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Mir-186-5p Regulates WNT Signaling Pathway by Targeting TCF4 Transcription Factor

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Abstract

The evolutionarily conserved Wnt signaling pathway plays essential roles during embryonic development, tissue homeostasis and differentiation. This pathway is deregulated in many cancers especially colorectal cancer. MiRNAs are a class of small noncoding RNAs that may play a major role in post transcriptional regulation of many genes and signaling pathway such as WNT signaling pathway. Here, we intended to investigate if miR-186-5p is capable of regulating WNT signaling pathway wia suppression TCF4 gene expression. miR-186-5p was bioinformatically predicted as a candidate regulator of TCF4 gene expression and then, in this experimental study, miR-186-5p was overexpressed in SW480 cell line and its increased expression was detected through quantitative reverse transcription polymerase chain reaction (RT-qPCR). The effect of miR-186-5p on WNT pathway was analysied with TOP/FOP flash assay in SW480 cell line. Finally, flow cytometery was used to inves tigate the effect of miR-186-5p overexpression on cell cycle progression in SW480 cell line. miR-186-5p was overexpressed in the SW480 cell line and its overexpression resulted in significant reduction of the TCF4 mRNA level. TOP/FOP flash assay, confirmed the negative effect of miR-186-5p on the Wnt pathway in SW480 cells. Finally, Overexpression of miR186-5p in SW480 cells resulted in cell cycle arrest in subG1 phase, detected by flow cytometry. Overall, accumulative results indi-cated that miR-186-5p by targeting TCF4 is potentially one of the regulators of the WNT signaling pathway.

Keywords: miR-186-5p, TCF4, WNT signaling pathway

1. Introduction

Wnt signaling has emerged during evolution as a highly conserved signaling pathway that regulates tissue morphogenesis and regeneration in various tissues of multicellular organisms. Hyperactivation of Beta-catenin-T cell factor (TCF)/lymphoid enhancer factor (LEF)-regulated gene transcription (the end point of Wnt signaling) is a hallmark of colorectal cancer (CRC) development. So the regulation of thease genes involve in development and progression of CRC (Suzuki et al., 2004; Bienz & Clevers 2000)

microRNAs (miRNAs) are endogenous single stranded non-coding RNAs (Wang et al., 2013; Nelson & Nusse 2004; Mosimann & Hausmann 2006; Chen et al., 2009) nucleotides. miRNAs can potentialy impresses the expression of genes in post transcriptional level (Krol et al., 2010). miRNAs do this regulatory function by imperfect binding to the miRNA regulatory elements (MREs) on the target mRNA and then cause mRNA translational repression or mRNA degradation (Wang, 2010). Current evidence indicate the direct link between miRNAs and varieties of human cancers (Yang & Belaguli, 2009).

miR-186-5p is locatet on 1p31.1 locus and is conserved in various specieses. According to recent observations miR-186-5p deregulate in many human cancers (Zhou et al., 2008; Zhang et al., 2009). Here by the using of bioinformatics target prediction analysis and experimental methods we indicated that miR-186-5p targets TCF4 genes and via this action can put the negative regulatory effect on WNT signaling pathway.

2. Materials and Method

2.1 Bioinformatics

The 3′-UTR sequence of human TCF4 gene was captured from Entrez (http://www.ncbi.nlm.nih.gov/Entrez/).online bioinformatic databases including DAVID (https://david.ncifcrf.gov/) and DIANA-mirPath (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath/index) were used to find the pathways

that miR-186-5p have a role in regulating of them. Prediction of miRNA targets was done by Targetscan (http://www.targetscan.org/), DIANA-microT (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index), miRmap (http://mirmap.ezlab.org/) and miRwalk (http://mirwalk.uni-hd.de/). for evaluating the MREs conservation in different organisms UCSC genome browser was used.

2.2 Cell Culture

SW480 cell line was cultured in DMEM-HG (Invitrogen). This media was supplemented with 100U/ml penicillin, 100µg/ml streptomycin (Sigma), and 10% fetal bovine serum (FBS) (Invitrogen), and followed by incubation at 37°C with 5% CO2. SW480 cell line was obtained from Pasteur Institute/Iran.

2.3 Transfection

For miRNA overexpression analysis, SW480 cells were seeded on 24 well plates (6×10^4 cells per well) and were allowed to adhere for 24 h. These cells were transiently transfected with pEGFP-miR-186-5p-C1 using Lipofectamine 2000 (Invitrogen). Transfected cells were then cultured for 6 h, and culture media were replaced with fresh media supplemented with 10% FBS. GFP expression was visually examined 36 h after transfection, using fluorescent microscope (Nikon eclipses Te2000-s). The cells were harvested hours after transfection and RNA extraction was performed.

2.4. RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from SW480 using Trizol reagent according to the manufacturer's protocol (Invitrogen). The RNA quality and yield of extracted RNA was analyzed by using agarose gel electro-phoresis and spectrophotometry, respectively. In order to remove geno-mic DNA contamination, DNaseI treatment was performed prior to cDNA synthesis by the following method: DNAaseI treatment (Takara) at 37 °C for 30 min followed by heat and EDTA inactivation, then cDNAs was synthesized using Prime Script II reverse transcriptase (Takara). For miRNA detection, polyA tail was added to 3′ end of RNAs before cDNA synthesis. Real-time PCR was performed using standard protocols by ABI PRISM 7500 instrument (Applied Biosystems). Relative changes of gene expression were calculated by 2^{-} Ct and 2^{-} Ct methods and analyzed by GraphPad PRISM software. GAPDH and U48 sno-RNA expression levels were used as the internal controls for nor-malization of TCF4 gene and miR-186-5p expression, respectively.

2.5 TOP/FOP Flash Assay

TOP/FOP flash assay was used for Wnt signaling pathway analysis and to this aim, the TCF/LEF-responsive luciferase construct was made under the control of minimal TK promoter and tandem repeats of the TCF/LEF transcriptional response element (TRE). The SW480 cells were plated in 48-well plates and transfected with 400ng miRNA-encoding vector and with 200ng of luciferase-encoding vector. The cells were harvested 48h post-transfection and luciferase activity was measured by using the Dual-Glo luciferase assay kit (Promega).

2.6 Cell Cycle Assay

Cells were harvested 38 h after transfection of miR-186-5p in SW480 cell line, and centrifuged at 1200 rpm for 5 min and washed twice in PBS. Subsequently, one ml ice-cold 70% ethanol was added and cells were fixed in this solution for at least 30 min. For each sample, 500 µl PI staining solution was added and incubated for 30 min at room temperature, subsequently analyzed by a FACS Calibur flow cytometer with Cell Quest software (BD Biosciences).

3. Results

3.1 Bioinformatic Analysis

Based on the bioinformatics databases DAVID, DIANA-mirPath, miR186-5p involved in regulation of WNT signaling pathway. the bioinformatic softwares Targetscan, DIANA microT, miRwalk, miRmap pre-dicted 3 miRNA recognition elements (MREs) within the TCF4 3′-UTR sequence for miR-186-5p.

3.2 Reduction of TCF4 mRNA Level after the miR-186-5p Overexoression

miR-186-5p overexpression effect on TCF4 mRNA level was investigated in SW480 cell line. To this aim, these cells were transfected with PEGFP-miR-186-5p-C1 vector ensuring miR-186-5p overexpression. RT-qPCR analysis indicated that miR-186-5p has been overexpressed more than 12 fold in the transfected cells (Figure 1A), compared to the mock and scrambled controls. In the same cells, RT-qPCR re-sults indicated that TCF4 mRNA level has been decreased about 5 fold, compared to the mock and scrambled transfected SW480 cells (Figure 1B).

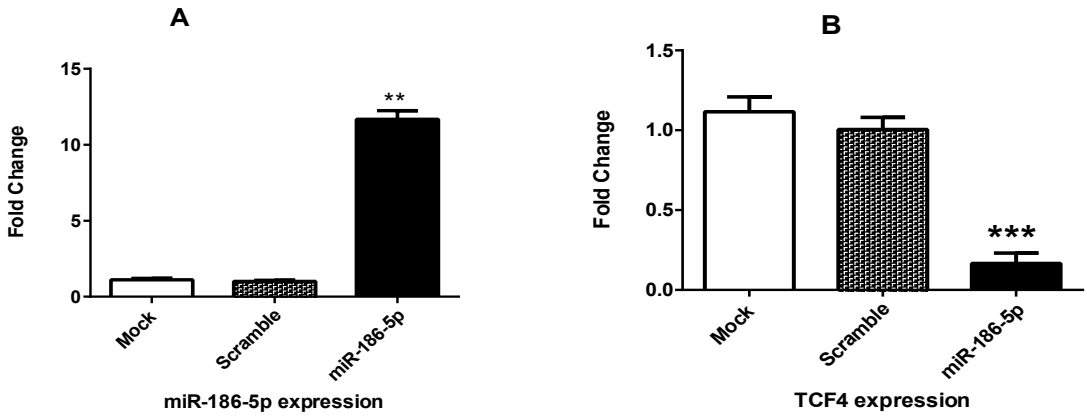

Figure 1. miR-186-5p overexpression effect on TCF4 gene expression. A) RT-qPCR result showed more than 12-fold increased miR-186-5p expression following the transfection of SW480 cells with PEGFP-C1 recombinant vector compared to the cells transfected with the empty vector (mock) and scrambled vector. Expression data were generally normalized against U48 as an internal control. Error bars indicate SD of triplicate experiments. **P b 0.01, versus the mock control by t-test. B) Shows RT-qPCR result of TCF4 transcript level in the cells transfected with vector, which ensured miR-186-5p overexpression. TCF4 downregulation is evident in the SW480 cells overexpressing miR-186-5p compared to the mock and scrambled controls. Expression data were generally normalized against GAPDH as an internal control. Error bars indicate SD of triplicate experiments. *P b 0.05 versus the mock control by t-test

3.3. miR-186-5p as a Regulator of WNT Signaling Pathway in SW480 Cell Line

TOP/FOP flash assay was done for analysis of miR-186-5p effect on WNT signaling pathway.TOP/FOP flash assay was done after co-transfection of SW480 cells with PGL-TOP and miR-186-5p over expressing vector and then results were compared with mock and scrambled controls. The activity of WNT signaling pathway were impressively suppressed compare with controls (Fig2).

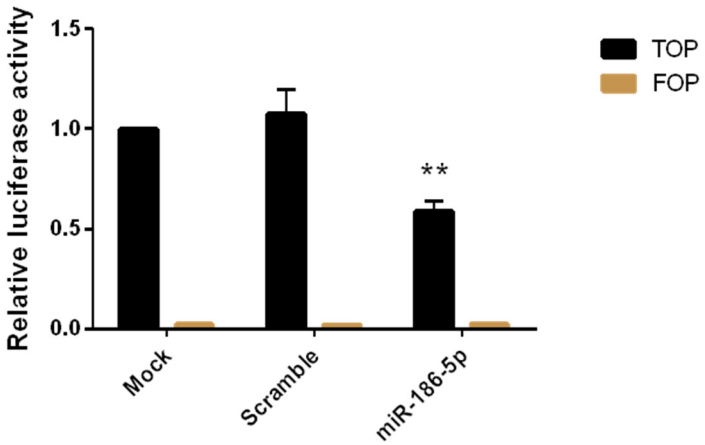

Figure 2. miR-186-5p expression alteration effect on the Wnt signaling pathway. Top/Fop flash assay after transfection of miR-186-5p in SW480 cells showed significant Wnt signaling reduction, compared to the transfected controls

3.4 Cell Cycle Effect of miR-186-5p Over Expression in SW480 Cell Line

The miR-186-5p overexpression effect on cell cycle was analyzed in transfected SW480 cells using PEGFP-miR-186-5p-C1 vector.The cells were harvested 38 h after transfection, and cell cycle analysis was performed by flow cytometry using PI-staining. The flow cytometry analysis indicate that the population of SW480 cells increased in subG1 phase of cell cycle compared to the mock and untransfected and scrambled controls (Figure 3).

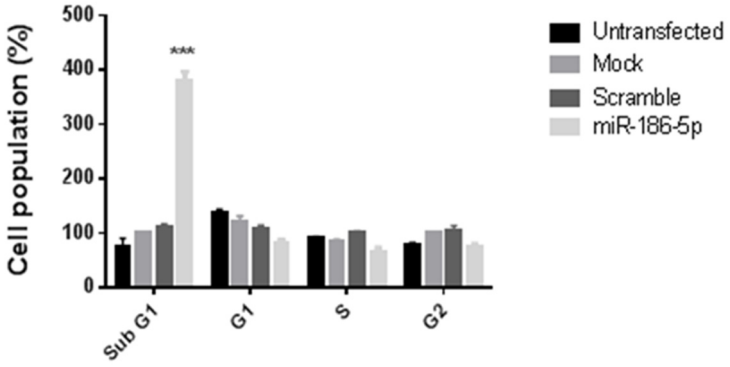

Figure 3. miR-186-5p overexpression effect on the cell cycle. PI staining of SW480 cells overexpressing miR-186-5p, 38 h after transfection. Flow cytometry analysis indicated that the population of SW480 cells increased in subG1 phase of the cell cycle compared to the untransfected and mock and scrambled transfected cells as negative controls. The percentages indicate the proportion of population in each phase

4. Discussion

Wnt signaling is one of the key cascades regulating development and stemness, and has also been tightly associated with cancer. The role of Wnt signaling in carcinogenesis has most prominently been described for colorectal cancer, but aberrant Wnt signaling is observed in many more cancer entities (Fodde & Brabletz 2004; Wielenga et al., 1999).

MiRNAs are now identified as a key regulator of biological processes (Winter, 2009; Lima et al., 2011; Hébert & Strooper 2009) including the WNT signaling pathway and consistently mis-expression of specific miRNAs is detected in many cancer types (Lu et al., 2005; Volinia et al., 2006). MiRNAs are short non-coding RNAs with 20–25 nucleotides long, which are generated from double stranded RNA precursors (Bartel, 2009). Most of the target sites of miRNAs are located within the 3′UTR of mRNAs. However, the functional MREs in 5′UTR and coding region have also been reported (Zhuo, 2011). The miRNA affects the target mRNAs expression ei-ther by translational repression or degradation of target transcripts (Stark et al., 2005).

MiRNAs are described as a master regulator of intracellu-lar signaling pathways and the aim of this study is the analysis of possible effect of miR-186-5p on WNT signaling pathway wia targeting of TCF4 gene.

Using bioinformatics analysis, 3 conserved MREs were predicted for miR-186-5p within the 3′-UTR sequence of TCF4, implying that miR-186-5p may repress the expression of this gene. It has been reported that the number of binding sites within a particular 3′-UTR can determine the degree of expression repression (18). Therefore, miR-186-5p was overexpressed through transfection of PEGFP-miR-186-5p-C1vector SW480 cell line and its effect on TCF4 expression was analyzed by qRT-PCR. Results suggested that miR-186-5p could reduce TCF4 expression level in respect to the mock and scrambled transfected controls in theSW480 cell line (Figure 1).

During the Wnt signaling, Wnt ligands interact with frizzled and LRP co-receptors leading to inactivation of the tumor suppressive genes APC, GSK-3β, and Axin and finally release of β-catenin oncogenic-protein (Nelson & Nusse, 2004). After nuclear translocation of β-catenin which complexes with TCF/LEF transcription factors, Wnt-responsive genes such as CyclinD1 (CCND1) is upregulated and cell cycle is motivated (Mosimann & Hausmann, 2006). Activity of the pathway was measured through TOP/FOPflash assay system following miR-186-5p overexpression. When miR-186-5p was overexpressed in SW480 cells (Chen et al., 2009), Wnt signaling was downregulated compared to the cells that were treated with mock and scrambled controls, that means miR-186-5p effect is downstream to the LRP receptor in the Wnt signaling pathway (Figure 2). This experiment again introduces miR-186-5p as a positive regulator of Wnt signaling pathway, potentially via targeting of TCF4.

We investigated miR186-5p overexpression effect on sw480 cells using flow cytometry. Results indicated that miR-186-5p has inhibited cell cycle progression in subG1 phase (Figure 3) which is consistent with its reported tumor suppressor function in hepatic, prostate and lung cell types. Our results emphasized on the negative regulatory effect of miR-186-5p against TCF4 transcripts, which suggest the possible role of this miRNA in regulation of WNT signaling pathway.

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