Overexpression of *hsa-miR-939* follows by NGFR down-regulation and apoptosis reduction

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Neurotrophin receptors play a crucial role in neuronal survival, differentiation and regeneration. Nerve growth factor receptor (NGFR) or $P75^{NTR}$ is a neurotrophin receptor that is involved in many pathological conditions including cancers. Genetic factors that are involved in regulation of neurotrophin receptors are under intense investigation. MiRNAs are novel regulators of signalling pathways that are candidates for regulation of neurotrophin receptors. Computational programs predicted that *NGFR* gene is a *bona fide* target for *hsa-miR-939*. RT-qPCR, Western analysis and dual luciferase assay evidences indicated that *NGFR* transcript is targeted by *hsa-miR-939*. Also, *hsa-miR-939* overexpression brought about down-regulation of *NGFR* expression in U87 cell line, followed by cell death rate reduction, detected by flow cytometry. Taken together, here for the first time, *hsa-miR-939* is introduced as a novel key regulator of *NGFR* expression and its involvement in cell death/survival processes is suggested.

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1. Introduction

NGFR (NC_000017.10 *NGFR*) has been defined as a multifunctional receptor that plays crucial roles in neuronal and non-neural cells (Gentry *et al.* 2004). Neurotrophins growth factors are regulators of neurons survival, development and function (Reichardt 2006). While each neurotrophin activates NGFR receptor, they may activate one or more of three members of receptor tyrosine kinases (TrkA, TrkB and TrkC) (Rodriguez-Tebar *et al.* 1990; Frade and Barde 1998; Reichardt 2006). NGFR may act independently or in cooperation with Trk receptors and activate signalling cascades, which lead to the induction of apoptosis or promotion of survival (Roux and Barker 2002). Overexpression of *NGFR* gene has been observed in several diseases including cancers and Alzheimer's (Cantarella *et al.* 2002; Salis *et al.* 2004; Rocha *et al.* 2006; Cheng *et al.* 2012). This gene can induce apoptosis and acts as a tumour suppressor in some cancers (Hefti and Mash 1989) (Krygier and Djakiew 2001), but may induce invasion and metastasis in some others (Johnston *et al.* 2007). The several biological actions of the neurotrophins via NGFR may reveal selectivity of ligand–receptor interactions and intracellular adaptor protein recruitment (Blöchl and Blöchl 2007; Gao *et al.* 2007). In addition to some known transcription factors that modulate *NGFR* expression, a group of noncoding microRNA(miRNA) genes are related to post-transcriptional regulation of this gene (Ramos *et al.* 2007).

MiRNAs are ~22-nucleotide noncoding RNAs that are derived from distinctive hairpin precursors in plants and animals and are known as regulators of gene expression (Friedman *et al.* 2009). Initially, miRNA genes are

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transcribed into long primary transcript (pri-miRNA) and are processed into ~70-nucleotide precursor miRNAs (pre-miRNAs). The pre-miRNAs are then transported to the cytoplasm and processed into ~22 nt miRNAs, which exert their regulatory functions via binding to their target genes (Ambros 2004, Khvorova *et al.* 2003). MiRNAs down-regulate their target genes expression by inhibition of translation or mRNA degradation. The interaction of miRNA and target mRNA mostly requires base-pairing between the seed sequence (positions 2–8) of the miRNA generally at the 5′-end and a sequence most frequently found in the 3′-untranslated region (UTR) of target mRNA called miRNA recognition element (MRE). Since binding of miRNA to its MRE is incomplete, a single miRNA usually has multiple targets and could regulate a large fraction of protein-coding genes (Du and Zamore 2005; Lewis *et al.* 2005).

Since disruption of *NGFR* regulatory mechanisms may lead to developmental disorders and diseases like cancers, it is crucial to identify the mechanisms that regulate this gene. To date, only an inverse expression correlation and direct interaction between *hsa-miR-592* and *NGFR* expression has been reported (Irmady *et al.* 2014). However, *NGFR* gene has a long 3′-UTRs and is prone to more unknown miRNA-mediated regulation.

Here, *hsa-miR-939* is predicted to target *NGFR* transcript and our experimental evidences support their direct interaction. *Hsa-miR-939* overexpression led to early apoptotic rate reduction in U87 cell lines, thus suggesting an approach to limit the *NGFR* pro-apoptotic actions.

2. Materials and methods

2.1 *Bioinformatics tools and studies*

To search for the miRNAs that are potentially capable of targeting human *NGFR*-3′-UTR sequence, DIANAmt, miRWALK and Targetscan programs were used. MiRWalk algorithm (*http://mirwalk.uni-hd.de/*) (Dweep *et al.* 2011) was used for identification of the longest consecutive complementary between miRNAs and *NGFR*-3′-UTR sequences. DIANA-microT (*www.microrna.gr/microT*) (Maragkakis *et al.* 2009) provides extensive information for predicting miRNA and target sequence interactions. Along with Blast search (Kent 2002) for miRNA and its MRE against human genome, mirbase database (*http://www.mirbase.org/index.shtml*) was also used to determine the degree of miRNA and its precursor sequence conservation. Gene Ontology Functional Analysis Tool (DAVID) (Dennis *et al.* 2003) was used for ontology analysis of *hsa-miR-939* potential target genes.

2.2 *Cell culture*

U87MG, SKNMC, and HeLa cell lines were cultured in RPMI-1640 media (Invitrogen), supplemented with 10% foetal bovine serum (FBS) (Invitrogen), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma), followed by incubation in 37°C with 5% CO_2. HUH7, HEK293t, HepG2, SW480, Fibroblast 1321N1, A172, and Daoy cells were cultured in DMEM-HG containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. All cell lines were obtained from Pasteur Institute, Iran.

2.3 *Plasmid constructs*

Human genomic DNA was extracted from a healthy blood donor and used as template for PCR amplification of ~200 bp DNA fragment covering *pre-hsa-miR-939* sequence using specific primers as follows: forward 5′CATGCGGC CGCGCGGTGTACAGCGTGGACTTCA3′ and reverse 5′ CTTACGCGTTGGAGGAAGCACTCACCTTC3′. The amplified fragment was cloned into a lentiviral vector (pLEX-JRed-TurboGFP) at Mlu1 and Not1 sites for expression of *hsa-miR-939*.

The luciferase-UTR reporter plasmids *(Renilla::NGFR, 3′ UTR*) were constructed by amplification of about 2000 bp DNA fragments covering *NGFR* 3′-UTR by using specific primers as follows: forward 5′CCCCTCGAGCCACA TTCCGACAACCGATGC3′ and reverse 5′GCCCAAGA AATGATTACACAGGAGG3′. The amplified fragment was then cloned into a psiCHECK-2 vector at Xho1 and Not1 cut sites. Accuracy of all recombinant vectors were confirmed by sequencing. Ampicillin antibiotic was used for selection of bacteria transformed by TA and psiCHECK-2 vectors. Also, kanamycin was used for selection of bacteria transformed by pEGFP-CI and pLEX-JRed-TurboGFP recombinant vectors.

2.4 *Transfection*

For miRNA overexpression analysis, U87MG and HUH7 cells were transfected using lipofectamin 2000 reagent (Invitrogen) in 24-well plates containing about 2×10^5 cells per well with 1µg DNA, following the manufacturer's protocol. GFP expression was visualized by a fluorescence microscope, 24 h post-transfection (Nikon eclipses Te2000-s).

Also, for co-transfection of *hsa-miR-939* and *NGFR*-3′-UTR in HEK293t, 1 µg DNA containing both constructs was engulfed in lipofectamin 2000 reagent in 48-well plates containing about 2×10^5 cells per well, and 48 h after transfection, dual luciferase assay was performed.

2.5 *Luciferase assay*

In order to analyse direct interaction of *hsa-miR-939* with the *NGFR*-3′-UTR and *SMAD4* (as a negative control), HEK293t cells were transfected with appropriate plasmids. The first plasmid was a psiCHECK-2 reporter construct that contained *Renilla luciferase* gene upstream of *NGFR*-3′-UTR and an independent *firefly luciferase* gene that served as an internal control. The second plasmid was pLEX-JRed-TurboGFP ensuring *pre-hsa-miR-939* overexpression. Both vectors were co-transfected into HEK293t cells that were used for Luciferase assay using DualGlo luciferase assay (Promega) 48 h post-transfection. Ratios of Renilla/firefly luminescence were calculated and compared to a control transfection sample containing *NGFR*-3′-UTR cassette alone, without overexpression of *hsa-miR-939*.

2.6 *Real-time PCR*

For each cell line, total RNA was extracted using Trizol reagent according to the manufacturer's protocol (Invitrogen). RNA quantity was measured by the Nano drop (Thermo) and residual DNA was removed using RNAase-free DNAaseI (Takara) at 37°C for 30 min followed by heating and EDTA inactivation. For detection of mature miRNA expression, cDNA synthesis and real time PCR reactions were performed using (Pars genome kit) according to the manufacturer's protocol. U6 small nuclear RNA Level was used as internal control. Reference sample between different cell lines was U87MG cell line. For detection of target gene expression level, 1μg of isolated RNA was used for cDNA synthesis by PrimeScript II reverse transcriptase (Takara) and oligo-dT primers. RT-qPCR was performed using standard protocols on an ABI PRISM 7500 instrument (Applied Biosystems). To confirm the authenticity and size of the amplified *hsa-miR-939*, RT-qPCR products were cloned in TA vector and sequenced. The fold change between the vector control and pre-microRNA expression vector was calculated using the $2^{-\Delta\Delta ct}$ method. Primers used in the present study are listed as follows:

NGFR-f: 5′CCGAGGCACCACCGACAACC3′,
NGFR-r: 5′GGCGTCTGGTTCACTGGCC3′,
GAPDH-f: 5′GCCACATCGCTCAGACAC3′,
GAPDH-r: 5′GGCAACAATATCCACTTTACCAG3′.

2.7 *Cell cycle analysis*

In brief, U87MG, HUH7 and HEK293 cells were first transfected with either control or *hsa-miR-939* expression vectors. Cells were harvested after 36 h and stained with propidium iodide (PI) and Annexin V-PI staining (Roche) according to the manufacturer's protocol. All samples were analysed with a FACS Calibur flow cytometer with Cell Quest software (BD Biosciences). All assays were carried out in duplicate.

2.8 *MTT assay analysis*

For cell proliferation analysis, HUH7 and U87 cells were seeded in 96-well plate and approximately 8000 cells/well were then transfected with either control or *hsa-miR-939* expression vectors. Culture media was harvested 36 h after transfection and then incubated with 20 μL of 5 mg/mL MTT solution (Sigma) in parallel with 180 μL of the fresh media at 37°C for 4 h, and the MTT formazon that resulted from the test was solubilized in 100 μL of DMSO. The absorbance at 490 nm was measured in BioTek instrument.

2.9 *Western blotting*

For Western blotting analysis, proteins were extracted and denatured. The solubilized proteins (25 μg/μL) were then subjected to electrophoresis on 12% polyacryl amide SDS gels, and transferred to PVDF membranes. The PVDF membrane was subsequently blocked by 5% skim milk in tris-buffered saline containing 0.1% Tween for 2 h at room temperature. They were then incubated overnight at 4°C with primary antibodies, NGFR (1:500, Abcam), B-actin (1:1000; Santa Cruz Biotechnology). Sheep anti-mouse IgG-HRP secondary antibodies (1:2000; Santa Cruz Biotechnology) were used on the second day for 1 h at RT. Actin was used as loading control. The Western blots were visualized with an ECL reaction kit (Beyotime, China), recorded on a Canon EOS 60D and quantitated using TotalLab Quant (Gramantieri *et al.* 2007).

2.10 *Statistical analyses*

Real time experiments were run in duplicates and were analysed using CT method by Data Assist software V3.0 and normalized by endogenous control U6 small nuclear RNA gene (Mestdagh *et al.* 2009). Other statistical analysis was performed using GraphPad Prism 5.04 (GraphPad, San Diego, CA). For apoptosis studies, data showing percent of the early apoptotic cell population within the negative group and the test group, compared with each other by Repeated Measures Student's *t*-test. Data were considered statistically significant, when P-values were ≤0. 05.

3. Results

3.1 *Bioinformatics prediction of miRNAs targeting NGFR transcript*

In order to predict miRNAs that may target human *NGFR*-3′UTR sequence, DIANAmt, miRWALK and Targetscan bioinformatics programs were applied (Watanabe *et al.* 2007). In total, 512 miRNAs were retrieved that may target *NGFR*-3′-UTR. *Hsa-miR-939* was predicted by all of these programs and had five predicted MREs in the *NGFR*-3′-UTR sequence. The fifth MRE was strongly conserved, while the second and the third MREs were less conserved, and the first and fourth MREs were not conserved across vertebrate species. *Hsa-miR-939* was predicted to target other neurotrophin receptors including *TrkB* and *TrkC*, 3′-UTR sequences. Also, it was predicted to target the genes involved in melanogenesis and diseases such as glioma, prostate cancer, and melanoma.

3.2 *Hsa-miR-939 expression profile in different cell lines*

The *hsa-miR-939* expression level in different cell lines was quantified through RT-qPCR and was normalized by U6 small nuclear RNA as internal control. Interestingly, RT-qPCR analysis revealed a high level of *hsa-miR-939* expression in HUH7 and HEK293t (non-brain cell lines) compared to the other tested cell lines (figure 1).

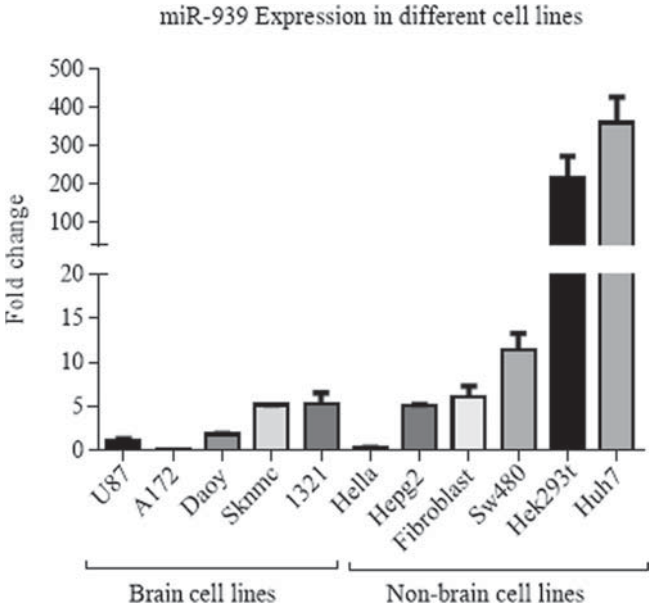

Figure 1. *Hsa-miR-939* relative expression in different cell lines. RT-qPCR result shows that *hsa-miR-939* transcript level is higher in HUH7 and HEK293t cells compared to the rest of non-brain cell lines (HeLa, HepG2, Fibroblast and SW480). Also, its level is higher in the 1321 compared to the rest of brain cell lines (U87, A172, Doay and Sknmc). Two technical replicates; error bars represent SEM; relative to U87 cell line. U48 has been used as internal control.

3.3 *Evidences supporting direct interaction of hsa-miR-939 with NGFR transcript*

NGFR expression was detectable in HUH7 cells which were transfected with more efficiency, compared to the neural origin cell lines. Therefore, pGFP::miR939 expression vector was transfected in HUH7 cells and *hsa-miR-939* overexpression was verified using RT-qPCR analysis (figure 2A). Following the overexpression of *hsa-miR-939, NGFR* mRNA level was reduced, compared to the cells transfected with mock vector as a negative control (figure 2B). On the other hand, Western blotting analysis indicated that *NGFR* protein level was also reduced following the overexpression of *hsa-miR-939* in hUH7 cell line, compared to the negative controls (figure 2C).

Direct interaction of *hsa-miR-939* with *NGFR* transcripts was investigated using cassettes containing 3′-UTR sequence of *NGFR* gene fused to Renilla luciferase reporter (Renilla::*NGFR*, 3′-UTR) and co-transfected in HEK293t cells with *hsa-miR-939* overexpressing vector. Results indicated significant reduction ($P<0.05$) of luciferase reporter signal 48 h after co-transfection of the cells (figure 1D). Renilla::*SMAD4*, 3′-UTR cassettes were used as a negative control for the specificity of *hsa-miR-939* interaction with *NGFR* 3′-UTR sequence.

3.4 *Cell death effect of hsa-miR-939 overexpression in U87 cell line*

U87 and HEK293t cell lines showed relatively low and high levels of endogenous *hsa-miR-939* expression, respectively (figure 1). Therefore, *hsa-miR-939* was overexpressed in HEK293t, HUH7 and U87 cell lines and cell death rate was measured using flow cytometry, 48 h post-transfection. No significant cell death alteration was detected in HEK293t (data not shown) and HUH7 cell lines (figure 3A), while early apoptosis rate was significantly reduced in U87 cell line and confirmed by Annexin test (figure 3B). On the other hand, survival rate of HUH7 (figure 3A) or U87 (figure 3B) cell lines was not significantly altered, following the *hsa-miR-939* overexpression, detected through MTT assay.

4. Discussion

NGFR has been identified as a multifunctional receptor with important roles in many different cell types including neuronal

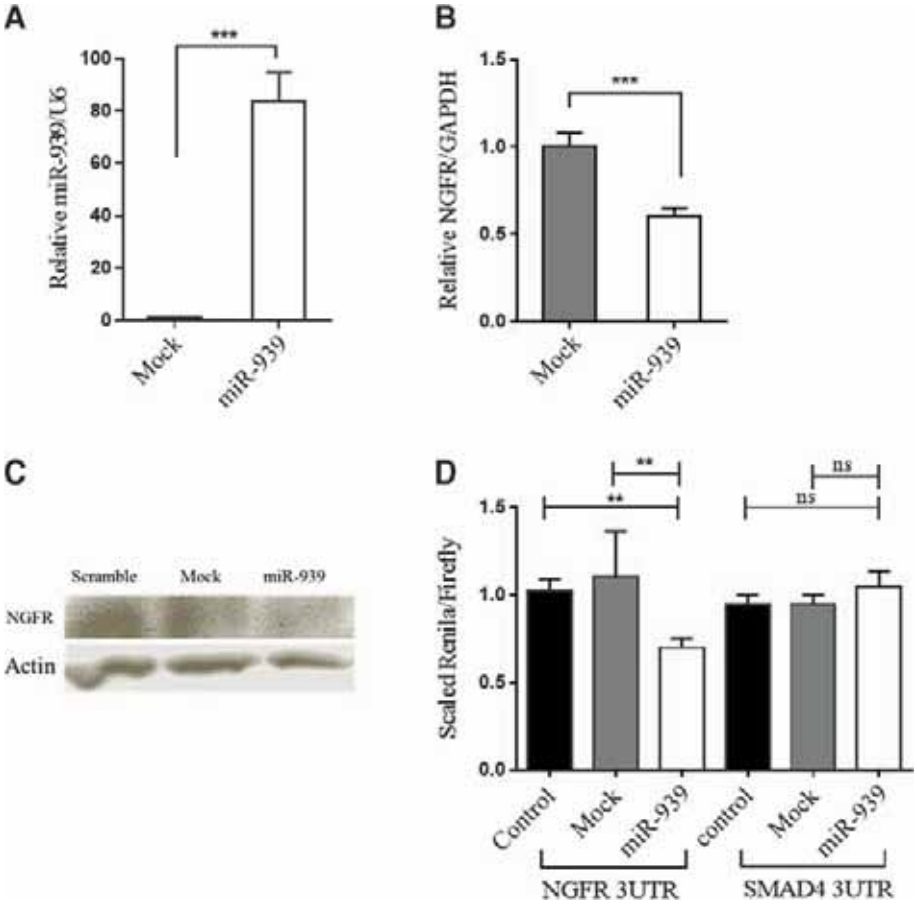

Figure 2. *Hsa-miR-939* regulates *NGFR* transcript and protein expression. (**A**) Efficient processing of *hsa-miR-939* precursor in the HUH7 cells and production of mature *hsa-miR-939*. (**B**) Significant down-regulation of *NGFR* transcription, following the *hsa-miR-939* overexpression, detected through RT-qPCR. Error bars represent SEM of two technical replicates, relative to mock-transfected cells. (**C**) Western blotting analysis against NGFR and β-actin proteins, 48 h after transfection of HUH7 cells. Compared to the mock and scrambled controls, Totallab Quant software confirmed reduction of NGFR protein level in the cells overexpressing *hsa-miR939*. (**D**) Renilla reporter::*NGFR*-3'UTR or ::SMAD4-3′UTR constructs were either transfected alone (control), or co-transfected into HEK293t cells with *hsa-miR-939* overexpressing vector or mock control vector. Luciferase assay indicated that *hsa-miR-939* has been interacting with *NGFR*-3′ UTR sequence cloned downstream of the luciferase ORF and resulted in luciferase expression reduction. Reporter expression is presented as scaled Renilla/firefly luciferase ratios. Luc=luciferase; prom=promoter; error Bars represent SEM. *P<0.05, n=3.

and non-neuronal lineages (Gentry 2004). Several studies show that *NGFR* may induce apoptosis and act as a tumour suppressor in some cancers (Frade 1998). Also, *NGFR* may induce invasion and metastasis. MiRNAs are known as important regulators of biological processes and the genes with long 3′-UTRs (such as *NGFR*) are more prone to miRNA-mediated regulation compared to the genes with shorter 3′-UTR sequences. Since regulatory network upstream of *NGFR* may justify its contradictory functions, we intended to investigate candidate miRNAs that may participate in the regulation of *NGFR*. Our comprehensive bioinformatics study candidate multiple miRNAs as regulators of *NGFR* gene expression. Among them only *hsa-miR-592* has been approved as a regulator of *NGFR* gene expression, to date. Along with *hsa-miR-592*, *hsa-miR-939* was predicted as a strong candidate miRNA capable of targeting *NGFR* transcript via multiple MREs in its 3′-UTR sequence. Moreover, it was predicted that this miRNA is capable of targeting other neurotrophin receptors such as *TrkB* and *TrkC* transcripts (data not shown). *Hsa-miR-939* is reported to be involved in lung cancer (Rani *et al.* 2013) and is functionally associated with the HBV life cycle (Zhang *et al.* 2012) and plays an essential role in the pathogenesis of aortic dissection formation (Hu *et al.* 2012). Also, it is a potential oncomiR via regulating the Wnt/β-catenin signalling pathway. To date, no relationship has been reported for *hsa-miR-939* with neurotrophin receptors.

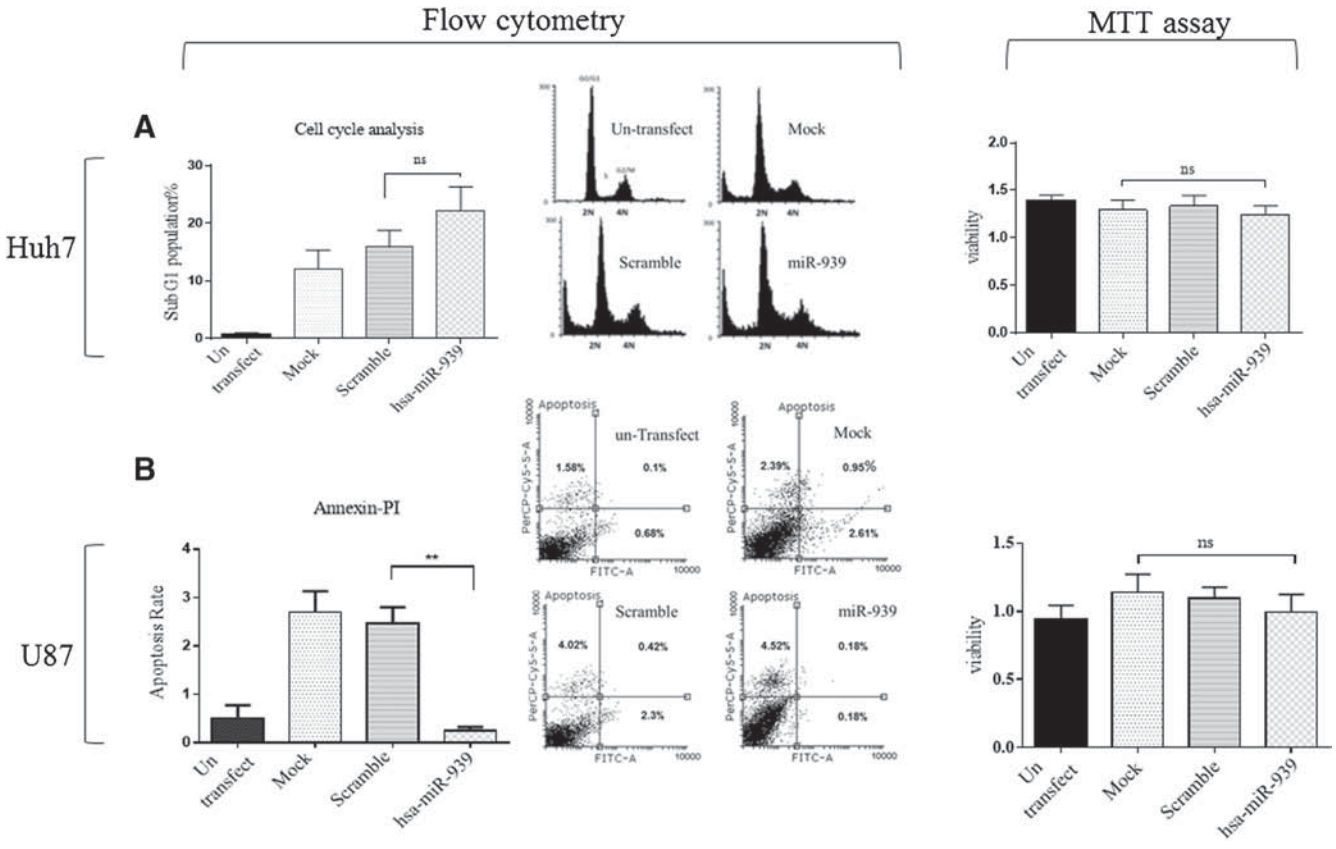

Figure 3. *Hsa-miR-939* overexpression effect on the cell death and survival rate. (**A**) PI staining and cell viability results of HUH7 cells transfected by *hsa-miR-939* overexpressing vector, or empty and scrambled control vectors. No significant cell death alteration was detected in HUH7 cell lines. (**B**) AnnexinV-PI and MTT result of U87 cell line transfected by *hsa-miR-939* overexpressing vector, compared to the cells transfected by empty and scrambled control vectors. AnnexinV-PI results indicate that *hsa-miR-939* resulted in significant reduction of apoptotic rate in U87 cells, while their survival rate has not been altered (detected by MTT assay). Two technical replicates; error Bars represent SEM; ***P<0.0001 relative to mock-transfected cells.

The expression level of *hsa-miR-939* was investigated in several cell lines in which HUH7 and HEK293t cells showed the highest *hsa-miR-939* expression level, while U87 was one of the lines with the lowest expression level (figure 1). HUH7 is a hepatocyte derived cellular carcinoma cell line. This epithelial-like tumorigenic cell was originally taken from a liver tumour (Nakabayashi *et al.* 1982). U87 cell line, a workhorse of brain-cancer research (Allen *et al.* 2016), has been derived from an astrocytoma primary brain tumour (Clark *et al.* 2010) and *NGFR* is reported as an inducer of apoptosis in these cells (Kumar *et al.* 2006). Since these cells are receptive to the transfection using lipofectamine, and *NGFR* expression is detectable in these cells, they were used for analysis of the effect of *hsa-miR-939* on *NGFR* gene expression.

To this aim, pGFP::miR939 expression vector was transfected into HUH7 cell line and elevated expression level of mature *hsa-miR-939* indicated proper processing of the cloned *hsa-miR-939* precursor (figure 2A). Then, down-regulation of endogenous *NGFR* expression, following *hsa-miR-939* overexpression, suggested targeting of *NGFR* transcript via this miRNA (figure 2B). Consistent with the changes in mRNA level, overexpression of *hsa-miR-939* resulted in reduction of *NGFR* protein's level, detected through Western blotting analysis (figure 2C).

Direct interaction of a candidate miRNA and its target sequences are supported through dual luciferase assay (Galardi *et al.* 2007). A significant decrease of luciferase activity was detected following the co-transfcetion of the cells with the vector carrying Luc::*NGFR*, 3′-UTR cassette and pGFP::miR939 vector (figure 2D). Overall, these results suggested *hsa-miR-939* as a key regulator of *NGFR* gene expression which exerts its effect via binding to *NGFR* 3′-UTR sequence.

MTT assay detected no significant difference between the HUH7 and U87 cells overexpressing *hsa-miR-939* compared with negative controls. Also, PI staining indicated that

hsa-miR-939 overexpression did not have a significant effect on the cell cycle status of HUH7 (figure 3A). However, *hsa-miR-939* overexpression resulted in apoptotic rate reduction and necrotic cell death elevation in U87 cell line, detected by PI annexin staining (figure 3B). The differences in the endogenous expression level of *hsa-miR-939* in HUH7 and U87 cell lines (figure 1) may explain different cell death rate in these cell lines, following *hsa-miR-939* overexpression. Stable survival rate of HUH7 cells following *hsa-miR-939* overexpression is consistent to the lack of subG1 cell population alteration in these cells (figure 3A). However, stable survival rate of U87 cells following *hsa-miR-939* overexpression (detected by MTT assay) is not consistent with the reduced apoptosis rate in these cells, which could be explained through elevation of necrosis rate in the same cells (figure 3B).

Following the introduction of *NGFR* into PC3 (prostate cancer cell line), cell cycle has been arrested. On the other hand, it is known that NF-κB and c-Jun N-terminal kinase (JNK) transcription factors are activated in PC3 cells, following *NGFR* up-regulation (Khwaja *et al.* 2006). It remains to be tested if *NGFR* expression alteration following the *hsa-miR-939* overexpression could affect apoptosis rate of PC3 cells through *NFkB* gene expression alteration as well. Relative low expression level of NGFR in PC3 cells has not been attributed to the point mutation in this gene; rather, involvement of unknown miRNAs has been suggested (Troy *et al.* 2002; Molloy *et al.* 2011). Likewise, cell death rate reduction in U87 cells following the *hsa-miR939* overexpression (figure 3B) might be attributed to the *NGFR* down-regulation (figure 2B).

Overall, current results provide strong evidences that *hsa-miR-939* regulates *NGFR* expression level via targeting its 3′ UTR. On the other hand, *NGFR* is a multi-functional cell surface neurotrophin receptor and is involved in many non-neural pathological conditions such as atherosclerosis, ischaemia, diabetes and cancer as well as neural development and diseases (Mufson and Kordower 1992; Scarpini *et al.* 1996; Chang *et al.* 2015). Therefore, *hsa-miR-939* as a novel regulator of *NGFR* expression introduces a key player in all these diseases as well.

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References

Allen M, Bjerke M, Edlund H, Nelander S and Westermark B 2016 Origin of the U87MG glioma cell line: good news and bad news. *Sci. Transl. Med.* **8** 354re3–re3

Ambros V 2004 The functions of animal microRNAs. *Nature* **431** 350–355

Blöchl A and Blöchl R 2007 A cell-biological model of p75NTR signaling. *J. Neurochem.* **102** 289–305

Cantarella G, Lempereur L, Presta M, Ribatti D, Lombardo G, Lazarovici P, *et al*. 2002 Nerve growth factor-endothelial cell interaction leads to angiogenesis in vitro and in vivo. *FASEB J.* **16** 1307–1309

Chang C-C, Fang W-H, Chang H-A and Huang S-Y 2015 Functional Ser205Leu polymorphism of the nerve growth factor receptor (NGFR) gene is associated with vagal autonomic dysregulation in humans. *Sci Rep* **5** 13136

Cheng H-C, Sun Y, Lai L-C, Chen S-Y, Lee W-C, Chen J-H, *et al*. 2012 Genetic polymorphisms of nerve growth factor receptor (NGFR) and the risk of Alzheimer's disease. *J. Negat. Results Biomed.* **11** 5

Clark MJ, Homer N, O'Connor BD, Chen Z, Eskin A, Lee H, *et al*. 2010 U87MG decoded: the genomic sequence of a cytogenetically aberrant human cancer cell line. *PLoS Genet.* **6** e1000832

Dennis G, Sherman B, Hosack D, Yang J, Gao W, Lane HC, *et al*. 2003 DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol.* **4** P3

Du T and Zamore PD 2005 microPrimer: the biogenesis and function of microRNA. *Development* **132** 4645–52

Dweep H, Sticht C, Pandey P and Gretz N 2011 miRWalk – database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. *J. Biomed. Inform.* **44** 839–47

Frade JM and Barde YA 1998 Nerve growth factor: two receptors, multiple functions. *Bioessays* **20** 137–45

Friedman RC, Farh KK-H, Burge CB and Bartel DP 2009 Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **19** 92–105

Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafrè SA, *et al*. 2007 miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. *J. Biol. Chem.* **282** 23716–24

Gao X, Daugherty RL and Tourtellotte WG 2007 Regulation of low affinity neurotrophin receptor (p75NTR) by early growth response (Egr) transcriptional regulators. *Mol. Cell. Neurosci.* **36** 501–14

Gentry JJ, Barker PA, Carter BD 2004 The p75 neurotrophin receptor: multiple interactors and numerous functions; in *Progress in brain research* (eds) A Luigi, C Laura (Elsevier) pp 25–39

Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu C-G, *et al*. 2007 Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res.* **67** 6092–9

Hefti F and Mash DC 1989 Localization of nerve growth factor receptors in the normal human brain and in Alzheimer's disease. *Neurobiol. Aging* **10** 75–87

Hu Z, Luo J, Zhong S, Xue L, Chen Y and Fan R 2012 MicroRNAs expression in normal and dissected aortic tissue. *Zhonghua Xin Xue Guan Bing Za Zhi.* **40** 406

Irmady K, Jackman KA, Padow VA, Shahani N, Martin LA, Cerchietti L, *et al*. 2014 MiR-592 regulates the induction and cell death-promoting activity of p75NTR in neuronal ischemic injury. *J. Neurosci.* **34** 3419–28

Johnston ALM, Lun X, Rahn JJ, Liacini A, Wang L, Hamilton MG, *et al*. 2007 The p75 neurotrophin receptor is a central regulator of glioma invasion. *PLoS Biol.* **5** e212

Kent WJ 2002 BLAT—the BLAST-like alignment tool. *Genome Res.* **12** 656–64

Khvorova A, Reynolds A and Jayasena SD 2003 Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115** 209–16

Khwaja F, Tabassum A, Allen J and Djakiew D 2006 The p75 NTR tumor suppressor induces cell cycle arrest facilitating caspase mediated apoptosis in prostate tumor cells. *Biochem. Biophys. Res. Commun.* **341** 1184–92

Krygier S and Djakiew D 2001 Molecular characterization of the loss of p75NTR expression in human prostate tumor cells. *Mol. Carcinog.* **31** 46–55

Kumar A, Sinha RA, Tiwari M, Pal L, Shrivastava A, Singh R, *et al*. 2006 Increased pro-nerve growth factor and p75 neurotrophin receptor levels in developing hypothyroid rat cerebral cortex are associated with enhanced apoptosis. *Endocrinology* **147** 4893–903

Lewis BP, Burge CB and Bartel DP 2005 Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are MicroRNA targets. *Cell* **120** 15–20

Maragkakis M, Reczko M, Simossis VA, Alexiou P, Papadopoulos GL, Dalamagas T, *et al*. 2009 DIANA-microT web server: elucidating microRNA functions through target prediction. *Nucleic Acids Res.* **37** W273–W6

Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, *et al*. 2009 A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol.* **10** R64

Molloy NH, Read DE and Gorman AM 2011 Nerve growth factor in cancer cell death and survival. *Cancer* **3** 510–30

Mufson EJ and Kordower JH 1992 Cortical neurons express nerve growth factor receptors in advanced age and Alzheimer disease. *Proc. Natl. Acad. Sci.* **89** 569–73

Nakabayashi H, Taketa K, Miyano K, Yamane T and Sato J 1982 Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. *Cancer Res.* **42** 3858–63

Ramos A, Chi Ho W, Forte S, Dickson K, Boutilier J, Favell K, *et al*. 2007 Hypo-Osmolar stress induces p75NTR expression by activating Sp1-dependent transcription. *J. Neurosci.* **27** 1498–506

Rani S, Gately K, Crown J, O'Byrne K and O'Driscoll L 2013 Global analysis of serum microRNAs as potential biomarkers for lung adenocarcinoma. *Cancer Biol. Ther.* **14** 1

Reichardt LF 2006 Neurotrophin-regulated signalling pathways. *Philos. Trans. R. Soc., B.* **361** 1545–64

Rocha AS, Risberg B, Magalhães J, Trovisco V, de Castro IV, Lazarovici P, *et al*. 2006 The p75 neurotrophin receptor is widely expressed in conventional papillary thyroid carcinoma. *Hum. Pathol.* **37** 562–8

Rodriguez-Tebar A, Dechant G and Barde Y-A 1990 Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron* **4** 487–92

Roux PP and Barker PA 2002 Neurotrophin signaling through the p75 neurotrophin receptor. *Prog. Neurobiol.* **67** 203–33

Salis MB, Graiani G, Desortes E, Caldwell RB, Madeddu P and Emanueli C 2004 Nerve growth factor supplementation reverses the impairment, induced by Type 1 diabetes, of hindlimb post-ischaemic recovery in mice. *Diabetologia* **47** 1055–63

Scarpini E, Conti G, Chianese L, Baron P, Pizzul S, Basellini A, *et al*. 1996 Induction of $p75^{NGFR}$ in human diabetic neuropathy. *J. Neurol. Sci.* **135** 55–62

Troy CM, Friedman JE and Friedman WJ 2002 Mechanisms of p75-mediated death of hippocampal neurons role of caspases. *J. Biol. Chem.* **277** 34295–302

Watanabe Y, Tomita M, Kanai A 2007 Computational methods for MicroRNA target prediction; in: *Methods in enzymology* (eds) JR John, JH Gregory (Academic Press) pp 65–86

Zhang X, Chen C, Wu M, Chen L, Zhang J, Zhang X, *et al*. 2012 Plasma microRNA profile as a predictor of early virological response to interferon treatment in chronic hepatitis B patients. *Antivir. Ther.* **17** 1243–53

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