Differentiation Induction Effect of Mir-429 Over-Expression in U251 Glioma Cell Line

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ABSTRACT

The purpose of this research was to investigate the effect of mir-429 ectopic over-expression on U251 glioblastoma cell line. It has been shown that mir-429 could target BMK1 and reduce glioma cells invasion. We cultured U251-mir-429 cells and observed changes in their morphology for 14 days. They seemed to become wider with expanded cytoplasm and go on differentiation. Neuronal (NEFM, NSE and neurogenin) and oligodendrocyte (MBP, OLIG2 and NKX2-2) gene markers expression level was examined with real-time PCR. NSE, neurogenin, MBP, OLIG2 and NKX2-2 were significantly over-expressed, giving an evidence of cellular differentiation in glioma cells.

Keywords: Glioma; Mir-429; BMK1; Invasion

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INTRODUCTION

Glioblastoma multiform (GBM) is a common and lethal malignancy of central nervous system, which leads to a survival rate of less than 14 months, despite remarkable progresses in cancer treatment [1]. The major reason for treatment failure is the infiltrative nature of glioblastoma tumors that makes problems in whole resection and causes tumor recurrence and resistance to chemo- and radiotherapy [2]. It seems that there is a critical and urgent need for novel strategies of fighting cancer like molecularly targeted therapies to improve GBM patients' treatment. Gene therapy and use of miRNAs are of these new approaches that have attracted scientists' attention [3].

MiRNAs are, endogenous non-coding 21-24 nucleotides RNAs, which play important roles in post-transcriptional gene expression regulation [4]. They bind to complementary regions of target mRNAs' 3'-untranslated region (3'-UTR) and induce their degradation or inhibit their translation initiation. MiRNAs are involved in major functions of cells like proliferation, differentiation and apoptosis [5]. They can also cause in (as oncogenes) or suppress (as tumor suppressors) tumor-genesis and development of different kinds of cancer [6]. For instance, researchers indicate that mir-125b-1 is correlated with lung, leukemia, breast, ovarian, and cervical cancers. Moreover, mir-15a and mir-16-1 are down regulated in B-cell chronic lymphocytic leukemia [7]. Zhou et al. demonstrated that down-regulation of miR-21 in glioblastoma cells prompts activation of caspases and increases apoptosis [8]. As well as Gabriely reported that microRNA-21 can stimulate glioma invasion by targeting matrix metalloproteinase regulators [9].

Chen et al. showed that Mir-429 is less expressed in glioma cells in comparison with normal tissues and its over-expression can inhibit glioma cells' migration and invasion by interfering with mesenchymal transition [10]. In this study

we investigated the fate of glioma cells transducted with mir-429 recombinant viruses and evaluated changes in neural lineage gene markers expression.

MATERIALS AND METHODS

Plasmid preparation and cloning

Mir-429 stem loop locus and its ~200 nucleotides up/down stream sequences (Figure 1) were amplified using PCR on a normal human genomic DNA and cloned in pCDH-CMV-MCS-EF1-cGFP-T2A-Puro vector (System Biosciences, USA). Mir-control vector was used as control. PAX and PMD virus packaging helper vectors were purchased from Addgene (USA).

Cell culture, virus packaging and transduction

Hek T293N and U251 cell lines were obtained from cell bank of Pasteur Institute of Iran and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 100 U/ml penicillin-streptomycin (Gibco, USA). Cells were maintained in a humidified incubator at 37C with 5% CO2.

Mir-429 recombinant and mir-control vectors were co-transfected separately with PAX and PMD to hek T293 cells via CaPhO4 protocol [11] and the culture media containing recombinant viruses was collected for 3 days then filtered with sterile 0.2 um filters and stocked in -70C freezer. The virus containing media was used in a ratio of 2:1 with fresh media to transduce U251 GBM cell line. Rate of transduction was investigated via fluorescent microscopy (Figure 2).

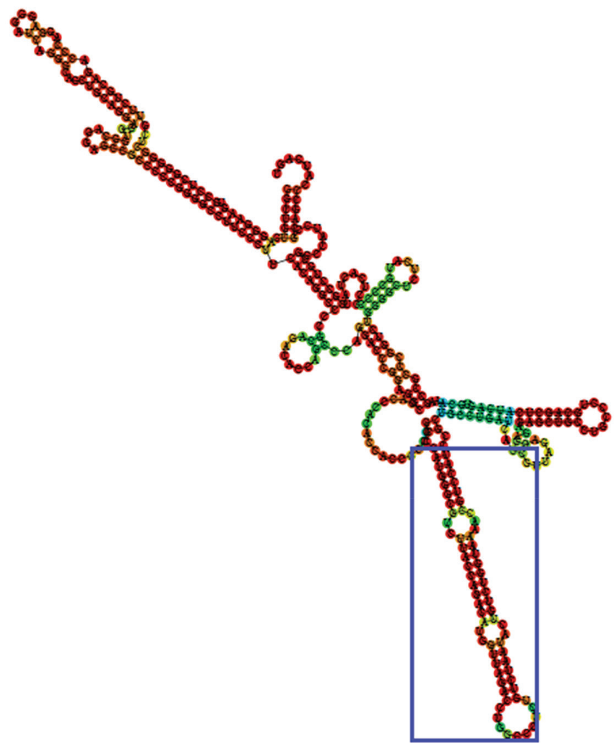
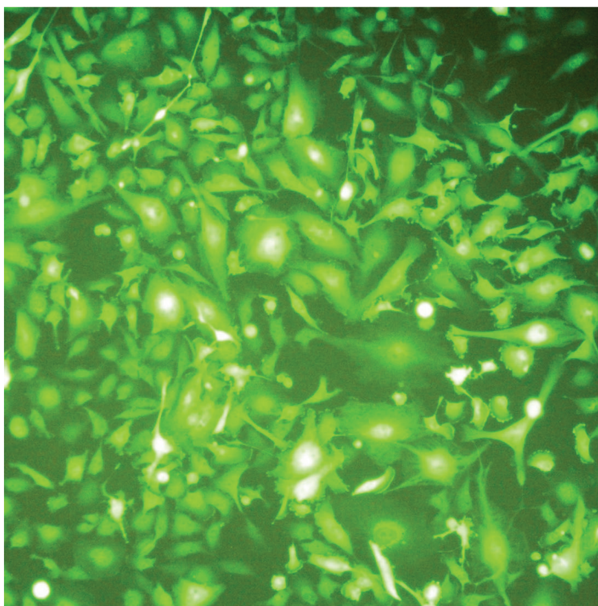
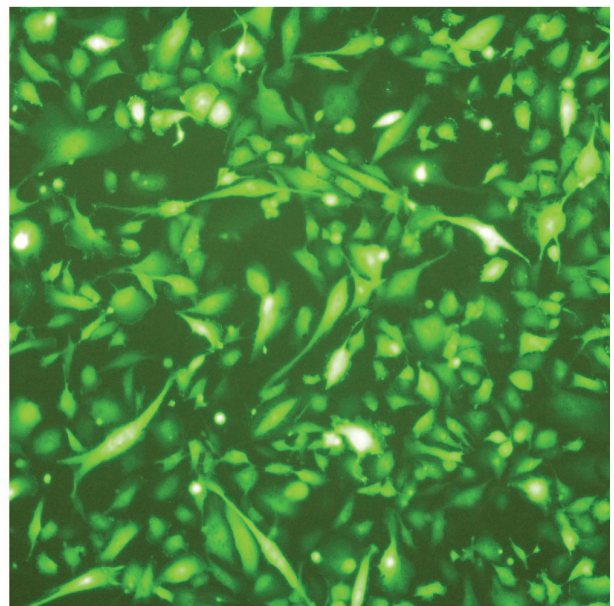


Figure 1. Mir-429 construct folding (Mir-429 main stem-loop is specified with a square).

RNA extraction and Real-time PCR

14 days after transduction, total RNA/miRNA was





a b

Figure 2. U251 cells transducted with a) mir-429 and b) mir-control recombinant viruses.

extracted from cells using RNAX-Plus reagent (Cinnagen, Iran). Mir-429-specific cDNA and snord47 (internal control)-specific cDNA were synthesized with reverse transcriptase from Fermentas (USA) using specific stem-loop primers (12). Real-time PCR was employed to detect mir-429 expression level. Its relative expression related to snord47 was calculated using ΔΔCt methods. Total cDNA was also made and expression level of NEFM, NSE and neurogenin as neuronal biomarkers and MBP, OLIG2 and NKX2-2 as oligodendrocyte biomarkers was detected via real-time PCR. B2M was used as internal control for normalization and quantification of expression.

RESULTS

Transduction of U251 cell line with mir-429 recombinant lentiviral vector induces mir-429 over-expression versus mir-control vector:

Relative expression of mir-429 was investigated in transducted U251 cells. Mir-429 was about 258 fold more expressed in U251-mir-429 cells than U251-mir-control cells (Figure 3).

Mir-429 over-expression in U251 cell line causes morphological changes:

Several cells with morphological changes were found in U251-mir-429 cultured cells. It seems that after 14 days some cells became wider with expanded cytoplasm (Figure 4).

Mir-429 over-expression in U251 cell line induces change in differentiation gene markers expression level:

After 14 days RNA extraction was done and relative expression of neuronal (NEFM, NSE and neurogenin) and oligodendrocyte (MBP, OLIG2 and NKX2-2) marker genes were investigated. Only NEFM was down-regulated (0.57 fold). NSE (1.91 fold), neurogenin (1.49 fold), MBP (2.76 fold), OLIG2 (2.13 fold) and NKX2-2 (10.02) were all up-regulated (Figure 5).

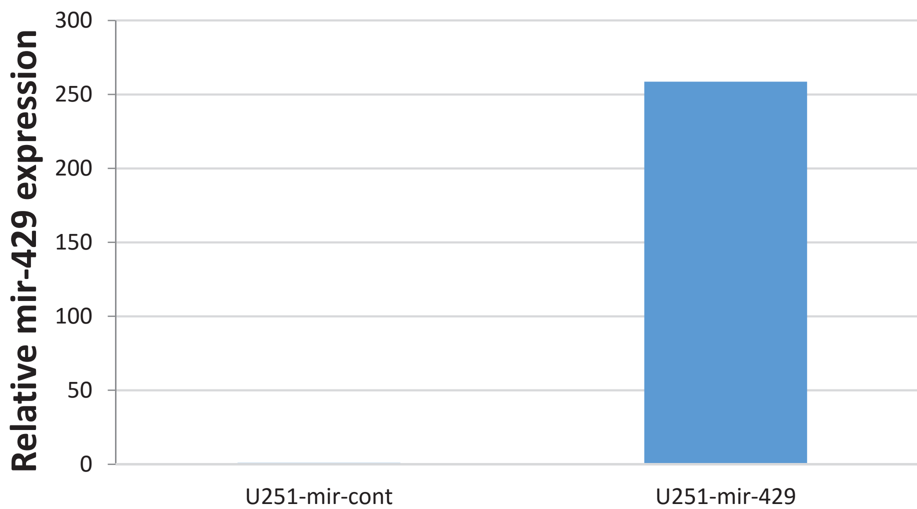


Figure 3. Relative expression of mir-429.

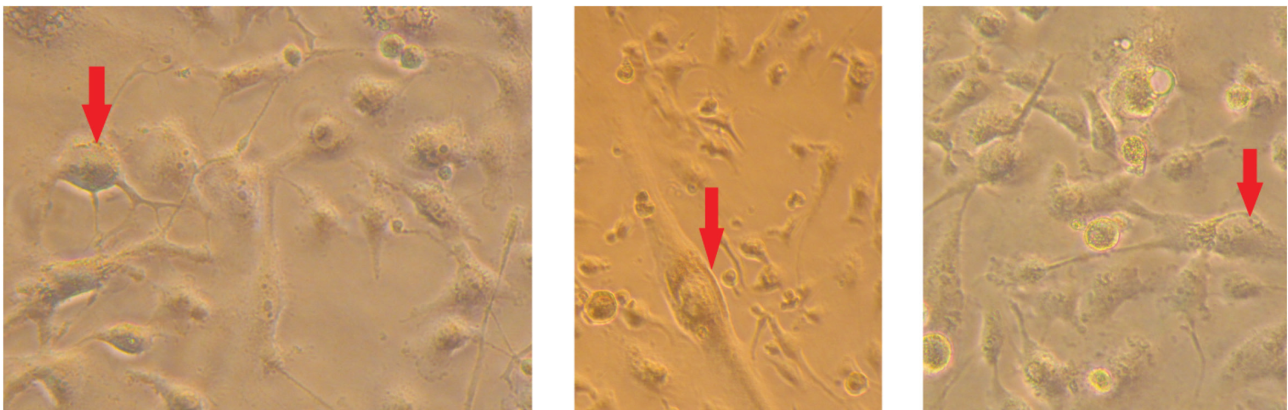


Figure 4. U251-mir-429 cells with expanded cytoplasm (specified with arrow).

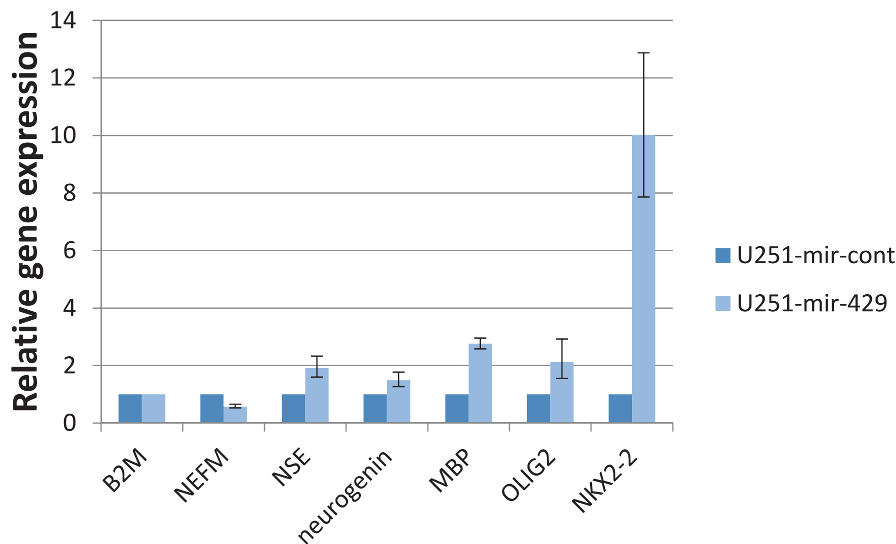


Figure 5. Relative expression of neuronal (NEFM, NSE and neurogenin) and oligodendrocyte (MBP, OLIG2 and NKX2-2) gene markers in U251-mir-429 versus U251-mir-control cells.

DISCUSSION

MiRNAs' regulatory role in major biological processes such as cell cycle, proliferation, differentiation and death has been investigated by different researcher groups [4]. In recent decays, miRNAs have also attracted great attention in different studies on cancer treatment, because of their proven role as oncogenes or tumor-suppressors [13]. Tumor-genesis function of mir-429 in lung cancer [14], colon cancer [15] and osteosarcoma [16] has been studied. In glioblastoma it has been showed that mir-429 expression becomes less in both glioma tissues and cell lines such as U251in comparison with normal brain tissues [17]. Via mir-429 ectopic over-expression, Chen et al. could significantly reduce glioma invasion. They demonstrated that mir-429 affects cancer cells invasion via targeting mitogen-activated protein kinase (BMK1). BMK1 induces mesenchymal transition (MT) followed by cancer cells invasion and is over-expressed in glioma [17].

In Our bioinformatics study we found that mir-429 is a potential regulator of EGFR pathway, which is a common active genetic pathway in most of gliomas [18]. Thus we predicted that ectopic over-expression of mir-429 may affect glioma cells proliferation and cancer promotion. We cultured U251 glioma cell line, transducted with mir-429 and mir-control recombinant viruses for 14 days and observed changes in cellular morphology and division. It seemed that in U251-mir-429 culture, cells were less proliferative and some cells showed a kind of expansion in their cytoplasm and look like they are differentiated. We examined the relative expression level of different marker genes of neural lineage differentiation in U251-mir-429 cells and found that neuronal markers i.e. NSE and neurogenin are over-expressed, unless NEFM is down-regulated. Also, oligodendrocyte markers i.e. MBP, OLIG2 and NKX2-2 seemed to be significantly over-expressed in U251-mir-429 cells. These evidences suggest that U251 cells have slowed down their proliferation and started to differentiate in consequence of mir-429 ectopic over-expression.

Further experiments are necessary to approve neuronal or oligodendrocyte differentiation like real-time PCR, immunocytochemistry and western blotting of other specific differentiation biomarkers [19]. If demonstrated, mir-429 could be a potential therapeutic gene-therapy element for glioma patients.

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