BACKGROUND

Neurotrophins, secretory growth factors, encourage existence, growth, development and function of neurons and other cell types via binding to multiple specific receptors including TrkA, TrkB, TrkC and NGFR. In some cell types, expression of Trk family receptors either stimulates cell proliferation or differentiation of neural cells. TrkC acts as an oncogene or a tumour suppressor and is associated with controlling of growth and survival of various cancer cells in humans. It is well known that TrkC signalling stimulates PI3K, Akt and MAPK pathways, where their mechanisms are in deep investigation. No intrinsic enzymatic activity has been reported for NGFR; however, its signal transduction depends on the recruitment of other cell surface receptors such as TrkC. Consequently, there is a diversity of consequences in NGFR signalling depending on the interplay between neurotrophin cell surface receptors. For example, NGFR is highly expressed in melanoma and thyroid carcinoma, whereas it is down-regulated in stomach, bladder, liver and prostate cancers. MicroRNAs or miRNAs are endogenous extremely conserved RNAs with ~21 nucleotides in length produced in many organisms and implicated in regulation of several crucial cell processes including cell death, survival and differentiation and also in many diseases. They may interplay between different receptors of the signalling pathways. We have previously introduced hsa-miR-6165 located in NGFR gene intron, with functionality during the course of NT2 cell differentiation.
differentiation. We have also introduced TrkC-miR2 at the vicinity of *hsa-miR-115181* regulating Wnt pathway. Here, we hypothesized interplay between neurotrophin receptors through the TrkC-miR2 and confirmed the effect of TrkC-miR2 on NGFR gene transcript.

### 2 METHODS

#### 2.1 In silico analysis for prediction of TrkC-miR2 candidate target genes

In order to predict TrkC-miR2 potential target genes, we used DIANA-microT and RNAHybrid online tools. DAVID, Geneset2 and Diana-miRPath online software packages were applied to find the pathways are affected by TrkC-miR2.

#### 2.2 Cell lines and tissue samples

RPMI 1640 was used as the media for culturing HeLa, HepG2, U87MG, 1321N1, Daoy, A172 and SK-N-MC cell lines. SW480, HEK293 t and NT2 cells were maintained in DMEM-HG. These media were supplemented with 10% foetal bovine serum (FBS). Tissue samples were freshly obtained from Imam Hospital, in Tehran, Iran, and then stored in −80 for until used.

#### 2.3 DNA constructs

Human genomic DNA extraction, TrkC-miR2 precursor cloning using the primers named Int-F and Int-R both in sense and in antisense directions, cloning of the scrambled sequence as a control construct, and the strategy used for NGFR 3’UTR cloning have been previously reported. In order to clone the region corresponding to TrkC-premir2 sense and antisense sequences, about 802bp of human TrkC-intron-14 was PCR-amplified using Int-F and Int-R primers (Table 1) and cloned into pEGFP-C1 expression vector (Clontech) downstream of GFP sequence, both in sense and in antisense directions.

#### 2.4 RNA extraction

TRizol kit (Invitrogen) was used for total RNA extraction according to the protocol reported by its manufacturer and then was treated with DNase I purchased from Fermentas.

#### 2.5 RNA polyA adenylation, cDNA synthesis and RT-qPCR

In order to examine TrkC-miR2 expression level, polyA tail was initially added to the extracted total RNA by using polyA polymerase enzyme (NEB), and cDNA was then synthesized by using two anchored oligodT (Table 1) primers against both TrkC-miR2 isomiRs according to the previously reported protocol. TrkC-miR2 has two isomiRs, which are different in 2 last nucleotides at their 3’-ends. These two isomiRs were identified in our previous study. The sequences of TrkC-miR2-5p-GC and TrkC-miR2-5p-CT are GGCTGGGGATTCTGAGCTGC and GGCTGGGGATTCTGAGCT, respectively. 1 μL of cDNA products was then applied for RT-qPCR. U48 and GAPDH were used as the control genes. Expression data normalization was performed using 2^ΔCt and 2^ΔΔCt method.

#### Table 1 The list of used oligo sequences

| Primer name | Primer sequence, 5’ to 3’ | Amplicon size (base pairs) |
|-------------|--------------------------|---------------------------|
| TrkC-real time | Forward: CCTGTGTCCTGTTGGTGGTTCTC | 195 |
| | Reverse: GAGTCATGCCAACAGACCACAGTGC | |
| TrkC-miR2-5p | GGCTGGGGATTCTGAGCT | |
| U48 | Forward: TGACCCCAGGTAACTCTGAGTGTGT | |
| Anchored OligodT-CT | GGCCTGACTAGTACAAACCGAGCTTCACGACG(T)18AG | |
| Anchored OligodT-CT-GC | GGCCTGACTAGTACAAACCGAGCTTCACGACG(T)18GC | |
| Universal-inner | AACTCAAGGTTCCTCATCCAGTAG | |
| NGFR-real time | Forward: CCGAGGGACCACAGCACAACC | 151 |
| | Reverse: GGGCGTCTGTTCACCTGGCC | |
| GAPDH | Forward: GCCACATCGCTCAGACAC | 115 |
| | Reverse: GGCAACAATATCCACTTTACCAG | |
| TrkC-Intron | Int-F: CTCGGGCGCCCTGGAACAAAGAGATGGGTCAGTGGA | 802 |
| | Int-R: TAGACGGCGTTGCTTCCCACGGCTCAGG | |
| NGFR-3’UTR | Forward: CCCCTCGAGGACCCACTTCCAGACAC | 1920 |
| | Reverse: GCCCAAGAAATGATTACACAGGAG | |
2.6 | Dual-luciferase assay

A Dual-Luciferase Reporter Assay System (Promega) was utilized for measurement of luciferase activity two days post-HEK293-T cell transfection. The controls and experimental conditions for performing this experiment have been reported previously.\textsuperscript{10,11,15}

2.7 | Primer designing

Primer sequences that were designed using IDT, NCBI Primer-blast and MWG tools are listed in table 1.

2.8 | NT2 cell differentiation

In order to differentiate NT2 cells into neural-like cells, retinoic acid (RA) treatment was applied, according to the previously reported procedure.\textsuperscript{17} Also, NT2 cells were transfected with the vector overexpressing anti-TrkC-premir2, 10 days after beginning differentiation. Expression alteration of Oct4, PAX6, hsa-miR-302 and hsa-miR-145 differentiation markers along with morphological changes was used for following up the successful differentiation process.

2.9 | Overexpression of TrkC-premir2

A pEGFP-C1 vector containing and expressing TrkC-miR2 pre-cursor was surrounded in Lipofectamine 2000 purchased from Invitrogen, and utilized for transfection of the studied cell lines. Successful transfection was then ensured via GFP microscopy one day post-transfection.

2.10 | Western blotting

After loading of 30 μg of each protein on to 12% SDS-PAGE, protein transferring was performed into PVDF membrane. 5% skim milk was used for membrane blocking for 1h at room temperature. The primary antibody incubation was done for 12h at 4°C and then followed by secondary antibody incubation for 1h at RT. Amersham ECL Prime Western Blotting Detection Reagent Kit was used for visualization of blot. Western blotting data were quantitated using the TotalLab Quant software.

2.11 | Cell Cycle Analysis

The protocol performed for cell cycle analysis has been described in our previous paper.\textsuperscript{11}

2.12 | Statistical Analysis

Statistical study was completed by GraphPad Prism 5.04. In order to analyse the apoptosis experiment statistically, the Bonferroni test was employed following the repeated-measures ANOVA test.

3 | RESULTS

3.1 | Regulation of neurotrophin signalling pathway by TrkC-miR2

Using Dianna lab software, about 700 target genes were predicted for TrkC-miR2. There are three poorly conserved MREs within the 3'UTR sequence of NGFR (ENSG00000064300) gene (Figure 1A). Following the RT-qPCR assessment of NGFR endogenous expression level within SW480 cells (data not shown), TrkC-premir2 was over-expressed in these cells, which resulted in 50% down-regulation of NGFR expression (Figure 1B). Furthermore, Western blotting verified about 8% decrease in NGFR protein level following the TrkC-premir2 overexpression in comparison with the cells transfected by scrambled construct. Consistently, overexpression of a vector containing an antisense sequence against TrkC-premir2 resulted in NGFR protein level elevation (Figure 1C). When NGFR 3'UTR was cloned in a vector at downstream of luciferase ORF, and coexpressed with TrkC-premir2, dual-luciferase assay showed about 50% reduction in luciferase counts supporting a direct interaction between TrkC-miR2 and 3'UTR of NGFR (Figure 1D).

3.2 | TrkC-premir2 overexpression effect on the expression of the genes implicated in neurotrophin signalling pathway

The global consequence of TrkC-premir2 overexpression effect on downstream genes of neurotrophin signalling in U87 cell line was additionally examined using RT-qPCR. Results indicated that the expression levels of TrkA, Akt2, NF-XB and BCL2 genes have been highly elevated following the TrkC-premir2 overexpression, compared with the mock control. Nevertheless, TrkC gene expression level has been reduced within the same cells (Figure 2).

3.3 | Detection of endogenous TrkC-miR2 in human cell lines and brain tumour specimens

Status of endogenous expression level of TrkC-miR2 was identified through RT-qPCR in astrocytoma (1321N1), glioblastoma (A172 and U87MG), medulloblastoma (Daoy) and neuroblastoma (SK-N-MC) human brain tumour cell lines (Figure 3A). The highest expression level of TrkC-miR2 was identified in A172.
The endogenous TrkC-miR2-5p-GC isomiR was also detected in primary brain tumour specimens (Figure 3B,C). Although, TrkC-miR2-5p-GC was relatively expressed at low level in most of the examined brain cancer biopsies, the highest expression level of it was detected in glioma biopsies (Figure 3B,C) compared with meningoia transition type 1 tissue samples as the control. On the other hand, both TrkC (significant) and NGFR (non-significant) genes were down-regulated in the examined tumour samples (Figure 3B). Interestingly, it seemed that TrkC-miR2-5p-GC is expressed independent of TrkC (as the TrkC-miR2 host gene) in all of the tested tumour samples (Figure 3C).

### 3.4 Anti-apoptotic effect of TrkC-premir2 in cell lines

In order to look at the outcome of TrkC-premir2 overexpression on the cell cycle status, U87 and HEK293 t cell lines were transfected
by a vector overexpressing TrkC-premir2. A significant decrease in sub-G1 cell population was observed following TrkC-premir2 overexpression in HEK293 t and U87, in comparison with the cells containing the negative control vector. Inversely, knockdown of this miRNA within the above-mentioned cell lines attenuated its cell survival effect (Figure 4A,B). An anti-apoptotic influence of TrkC-premir2 overexpression in U87 was also confirmed by using annexin V test (Figure 4C). Further, MTT assay results confirmed survival effect of TrkC-premir2 overexpression in U87 and HEK293 t cells (Figure 4D).

3.5 | TrkC-miR2 expression alteration for the duration of NT2 cell differentiation

As TrkC is primarily expressed in neural cells, the expression status of TrkC-miR2 was explored for the period of NT2 cell differentiation towards the neural-like cells (Figure 5). This process was successfully accomplished in three weeks, and then Sox2, Oct4A, hsa-miR-145, hsa-miR-302, PAX6, TrkC, TrkC-miR2 and NGFR gene expression levels were weekly investigated. The expression of Sox2, Oct4A and hsa-miR-302, as the pluripotent markers, was significantly declined throughout the NT2 differentiation progress (Figure 5A). Unlike TrkC-miR2-5p-GC, a major TrkC-miR2-5p-CT expression elevation was noticed at the second week of NT2 differentiation course, which was coincident with notable TrkC expression decline (Figure 5B). Consistently, the expression of NGFR was reduced at the time that the expression of TrkC-miR2-5p-CT was increased (Figure 5C).

TrkC-miR2 down-regulation effect against the neural cell-like differentiation was also investigated. To this aim, NT2 cells were first treated with RA (retinoic acid) in order to convince the cell differentiation and then were transfected with the vector containing anti-TrkC-premir2, 10 days after beginning of differentiation induction. Real-time PCR results revealed a significant reduction (about 35% ) in TrkC-miR2 expression in these cells compared with the NT2 cells only treated with RA, as a control (Figure 5D). Following TrkC-miR2 suppression via anti-TrkC-premir2, PAX6 and hsa-miR-145 differentiation markers were significantly down-regulated, whereas OCT4A and hsa-miR-302 pluripotent markers were up-regulated, 21 days after starting differentiation (Figure 5E).

4 | DISCUSSION

MiRNAs are small non-coding RNAs regulating many important cell processes such as differentiation via translation inhibition or mRNA degradation. It has been reported that TrkC receptor is implicated in neurotrophin signalling associated with various functions such as differentiation, cell death and cell survival. On the other hand, the mechanism(s) explaining the TrkC contradictory functions is/are not well known yet. Hence, finding novel factors regulating neurotrophin signalling pathway may make available the cell fate manipulation opportunity in some diseases such as cancers and tissue regeneration. Our prior attempt led to the prediction of a unique bona fide stem loop structure named TrkC-premir2 using multiple software (SSC profiler, miPRED, CID-miRNA software along with Microprocessor SVM program, Mireval, MatureBayes, Pmirp, miRNA Spotter, MiRmat, and MirZ, and RNAfold algorithm) and discovery of a novel miRNA, named TrkC-miR2, which is located in TrkC gene and implicated in Wnt signalling pathway regulation. Also, in our previous research, following the overexpression of TrkC-miR2 precursor, we tested the production of both predicted TrkC-miR2-5p and TrkC-miR2-3p levels using specific RT-qPCR. However, only TrkC-miR2-5p was amplifiable, probably due to the more stability of it. Consistently, the number of reads for TrkC-miR2-3p sequence in the RNAseq data
was much lower than TrkC-miR2-5p.\(^1\) Here, we presented in silico study and supportive experimental evidence revealing TrkC-miR2 has the potential to be under consideration as a main regulator implicated in neurotrophin signalling pathway.

### 4.1 Association between neurotrophin signalling pathway and TrkC-miR2

MiRNAs, as the key regulatory factors in the cells, apply their effects via target mRNAs degradation or their translation inhibition.\(^2\) RT-qPCR data revealed that NGFR (a key gene in neurotrophin signalling pathway) is down-regulated following the overexpression of TrkC-premir2 (Figure 1B), which was then verified by performing Western blotting against NGFR (Figure 1C). Furthermore, direct interaction of NGFR 3' UTR with TrkC-miR2 was supported by dual-luciferase assay (Figure 1D).

NGFR, TrkA, TrkB and TrkC are categorized as the neurotrophin receptors, which function alone or in cooperation with each other.\(^1\) TrkA with the help of its downstream gene, Akt, leads the cell to survival fate, whereas NGFR is a cell surface receptor with multi-functional role, which is implicated in injury, regeneration and development of nervous system, and also acts as a tumour suppressor.\(^8,22\) BCl2 is an anti-apoptotic gene working downstream of NF-kB, which in turn results in survival of the cell.\(^23\) Overexpression of TrkC-miR2 resulted in NGFR down-regulation (Figure 1) and up-regulation of TrkA, Akt and BCl2 (Figure 2), which is expected to result in more survival of transfected cells. Up-regulation of neurotrophin downstream genes is consistent with increased survival effect, which is shown by flow cytometry and MTT assay results against the cells overexpressing...
FIGURE 4  Implication of TrkC-premir2 overexpression in the cell cycle status alteration of the cells. A and B, PI staining of the HEK293 t and U87 cells after the overexpression and knock down of TrkC-premir2. Significant decrease in the distribution of sub-G1 cell population was documented in HEK293 t (A) and U87 (B) cells following overexpression of TrkC-premir2. Consistently, knockdown of TrkC-premir2 in the same cells had a reverse effect on sub-G1 percentage. C, The figure displays annexin-PI analysis of transfected U87 cells. The gate setting revealed that most of the cells that overexpress TrkC-premir2 have less distribution in early apoptosis stage (bottom right), compared with the negative controls. D, The figure illustrates the outcome of MTT test in the HEK293 t and U87 cells containing a vector encoding TrkC-premir2. Survival rate of the transfected U87 and HEK293 t cells was meaningfully raised following TrkC-premir2 overexpression. SD of triplicate experiments is shown by error bar.
TrkC-miR2 (Figure 4). The result is consistent with the previously described survival influence of TrkC,24,25 which emphasizes on the cellular functionality of TrkC-miR2 corresponding to the function of its host gene, TrkC. Interestingly, NF-κB was up-regulated (Figure 2) following TrkC-premir2 overexpression, whereas NGFR was down-regulated (Figure 1). It suggests that TrkC-miR2 might be involved in neurotrophin signalling pathway in an NGFR-independent pathway.26

4.2 Uncovering of TrkC-miR2 expression in brain cell lines and tumour specimens

Both isomiRs of TrkC-miR2 were identified in several cancer tissues and cell lines (Figure 3A, B, C), similar to the TrkC as the host gene of this miRNA.27-29 The comparative higher expression level of this miRNA in glioblastoma samples and cell lines may identify this
miRNA candidate as a glioma biomarker. On the other hand, opposite expression level of TrkC-miR2-5p-GC related to the NGFR and TrkC genes in tumour tissue samples supports the functionality of this miRNA against NGFR and TrkC (Figure 3B,C).

4.3 | Induction of cell survival through ectopic expression of TrkC-premir2

Flow cytometry, annexin V test and also MTT assay in U87 and HEK293 t cells transfected with a construct overexpressing TrkC-premir2 showed significant increase in the rate of cell survival (Figure 4). These results were consistent with the survival effect of TrkC gene, which has been previously described that emphasizes the effectiveness of TrkC-miR2 along with its host gene, TrkC. U87 cell line expresses the genes that are implicated in neurotrophin signalling pathway actively. Up-regulation of neurotrophin signalling pathway genes following TrkC-miR2 overexpression (Figure 2) is consistent with its survival effect in U87 cells overexpressing TrkC-premir2 (Figure 4). This is also consistent with the effect of TrkC-premir2 overexpression in SW480 cell line in which Wnt signalling pathway is prominent.

4.4 | TrkC-miR2 expression is altered in the course of NT2 differentiation into neural-like cells

As Trk genes are identified to be implicated in neural cell differentiation and also TrkC-miR2 is significantly expressed in glioma-originated cancers and cell lines (Figure 3) and targets NGFR (Figure 1), the expression effect of TrkC-miR2 was investigated for the duration of NT2 cell differentiation into neural-like cells. Results showed that TrkC-miR2 expression alteration was in reverse correlation with NGFR expression particularly since day 14 of the differentiation (Figure 5C). Down-regulation of TrkC-miR2 (Figure 5D) attenuated differentiation outcome (Figure 5E) supporting the fundamental role of this miRNA during the differentiation of NT2 cells possibly through targeting of NGFR. It remained to be examined whether further TrkC-miR2-predicted target genes are expressed in opposite association.

5 | CONCLUSION

In conclusion, here we introduced accumulative evidence showing the function of TrkC-miR2 against the components of neurotrophin signalling pathway. Altogether, the presented evidence identifies this miRNA candidate as a controller of neurotrophin pathway and its implication in differentiation of neural cells.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

AUTHOR CONTRIBUTION

Sadat Dokaneheifard: Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Project administration (equal); Software (equal); Validation (equal); Writing-original draft (equal); Writing-review & editing (equal). Bahram Mohammad Soltani: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Methodology (equal); Project administration (equal); Resources (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing-review & editing (equal).

ETHICAL APPROVAL

Tissue samples were obtained from Imam Hospitals, Tehran/Iran. All these samples have been used with getting satisfying with all donors. The Tarbiat Modares University Ethics Committee approved the study.

DATA AVAILABILITY STATEMENT

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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