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# MiR-221/222 Promote the Growth of Malignant Glioma Cells by Regulating Its Target Genes

Zhang Chunzhi<sup>1,2</sup>, Jiang Tao<sup>3</sup>, Wang Jinhuan<sup>4</sup>, Cheng Jinqian<sup>5</sup>,  
Pu Peiyu<sup>2</sup> and Kang Chunsheng<sup>2</sup>

<sup>1</sup>Department of Radiation Oncology, Tianjin Huanhu hospital, Tianjin,

<sup>2</sup>Department of Neurosurgery, Laboratory of Neuro-Oncology, Tianjin Medical University  
General Hospital, Tianjin Key Laboratory of Nerve Injury,  
Variation and Regeneration, Tianjin,

<sup>3</sup>Department of Neurosurgery, Tiantan Hospital, Capital Medical University, Beijing,

<sup>4</sup>Department of Neurosurgery, Tianjin Huanhu hospital, Tianjin

<sup>5</sup>Department of Molecular Oncology, H.Lee Moffitt Cancer and Research Institute, Tampa,  
<sup>1,2,3,4</sup>China  
<sup>5</sup>USA

## 1. Introduction

MicroRNAs (miRNAs or miRs) are a class of small noncoding RNAs of 19~22 nucleotides, and repress protein expression at the posttranscriptional level through imperfect or perfect base pairing with 3' untranslated region of the target mRNA in mammalian cells, leading to its reduced translation or mRNA degradation. Although miRs are thought to be involved in various biological processes, including development, differentiation, cell proliferation, cell death, and so on, accumulating evidence suggests that alterations of their expression may play a role in the development of human cancers(1-4). Specific miRNAs have been demonstrated to be deregulated in diverse cancer subtypes including lymphoma, colorectal cancer, lung cancer, breast cancer, papillary thyroid carcinoma, hepatocellular carcinoma, and glioblastoma(5-13). Ciafre et al. used microarray method to compare the global expression levels of 245 miRNAs in glioblastoma tissue with normal brain tissue and found that the expression of miR-221 was strongly upregulated in glioblastoma cells. They identified nine overexpression miRNAs including miR-221 and miR-222(miR-221/222) cluster (13). This chapter review how miR-221/222 cluster regulated glioma cell phenotype and it's mechanism.

## 2. miR-221 and miR-222 cluster regulated glioma cell phenotype and it's mechanism

In Ciafre study, they found that miR-221 and miR-222 overexpress in glioma tumor tissue and glioma cell line compare to normal brain tissue, as well as our microarray results. So, we question what biogenesis of miR-221 and miR-222 and this cluster miRs function.

2.1 Biogenesis of miR-221 and miR-222 and this tow miRs location

MiR-221, miR-222 genes are clustered chromosome Xp11.3 in tandem. When zhanglilei research retinitis pigmentosa, they discover miR-221 and miR-222 genes. Two miR genes are located near the telomeric end of deletion ~700bp apart(mir-221 : 45,361,839-45,361,948 and mir-222 : 45,362,675-45,362,784) (14). Like some other clustered miRNA genes, two miRNA genes is high similariy in sequence and they might be transcribed as polycistrons (Fig 1).

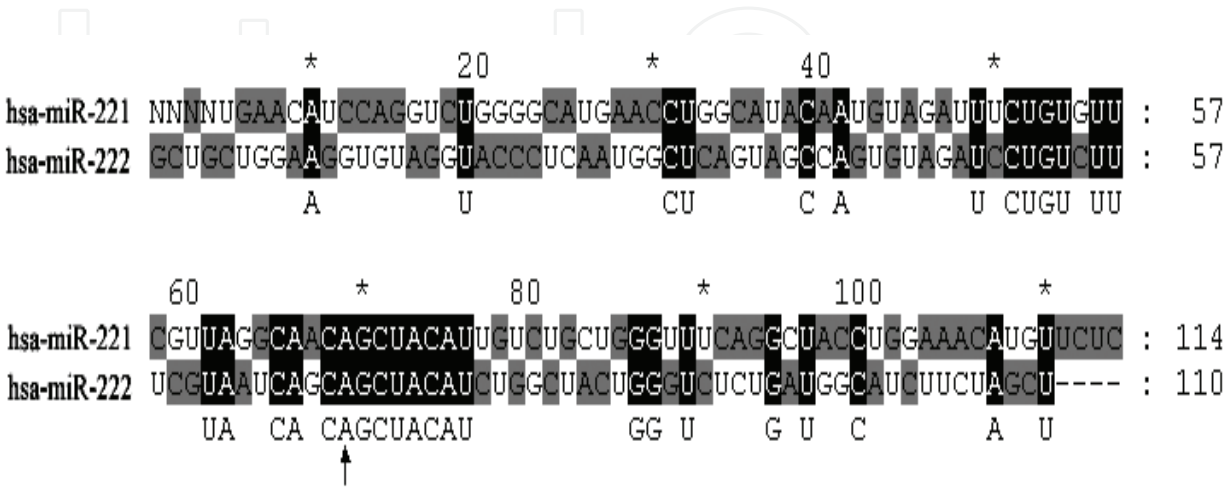


Fig. 1. A schematic representation defines the miR-221 and miR-222 sequences (arrow indicates identical seed sequences AGCUACAU as shown in GeneDoc, [www.psc.edu/biomed/genedoc](http://www.psc.edu/biomed/genedoc)).

As well as the other miRs, biogenesis of miR-221 and miR-222 are a multi-step process beginning with transcription by RNA polymerase II of a primary transcript, called pri-miRNA. This precursor is cleaved to form a 70nt long stem-loop precursor RNA(pre-miRNA) by an RNase III type endonuclease called Drosha inside the nucleus. RanGTP exportin 5 transports pre- miRNA to the cytoplasm, where another RNase III, Dicer, cleaves the stem-loop structure to produce 19~23 nucleotide miRNA duplexes. That one of the two RNA strands is then included into the RNA-induced silencing complex(RISC) form asymmetric RISC assembly. Asymmetric RISC assembly is also called miRNP. It inhibit translation or mRNA degradation by miRNA complete pairing or incomplete pairing with mRNA 3'UTRs.(15-17)

2.2 miR-221 and miR-222 cluster regulated glioma cell phenotype in vitro

We already proved that miR-221 and miR-222 upregulated in glioma compared to normal brain tissue, and knowed this two miRs biogenesis and location in chromosome. Furthermore, we ready to research how the cluster miRs affect glioma cell phenotype, including proliferation, cell cycle, apoptosis, invasion and radiosensitivity, *in vitro*. Our previous work identified that a functional overlap between miR-221/222 in modulating glioma cell phenotype. So, we knocked down or ectopic express miR-221/222 to study their function. Firstly, by northern blot, it is identified that pMSCV-miR-221/222 increased miR-221 and miR-222 level and anti-miR-221/222(AS-miR-221/222 or As-miR-221/222) knocked down miR-221 and miR-222 level (not show).By MTT assay, the glioma U251 cell proliferation rate in different transfected cells was measured. It was shown that the cells transfected with anti-miR-221/222 were proliferated at a significantly lower rate than the other three groups (Figure 2).

Moreover, the cells transfected with pMSCV-miR-221/222 proliferated at a significantly higher rate than the control and the scramble groups (Figure 2). These data demonstrated that the growth rate of glioma cell could be co-modulated by miR-221 and miR-222. The result were also proved in the other tumors. Agami R et al found that miR-221 and miR-222 promote cancer cell proliferation(18). Frenquelli M et al also identified that miR-221 and miR-222 promote cancer cell proliferation in chronic lymphocytic leukemia(19). The same result were also proved in human prostate carcinoma cell lines, human thyroid papillary carcinomas, human colorectal carcinoma, and so on(20-22).

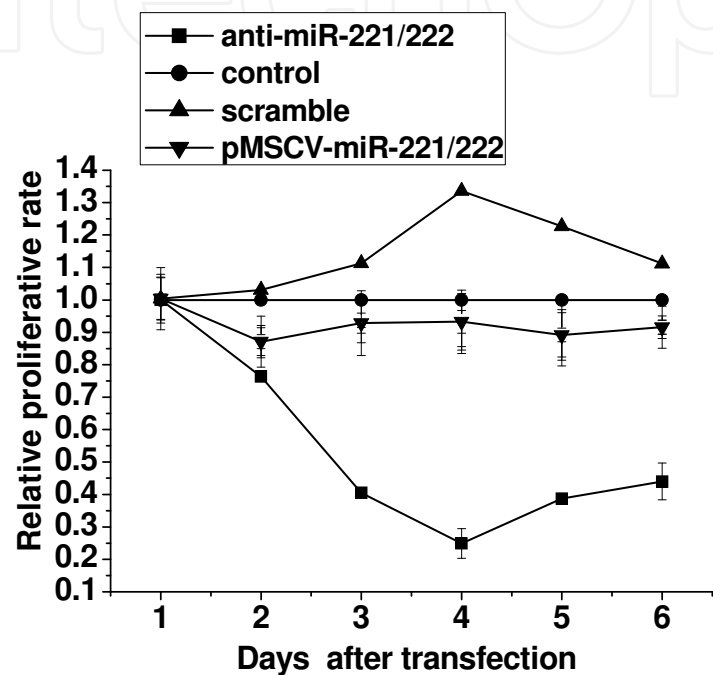


Fig. 2. As-miR-221/222 decrease cell proliferation. MTT assay show that the cells transfected with As-miR-221/222 were proliferated at a significantly lower rate than the other four groups. The latitudinal axis represented days after cell implantation in the 96-well plate. The cell proliferation rate in parental U251 glioma cells was presented as 100%.

The cell cycle distribution and cell apoptosis were analyzed by flow cytometry. The cell cycle distribution showed that the G0/G1 phase percentage of control, scramble treated cells was 28.6%, 26.0%, respectively; while the fraction of anti-miR-221/222 treated cells was 46.2% and the fraction of pMSCV-miR-221/222 treated cells was 22.3%. The S phase fraction in control, scramble treated cells, anti-miR-221/222 treated cells, pMSCV-miR-221/222 treated cells was 56.8%, 57.2%, 26.8%, 60%, respectively. No statistical significance of G2/M phase percentage was detected among the four groups (Fig. 3). Frenquelli M et al also identified that miR-221/222 promote cancer cell into S phase in chronic lymphocytic leukemia. They synchronized cells in the G1 phase and analyzed by flow cytometry the cell-cycle distribution at 4, 6, and 24 hours after cell-cycle block release. The result document that miR-221- and miR-222-transduced cells progressed more quickly through the cell-cycle phases (19). The same result was also proved in human prostate carcinoma cell lines, human thyroid papillary carcinomas, human colorectal carcinoma, and so on (20-22).

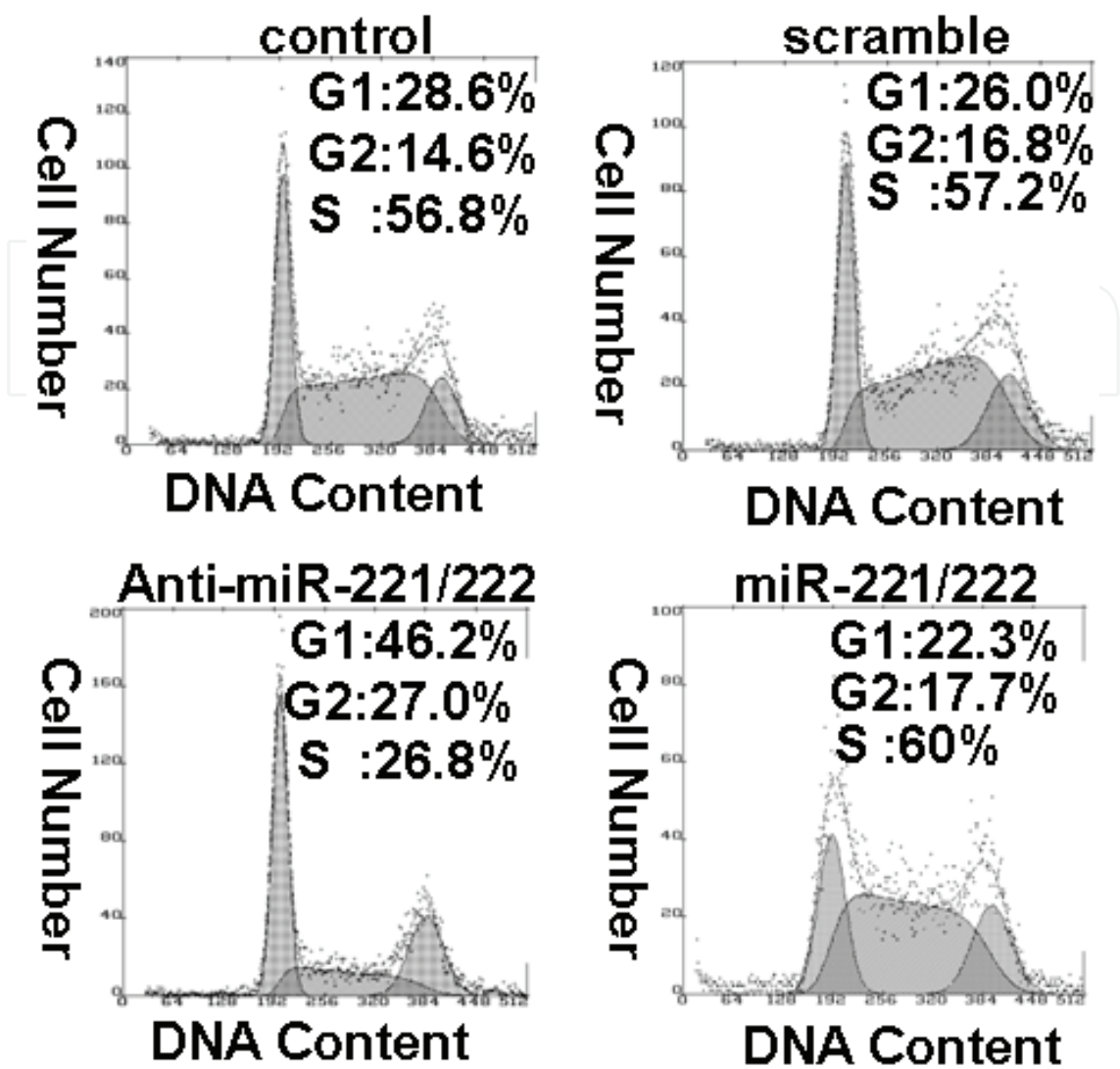


Fig. 3. Co-suppression of miR-221/222 expression by as-miR-221/222 induce G1 arrest in U251 cell. Transfected and control cells were harvested by trypsinization, washed and fixed overnight. Nuclei of cells were stained with propidium iodide, a total of 10,000 nuclei were examined. By flow cytometry analysis, the rate of cell in G1 in anti-miR-221/222 group was higher significantly than the other four groups.

Moreover, miR-221 and miR-222 inhibit glioma cell apoptosis. To examine biological significance of miR-221 and miR-222 in glioma, glioma U251 cells were treated with anti-miR-221 /222. Interestingly, annexin V-labeling revealed that knockdown of miR-221 and miR-222 significantly increased cell apoptosis compared to the cells treated with scramble oligonucleotide (Fig. 4). In addition, caspase 3/7 activity was also considerably elevated in miR-221 and miR-222 knocked down cells (Fig. 5). Since collapse of the mitochondrial membrane potential is one of the early events in apoptosis (23), we next examined whether miR-221 and miR-222 regulate mitochondrial membrane potential. The cells with depletion of miR-221 and miR-222 were stained with cationic dye JC-1. FACSCalibur analysis showed that the mitochondrial membrane potential was largely damaged when miR-221 and miR-222 were depleted (Fig. 6). These findings indicate that miR-221 and miR-222 play an important role in initiation of cell apoptosis(24).

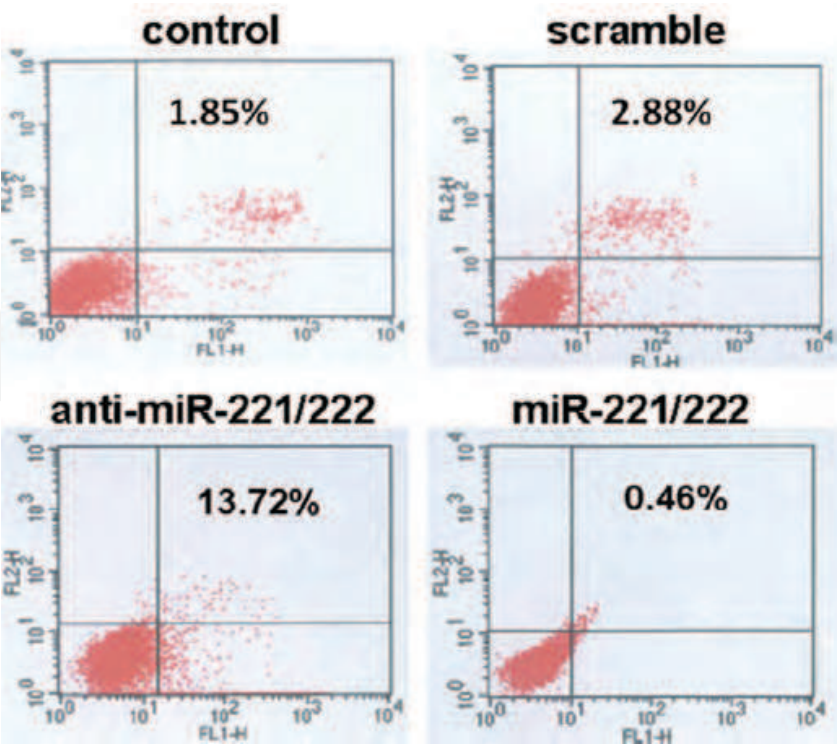


Fig. 4. Annexin V analysis showed that U251 cells transfected with As-miR-221/222 displayed significantly more apoptosis than the other three groups.

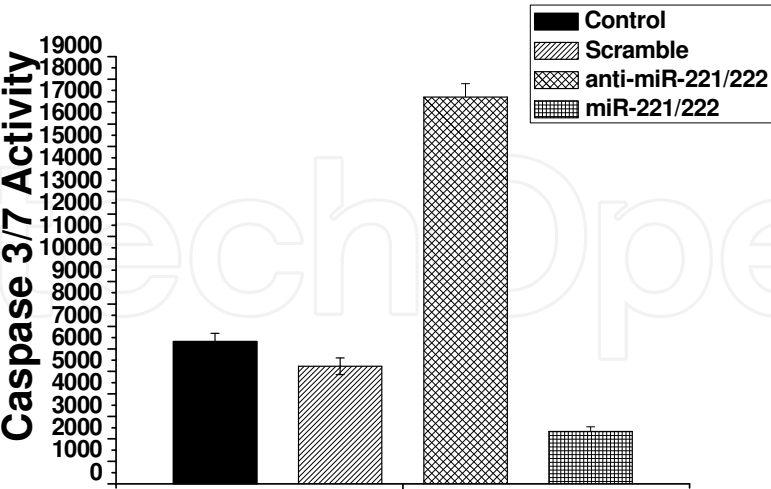


Fig. 5. Caspase 3/7 activity was considerably elevated in miR-221 and miR-222 knocked down cells compared to the other three group.



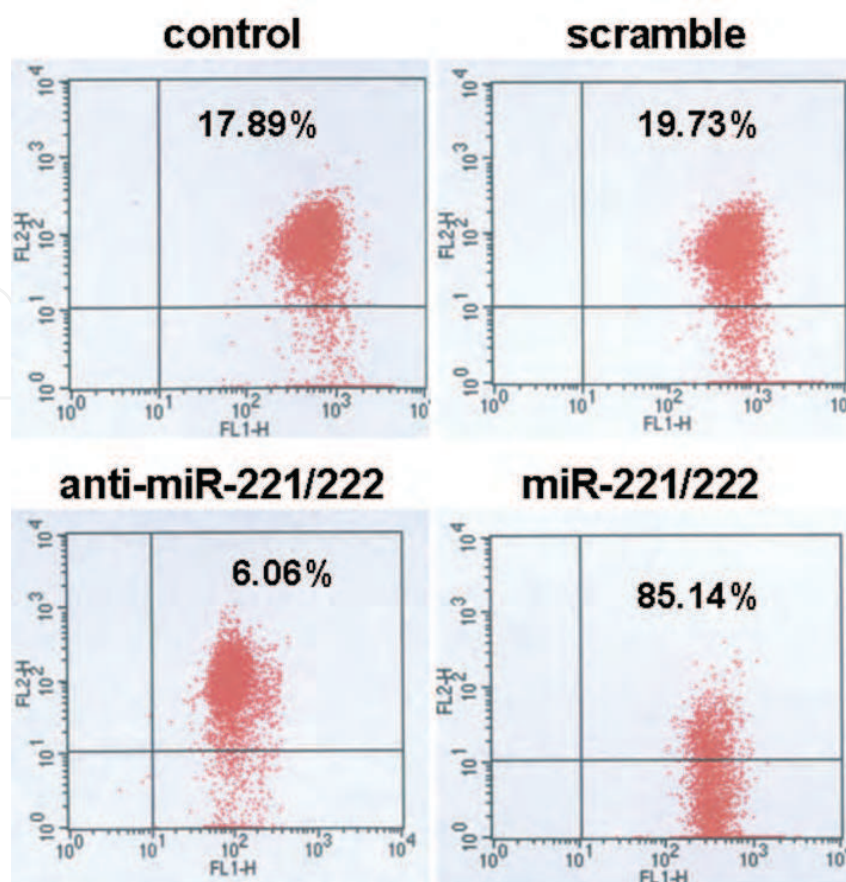


Fig. 6. U251 cells transfected with As-miR-221/222 showed a significantly greater collapse in mitochondrial membrane potential compared with the other four groups.

Furthermore, Garofalo M et al proved that miR-221/222 modulated TIMP3 expression by binding its 3'UTR (25). Whether miR-221/222 modulated TIMP3 expression to affect glioma cell invasion or not. To further explore the role of miR-221/222 in cell invasion, we performed gain-of-function and loss-of-function analyses by over-expressing or suppressing miR-221/222 with anti-miR-221/222 or pMSCV-miR-221/222, respectively. Interestingly, the transwell assay revealed that knockdown of miR-221/222 significantly decreased cell invasion potential compared with cells treated with scrambled oligonucleotide, whereas over-expression of miR-221/222 increased cell invasion (Fig. 7). Consistent with the results of the transwell assay, in the wound healing assay, repression of miR-221/222 significantly inhibited cell migration, while over-expression of miR-221/222 increased migration in U251 cells (Fig. 8). Therefore, miR-221 and miR-222 are required for glioma cell invasion.

In addition to modulating proliferation, cell cycle, apoptosis and invasion, whether miR-221 and miR-222 regulated glioma cell radiosensitivity or not. To test this hypothesis, we transfected AS-miR-221/-222 into U251 cells, then performed the colony formation assay to elevated radiosensitivity. Figure 9 shows the survival curves for U251 cells transfected with control, anti-miR-221/-222, and scrambled oligonucleotides and then exposed to IR over a dose range of 0 to 6 Gy. We found that whereas scrambled oligonucleotides did not affect radiosensitivity, anti-miR-221/-222 transfection significantly decreased IR-induced survival. Radiation survival curves were characterized based on parameters such as the dose required to reduce survival to 37% of its value (D0), the surviving fraction at 2 Gy (SF2), and the sensitization enhancement ratio at 10% (SER10). The SER10 value for the U251 cells

transfected with AS-miR-221/-222 was 1.97 (Table 1) ( $p < 0.01$  compared to the controls), indicating a radiosensitization potential for targeting miR-221/-222. Collectively, these observations provide strong evidence for the role of miR-221/-222 in regulation of radiosensitivity.

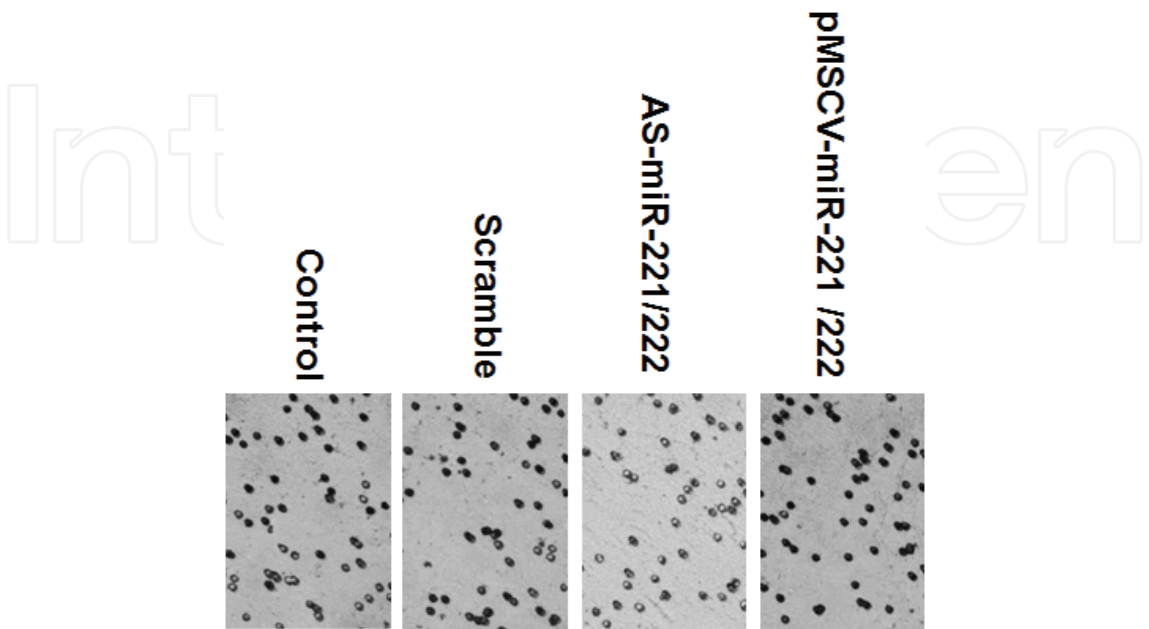


Fig. 7. The transwell assay revealed that knockdown of miR-221/222 significantly decreased cell invasion potential compared with cells treated with scrambled oligonucleotide, whereas over-expression of miR-221/222 increased cell invasion

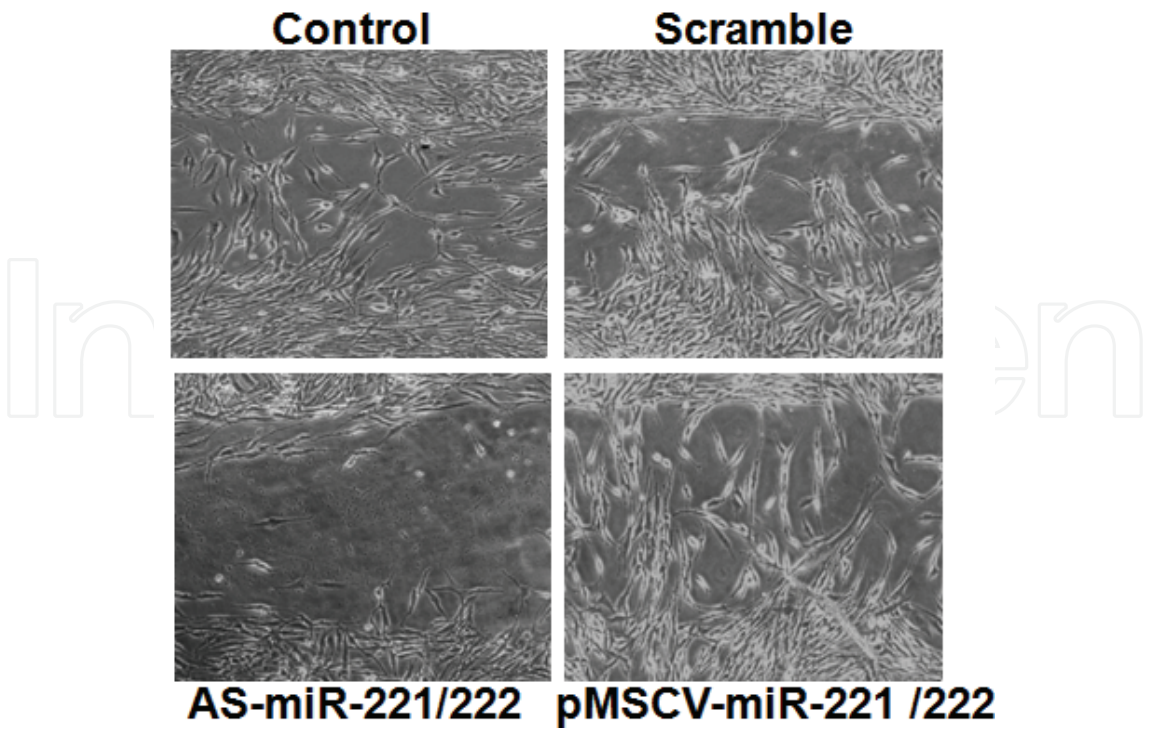


Fig. 8. The wound healing assay, repression of miR-221/222 significantly inhibited cell migration, while over-expression of miR-221/222 increased migration in U251 cells



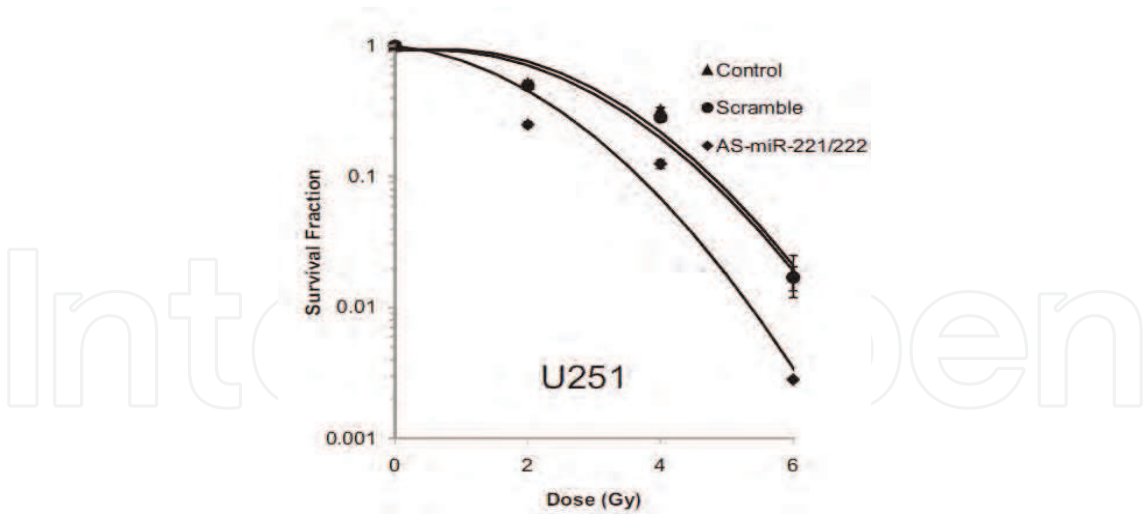


Fig. 9. miR-221/-222 knockdown leads to an increase of radiosensitivity. U251 cells stably transfected with control, AS-miR-221/-222, or scrambled ODN were exposed to 0 to 6 Gy IR and incubated for 2 weeks prior to fixation, staining, and assessment of colony formation. The clonogenic survival assays were performed in triplicate. Standard errors of the mean are shown by error bars.

Cell	Group	D <sub>0</sub>	SF2	SER10
U251	Control	2.8827	0.51	1
	Scramble	2.649	0.49	1.0731
	AS-miR-221/-222	1.3656*	0.25*	1.9705*

Abbreviations: D0 = dose to reduce survival to 37% of its value; SF2 = surviving fraction at 2 Gy; SER10 = sensitizer enhancement ratio at 10% survival.\* p<0.01.

Table 1. Radiosensitizing effects of respective cell lines

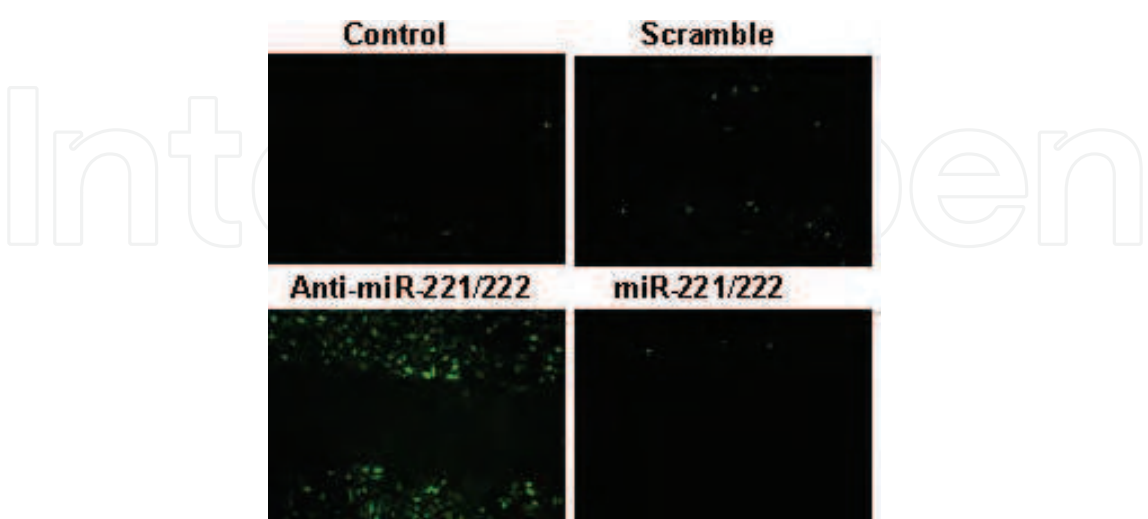


Fig. 10. SLDT assay showed that the functional GJIC in AS-miR-221/222 treated U251 cells were re-established, lucifer yellow was transferred to multiple rows of cells nearby the scrape border.

In addition, by Scrape loading and dye transfer (SLDT) assay, we found that miR-221 and miR-222 also regulated gap junction intercellular communication (GJIC) of glioma cell.

2.3 miR-221 and miR-222 cluster promote glioma growth in vivo

In vitro, miR-221/222 modulated glioma cell growth by regulating cell cycle, apoptosis, and invasion, and so on. Whether these results are right or not in vivo. Ciafre et al firstly proved that the anti-miR-221/222 antagomir treatment of established subcutaneous tumors derived from the highly aggressive PC3 cell line, naturally expressing high levels of miR-221/222, reduces tumor growth by increasing intratumoral p27 amount; this effect is long lasting, as it is detectable as long as 25 days after the treatment(26). We used 5-week-old female immune-deficient nude mice (BALB/C-nu) to perform related experiment. It were housed in microisolator individually ventilated cages with water and food. All experimental procedures were carried out according to the regulations and internal biosafety and bioethics guidelines of Tianjin Medical University and the Tianjin Municipal Science and Technology Commission. U251 glioma subcutaneous model was established as previously described (27). When the volumn of the subcutaneous tumor reached 50 mm<sup>3</sup>, the mice were randomly divided into four groups (ten mice/group). A mixture of 5 μl oligonucleotides containing Scr, AS-miR-221, AS-miR-222 or equal mount of AS-miR-221/222 cluster and 10 μl Lipofectamine was injected into the xenograft tumor model using a multi-site injection manner. Mice in U251 control group received 10 μl of PBS only. Treatment was conducted one time every three days and five times in all (Please confirm if it is correct). The tumor volume was measured with a caliper every two days, using the formula volume = length × width<sup>2</sup>/2.

Fluorescence in situ hybridization proved that As-miR-221 and As-miR-222 efficiently and specifically silenced endogenous miR-221 and miR-222, respectively, in xenograft tumor in vivo (Figure 11).Moreover, we have found that transfection of U251 cells with As-miR-221/222 mediated significant inhibition of tumor growth in a xenograft tumor mouse model (Figure 12). Tumor pathological examination of xenograft tumors demonstrated that, in control and scramble group, more neonatal micro-vessels, bigger tumor cell nucleus, deeper blue staining chromosome, more mitotic tumor cells and lesser necrosis focuses were

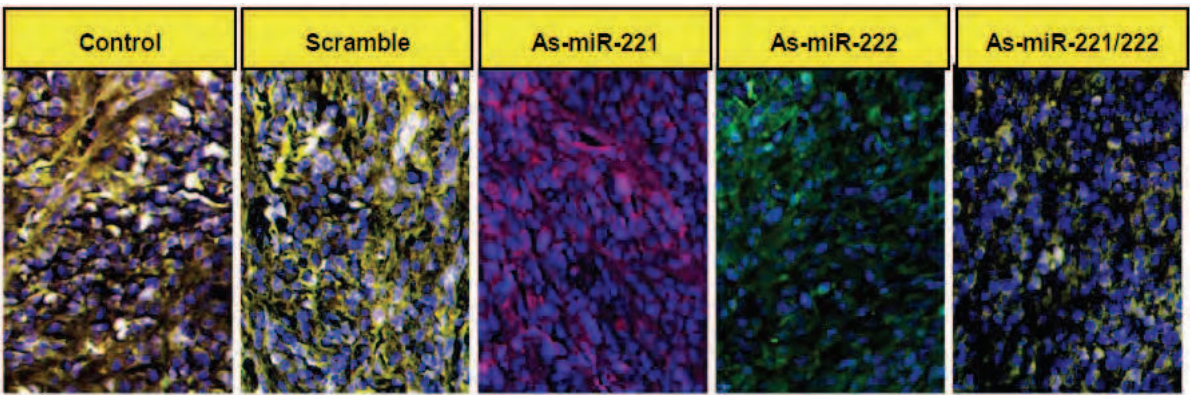


Fig. 11. Tumor pathological section of xenograft tumors show that miR-221 and miR-222 expression were knocked down by As-miR-221/222 in vivo by fluorescence in situ hybridization .

observed than in As-miR-221/222 treatment group (Figure13). All these morphological changes among the three different treated groups indicate that the xenograft tumor without effective As-miR-221/222 therapy can perform relative more malignant pathological manifestation. TUNEL assay analysis of xenograft tumor taken at 28 days after treatment revealed much more apoptosis in As-miR221/222 group when compared to tumors from scramble and control groups (Figure14). Also, the As-miR-221/222 treated tumors had a lower level of Ki-67 expression compared to the other four groups group. Tumor immunochemistry examination of xenograft tumors demonstrated that some protein expression level in As-miR-221/222 group was up-regulated significantly than the other four groups, such as p27, puma, PTEN, TIMP3, and so on. Some protein expression levels in As-miR-221/222 group was down-regulated significantly than the other four groups, such as CyclinD, MMP2, MMP9, pAkt, and so on (Figure 15).

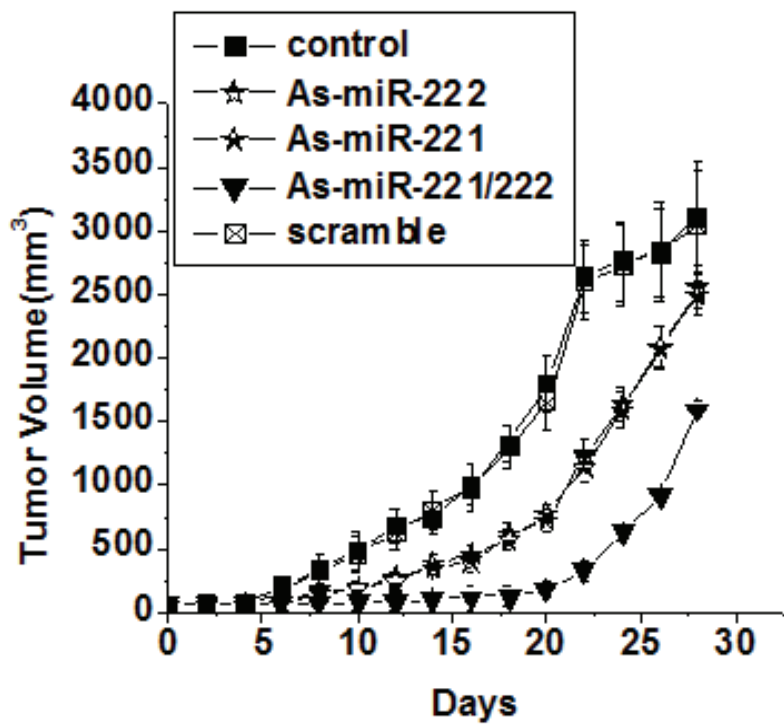


Fig. 12. Transfection of U251 cells with As-miR-221/222 mediated significant inhibition of tumor growth in a xenograft tumor mouse model.



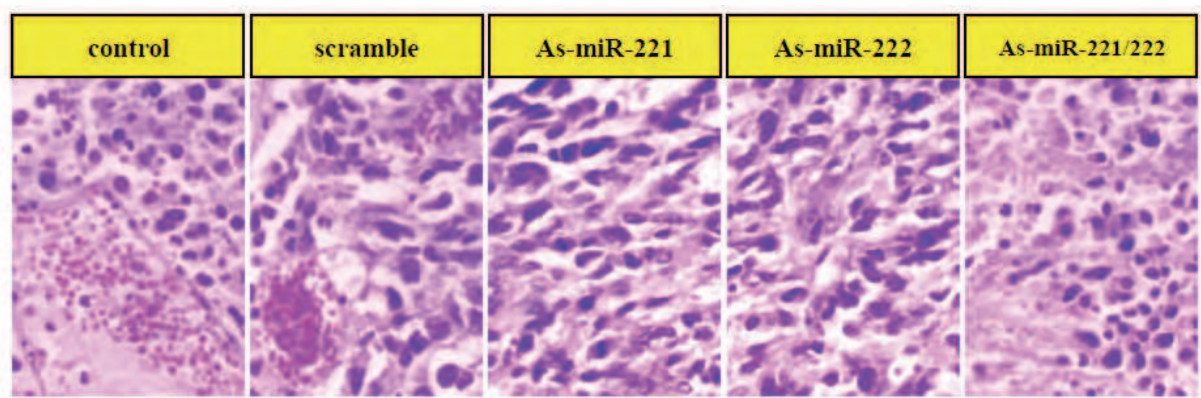


Fig. 13. Tumor pathological examination of xenograft tumors demonstrated that, in control and scramble group, more neonatal micro-vessels, bigger tumor cell nucleus, deeper blue staining chromosome, more mitotic tumor cells and lesser necrosis focuses were observed than in As-miR-221/222 treatment group.

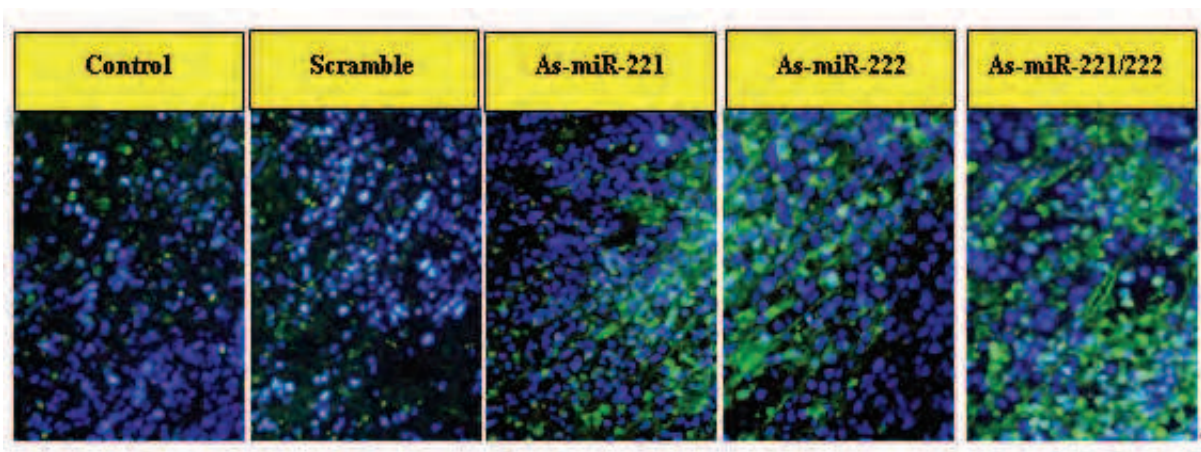
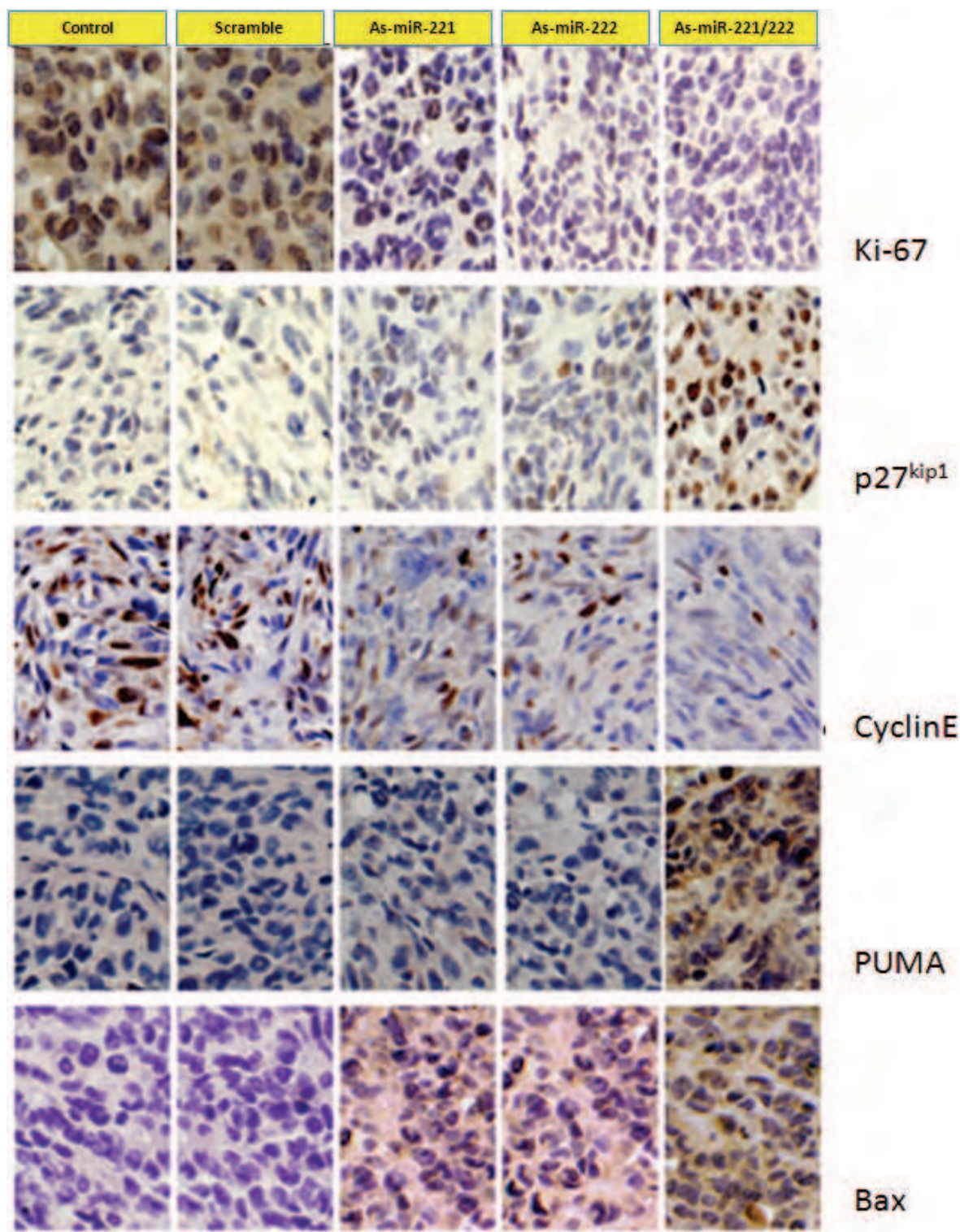
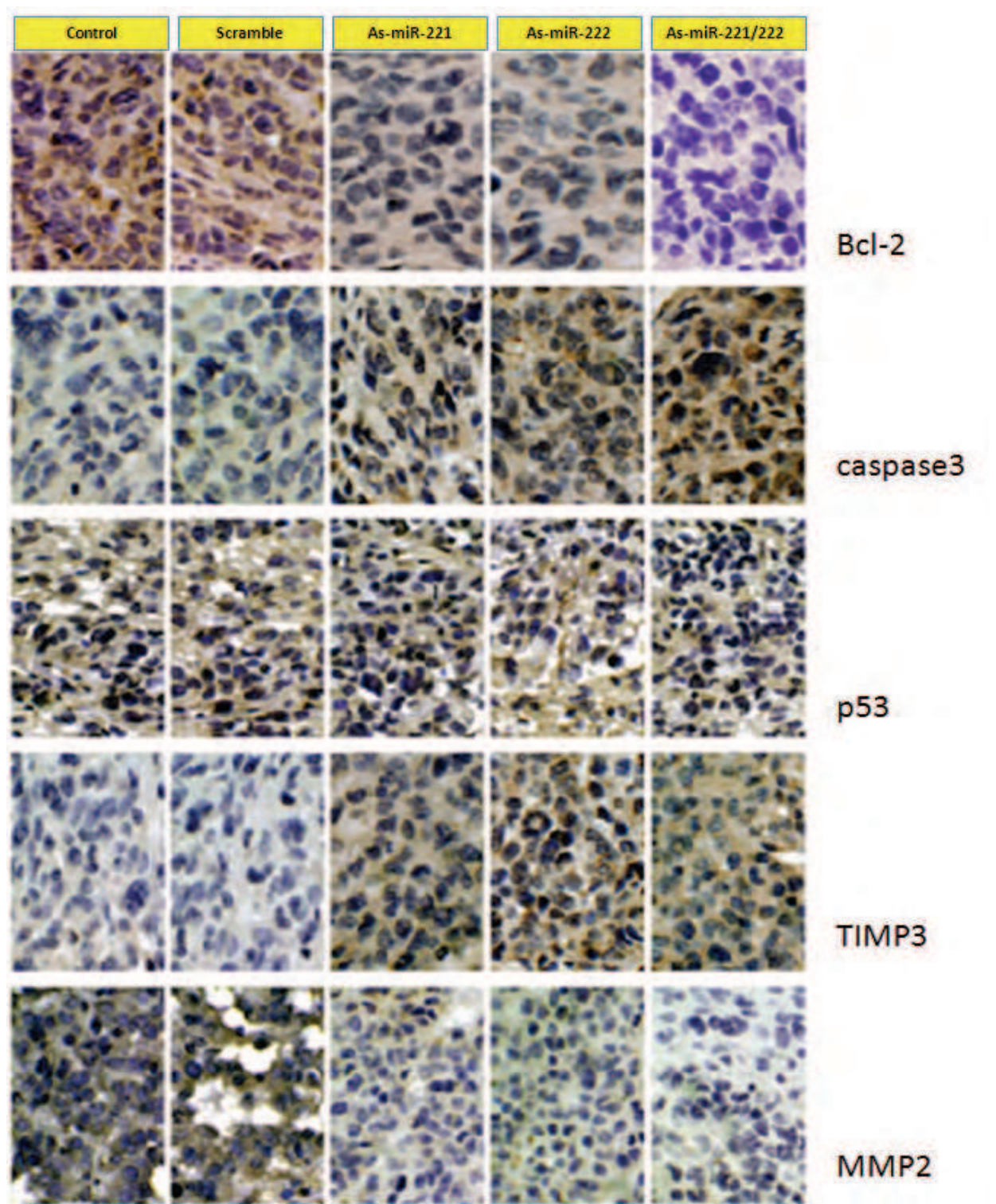


Fig. 14. TUNEL assay revealed much more apoptosis in As-miR221/222 group when compared to tumors from scramble and control groups.









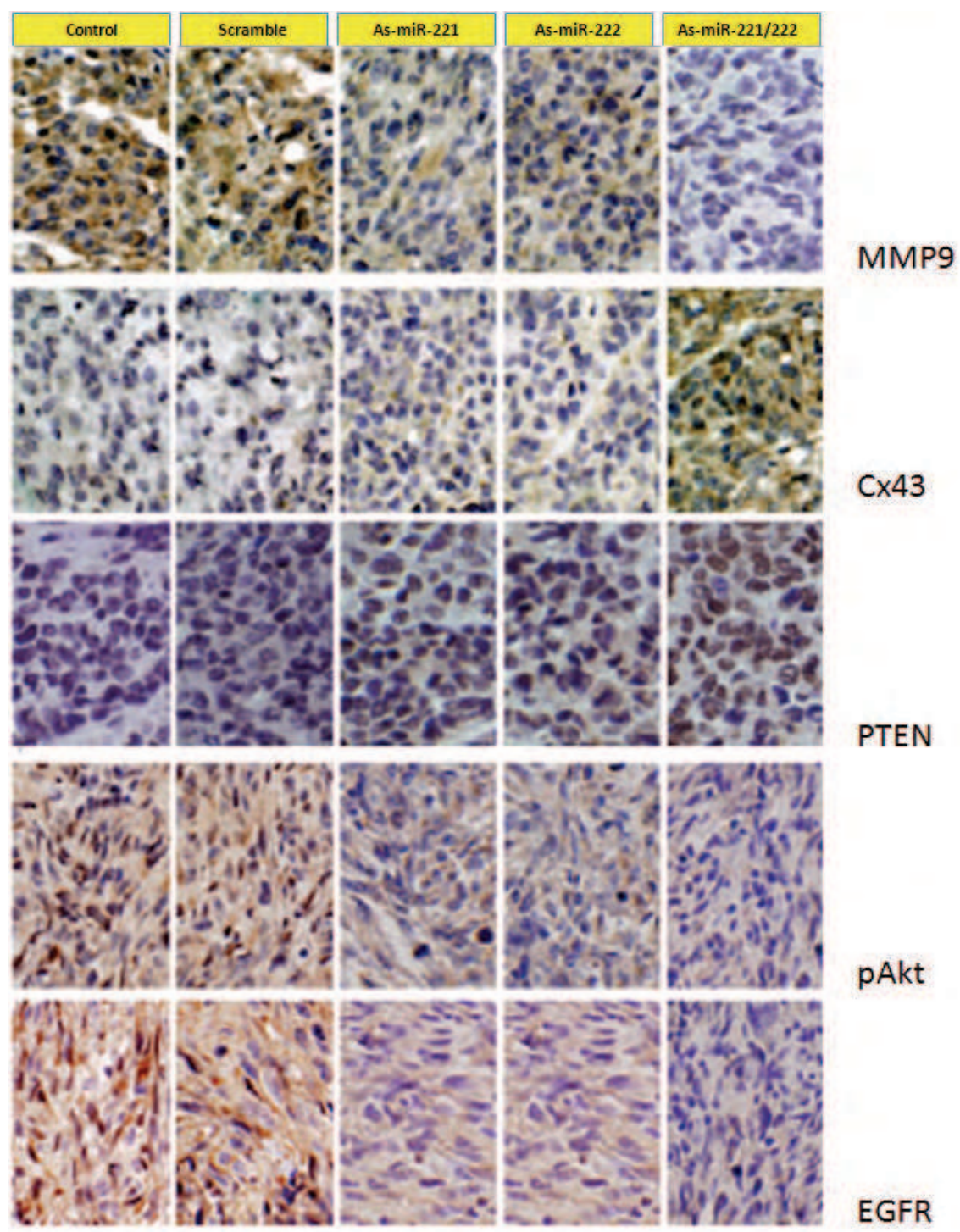


Fig. 15. Tumor immunochemistry examination of xenograft tumors demonstrated that some protein expression level in As-miR-221/222 group was up-regulated significantly than the other four groups and the other proteins expression level in As-miR-221/222 group was down-regulated.

## 2.4 The mechanism on miR-221 and miR-222 cluster modulate glioma cell malignant phenotype

MiRs repress protein expression at the posttranscriptional level through imperfect base pairing with 3' untranslated region of the target mRNA in mammalian cells, leading to its reduced translation in order to affect cell function. That is, function of miRs depend on function of its target. So, it is necessary to identify targets of miRs. Bioinformatic analysis is the most effective approach to study miRNAs. We search some target by miRs target database, such as PicTar, TargetScan, and so on. Then, by luciferase assay, these targets were identified. As for miR-221 and miR-222, we proved that p27, PUMA, PTEN, TIMP3 and Cx43 were some targets of miR-221 and miR-222 in glioma.

### 2.4.1 p27<sup>Kip1</sup>

The p27<sup>Kip1</sup> gene is a member of the cip/kip family of cyclin-dependent kinase (CDK) inhibitors that negatively control the cell cycle progression. The p27<sup>Kip1</sup> binds to CDK2 and cyclin E complexes to prevent cell cycle progression from G1 to S phase (28,29). The p27<sup>Kip1</sup> does not follow Knudson's classic 'two-hit hypothesis' of tumor suppression. The inactivation of p27<sup>Kip1</sup> is not caused by direct mutations in the gene encoding p27<sup>Kip1</sup>. The homozygous loss or silencing of the locus in human tumors is extremely rare (30). A recent study demonstrated that the miR-221/222 cluster is an endogenous regulator of p27<sup>Kip1</sup>, confirming the miRNA modulation may play a key role in the inactivation of p27<sup>Kip1</sup> (31-34). However, that the miR-221/222 cluster affects tumor cell proliferation by regulating p27<sup>Kip1</sup> in vivo has not been well documented.

### 2.4.2 PUMA

Puma was originally identified during large-scale analysis for novel p53-inducible genes and Bcl-2-interacting proteins. Puma is an extremely potent BH3-only protein, having the capacity to bind with high affinity to all known anti-apoptotic Bcl-2 proteins. In addition, Puma may also directly interact with and activate Bax/Bak, for example, through an interaction with the first  $\alpha$ -helix of Bax. In keeping with its highly pro-apoptotic nature, Puma has been shown to have an essential role in the induction of apoptosis in numerous in vitro and in vivo systems and cell types including neurons, lymphocytes, thymocytes, fibroblasts and cardiomyocytes. Puma expression is regulated by transcription, and factors regulating Puma transcription include p53, p73, E2F and FOXO3a (24,35).

### 2.4.3 PTEN

The PTEN (phosphatase and tensin homology) gene, located at 10q23.3, encodes a central domain with homology to the catalytic region of protein tyrosine phosphatases. It is important in the function of protein phosphatases and 3' -phosphoinositol phosphatase activities. It dephosphorylates phosphatidylinositol-3,4,5-triphosphate(PIP3), the second messenger produced by PI3K. In doing so, PTEN negatively regulates the activity of the serine/threonine protein kinase, AKT(36-37). PTEN is inactivated in some malignant tumors, including glioblastoma, prostate cancer, melanoma, endometrial carcinoma and lung carcinoma. To restore PTEN expression become an approach which enhances cancer radiosensitivity instead of suppressing Akt activity in PTEN-deficient tumor cells(38).

### 2.4.4 TIMP3

Malignant glioma cells express certain known proteinases such as matrix metalloproteinases (MMPs), which have been implicated as important factors in regulating cell invasiveness in

gliomas(39). MMPs are a family of at least 20 zinc-dependent endopeptidases collectively capable of degrading essentially all extracellular matrix(ECM) components, including collagens, fibronectin, laminin and basement membrane proteoglycans (40). Among the MMPs, attention has focused on gelatinases(MMP-2 and MMP-9) in human gliomas. A high level of MMP-2 expression was observed in the higher-grade gliomas, which correlated well with the malignant potential of human gliomas (41). A major mechanism for controlling MMPs activity in the pericellular space is mediated by the action of tissue inhibitors of metalloproteinases (TIMPs), which bind to active MMPs in a 1:1 molar stoichiometry (42). There are four family member, including TIMP-1, TIMP-2, TIMP-3, TIMP-4 (43). TIMP-3 is unique within the TIMP family as it remains closely associated to the extracellular matrix after being secreted by the cell (40, 44). In human gliomas, TIMP expression is reported to be down-regulated with increasing malignancy, including TIMP-3 (44).

#### **2.4.5 Cx43**

Cx genes have been demonstrated as tumor suppressor genes. Up to date, twenty members of Cx genes have been identified in mammalian tissues and they are expressed in tissue specific manner. Cx43 is the predominant form of Cxs expressed in astrocytes and also the constituent of connexons which compose the gap junction intercellular communication(GJIC) in brain (45, 46). Our previous study and other studies showed that Cx 43 expression was inversely correlated with the degree of malignancy of astrocytomas (47). In most malignant gliomas, Cx43 expression was reduced or deleted and GJIC was deficient (47).

In addition these target above, c-kit, p57, ER  $\alpha$ , Bmf are also is some target of miR-221 and miR-222, but they are not proved in glioma until now (48-51).

### **2.5 Inter-relationship between miR-221 and miR-222 expression and global mRNA expression**

Studies above have identified some targets of miR-221 and miR-222, such as p27, p57, and so on. Inter-relationship between miR-221 and miR-222 expression and global mRNA expression remains important as well. 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.

### **2.6 Transcription factor modulate miR-221 and miR-222 expression**

MiRNAs finely modulate virtually all physiological pathways in metazoans, and are deeply implicated in all main pathologies by regulating their taregets. But who regulate them expression. Ciafrè et al report that the ectopic modulation of NF-kB modifies miR-221/222 expression in prostate carcinoma and glioblastoma cell lines, where we had previously shown their oncogenic activity. We identify two separate distal regions upstream of miR-221/222 promoter which are bound by the NF-kB subunit p65 and drive efficient



transcription in luciferase reporter assays; consistently, the site-directed mutagenesis disrupting p65 binding sites or the ectopical inhibition of NF- $\kappa$ B activity significantly reduce luciferase activity. In the most distal enhancer region, we also define a binding site for c-Jun, and we show that the binding of this factor cooperates with that of p65, fully accounting for the observed upregulation of miR-221/222. Thus, our work uncovers an additional mechanism through which NF- $\kappa$ B and c-Jun, two transcription factors deeply involved in cancer onset and progression, contribute to oncogenesis, by inducing miR-221/222 transcription (52). Moreover, Garofalo et al also identified that c-jun regulated miR-221 and miR-222 expression level.

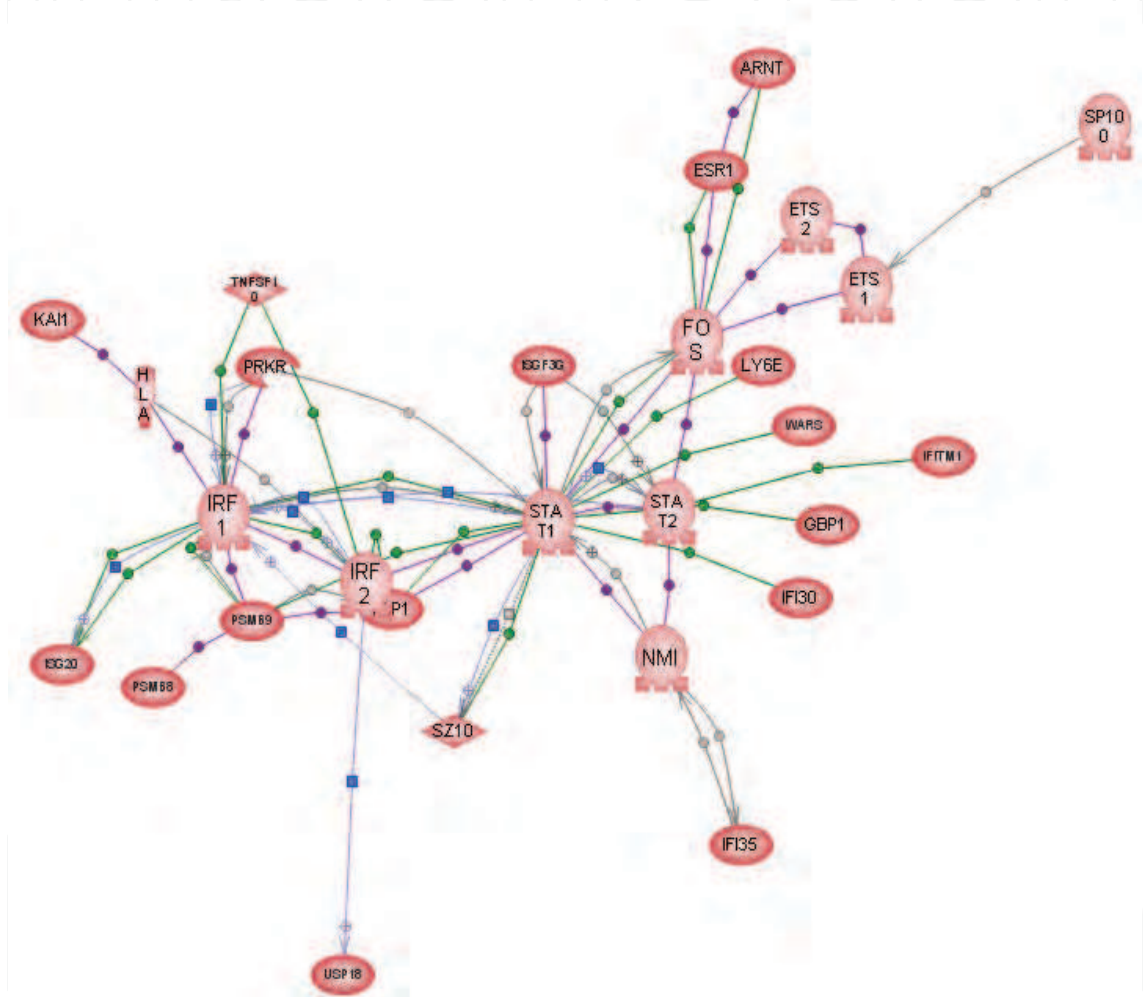


Fig. 16. Constructed network of miR-221/222-TFs-STAT1/2-gene expression. Genes involved in TF-gene expression in glioma U251 cells after repression of the miR-221/222 cluster, determining the cell death or survival through the INF- $\alpha$  pathway. Genes associated with eight TFs (FOS, ESR1,ETS2, IRF2, 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 INF- $\alpha$  pathway in glioma U251 cells. The INF- $\alpha$  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.



### 3. Conclusion

In summary, We first identified that miR-221 and miR-222 play an role in malignant phenotype of glioma by increasing or knocking down miR-221 and miR-222. It was found that miR-221/222 increased cell proliferation, increased S phase proportionality, inhibited cell apoptosis, increased cell invasion and migration, enhanced radioresistance and reduced gap junction intercellular communication (GJIC) in glioma U251 cell. Furthermore, knockedown miR-221/222 considerably decreases tumor growth in U251 cell xenograft model. Then, we identified that p27kip1, PUMA, PTEN, TIMP3 and Cx43 are some targets of miR-221 and miR-222. We also found that knocked down miR-221/222 changed some proteins expression. Taken together, our studies provide evidence that miR-221/222 regulate its target genes and its related transduction signal way to promote the growth of malignant glioma cells. So, we thought that miR-221 and miR-222 may serve as a novel therapeutic target for malignant gliomas.

### 4. Acknowledgment

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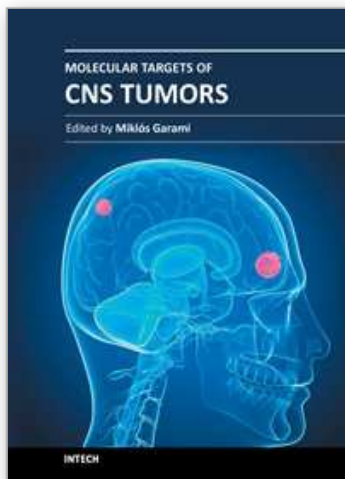
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Phone: +86-21-62489820  
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