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Hsa-miR-106b-5p Negatively Regulates LEF1

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Abstract: Aberrant expression of the genes involved in Wnt signaling pathway, one of the most important developing pathways, is observed in many malignancies. Reports show that Wnt/β-catenin activation is critical for cancer development, angiogenesis, migration, and invasion. LEF1 belongs to the T cell Factor (TCF)/LEF family of transcription factors and plays the role of nuclear effector in the Wnt/β-catenin signaling pathway. LEF1 has central role as a transcription factor in the Wnt/β-catenin signaling pathway which makes it an ideal target for therapeutic treatment in dealing with cancer proliferation. It can act as an oncogene or a tumor suppressor in cellular context dependent manner. miRNAs are aberrantly expressed in cancers and can act as tumor suppressors or oncomirs depending upon the type of carcinomas. Studies show that miRNAs can be used as novel agents for targeted cancer therapy. miR-106b, which belong to miR-17-92 paralog cluster, is reported to be overexpressed in multiple tumor types including medulloblastomas, breast, colon, kidney, gastric, lung cancer and HCC. In this study we have demonstrated that over-expression of miR-106b-5p down-regulates the endogenous expression of LEF1 in HEK293FT cells, thereby affecting the expression of N-Myc, downstream gene of Wnt signaling. Therefore, our results suggest that miR-106b-5p plays a significant role in suppressing the carcinomas resulted due to the over-expression of LEF1 and/or activation of Wnt pathway and may prove to be a potential target for novel cancer therapy. It may helpful in developing therapeutic strategies for cancer treatments.

Keywords: WNT Signaling Pathway, LEF1, MYCN, Hsa-miR-106b, HEK293FT Cells, Western Blotting, Luciferase Assay

1. Introduction

Wnt signaling pathway is one of the most important developmental pathways. The overexpression of Wnt signaling is common in many hematological malignancies and solid tumors. Clinical and experimental evidence suggests that Wnt/β-catenin activation is critical for cancer development, angiogenesis, migration, and invasion [1-3]. The antagonists of Wnt pathway, such as Wnt inhibitory factor 1 (WIF-1), Dickkopf proteins (DKKs), the secreted frizzled-related proteins (sFRPs), and Disheveled- axin domain containing 1 (DIXDC1), enhance the tumorigenic and metastatic processes of various cancer types in vitro and in vivo [4-6]

Wnt/β-catenin pathway is initiated by evolutionarily conserved growth factors of the wingless and integration site growth factor (Wnt) family. Wnts are encoded by 19 different Wnt genes and share a high degree of sequence homology [7]. They bind to cell surface receptors to activate the Wnt pathway and thus trigger signaling cascades that are important in many physiological settings [8]. Wnt signaling pathway actively functions in embryonic development and helps in homeostasis in mature tissues by regulating diverse processes including cell proliferation, survival, migration and polarity, specification of cell fate, and self-renewal property [9-10]. It is a critical step in β-catenin signal transduction and is responsible for maintaining its own unphosphorylated state. In its dephosphorylated state, β-catenin is localized in the nucleus, where it activates transcription factors in the T-cell factor (TCF)/lymphoid enhancing factor (LEF) family [11-13]. This results in the expression of the downstream target genes, c-jun, fra-1, c-myc, cyclin D1, etc., that are normally involved in developmental stages and adult tissue homeostasis. In the absence of Wnt activity, β-catenin is

phosphorylated by glycogen synthase kinase (GSK)-3β and marked for subsequent degradation by the ubiquitin/proteasome pathway [14-19]. In its active or dephosphorylated state, β-catenin has a profound effect on the regulation of stem cells [20-21]. When the Wnt pathway is upregulated, it consistently results in tumorigenesis in a variety of organs. Canonical Wnt pathway supports the formation and maintenance of CSCs and thereby cancer formation. Hence, the aberrant activation of this pathway and the target genes maintains the pluripotency of the stem cells rather than differentiating them and results in the neoplastic proliferation. Overexpression or mutation in any of the pathway components leads to malignant growth [22-24].

LEF1 belongs to the T cell Factor (TCF)/LEF family of transcription factors, containing a highly conserved high mobility group (HMG) DNA-binding domain and plays the role of nuclear effector in the Wnt/β-catenin signaling pathway [25-26]. In the absence of Wnt signaling, LEF1 is bound to Groucho-related co-repressors, thus negatively regulating the expression of Wnt signaling genes [27]. Upon stabilization from Wnt signals, β-catenin displaces the Groucho-related co-repressors and promotes LEF1 transcription factor activity (1). LEF1 has a central role as a Wnt/β-catenin signaling pathway mediator and on downstream cellular effects, making it a key regulatory factor for eliciting or preventing aberrant protein expression.

Reports show that aberrant expression of LEF1 is implicated in tumorigenesis and cancer cell proliferation, migration, and invasion. Increased β-catenin-TCF/LEF1 expression and localization in the nucleus is implicated in migratory, Vimentin-expressing oral squamous cancer cells (OSCC) and breast cancer cells [28-29]. Higher LEF1 expression is associated with lymphovascular invasion and reduced overall survival [30]. Overexpression of LEF1 has been identified to be a positive prognostic factor in pediatric acute lymphoblastic leukemia (ALL) [31]. Strong over expression of nuclear LEF1 is reported in chronic lymphocytic B cell leukemia [32-33]. Amongst CLL cases, patients exhibiting higher expression levels of LEF1 have poorer prognoses with lower overall survival times compared to patients with low LEF1 expression [34]. Similar to CLL, LEF1 is overexpressed in Burkitt's Lymphomas and in Colorectal cancers (CRCs), making it a valuable marker in differentiating lymphoma types [35]. Thus, LEF1 expression in above cancer cell types makes it a valuable biomarker in predicting patient prognosis.

LEF1 has central role as a transcription factor in the Wnt/β-catenin signaling pathway which makes it an ideal target for therapeutic treatment in dealing with cancer proliferation. Knockdown of LEF1 in colon cancer cells causes increased apoptosis compared to control cells in vitro, and reduced tumor growth compared to normal colon cancer cells in vivo [36]. Inhibition of LEF1expression using miR-218 as a treatment reduces metastatic potential of glioblastoma multiforme (GBM) cells [37]. Inhibiting LEF1 expression in vivo using 5-aza-2'-deoxycytidine (DAC) and paclitaxel (PTX), results in decreased renal cell carcinoma (RCC) proliferation [38]. Thus, knockdown and inhibition treatments designed to target LEF1

have proven effective in alleviating cancer growth, migration, and invasion.

In addition to its role as a transcriptional activator in the setting of active WNT/β-catenin signaling, LEF1 can also act as a transcriptional repressor in some cellular contexts. Experiments are currently underway to establish the mechanism mediating the tumor suppressor activity of LEF1. Reports show that LEF1 acts as a tumor suppressor in rhabdomyosarcoma, leukemia and acute lymphoblastic leukemia (ALL) [39-41]. LEF1 knockdown experiments in cell lines reveal that depending on the cellular context, LEF1 can induce pro-apoptotic signals. LEF1 can also suppress proliferation, migration and invasiveness of RMS cells both in vitro and in vivo. Furthermore, LEF1 can induce myodifferentiation of the tumor cells. This may involve regulation of other LEF1/TCF factors i.e. TCF1, whereas β-catenin activity plays a subordinate role. This data suggest that LEF1 rather has tumor suppressive functions and attenuates aggressiveness in a subset of RMS [39].

MicroRNAs (miRNAs) are short (19–25 nucleotides) RNA molecules that can modulate the expression of a wide range of target genes by pairing homologous sequences within the 3′-untranslated region (3′-UTR) of messenger RNAs (mRNAs), thus preventing or impairing their translation or promoting RNA degradation [42-44]. Therapeutic roles of miRNAs have been proven in in-vitro and in-vivo experiments. miRNAs are aberrantly expressed in cancer tissues and cancer cell lines. Therefore, tumor suppressive miRNAs, or their mimics, can be used as novel agents for targeted therapy; in contrast, the oncogenic miRNAs can be used as targets for personalized therapy, or correcting of the aberrant expression of miRNAs by either blocking or restoring miRNA levels and functions as therapeutic strategies for cancer treatment.

miR-17-92, a polycistronic miRNA cluster, has been reported to be overexpressed in a wide variety of human cancers. miR-106b and miR-25, which belong to miR-17-92 paralog cluster, are overexpressed in all medulloblastomas as compared to the normal cerebellar tissues [45]. miR-106 is overexpressed in multiple tumor types, including breast, colon, kidney, gastric, lung cancer and HCC. miR-106b gain of function promotes cell cycle progression by modulating checkpoint functions [46-49]. Recent reports show that miR-106b probably promotes hepatoma cell proliferation by directly targeting the 3′-UTR of the APC mRNA, consequently leading to activation of Wnt/β-catenin pathway, nuclear accumulation of β-catenin and upregulation of the cyclin D1 cell cycle regulator [49]. Therefore, it is particularly interesting to identify and study the role of miR-106 in Wnt driven cancers and find its target genes for better diagnosis. It may provide considerable therapeutic strategies for cancer treatments.

2. Materials and Methods

2.1. Identification of Genes Targeting miR-106b

Three computer-aided algorithms including TargetScan,

Targetminer and miRDB were used to identify the putative targets of miR-106b. These bioinformatic approaches use several common criteria to judge whether certain transcript is a target for certain miRNA. The most important criterion for target recognition is base-pairing between the 'seeds' (the core sequence that encompasses the first 2–8 bases of the mature miRNA) and target. Another important rule for target prediction relies on strict requirements of interspecies conservation. Moreover, because individual computer-aided algorithm can generate a high number of false positives, the combination of these three approaches will provide more accurate assessment of the real miRNA targets than a single approach would do. With these bioinformatic approaches a few WNT pathway genes and their downstream targets were selected namely, TCF4, LEF1, FZD4, MYC, MYCN and MNT.

2.2. Cloning miR-106b-5p in Mammalian Expression Vector

Primers for the genomic region encoding for miR-106b-5p were synthesized and obtained from Sigma Genosys. Genomic DNA extracted from peripheral blood lymphocytes of a healthy individual was used as a template for PCR. Fragment of miR-106b-5p with ~200 bp flanking regions at the 5' and 3' end of the miRNA was amplified using Taq DNA Polymerase. It was cloned directionally under CMV promoter in pcDNA4/Myc-HisB (Addgene). The constructs so obtained were checked for the correct insert and its orientation by restriction analysis.

2.3. Cloning 3' UTRs of WNT Pathway Genes in Luciferase Reporter Vector

Specific set of primers for the genomic regions for 3' UTRs of WNT pathway genes namely, FZD4, TCF4, LEF1, MYC, MYCN and MNT were synthesized and obtained from Sigma Genosys. Genomic DNA extracted from peripheral blood lymphocytes of a healthy individual was used as a template for PCR. Amplified fragment of all the 3' UTRs were cloned downstream of Luciferase gene under CMV promoter in pcDNA3-Luciferase Reporter Vector (will be denoted as pLuc hereafter). The constructs so obtained were checked for the correct insert and its orientation by restriction analysis.

2.4. Transient Transfection of miR-106b-5p and 3'UTRs in HEK 293FT Cell Line

The constructs containing miR-106b-5p and 3' UTRs were co-transfected in HEK293FT cells using CaCl$_2$ and BES Buffer method. Plasmid containing EGFP was also transfected simultaneously for normalizing luminescence values. DNA was taken in the ratio of 4:1:1 for miR-106b-5p: 3' UTR: pEGFPN1 respectively. As negative controls, empty plasmid vectors for miR-106b-5p and/or 3' UTR were used. Protein was extracted 72hrs of transfection.

2.5. Luciferase Reporter Assay

Protein was extracted after 72hrs of transfection. Each protein sample was assayed in triplicates on Mithras Berthold LB940 multimode Plate Reader. Fluorescence for the protein samples was measured at the excitation wavelength of 485nm and emission wavelength of 515nm. Then luciferin substrate was added to the same protein samples and the luminescence was measured immediately at exposure time of 0.1 sec. Luminescence values were normalized with the corresponding fluorescence values. Average of the normalized readings was compared to see any reduction in Luciferase activity in presence of miRNA and percentage of down regulation was calculated.

2.6. Western Blotting

Plasmid construct containing miR-106b-5p was transiently transfected using CaCl$_2$ and BES Buffer in HEK293FT cells to check its effect on endogenous levels of target genes. Protein was extracted from the transfected cells after 72hrs of transfection. 25µg of protein was used to run on SDS-PAGE gel which was then transferred on to a nitro cellulose membrane and probed with 1:1000 diluted (in 5% BSA) primary antibodies for TCF4 (Abcam) LEF1 (Abcam), MYC (SantaCruz Biotechnology) and MYCN (SantaCruz Biotechnology). 1:5000 diluted (3% BSA) anti γ-tubulin antibody (Sigma) was used as house keeping control 1:2000 diluted (in 2% milk) anti Rabbit HRP conjugated secondary antibody (Thermo Scientific) was used. Blots were incubated overnight with primary antibodies and secondary antibody was kept for one hour. Blots were developed using SuperSignal® West Pico chemiluminescent substrate. The blots were developed using BioRad ChemiDoc imaging system and quantitated using Image Lab version 6.0.

3. Results

Putative gene targets for miR-106b-5p were identified using three in silico prediction algorithms (miRDB, TargetScan and Targetminer). By comparing the complementarity of the m-RNA-miRNA sequence, a few Wnt pathway genes and their downstream targets were chosen for further investigation including FZD4, TCF4, LEF1, MYC, MYCN and MNT. First we PCR amplified the genomic sequence encoding for miR-106b-5p with 200bp flanking on both the ends (for proper processing of miRNA in vitro) and cloned it in mammalian expression pcDNA4/myc-HisB vector under CMV promoter. The expression was confirmed by real time PCR. We also cloned 3′UTRs of the above genes that contain putative miR-106b-5p binding sites into the Luciferase Reporter vector (pLUC). Each of the cloned UTR then was co-transfected with a miR-106-b-5p expression plasmid into HEK293FT. Overexpressing miR-106b-5p resulted in significant reduction of luciferase reporter activity (normalized against fluorescence) of FZD4, LEF1, MYCN and MNT transfected cells as compared to the vector control transfected cells, whereas, there is no significance difference between the luciferase activity (normalized against fluorescence) of MYC and TCF4 on transfecting miR-106b-5p (Figure: 1). However, because HEK293FT

cells expressed moderate level of endogenous miR-106b-5p, the complete effect of miR-106b-5p might be masked in a background. The effect is only ~20-30% reduction, which might be actually more. Taken together, our results unequivocally demonstrate that miR-106b-5p directly recognize the 3′ UTR of FZD4, LEF1, MYCN and MNT transcripts.
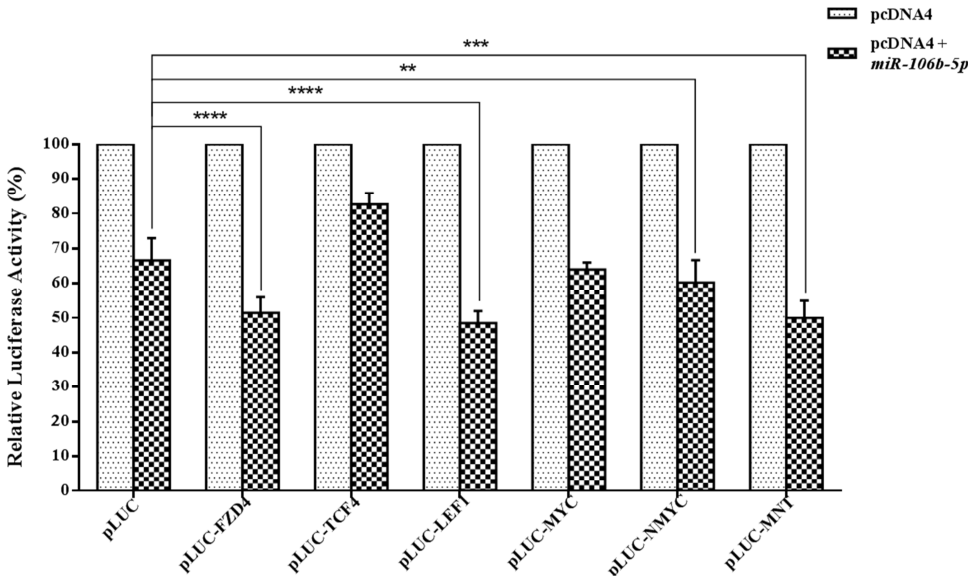

Figure 1. *LEF1 is a direct downstream target of miR-106b-5p.*

Luciferase reporter assay analysis of the effects of miR-106b-5p overexpression on the activities of 3′UTRs of predicted target genes in HEK293T (A). These results are representative of at least three independent experiments. **p < 0.01, ***p<0.001, ****p < 0.0001.
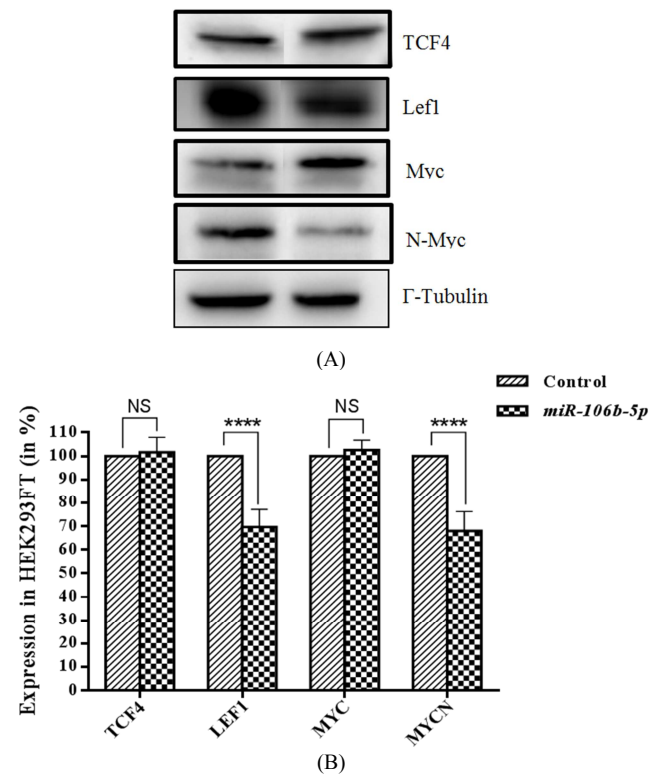

Figure 2. *(A) Western blot analysis of the levels of Wnt pathways related proteins Tcf4, Lef1, Myc and N-Myc in HEK293FT cells after overexpressing miR-106b-5p. House keeping gene γ-Tubulin is used as a control. (B) Percentage expression of Tcf4, Lef1, Myc and N-Myc in HEK293FT after overexpressing miR-106b-5p. These results are representative of at least three independent experiments. p < 0.0001, NS: No significance.*

Western Blot analysis of HEK293FT cells further revealed that miR-106b-5p overexpression decreased the expression of LEF1 and MYCN as compared with controls (Figure 2A). On quantitating the endogenous levels of these genes using BioRad image lab software, it is observed that levels of LEF1 and MYCN are reduced significantly whereas, there was no significant effect on TCF4 and MYC expression (Figure 2B). Together, these results indicate that miR-106b-5p targets LEF1 and MYCN directly.

4. Discussion

Wnt/β-catenin signaling controls fundamental cellular processes during tissue homeostasis, including proliferation, and aberrant activation of this pathway is implicated in a wide range of human cancers [1-3]. Aberrant activation of Wnt/β-catenin signaling results in enhanced cell growth and malignant cellular transformation. Although Wnt/β- catenin signaling is frequently activated in many carcinomas, causes of its activation are not well understood. Oncogenic β-catenin mutations, inactivating APC mutations, upregulation of frizzled-type receptors and/or other alterations in Wnt signaling pathway are most common and are supposed to play important roles in malignancies. Upon Wnt activation, accumulated β-catenin enters the nucleus and binds to TCF/lymphoid-enhancer-factor family transcriptional factors to induce target gene expression [11-13]. A key event in both Wnt signaling transduction and cancer cell proliferation is the regulation of β-catenin stability and activity. LEF1 acts as a tumor suppressor in rhabdomyosarcomas, leukemia and acute

lymphoblastic leukemia (ALL) [39-41].

miRNAs, a class of small regulatory RNA molecules that negatively regulate target mRNAs in a sequence-specific manner, have been demonstrated to play important roles in multiple biological processes, such as cellular differentiation, proliferation, oncogenesis, angiogenesis, invasion and metastasis, and can function as either tumor suppressors or oncogenes. Recent evidences indicate that miR-106b, a member of the miR-17/92 cluster, participates in the development and progression of human cancers, such as breast, colon, kidney, gastric, lung cancer and HCC [43-46].

Through bioinformatics analysis, the LEF1, context dependent tumor suppressor gene, was indicated as a theoretical miR-106b target gene. We were able to demonstrate LEF1 as a bonafide target of miR-106b-5p using Luciferase Assay. Western blotting analysis showed that exogenous miR-106b-5p expression reduces the level of APC protein. Together it indicates that LEf1 downregulation is mediated by miR-106b-5p through the its 3′- UTR. The biological function of miR-106b in protection against apoptosis and in cell survival, is a topic of further study in our laboratory.

5. Conclusion

To conclude, we have showed that miR-106b, an oncogenic miRNA, directly targets 3'UTR of LEF1. We have also shown the miRNA-mRNA interaction between miR-106b-5p and 3' UTRs of FZD4, MNT and MYCN. LEF1 and NMYC are, therefore, novel and critical targets of miR-106b. Therefore, miR-106b might be a potential therapeutic target for Wnt activated cancers. The role of this oncomir in the pathogenesis of such cancers still requires more in-depth analysis.

Acknowledgements

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