	0.006±0.005	AMOTL1
miR-338	17q25.3	0.006±0.003	FGFR2
miR-451	17q11.2	0.004±0.003	CAB39, VAPA

of total RNA was used to produce fluorescent dye-labeled cDNA with Eberwine's linear RNA amplification method and a subsequent enzymatic reaction according to our previously published protocol (16). The labeled DNA was purified with PCR Clean-up NucleoSpin kits (Macherey Nagel, Germany), resuspended in Elution buffer, and quantified by UV spectrophotometry. Labeled control and test samples were quantitatively adjusted based on the efficiency of Cy-dye incorporation and mixed into 80°C hybridization solution (3X SSC, 0.2% SDS, 25% formamide and 5% Denhart's). DNA in hybridization solution was denatured at 95°C for 3 min prior loading on a microarray. Hybridization was performed under LifterSlip™ (Erie, USA), which allows for even dispersal of hybridization solutions between the microarray and coverslip. The hybridization chamber was placed on a 3-D tilting agitator BioMixer™ II (CapitalBio) to facilitate the microfluidic circulation under the coverslip. The array was hybridized at 42°C overnight and washed with two consecutive washing solutions (0.2% SDS, 2X SSC at 42°C for 5 min, and 0.2% SSC for 5 min at room temperature). After hybridization, microarrays were scanned with a confocal scanner LuxScan™ 10KA

(CapitalBio), and the data from the obtained images were extracted with LuxScan 3.0 Software (CapitalBio). The raw data were normalized using a space and intensity-dependent Lowess program. The data from faint spots were removed, in which the intensity was lower than the average intensity plus 2 standard deviations of the negative controls on the array. The fidelity of the microarray had been previously evaluated (17,18). Considering the very low false-positive ratio (data not shown), the ratio cut-off value of 2-fold was chosen for significant differential expression.

*Functional group analysis.* The microarray data were input into the Database for Annotation, Visualization and Integrated Discovery (DAVID, <http://david.abcc.ncifcrf.gov/>), which leveraged the Gene Ontology (GO) to identify the molecular function represented in the gene profile. In addition, we used the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (<http://www.genome.ad.jp/kegg/>) and BioCarta (<http://www.biocarta.com>) to analyze the roles of these genes in the pathways. Literature mining was performed manually on the 145 genes and illustrated by graph-medusa (V 1.041, <http://coot.embl.de/medusa>).

Table II. Functional classification of the 158 differentially expressed genes, by GO, and their over-representation.

System	Term	Count	%	P-Value	Benjamini
Biological process	Protein kinase cascade	7	4.5	8.4E-2	1.0E0
	Induction of programmed cell death	5	3.2	8.4E-2	1.0E0
	Modification-dependent macromolecule catabolic process	5	3.2	6.0E-2	1.0E0
	Regulation of apoptosis	9	5.7	5.7E-2	1.0E0
	Regulation of progression through cell cycle	9	5.7	5.1E-2	1.0E0
	Protein catabolic process	6	3.8	4.6E-2	1.0E0
	Ectoderm development	5	3.2	3.5E-2	1.0E0
	Epidermis development	5	3.2	2.8E-2	1.0E0
	Regulation of programmed cell death	10	6.4	2.5E-2	1.0E0
	Cellular protein catabolic process	6	3.8	1.7E-2	1.0E0
	Cell death	14	8.9	1.2E-2	9.9E-1
	Apoptosis	14	8.9	7.3E-3	9.8E-1
	DNA packaging	10	6.4	8.4E-4	4.2E-1
	Establishment and/or maintenance of chromatin architecture	10	6.4	7.4E-4	4.7E-1
	Potein-DNA complex assembly	8	5.1	1.9E-4	2.2E-1
	Nucleosome assembly	8	5.1	5.5E-6	1.4E-2
	Cellular component	Proteasome complex ( <i>sensu Eukaryota</i> )	3	1.9	7.9E-2
Intermediate filament cytoskeleton		5	3.2	7.2E-2	1.0E0
Intermediate filament		5	3.2	7.2E-2	1.0E0
Chromosome		10	6.4	5.5E-3	6.1E-1
Chromosomal part		10	6.4	1.8E-3	3.4E-1
Chromatin		10	6.4	4.0E-5	1.4E-2
Nucleosome		8	5.1	2.5E-6	1.7E-3
Molecular function	GPI anchor binding	4	2.5	6.9E-2	1.0E0
	NAD+ADP-ribosyltransferase activity	3	1.9	1.4E-2	9.9E-1
	Copper ion binding	5	3.2	4.6E-3	8.9E-1
	Cadmium ion binding	4	2.5	2.4E-5	2.3E-2

**Immunofluorescence analysis.** Transfected cells were seeded on coverslips and fixed with 4% paraformaldehyde (PFA, Sigma), treated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min and incubated with the PUMA or Bax antibodies (Santa Cruz Biotechnology, CA, USA) overnight at 4°C. FITC- or TRITC-labeled secondary antibody was then added (1:200 dilution) for 2 h at 37°C. After staining with DAPI dye for 5 min, the cells were visualized on a FV-1000 laser scanning confocal microscope and analyzed by IPP5.1 software (Olympus, Japan).

**Western blot analysis.** Western blot analysis was used to characterize the expression of proteins after gene knockdown by co-transfection with AS-miR-221 and AS-miR-222 in U251 cells (19). Equal amounts (20 µg) of protein extracts from each sample were resolved by SDS-PAGE on 8% gels. Proteins were probed with mouse anti-STAT1, STAT2 and phospho-STAT1, STAT2 monoclonal antibodies (1:1,000 dilution, Santa Cruz Biotechnology), and β-actin (1:2,000 dilution, Santa Cruz Biotechnology) followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:2,500 dilution, Zhongshan, China). Proteins were visualized with a chemiluminescence detection system using the Super Signal substrate (Pierce, USA).

**Statistical analysis.** Data are expressed as means ± SE, and P<0.01 is considered as statistically significant by ANOVA,  $\chi^2$  test and SNK t-test.

## Results

**Knockdown of the miR-221/222 cluster in U251 cell line.** To investigate whether miRNAs are differentially expressed in glioma cells versus normal brain tissue, we performed a microarray analysis using a chip able to examine the global expression levels of 469 miRNAs. The results showed that miR-15b, miR-106b, miR-221, miR-27a, miR-21, miR-222, miR-23b and miR-23a were upregulated in glioma cells (Table I). In particular, miR-221 and miR-222 were strongly upregulated in glioma cells (Fig. 2). This result was similar to the result of Ciafre *et al* (15).

Our study indicated a single miR-221 antagomiR did not affect cellular growth in U251 glioblastoma cells *in vitro*, indicating a functional overlap between miR-221 and miR-222 in controlling cell survival (20). Noting this, we used an antisense method to co-suppress the expression of miR-221/222 in U251 cells. We found that miR-221/222 were downregulated by co-transfecting U251 cells with AS-miR-

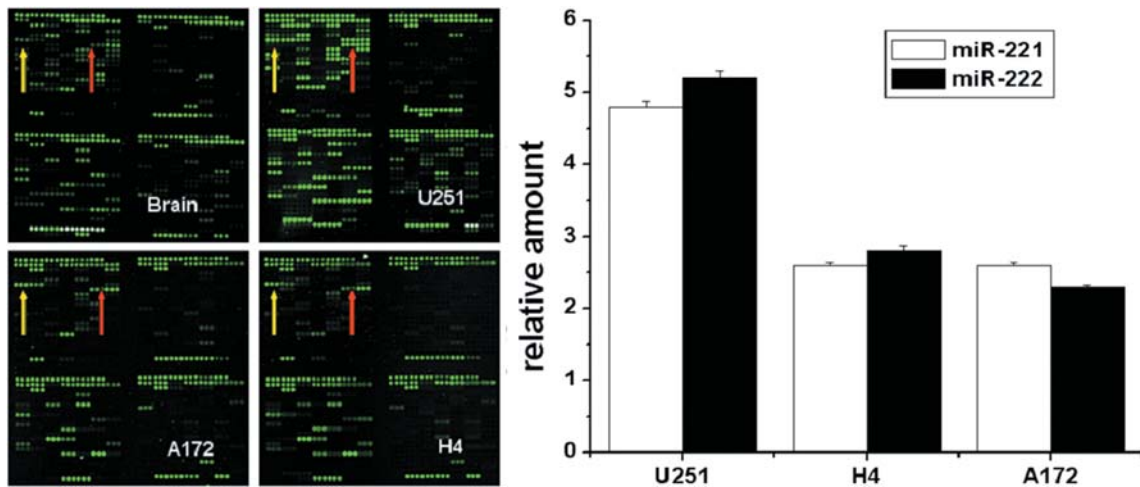


Figure 2. miRNA microarray analyzed miRNAs proliferate in glioma cell lines. The cluster analysis of miRNA microarray data showed miR-221 and miR-222 overexpression in four human glioma cell lines.

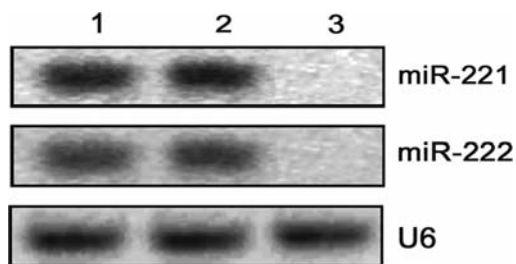


Figure 3. Northern blot analysis of miR-221 and miR-222 expression. Northern blot detection of miR-221/222 expression in U251 glioblastoma cells after co-suppression by antisense oligonucleotides (1, control; 2, scrambled miR; 3, As-miR-221/222).

221/222. Northern blotting using non-isotopic detection of miRNA with digoxigenin-labeled RNA probes showed that AS-miR-221/222 was effectively blocking miR-221/222 expression, while scramble transfection yielded no effect on miRNA expression (Fig. 3). All of the tested microRNAs were predominantly present in their mature form in U251 cells.

*Microarray analysis of expression of INF- $\alpha$  pathway by repression of miR-221/222 in U251 glioma cells.* Previously, Guo *et al* compared CapitalBio's long oligonucleotide (Operon 70-mer) microarray with Affymetrix's short oligonucleotide (25-mer) HG U133A 2.0 array (21). Employing the same batch of RNA extracted from HeLa and HEK293 cell lines, the correlation coefficient (R-value) between the two platforms was 0.787, and the reverse fluorescence strategy in CapitalBio's platform was proved to give a high level of reproducibility between two independent experiments (21,22). A large-scale microarray analysis was used to investigate the changes in mRNA expression profiles after repression of the miR-221/222 cluster in U251 glioma cells.

We identified 158 differentially expressed genes with 2-fold changes after repression of the miR-221/222 cluster in U251 glioma cells. Among them, 145 were upregulated and 13 were downregulated. We took several approaches to

identify the potential functional significance of the differentially expressed genes. We used gene ontology (GO) to classify each differentially expressed gene into functional categories and determined if any categories were over-represented compared to all genes on the array. The most over-represented GO molecular function categories related to the protein kinase cascade and induction of programmed cell death (Table II). Other significant GO-defined categories included modification-dependent macromolecule catabolic processes, regulation of apoptosis, and regulation of progression through the cell cycle (Table II).

We also evaluated the pathways linking the differentially expressed genes. Using the KEGG pathway databases and BioCarta, we found that the antigen processing and presentation in KEGG and the IFN- $\alpha$  signaling pathway in KEGG and BioCarta were the most significant pathways modulated by the differentially expressed genes. The IFN- $\alpha$  signaling pathway contained 17 genes that were upregulated in miR-221/222 knocked-down U251 cells compared to parental U251 cells (Table III).

We then determined whether the differentially expressed genes were the target genes of miR-221/222. Three databases (TargetScan, PicTar, miRanda) were used for miRNA target prediction. A total of 70 candidate genes were potential targets of the miR-221/222 cluster (Table IV). Intriguingly, no intersection was found between differentially expressed genes and the predicted target genes, suggesting an indirect regulation between the miR-221/222 cluster and mRNA expression, possibly via transcription factors (TFs).

We chose TRANSFAC<sup>®</sup> 7.0 Public (<http://www.gene-regulation.com/pub/databases.html>) as the transcription factor database to identify the TFs potentially regulated by the miR-221/222 cluster. Eight TFs were found to be regulated by the miR-221/222 cluster (Table V). Literature data mining was used to predict relationships between the genes, identifying 28 genes and 64 verified modulation nodes. Our results support the view that IRF-1, STAT1 and STAT2 regulate the balance between miR-221/222-TFs and TF-gene expression, determining the cell death or survival through the INF- $\alpha$  pathway in glioma U251 cells (Fig. 4).

Table III. Upregulation of members of the IFN- $\alpha$  signaling pathway in miR-221/222 knocked-down U251 cells.<sup>a</sup>

Name	WT <sup>+</sup> /WT <sup>-b</sup>	Description of RefSeq <sup>c</sup>
CXCL10	7.5706	Small inducible cytokine B10 precursor (CXCL10)
GBP1	3.7516	Interferon-induced guanylate-binding protein 1
IFI16	2.5931	$\gamma$ -interferon-inducible protein Ifi-16
IFI27	10.5238	Interferon- $\alpha$ -induced 11.5 kDa protein
IFI30	4.2318	$\gamma$ -interferon inducible lysosomal thiol reductase precursor
IFI35	6.8096	Interferon-induced 35 kDa protein (IFP 35)
IFI44	9.8163	Interferon-induced protein 44
IFIT1	18.1824	Interferon-induced protein with tetratricopeptide repeats 1
IFIT2	21.1874	Interferon-induced protein with tetratricopeptide repeats 2
IFIT3	39.4750	Interferon-induced protein with tetratricopeptide repeats 3
IFIT5	4.2232	Interferon-induced protein with tetratricopeptide repeats 5
IFITM1	13.7500	Interferon-induced transmembrane protein 1
IFITM2	8.8921	Interferon-induced transmembrane protein 2
IRF1	2.8741	Interferon regulatory factor 1
ISGF3G	2.6395	Transcriptional regulator ISGF3 $\gamma$ subunit
STAT1	5.5881	Signal transducer and activator of transcription 1
STAT2	2.4067	Signal transducer and activator of transcription 2

<sup>a</sup>The genes whose expression was upregulated 2-fold. <sup>b</sup>WT<sup>+</sup>, U251 cell; WT<sup>-</sup>, U251 cells with miR-221/222 knockdown. <sup>c</sup>Reference sequence (RefSeq).

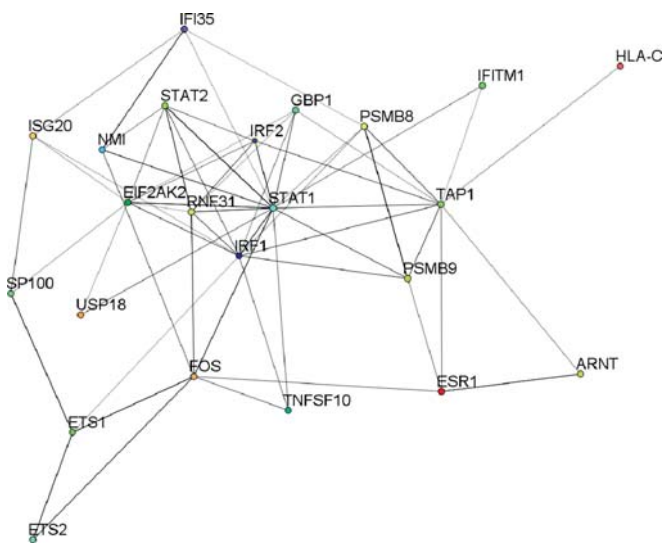


Figure 4. Constructed network of miR-221/222-TFs-STAT1/2-gene expression. Genes involved in TF-gene expression in glioma U251 cells after repression of the miR-221/222 cluster, determining the cell death or survival through the IFN- $\alpha$  pathway. Genes associated with eight TFs (FOS, ESR1, ETS2, IRF2, ARNT, ETS1, HOXC1, FOXN2) were manually identified via literature mining. IRF-1, STAT1 and STAT2 regulate the balance between miR-221/222-TFs and TF-gene expression, determining the cell death or survival through the IFN- $\alpha$  pathway in glioma U251 cells. The IFN- $\alpha$  pathway regulates cell survival negatively, via processes occurring in multiple intracellular compartments. Regulatory relationships are denoted by line colors, including the 28 genes and 64 verified modulation nodes.

*miR-221/222 modulate STAT1 and STAT2 tyrosine phosphorylation and nuclear translocation.* Dephosphorylation and tyrosine phosphorylation of STAT1 and STAT2 were detected

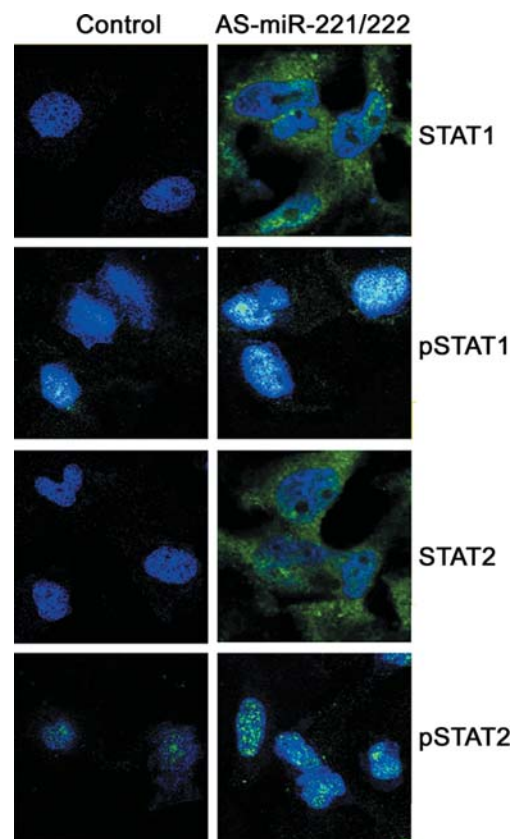


Figure 5. Immunofluorescence indicates dephosphorylation and phosphorylation of STAT1 and STAT2 expression and location. After co-suppression of the miR-221/222 cluster in U251 glioblastoma cells, dephosphorylation of STAT1 and STAT2 expression increased in the cytoplasm, tyrosine phosphorylation of STAT1 and STAT2 also increased in the nucleus.

Table IV. Target prediction of miR-221/222 (TargetScan, PicTar, miRanda).

Gene symbol	Gene name
CDC2L6	Cell division cycle 2-like 6 (cdk8-like)
HOXC10	Homeobox c10
ETS1	v-ets erythroblastosis virus e26 oncogene homolog 1 (avian)
KIAA1267	dkfzp727c091 protein
RALA	v-ral simian leukemia viral oncogene homolog a (ras related)
MYLIP	Myosin regulatory light chain interacting protein
VAPB	Vamp (vesicle-associated membrane protein)-associated protein b and c
ESR1	Estrogen receptor 1
PAIP2	Poly(a) binding protein interacting protein 2
RAB1A	rab1a, member of the ras oncogene family
IGF2BP2	Insulin-like growth factor 2 mRNA-binding protein 2
RBM24	RNA-binding motif protein 24
SNCB	Synuclein, $\beta$
PPP3R1	Protein phosphatase 3 (formerly 2b), regulatory subunit b, 19kda, $\alpha$ isoform (calcineurin b, type i)
TRPS1	Trichorhinophalangeal syndrome i
HECTD2	Hect domain containing 2
ADAM11	Adam metallopeptidase domain 11
ARID1A	At rich interactive domain 1a (swi-like)
PPARGC1A	Peroxisome proliferative-activated receptor, $\gamma$ , coactivator 1, $\alpha$
ETS2	v-ets erythroblastosis virus e26 oncogene homolog 2 (avian)
TMCC1	Transmembrane and coiled-coil domain family 1
POGZ	Pogo transposable element with znf domain
DNAJC14	Cytokine-induced protein 29 kda
C12orf30	Chromosome 12 open reading frame 30
TIMP3	Timp metallopeptidase inhibitor 3 (sorsby fundus dystrophy, pseudoinflammatory)
MAP3K10	Mitogen-activated protein kinase kinase kinase 10
FOS	v-fos fbj murine osteosarcoma viral oncogene homolog
PPP6C	Protein phosphatase 6, catalytic subunit
CDV3	cdv3 homolog (mouse)
FNDC3A	Fibronectin type iii domain containing 3a
GNAI2	Guanine nucleotide-binding protein (g protein), $\alpha$ -inhibiting activity polypeptide 2
ZFPM2	Zinc finger protein, multitype 2
VGLL4	Vestigial like 4 ( <i>Drosophila</i> )
FAT2	Fat tumor suppressor homolog 2 ( <i>Drosophila</i> )
ANKHD1	Ankyrin repeat and kh domain containing 1
KIAA1598	kiaa1598
NAP1L5	Nucleosome assembly protein 1-like 5
PHF2	phd finger protein 2
INSIG1	Insulin-induced gene 1
PAIP1	Poly(a) binding protein interacting protein 1
BMF	bcl2 modifying factor
IRX5	Iroquois homeobox protein 5
YWHAG	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, $\gamma$ polypeptide
HRB	hiv-1 rev binding protein
PDCD10	Programmed cell death 10
CDKN1C	Cyclin-dependent kinase inhibitor 1c (p57, kip2)
HIPK1	kiaa0630 protein
CCDC64	Coiled-coil domain containing 64
ASB7	Hypothetical protein flj22551
RSBN1L	Round spermatid basic protein 1-like
MAPK10	Mitogen-activated protein kinase 10
IRF2	Interferon regulatory factor 2

Table IV. Continued.

Gene symbol	Gene name
MESDC1	Mesoderm development candidate 1
NLK	Nemo-like kinase
ARF4	adp-ribosylation factor 4
RIMS3	Regulating synaptic membrane exocytosis 3
KHDRBS2	kh domain containing, RNA binding, signal transduction associated 2
PBX3	Pre-b-cell leukemia transcription factor 3
DMRT3	Doublesex and mab-3 related transcription factor 3
GARNL1	kiaa0884 protein
DACH1	Dachshund homolog 1 ( <i>Drosophila</i> )
ANGPTL2	Angiopoietin-like 2
MSL2L1	Male-specific lethal 2-like 1 ( <i>Drosophila</i> )
TOX	Thymus high mobility group box protein tox
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATXN1	Ataxin 1
INA	Internexin neuronal intermediate filament protein, $\alpha$
SLC25A37	Solute carrier family 25, member 37
MIER3	
ZEB2	

Table V. miR-221 and miR-222 co-regulated transcription factors.

Gene symbol	Gene name
FOS	v-fos fbj murine osteosarcoma viral oncogene homolog
ESR1	Estrogen receptor 1
ETS2	v-ets erythroblastosis virus e26 oncogene homolog 2 (avian)
IRF2	Interferon regulatory factor 2
ARNT	Aryl hydrocarbon receptor nuclear translocator
ETS1	v-ets erythroblastosis virus e26 oncogene homolog 1 (avian)
HOXC1	Homeobox protein HOXC1
FOXP2	Forkhead box N2

using immunostaining and immunoblotting techniques. Fig. 5 shows that dephosphorylation of STAT1 and STAT2 occurs in the cytoplasm. Fig. 5 also shows that tyrosine phosphorylation of STAT1 and STAT2 was present in the nucleus after repression of miR-221/222 expression in U251 cells. This rapid onset in STAT1 and STAT2 immuno-reactivity after miR-221/222 repression probably represents increased protein affinity and/or accessibility to the antibody consequent to STAT1/STAT2 mobilization and dimerization. Finally, Western blotting confirmed these results (Fig. 6).

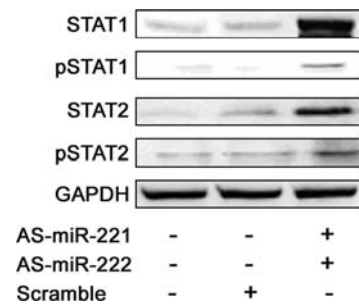


Figure 6. Western blot analysis of dephosphorylation and phosphorylation of STAT1 and STAT2 expression. After co-suppression of the miR-221/222 cluster in U251 glioblastoma cells, dephosphorylation of STAT1 and STAT2 expression increased, and tyrosine phosphorylation of STAT1 and STAT2 also increased.

Not unexpectedly, the expression of STAT1 and STAT2 and their activated form (phosphorylated STAT1 and STAT2) were greater after repression of the miR-221/222 cluster than that in parental U251 cells (Fig. 6).

Our results revealed that knockdown of miR-221/222 changed the mRNA expression profiles of genes that are not target genes of miR-221/222 in U251 cells. In the regulatory networks that are affected by knockdown of miR-221/222, the mRNA expression profiles of STAT1 and STAT2 are central to the effect. That is, knockdown of miR-221/222 upregulated STAT1 and STAT2 expression and their phosphorylation to change mRNA expression profiles.

## Discussion

A growing number of papers have been published describing a link between several forms of human cancer and the



expression of miRNAs (3). The majority of the studies identified targets of the miRNAs that were shown to be specifically modulated in tumors. Compared with the regulation of transcription, the study of the regulatory networks influenced by miRNA is only just beginning (23). In this study, we focused on miRNAs, miR-221/222, that are clustered at Xp11.3, which had been demonstrated to overexpress in the specific context of cancers (2,15,24,25). Both miRNAs are clustered within a 1-kb genomic interval and are transcribed from the same strand (EMBL: AJ550425 and AJ550426, respectively). They also share an identical seed sequence (20). Thus, miRNA221/222 might be generated from the same primary transcript, which could account for the simultaneous enhancement of their expression in tumor cells observed in previous studies (26,27). Importantly, our results show that co-repression of the miR-221/222 cluster compromises cell survival (20). This indicates a functional overlap between miR-221 and miR-222 in controlling cell survival. There are four reports that provide evidence not only on the carcinogenic role of miR-221/222 regulating p27, but also conferring tamoxifen resistance in breast cancer by targeting p27 (26-29). However, to the best of our knowledge, indirect regulation of miR-221/222 by TFs in the context of cancer cells has not yet been reported.

Using a bioinformatic approach, Shalg *et al* proposed the concept of miRNA target hubs, that were genes regulated by dozens of miRNA and that were involved in a diversity of developmental processes and in transcription regulation. The miRNA target hubs were highly enriched for developmental processes, specifically for muscle development and nervous system development, as well as for TFs and transcription regulators. A potential regulatory model of the gene expression network is that genes belonging to the same regulon will be coregulated, not only at the transcriptional level, but also posttranscriptionally (23). We think that our results, which identify STAT1 and STAT2 as indirect targets for miR-221/222 in the context of glioma cell lines, demonstrated another regulatory model. Based on our results, after repression of miR-221/222, eight direct mRNA targets of TFs were upregulated (released from the mRNA-3' UTR binding effect), then the TFs interacted with other TFs (such as STAT1 and STAT2), finally regulating gene expression. The miRNA-TF-TF-genes model of miR-221/222-TFs-STAT1/2-gene expression has uncovered another anti-cancer mechanism via the repression of miR-221/222 in U251 glioma cells. It is well known that miRNAs function to fine tune gene expression through their target mRNAs; however, the changes in the expression levels of the eight mRNAs remain undetectable in our study.

Type I IFNs (IFN- $\alpha$  and IFN- $\beta$ ) bind to a common receptor and are crucial effector cytokines in many antiviral, antiproliferative, and immune responses (30,31). Binding of IFN $\alpha/\beta$  to the type I receptor results in transphosphorylation and activation of the Jak1/Tyk 2 kinase (32,33). Type I IFN receptor (IFNAR) activation triggers the JAK-mediated tyrosine phosphorylation of STAT2 and STAT1 (34). The activated kinases elicit tyrosine phosphorylation and dimerization of STAT1 and STAT2, which subsequently form a heterotrimeric transcription factor complex with ISGF-3, and activate genes that contain an ISRE (interferon-stimulated

response element) site to modulate the transcription of over 300 genes (35). The interferon-induced protein with tetratricopeptide repeats (IFIT) 1, IFIT2, IFIT3, and IFIT5 were upregulated 4.22- to 39.48-fold, as indicated by the microarray results.

We also observed upregulation of STAT1, STAT2 and ISGF3- $\gamma$  subunit expression after repression of the miR-221/222 cluster in U251 glioma cells. STAT1 is a key factor not only in signal transduction coupled to IFNAR, but also coupled to the type II IFN (i.e., IFN- $\gamma$ ) receptor (36). In addition, activation of STAT1 can occur in response to other cytokines, such as IL-6 (37). STAT1 promotes apoptosis and negatively regulates cell growth, metastasis and angiogenesis (38-41). STAT1 is deficient or inactive in many types of human tumors, although some tumors have activated STAT1 (38-40). The reconstitution of STAT1 suppressed the tumorigenicity of RAD-105 cells *in vivo*, which correlated with decreased expression of proangiogenic molecules, such as bFGF, MMP-2 and MMP-9 (41). Consequently, STAT1 might act as an important inhibitor of tumor angiogenesis and therefore negatively regulates tumor growth and metastasis, and tumor-intrinsic STAT1 is an important mediator for antiangiogenic signals, such as IFN (41). By contrast, STAT2 is implicated in only IFNAR-coupled signal transduction (36).

In conclusion, our results suggest that repression of the miR-221/222 cluster suppresses the growth and progression of glioma, at least in part by upregulating STAT1 and STAT2 mRNA translation and activating their function. These findings provide a mechanistic rationale for the overexpression miR-221/222 cluster in glial carcinogenesis. Additional functional studies are now required to understand the molecular basis of the formation of glioma, and to allow the development of innovatively tailored therapies targeting specific tumor markers, and that act as vaccine adjuvants for cancer therapy.

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