hsa-miR-543 Acts as a Tumor Suppressor by Targeting NMYC

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Abstract: Cancer is a complex human disease involving de-regulation of one or many developmental pathways. Aberrant activation of canonical Wnt signaling pathway, one of the most important developmental pathways, is a common cause of various carcinomas. Therefore, it is possible that potential cancer drugs can be developed by targeting the different nodal points of this signaling pathway. MYCN is a transcription factor of MYC family proto-oncogene. N-Myc over expression is known to be associated with various childhood tumors like neuroblastomas, medulloblastomas and prostate and lungs cancers in adults. MicroRNAs are short non-protein coding RNAs that bring about translational repression of the target gene by binding to its 3'UTR. Reports show that microRNAs play a significant role in carcinogenesis by acting as oncogenes or tumor suppressors. Oncogenic potential of hsa-miR-543 has been shown in prostate and cervical cancers, whereas, it tumor suppressive role has been reported in gliomas and colorectal cancers. Neuroblastoma patients show allelic loss of chromosome 14q, where miR-543 is located indicating the possibility of miR-543 playing an important role in neuroblastoma progression and prognosis. In the current study, we demonstrated that over-expression of miR-543 down-regulates the endogenous expression of N-Myc in HEP293FT cells. Also, it is shown to target FZD4, thereby, indirectly affecting the expression of other downstream genes of Wnt signaling including CTNNB1, TCF4 and LEF1. Therefore, our results suggest that miR-543 plays a significant role in suppressing the carcinomas resulted due to the over-expression of N-Myc and/or activation of Wnt pathway and may prove to be a potential target for novel cancer therapy.

Keywords: WNT Signaling Pathway, MYCN Proto-oncogene (MYCN), hsa-miR-543

1. Introduction

Cancer is one of the most complex dynamic human diseases. Despite of extensive research, very little is understood about the physics underlying human carcinogenesis [1]. Cancer is viewed as a multi-stage disease caused by the accumulation of genetic alterations in tumor suppressor genes and/or oncogenes. Aberrant regulation of the Wnt/β-catenin signaling pathway is a prevalent theme in cancer biology. Reports show activation of the Wnt/β-catenin signaling pathway plays an important role in human tumorigenesis [2-4]. Therefore, many components of this signaling pathway may serve as rational targets of cancer drug development.

Wnt proteins are a large family of secreted glycoproteins with atleast 19 known human members [5]. Wnt signals are transduced by atleast two distinct pathways: canonical Wnt/β-catenin pathway and the β-catenin independent non-canonical pathway. The non-canonical Wnt signaling is very diverse and is still evolving into more and more branches [6]. Canonical Wnt signaling initiated by the secreted wnt proteins, which bind to a class of seven-pass transmembrane receptors encoded by the frizzled genes [7-9]. Activation of the receptor leads to the phosphorylation of the dishevelled protein which, through its association with axin, prevents glycogen synthase kinase 3β (GSK3β) from phosphorylating critical substrates [10-14]. The GSK3β substrates include the negative regulators axin and APC, as well as β-catenin itself [15-17]. Unphosphorylated β-catenin escapes recognition by
β-TRCP, a component of an E3 ubiquitin ligase, and translocates to the nucleus where it engages transcription factors such as TCF and LEF [18-20].

Abnormal activation of the Wnt/β-catenin signaling pathway is associated with various types of cancers [21-23]. Given the complexity of Wnt/β-catenin signaling pathway, it is possible that potential cancer drugs can be developed by targeting the different nodal points of this signaling pathway. Deregulation of the canonical Wnt/β-catenin signaling pathway, mostly by hyper-activation of β-catenin caused by the over expression of Wnt or mutation of CTNNB1 (the gene which encodes β-catenin) is a common cause of carcinoma [24-25].

MYCN, transcriptional factor and member of the MYC family of proto-oncogenes, is known for controlling fundamental processes during embryonal development. The MYCN protein is situated downstream of several signaling pathways promoting cell growth, proliferation and metabolism of progenitor cells in different developing organs and tissues [26-27]. Deregulated MYCN signaling supports the development of several different tumors, mainly with a childhood onset, including neuroblastoma, medulloblastoma, rhabdomyosarcoma and Wilms’ tumor, but it is also associated with some cancers occurring during adulthood such as prostate and lung cancer. In neuroblastoma, MYCN-amplification is the most consistent genetic aberration associated with poor prognosis and treatment failure [28-29]. Targeting MYCN has been proposed as a therapeutic strategy for the treatment of these tumors and great efforts have allowed the development of direct and indirect MYCN inhibitors with potential clinical use.

Cancer studies have been traditionally focused on protein-coding genes, considering these as the principle effectors and regulators of tumorigenesis. Recent advances have brought small non-protein-coding RNAs (microRNAs) into the spotlight, which regulate gene expression at the post-transcriptional level. MicroRNAs are endogenous, short (18-24 nucleotides), non-protein-coding RNAs that regulate cell processes by binding to the 3’ untranslated region (UTR) of mRNAs resulting in its translational repression [30]. To date, more than 500 miRNAs have been identified in human cells which have been shown to regulate a wide array of cellular functions, ranging from cell proliferation, differentiation, death and stress resistance [31]. Most microRNA expression analyses of human cancers show that miRNAs are deregulated in cancer. miRNAs may function as tumor suppressors, oncogenes, or in some cases, both. Increased miRNA expression in cancer may be associated with down-regulation of tumor suppressors while miRNAs that are reduced in cancer may normally suppress oncogenes. Therefore, the study of such miRNAs which target the deregulated tumor suppressor genes and/or oncogenes in canonical Wnt signaling pathway may be helpful in the development of specific cancer therapy.

miR-543, located on chromosome 14q. 32, is known to promote cell proliferation in prostate cancers [32] and cervical cancers [33]. It has been reported as tumor suppressor in gliomas [34] and colorectal carcinomas [35]. Reports show an allelic loss of long arm of chromosome 14 (14q) in neuroblastomas, thereby, repressing the miRNAs located on it including miR-543 [36-38]. This suggests a probable role of miR-543 in neuroblastoma progression and prognosis. Thus, it is particularly interesting to identify role of miR-543 in one of the most important developmental pathway, Wnt/β-catenin signaling, which may help in developing targeted therapies in future.

2. Materials and Methods

2.1. Cloning miR-543 in Mammalian Expression Vector

Genomic region encoding for miR-543 was PCR amplified using Taq DNA Polymerase. Genomic DNA extracted from peripheral blood lymphocytes of a healthy individual was used as a template for PCR. Set of primers was synthesized and obtained from Sigma Genosys. Amplified fragment of miR-543 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.

2.2. Cloning 3’ UTRs of WNT Pathway Genes in Luciferase Reporter Vector

Genomic regions for 3’ UTRs of WNT pathway genes namely, MYC, MYCN, CTNNB1, TCF4, FZD4, BTRC and ERBB4 were PCR amplified using Taq DNA Polymerase. Genomic DNA extracted from peripheral blood lymphocytes of a healthy individual was used as a template for PCR. Set of primers was synthesized and obtained from Sigma Genosys. 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.

2.3. Transient Transfection of miR-543 and 3’UTRs in HEK 293FT Cell Line

miR-543 was co-transfected with 3’ UTRs in HEK293FT cells along with EGFP (for normalization) using CaCl2 and BES Buffer. DNA was taken in the ratio of 4:1:1 for miR-543: 3’ UTR: pEGFPN1 respectively. As negative controls, empty plasmid vectors for miR-543 and/or 3’ UTR were used. Protein was extracted 72hrs of transfection.

2.4. Luciferase Reporter Assay

After 72hrs of transfection proteins were extracted and each protein sample was assayed in triplicates on Mithra’s 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.

2.5. Western Blotting

miR-543 was transiently transfected using CaCl₂ and BES Buffer in HEK293FT cells to check the 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 MYC (SantaCruz Biotechnology), MYCN (SantaCruz Biotechnology), CTNNB1 (Sigma), TCF4 (Abcam) and LEF1 (Abcam). 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

To explore the tumor-suppressive roles of miR-543, we examined the putative downstream targets of miR-543 by three in silico prediction algorithms (miRanda, TargetScan and miRWalk). Several in silico prediction algorithm-identified Wnt pathway genes including MYC, MYCN, CTNNB1, TCF4, FZD4, BTRC and ERBB4 which contain putative binding sites for miR-543 in their 3′UTRs, were chosen for further investigation. First we PCR amplified the genomic sequence encoding for miR-543 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-543 binding sites into the Luciferase Reporter vector (pLUC). Each of the cloned UTR then was co-transfected with a miR-543 expression plasmid into HEK293FT. Dual-luciferase reporter assays revealed that the luciferase activities of MYCN and FZD4 significantly reduced in HEK293T but there was no effect on MYC, CTNNB1, TCF4, BTRC and ERBB4 (Figure 1) upon miR-543 overexpression. This data demonstrate that MYCN and FZD4 are direct the targets of miR-543.

Western Blot analysis of HEK293FT cells further revealed that miR-543 overexpression decreased the expression of MYCN, CTNNB1, TCF4 and LEF1 compared with controls (Figure 2A). On quantititating the endogenous levels of these genes using BioRad image lab software, it is observed that levels of MYCN, CTNNB1, TCF4 and LEF1 are reduced significantly whereas, there was no significant effect of MYC expression (Figure 2B). Together, these results indicate that miR-543 targets MYCN directly and CTNNB1, TCF4 and LEF1 indirectly.

Figure 1. MYCN is a direct downstream target of miR-543. Luciferase reporter assay analysis of the effects of miR-543 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 < 0.0001.
4. Discussion

It has been reported that miR-543 promotes prostate cancer [32] and cervical cancer [33] but functions as a tumor suppressor in gliomas [34] and colorectal carcinomas [35]. Thus, it can be said that miR-543 may function as a tumor suppressor or oncogene in a context-dependent manner; therefore, the discrepancy that miR-543 exhibits opposing effects in different tumors needs further investigation. Using *in silico* analysis and luciferase reporter assays, we identified N-Myc as a new direct target of miR-543. Thus, the present study shows that miR-543 targets MYCN directly thereby suppressing its expression in HEK293FT cells suggesting its tumor suppressive role in cancers resulted due to over expression of N-Myc. Also, we have shown that miR-543 overexpression results into reduced endogenous expression of several downstream genes of Wnt pathway signaling including CTNNB1, LEF1 and TCF4 possibly resulted due to direct down regulation of FZD4 by miR-543. MYCN is an oncogene that was found to be aberrantly activated in many carcinomas including neuroblastoma and medulloblastoma along with down regulated miR-543 [36-38]. Therefore, it would be interesting to study the role of miR-543 in these cancers along with other Wnt related carcinomas.

In conclusion, our study highlights a pivotal role for miR-543 as a tumor suppressor by directly targeting MYCN and indirectly affecting the expressions of downstream Wnt pathway genes like CTNNB1, TCF and LEF1 by targeting FZD4. Further investigations are needed to determine the role of mir-543 in pathogenesis of Wnt pathway driven cancers as it may serve as a novel diagnostic and prognostic biomarker. Also it may prove helpful to develop target based therapy in various cancers.

5. Conclusion

In the present investigation we have found that MYCN is a novel direct target of miR-543. Our study shows that over expression of miR-543 in HEK293FT cells affects the expression of Wnt pathway genes. MYCN is significantly down regulated on exogenous miR-543 over expression. This result was again confirmed by comparing the endogenous levels of MYCN using western blotting analysis. This proves mRNA-miRNA interaction between MYCN and miR-543 indicating that miR-543 is a direct target of MYCN.

We have found that there is a significant decrease in the expression of FZD4 on miR-543 over expression indicating that miR-543 targets FZD4 too. But further confirmation by western blotting needs to be done.

Our study highlights a pivotal role for miR-543 as a tumor suppressor by directly targeting MYCN and indirectly affecting the expressions of downstream Wnt pathway genes like CTNNB1, TCF and LEF1 by targeting FZD4. Further investigations are needed to determine the role of mir-543 in pathogenesis of Wnt pathway driven cancers as it may serve as a novel diagnostic and prognostic biomarker. Also it may prove helpful to develop target based therapy in various cancers.

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