Gene 552 (2014) 255–264

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

miR-15/16 complex targets p70S6 kinase1 and controls cell proliferation in MDA-MB-231 breast cancer cells

M. Janaki Ramaiah [a,c,1], A. Lavanya [a,1], Mohsen Honarpisheh [a], Mojtaba Zarea [a], Utpal Bhadra [b], Manika Pal Bhadra [a,*]

[a] Centre for Chemical Biology, Indian Institute of Chemical Technology (IICT), Tarnaka, Hyderabad, India
[b] Centre For Cellular and Molecular Biology (CCMB), Uppal Road, Hyderabad, India
[c] School of Chemical and Biotechnology, SASTRA University, Tirumalaisamudram, Thanjavur, India

ARTICLE INFO

Article history:
Received 24 June 2014
Received in revised form 18 September 2014
Accepted 22 September 2014
Available online 26 September 2014

Keywords:
miR-15
miR-16
RPS6KB1
mTOR
MDA-MB-231

ABSTRACT

Background: MicroRNAs are small non-coding RNAs that regulate post-transcriptional mRNA expression by binding to 3′ untranslated region (3′-UTR) of the complementary mRNA sequence resulting in translational repression and gene silencing. They act as negative regulators of gene expression and play a pivotal role in regulating apoptosis and cell proliferation. Studies have shown that miRNAs interact with p53 by regulating the activity and function of p53 through direct repression or its regulators. Mammalian target of rapamycin (mTOR) is an evolutionary conserved check point protein kinase that plays a major effect in the control of cell division via protein synthesis regulation. mTOR regulates protein synthesis through phosphorylation and inactivation of 4E-BP1 and through phosphorylation and activation of S6 kinase 1 (S6K1). These two downstream effectors of mTOR control cell growth and metabolism. In mammals, mTOR protein kinase is the central node in the nutrient and growth factor signaling and p53 plays a critical role in sensing genotoxic stress. Activation of p53 inhibits mTOR activity, which in turn regulates its downstream targets providing a cross talk among both the signaling machinery. MicroRNA-15 and 16 belong to a common precursor family and are highly conserved. Deletion or downregulation of these two microRNAs has been shown to accelerate cell division by modulating the expression of the genes involved in controlling cell cycle progression. These microRNAs may function as tumor suppressors and act on the downstream targets of p53 signaling pathway. To have a better insight of the role of miR-15/16 in regulating the cross talk of p53 and mTOR, we performed an in depth study in MDA-MB-231 breast cancer cells by performing a gain-of-function analysis with lentiviral plasmids expressing microRNA-15 and 16.
Methods: The effect of individual microRNAs on RPS6KB1 was examined by using 3′-UTR clones via luciferase based assays. The cell cycle effects were observed by flow-cytometric analysis. Reverse transcription PCR was used to explore the expression of mTOR and RPS6KB1 in cells transfected with miR-15/16.
Results: Overexpression of miR-15/16 led to inhibition of cell proliferation causing G1 cell cycle arrest as well as caspase-3 dependent apoptosis. Forced expression of miR-15/16 might lead to decrease in mRNA level of RPS6KB1, mTOR. The effect was a complete reversal after treatment with anti-miRs against miR-15/16 proving the specificity of the expression. In addition, the dual luciferase reporter assays indicated a clear decrease in luciferase gene expression in cells transfected with lentiviral based miR-15 and 16 plasmids indicating that miR-15/16 directly targets RPS6KB1 through its 3′-UTR binding. Further, these microRNAs also inhibit epithelial to mesenchymal transition (EMT) by targeting key proteins such as Twist1 and EZH2 clearly demonstrating its crucial role in controlling cell proliferation.
Conclusion: This study suggests that exogenous microRNA-15/16 can target RPS6KB1, control cell proliferation and cause apoptosis in caspase-dependent manner even in the absence of functional p53.

1. Introduction

Cell growth and proliferation require an intricate coordination between signals from intracellular and extracellular environments. Pertubations in this coordination result in cancer. Prevention or elimination of cancer progression could have a major impact on cancer

Abbreviations: 3′-UTR, 3′ untranslated region; EMT, epithelial to mesenchymal transition; mTOR, mammalian target of rapamycin; miR-15, microRNA-15; miR-16, microRNA-16.

* Corresponding author at: Centre for Chemical Biology, Indian Institute of Chemical Technology (IICT), Uppal Road, Hyderabad, India.
E-mail addresses: janaki7777@gmail.com (M. Janaki Ramaiah), lavanyaiict@gmail.com (A. Lavanya), mohsen.honarpisheh@gmail.com (M. Honarpisheh), moj.zarea@gmail.com (M. Zarea), Utpal@ccmb.res.in (U. Bhadra), manikapb@gmail.com, manika@iict.res.in (M.P. Bhadra).
[1] Joint first authors.

http://dx.doi.org/10.1016/j.gene.2014.09.052
0378-1119/

mortality. MicroRNAs (miRNAs) are endogenously expressed small non-coding RNAs that play a regulatory role in post-transcriptional gene products (Hwang and Mendell, 2006). miRNA binds with 3′UTR of target mRNAs leading to its degradation and translational repression. Aberrant expression of miRNA is observed in various human tumors. Studies have also shown that regulation of the expression of specific miRNAs could serve as an additional mechanism for tumor suppression. Therefore, targeted therapies that use microRNAs to stop metastasis may be an effective approach to treat cancer.

Genome instability is a hallmark for cancer. One of the major mechanisms for controlling tumorigenesis is to inhibit mTOR signaling by several check point proteins. S6 Kinase 1 (S6K1) is a major mTOR downstream signaling molecule that regulates fundamental cellular processes including transcription, translation, protein and lipid synthesis, cell growth, size and cell metabolism (Magnuson et al., 2012). Several isoforms of S6K1 are overexpressed in breast cancer cell lines and tumors. S6K1 gene (RPS6KB1) is located in human chromosome 17q23, a region that is amplified in 20% of primary breast cancers (Courjal and Theillet, 1997; Kim et al., 2009), non-small cell lung cancer (Zhang et al., 2013), HCC patients (Li et al., 2012), HIV/EBV + diffuse large B-cell lymphoma (Zhao et al., 2013), and gastric cancer (Sun et al., 2014) and is associated with poor prognosis. Studies by Lai et al. (2010) have revealed that the mTOR-S6K1 was found to be a novel regulator of p53 in DNA damage response and plays a key role in tumorigenesis (Lai et al., 2010). It is reported that microRNAs regulate cell growth and apoptosis in which miRNAs themselves function as oncogenes or tumor suppressors (Cheng et al., 2005). MicroRNA-15 family includes six highly conserved miRNAs (miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195, miR-497) that are clustered on three separate chromosomes (Porrello et al., 2011). Deletion of miR-15a/16-1 accelerates cell growth through modulation of cell cycle proteins such Cyclin E and Cyclins D1, D3 and Cdk6 involving E2F (Calin et al., 2002; Wang et al., 2009; Lerner et al., 2009). It has been reported that miR-15 and miR-16 are regulated by p53 (Brosh et al., 2008; Suzuki et al., 2009; Boominathan, 2010). Since E2F1 activates p53 via numerous pathways (Polager and Ginsberg, 2009), it is possible that E2F1 regulation of miR-15 and miR-16 expression is mediated by p53. Studies by Klein et al. (2010) have demonstrated that these microRNAs negatively regulate Bcl2 oncogene, modulate proliferation and promote apoptosis. Till date, there is no report of its role in understanding the mechanism of action in the signaling pathway. Thus understanding the link of both these miRNAs in regulating the major signaling players of ATM check point pathway with respect to p53 may enable to develop better therapeutics. Reports on drug discovery have indicated that mTOR inhibitors have limited success (Sridharan and Basu, 2011). The role of miR-15/16 in breast cancer targeting RPS6KB1 has not been reported. Here, in this study we examined the effects of miR-15/16 on RPS6KB1 gene regulation and its action on cell proliferation and apoptosis.

2. Materials and methods

2.1. Cell culture

Human breast carcinoma cells (MCF-7, MDA-MB-231), lung cancer cells (A549), cervical cancer cells (HeLa), neuroblastoma (IMR-32, SK-N-SH, Neuro-2a) were purchased from American Type culture collection centre. MCF-7, A549, HeLa, IMR-32 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), supplemented with 2 mM glutamax (Invitrogen), 10% fetal calf serum and 100 U/ml penicillin and 100 mg/ml streptomycin sulfate (Sigma). EMEM was used for growing SK-N-SH and Neuro-2a cell lines and RPMI was used for MDA-MB-231 cells. All cell lines were grown on 60 mm cell culture dishes and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in the incubator.

2.2. Cloning of 3′-UTR of RPS6KB1

RNA was isolated from MCF7 cell line using Trizol-Chloroform (Invitrogen). cDNA was synthesized using Invitrogen SuperScript® III by following manufacturer's protocol. Platinum® Taq DNA Polymerase having high Fidelity was used for PCR (Thermo scientific). 3′-UTR region of p70S6K1 (NCBI Reference Sequence: NM_003161.2) containing full length as well as partial1 (P1) and partial 2 (P2) was cloned in psiCheck2 vector (Promega) using XhoI and NotI restriction enzymes. Full length (FL) region comprises of 3439 bp, and partial 1 (P1) and partial 2 (P2) are 1213 bp and 1562 bp respectively. Primers used for cloning were listed in Table 2.

2.3. Luciferase activity assay

Cells were seeded in 6 well plates 24 h prior to transfection. Co-transfection of plasmid DNAs (i.e. 2 μg of 3′-UTR constructs of RPS6KB1 cloned in psiCHECK2 vector and 2 μg lentiviral based microRNA expression plasmids [miR-15, miR-16, miR-17], System Biosciences) was performed using lipofectamine 2000. Luciferase assays were performed 48 h after transfection using dual luciferase assay substrate (Promega). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. Here the ratio of F/R readings will provide the expression of particular gene in microRNA transfected cells. Three independent experiments were performed in triplicate.

2.4. P53 siRNA transfection

P53 siRNA (Dharmacon) transfection was performed by the Lipofectamine RNAiMAX transfection reagent following the manufacturer's method. Briefly MCF-7 cells were plated in 6-well plates at density of 1, 00,000/well and transfected with *p53* siRNA at a final concentration of 25 nM in serum/antibiotic free media. Scrambled siRNA was used as control. After 6 h of transfection, the media was replaced with complete growth media following which the cells were allowed to recover for 48 h and assayed for microRNA expression using microRNA specific primers obtained from kit [RA-610A-1-System Biosciences].

2.5. P53 overexpression study

Briefly MDA-MB-231 cells were plated in 6-well plates at a density of 1, 00,000/well and transfected with p53 overexpression plasmid (2 μg of CMV-p53) or vector in serum/antibiotic free media. After 6 h of transfection, the media was replaced with complete growth media following which the cells were allowed to recover for 48 h. Cells were extracted 48 h after transfection and then the RNA isolated was used for microRNA and gene expression studies.

2.6. Reverse transcription PCR (RT-PCR)

Total RNA was extracted using RNeasy mini kit (Qiagen) and reverse transcribed into cDNA using superscript II reverse transcriptase

Table 1
Primers used in RT-PCR experiment.

S. no	Primer name	Sequence 5′–3′	Product size
1.	RPS6KB1 FW	CCTGAAGCCGGAGAATATCA	186 bp
2.	RPS6KB1 RV	AAACTCCACCAATCCACAGC	
3.	mTOR FW	CCAACAGTTCACCCTCAGGT	208 bp
4.	mTOR RV	GCTGCCACTCTCCAAGTTTC	
5.	Twist1 FW	GTCCGCAGTCTTACGAGGAG	145 bp
6.	Twist1 RV	CTAGTGGGACGCGGACAT	
7.	EZH2 FW	AGGACGGCTCCTCTAACCAT	179 bp
8.	EZH2 RV	CTTGGTGTTGCACTGTGCTT	
9.	GAPDH FW	GGG AAG GTG AAG GTC GGA GT	110 bp
10.	GAPDH RV	TTG AGG TCA ATG AAG GGG TCA	

FW = forward primer, RV = reverse primer, bp = base pairs.