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Micro RNA-214 contributes to proteasome independent downregulation of beta catenin in Huntington's disease knock-in striatal cell model STHdhQ111/Q111

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ABSTRACT

Role of beta catenin in Huntington's disease (HD) is not clear. Previous studies on HD reported varied levels of beta catenin. In the present study we showed that beta catenin is post transcriptionally down-regulated in mutant huntingtin knock-in cell model STHdhQ111/Q111. This in turn leads to decreased level of wnt/beta catenin responsive genes. We observed that Gsk3beta or Gsk3beta (phospho Ser 9) is unaltered in HD and this down-regulation of beta catenin is independent of proteasomal degradation. Finally, we showed that the overexpression of miR-214 leads to the down-regulation of beta catenin at protein level only and reduces its transcriptional activity. We concluded that, miR-214 contributes to the processes that result in proteasome independent post transcriptional down-regulation of beta catenin in STHdhQ111/Q111, probably through inhibition of protein synthesis from beta catenin mRNA.

1. Introduction

Huntington's disease (HD) is a fatal neurodegenerative disease in which protein huntingtin is mutated with an expanded poly glutamine stretch (more than 36 glutamine residues) in its N-terminal region due to CAG trinucleotide expansion in exon 1 of huntingtin gene*HTT* [1]. Medium spiny neurons, such as striatal neurons of basal ganglia of the brain are mostly affected in this disease [2]. Overtime, HD results in cognitive and motor functional abnormalities like speech, thought and psychiatric problems and involuntary muscle movements [2–4]. Mutated huntingtin protein forms aggregates through intermediate monomer/oligomers. It remains unknown whether the visible aggregates or intermediate oligomers result in neuronal dysfunction and death.

Beta catenin is an 88 kDa multifunctional protein which on the one hand acts as a cell-cell adhesion molecule in adherens junction and on the other it is also employed as intracellular signal relaying molecule in the wnt/beta catenin signaling [5]. Thus beta catenin is involved in two distinct cellular processes.

Wnt/beta catenin signaling is a cell survival pathway that is mostly involved in animal development and is known to undergo changes in disease processes [6]. This signaling is triggered by extracellular wnt ligands. When wnt ligands are absent, cytoplasmic pool of beta catenin is captured by a protein complex called 'beta catenin destruction complex' that includes Gsk3beta, APC, Axin etc. Here, Gsk3beta phosphorylates beta catenin which is then ubiquitinated and eventually degraded in proteasome [7]. But when wnt signal ligands bind to receptor frizzled and co-receptor LRP5/6, intracellular signaling cascade dismantles beta catenin destruction complex. Now free cytoplasmic beta catenin enters the nucleus and replaces repressor protein Groucho from transcription activator LEF/TCF and together with LEF/TCF, beta catenin acts as transcription co-activator to switch on the expression of wnt responsive genes [8,9].

Studies on HD done previously in cell models and tissues have demonstrated full length mutant *HTT* expressing at physiological level [10,11] or mutant *HTT* exon 1 expressing at physiological level [12] or mutant *HTT* exon 1 overexpressed in a cell [13]. Results obtained in these studies are contradictory in respect of beta catenin. It has been reported that beta catenin is decreased [12], unaltered [11] or increased [10,13] in HD. Again, studies documenting

Abbreviations: HD, Huntington's disease; miR, micro RNA; Gsk3beta, glycogen synthase kinase 3 beta; APC, adenomatous polyposis coli; LRP5/6, low-density lipoprotein receptor-related protein 5/6; LEF, lymphoid enhancer binding factor; TCF, T-cell factor; STHdhQ111/Q111, Q111 cell; STHdhQ7/Q7, Q7 cell.

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high beta catenin level in HD also proposed varied reasons like Akt activation [10] or impairment of ubiquitin-proteasome system [13]. On the other hand the study that showed association between mutant *HTT* and decreased beta catenin did not adequately elucidate the reason behind such observation [12]. Similarly, no elaborate discussion on the underlying mechanism was provided in the study by Reis and colleagues observing unchanged beta catenin levels [11]. Moreover, there was no significant follow up (after Carmichael and colleagues [12]) of the status of wnt/beta catenin signaling activity in terms of TCF mediated gene transcription which is mediated by beta catenin itself. Hence, our aim was to further investigate the status of beta catenin and downstream transcriptional activity of wnt/beta catenin signaling in Huntington's disease cell model in normal condition and under exposure to different treatments and to throw light on the probable reasons behind it. In this study, we used a well established mutant huntingtin knocked in murine cell model STHdhQ111/Q111 as HD cell model. Briefly, this is a conditionally immortalized cell line expressing at physiological level a full length HTT gene (having 111 CAG trineucleotide repeat) corresponding mutant huntingtin protein having an N-terminal poly glutamine tract with 111 glutamine (Q) residues [14]. As a control cell line, we used STHdhQ7/Q7 which is also a conditionally immortalized murine cell line which has 7 CAG repeat HTT knock-in corresponding to wild type huntingtin protein with an N-terminal tract with 7 glutamine residues [10].

Here we reported decreased level of beta catenin protein in Huntington's disease cell STHdhQ111/Q111 as compared to normal cell STHdhQ7/Q7 and studied downstream activity of wnt/beta catenin signaling in terms of target gene transcription. We also probed the involvement of Gsk3beta and proteasome in this altered beta catenin level. We are the first to report micro RNA involvement in beta catenin level and wnt/beta catenin signaling activity in HD cell model. We showed the involvement of micro RNA-214 in the observed low beta catenin level and low downstream activity of wnt/beta catenin signaling in STHdhQ111/Q111.

2. Materials and methods

2.1. Antibodies and reagents

A list of antibodies and other reagents used in this study is given in Supplementary Table S1.

2.2. Cell culture and treatments

STHdhQ7/Q7 and STHdhQ111/Q111 cells (Now onwards these cells will be referred to as Q7 and Q111 respectively except in the result headings) were grown in DMEM supplemented with 10% fetal bovine serum, penicillin/streptomycin and 400 μg/ml G418 and incubated at 33 °C in a humidified incubator with 5% CO2. For drug treatment, cells grown in 60 mm dish were washed with PBS and then fresh medium is added to cells and drug/s with indicated doses added to cells and incubated for desired time. Both the drugs (Lactacystin and LiCl) used in this study were dissolved in suitable vehicle (DMSO).For transfection (cells to be used in western blots), cells were grown in 60 mm dishes, washed twice with PBS, fresh medium was added and cells were transfected with 3ug pRNA-U61/Hygro empty vector control (Genescript, USA) or 3ug pRNA-U61-pre-miR-214 [15]and incubated for 72 h. Transfections were done with 10ul Lipofectamine2000 per dish.

2.3. Western blot

Cells were lysed and protein concentration in each lysate was measured by Lowry assay [16]. Approximately 10–30 μg of lysates for each sample were boiled with loading dye containing SDS and beta mercaptoethanol and resolved in 10% SDS- polyacrylamide gel, transferred to PVDF membrane, blocked with BSA and probed with appropriate primary antibody overnight at 4 °C. Actin was used as loading control. Blots were developed either by chemiluminescent method or by colorimetric method and integrated optical density of the bands were calculated using Image J software.

2.4. RNA isolation, cDNA synthesis and quantitative real time RT-PCR (qRT-PCR)

Total RNA from cell isolated with TRIzol reagent according to manufacturer's protocol. RNA was quantified in NanoDrop 2000 (Thermo Scientific). Total 1ug of RNA was reverse transcribed to cDNA using MuLv-Reverse Transcriptase and random hexamer primer and subjected to real time quantitative PCR analysis using SYBR Green mastermix on Eppendorf Mastercycler Ep Realplex (Eppendorf, Germany) using the primers of: Akt1, Gsk3b, Ctnnb1, Myc, Ccnd1, Jun, FosL1 (also called Fra 1) and Actb (beta-actin). Actb expression was taken as internal control.Ct values of targets normalized to that of reference (control) gene (Actb). $\mathbf{2}^{-\Delta\Delta\mathbf{Ct}}$method was used to calculate fold change in gene expression. A list of the sequences of the primers used in this study is shown in Supplementary Table S2.

2.5. Luciferase assay

For luciferase assay, TOPFLASH TCF-reporter plasmid was used. The plasmid contains two sets of three copies of wild type TCF binding sites upstream of Thymidine Kinase promoter and luciferase reporter gene. When wnt/beta catenin signaling pathway is active, then beta catenin translocates into the nucleus, associates with LEF/TCF transcription factors and switch on the expression of target genes, which in this assay is *Luc* (coding for firefly luciferase). So, TCF-reporter luciferase assay is a convenient method to detect the activity of beta catenin as transcription co-activator in the nucleus of the cell. Briefly, cells were grown in 24-well plate, transfected with 200 ng TOPFLASH TCF-reporter plasmid or co-transfected with 300 ng empty pRNA U61 vector or 300 ng pRNA-U61-pre-miR-214 and 200 ng TOPFLASH and incubated for 72 h. 5ug of protein from each sample was subjected to luciferase assay using luciferase reporter assay reagents (Promega) in Sirius Luminometer (Berthold detection systems). Fold changes (Relative Luciferase Activity) were calculated from obtained data in relative light units/sec (RLU/S).

2.6. Statistical analysis

Unpaired t-test was done for statistical analysis by comparing the mean values of two experimental groups using online tool GraphPad QuickCals.

3. Results and discussion

3.1. Beta catenin is post transcriptionally downregulated in STHdhQ111/Q111 cells resulting in decreased transcriptional activity of wnt/beta catenin signaling

Beta catenin level from whole cell extract was found to be significantly decreased in HD cell model Q111 as compared to normal control cell Q7 (Fig. 1A, B). This data was confirmed with a different type of beta catenin antibody (Supplementary Figure S1).

To identify the downstream transcriptional effect/s of decreased beta catenin, we used TOPFLASH TCF-reporter luciferase assay. For this, Q7 and Q111 cells were transiently transfected with TCF reporter plasmid TOPFLASH and 48 h after transfection, cells were

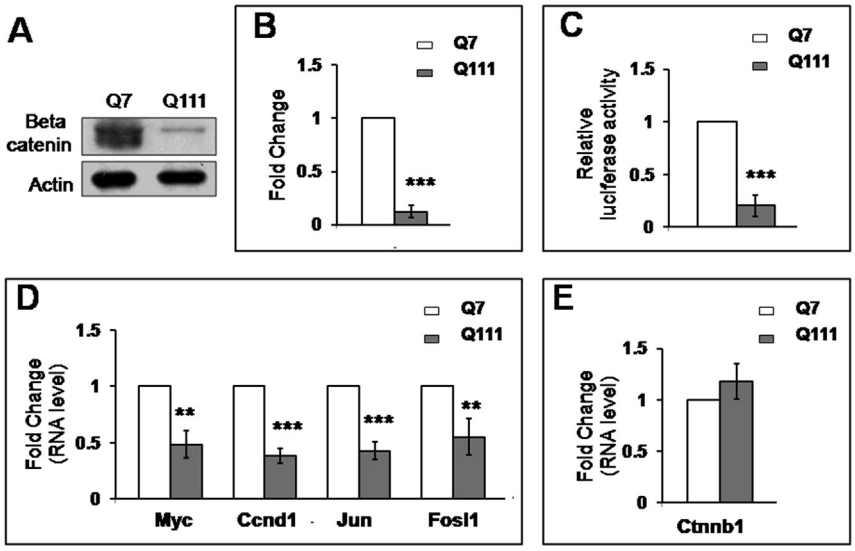

Fig. 1. Beta catenin was post transcriptionally downregulated in STHdhQ111/Q111and it resulted in lower transcriptional activity of wnt/beta catenin signaling in cell. (A) Western blot shows beta catenin levels in Q7 and Q111 cells. Intensity analysis is normalized to actin and displayed in bar diagram (n = 5) (B). (C) TCF reporter activity in Q7 and Q111 cells shown in terms of relative luciferase activity (n = 3). (D) Normalized fold changes of Myc, Ccnd1, Jun and Fosl1 (Fra 1) RNA levels in Q111 with respect to Q7, Actb (Actin) was the internal control (n = 3). (E) Normalized fold change of Ctnnb1 RNA level in Q111 with respect to Q7, Actb (actin) taken as internal control (n = 3). Error bar represents standard deviation. Statistical significance, $^{**}P < 0.01$, $^{***}P < 0.001$.

harvested and luciferase assay was performed. We observed that luciferase reporter activity was significantly decreased in Q111 cell as compared to that of Q7 cell (Fig. 1C). So, low level of beta catenin in Q111 cell correlated with the decreased beta catenin/TCF mediated transcriptional activity. This result indicated that decreased beta catenin level in Q111 cells might result in a lower expression of the downstream target genes.

We further checked the expression of some target genes of wnt/beta catenin signaling at RNA level. We observed that wnt/beta catenin target genes Myc [17], Ccnd1 [18,19], Jun [20], Fosl1 (also called Fra 1) [20] were downregulated in Q111 (Fig. 1D).

We wanted to check whether poor transcription of Ctnnb1 gene (codes for beta catenin) was the reason behind the decreased beta catenin level in Q111 cell. But we found that Ctnnb1 RNA levels in Q7 and Q111 are unaltered (Fig. 1E). This gives a clear impression that beta catenin is post transcriptionally downregulated by some mechanism in HD cell model Q111 that reduced the expression of wnt/beta catenin responsive genes.

3.2. Downregulation of beta catenin in STHdhQ111/Q111 is not by Gsk3beta dependent proteasomal degradation

A well known mechanism of post transcriptional downregulation of beta catenin in a cell is its degradation in proteasome in a Gsk3beta dependent manner. We tested if this mechanism was playing a role to downregulate beta catenin in Q111. Typically, the inhibition of proteasome by a proteasomal inhibitor drug should increase overall beta catenin level in the cell. Lactacystin is a cyclic amide and commercially available well known drug to inhibit proteasome which acts by inhibiting specific catalytic subunits in proteasome [21].So, in this study, both Q7 and Q111 cells were treated with standardized dose of 10uM Lactacystin for 0 h, 16 h, 24 h and 48 h. Beta catenin level increased in Q7 in time dependent manner and the result was statistically highly significant and the increments were very high in 24 h and 48 h treated samples (Fig. 2A, B). But in Q111, changes in beta catenin level upon lactacystin treatment were insignificant except only 48 h treated sample and the extent of increment in 48 h treated sample was also very low as compared to corresponding Q7 sample (Fig. 2C, D). So the proteasome inhibition data indicated that extreme post transcriptional downregulation of beta catenin as observed in Q111 is not because of its proteasomal degradation.

AS Gsk3beta is known to modulate proteasomal degradation of beta catenin, we tested the effect of Gsk3beta inhibition by Lithium Chloride (LiCl), a potent Gsk3beta inhibitor. Q7 and Q111 cells were treated with 10 mM LiCl for 24 h Gsk3beta inhibition by LiCl increased beta catenin significantly in Q7 cell (Fig. 2E, F) but not in Q111 (Fig. 2G, H). This observation indicated that the Gsk3beta hardly has any involvement in the observed downregulation of beta catenin in Q111 cell.

3.3. Gsk3beta and Akt level unaltered in STHdhQ7/Q7 and STHdhQ111/Q111

To confirm our data of proteasome and Gsk3beta inhibition, it was necessary to check the status of beta catenin degradation modulator Gsk3beta. Gsk3beta and Gsk3beta (phospho Ser 9) levels were unaltered in Q7 and Q111 (Fig. 3A, B) so was the Gsk3b RNA levels (Fig. 3C). As Gsk3beta levels (protein, phospho protein, RNA) were found unaltered in Q7 and Q111 cell and as Gsk3beta inhibition did not significantly increase beta catenin in Q111 but did so in Q7 cell, Gsk3beta seems not to have an instrumental role in extreme post transcriptional downregulation of beta catenin in Q111.

Akt indirectly modulates beta catenin degradation by inhibiting Gsk3beta by phosphorylating it on serine-9 position. So it was necessary to observe Akt status to validate the Gsk3beta and beta catenin data. We found no statistically significant change in total Akt protein, activated Akt (Akt (phospho Ser 473)) and Akt1 RNA levels in Q7 and Q111 (Fig. 3D–F). So the Akt status (especially Akt (phospho Ser 473)) support and is well correlated with unaltered Gsk3beta (phospho Serine 9) levels.

3.4. Micro RNA-214 overexpression downregulates beta catenin and its transcriptional activity (TCF mediated) in STHdhQ7/Q7 and STHdhQ111/Q111 cell

Since proteasomal inhibition did not increase beta catenin level significantly throughout the time points in Q111 cell unlike that in

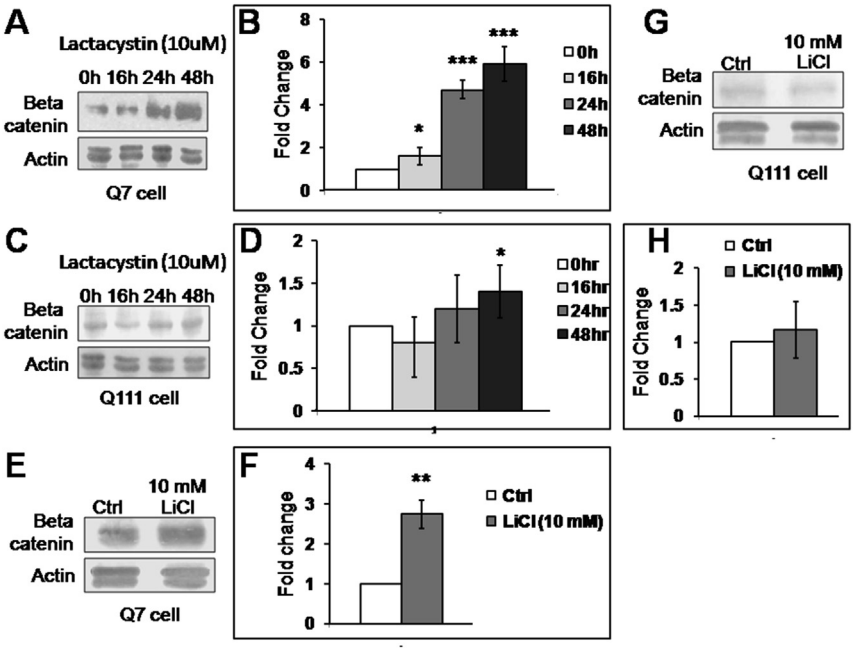

Fig. 2. Extreme downregulation of beta catenin in STHdhQ111/Q111 was not by Gsk3beta dependent proteasomal degradation. (A) Western blot of beta catenin in Q7 cell undergoing exposure of 10 μM lactacystin for 0 h, 16 h, 24 h and 48 h. Intensity analysis is normalized to actin and displayed in bar diagram (n = 3) (B). (C) Western blot of beta catenin in Q111 cell undergoing exposure of 10 μM lactacystin for 0 h, 16 h, 24 h and 48 h. Intensity analysis is normalized to actin and displayed in bar diagram (n = 3) (D). (E) Western blot image of beta catenin in control (vehicle DMSO treated) and 10 mM LiCl treated Q7 cell for 24 h. Intensity analysis is normalized to actin and displayed in bar diagram (n = 3) (F). (G) Western blot image of beta catenin in control (vehicle DMSO treated) and 10 mM LiCl treated Q111 cell for 24 h. Intensity analysis is normalized to actin and displayed in bar diagram (n = 3) (H). Error bar represents standard deviation. Statistical significance, *P ≤ 0.05, **P < 0.01, ***P < 0.001.

Q7 cell, indicating that proteasomal function in Q111 might have been impaired, at least to some extent by knocked-in poly-Q mutant huntingtin. A previous study has showed ubiquitin-proteasome system is globally impaired by poly-Q mutant huntingtin [22]. Proteasomal impairment should lead to accumulation of beta catenin [13], but we found the opposite which is downregulation of beta catenin in Q111 cell. So there must be some other mechanism behind this observation.

MicroRNAs (miRNAs) are small about 21 nucleotides long non-coding RNAs that can regulate gene expression by multiple ways such as by degrading the mRNA or inhibiting translation from mRNA without degrading the mRNA [23–25].

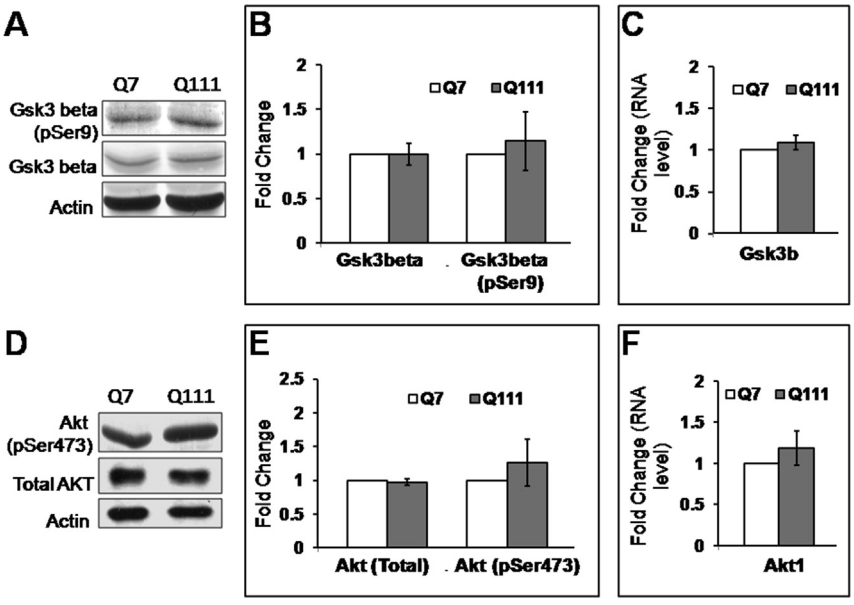

Fig. 3. Gsk3beta and Akt level were unaltered in STHdhQ7/Q7 and STHdhQ111/Q111. (A) Western blots of total and phosphorylated (Ser 9) Gsk3beta in Q7 and Q111 cells. Intensity analysis is normalized to actin and displayed in bar diagram (n = 3) (B). (C) Normalized fold change of Gsk3b RNA level in Q111 with respect to Q7, Actb (actin) taken as internal control (n = 3). (D) Western blots of total and phosphorylated (Ser 473) Akt in Q7 and Q111 cells. Intensity analysis is normalized to actin and displayed in bar diagram (n = 3) (E). (F) Normalized fold change of Akt1 RNA level in Q111 with respect to Q7, Actb (actin) taken as internal control (n = 3). Error bar represents standard deviation.

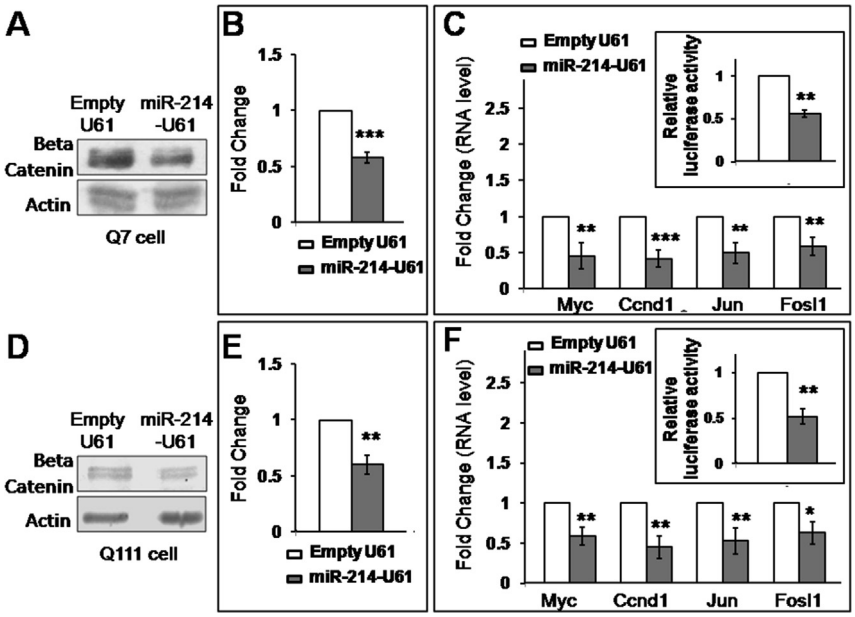

Fig. 4. MiR-214 overexpression downregulated beta catenin and its transcriptional activity (TCF mediated) in STHdhQ7/Q7 and STHdhQ111/Q111 cell. (A) Western blot shows beta catenin expression in empty U61 vector and miR-214-U61 plasmid transfected Q7 cell. 72 h post transfection samples were assayed. Intensity analysis is normalized to actin and displayed in bar diagram (n = 5) (B). (C) Normalized fold change of Myc, Ccnd1, Jun, Fosl1 (Fra 1) RNA levels in Q7 cells transfected with empty U61 vector and miR-214-U61 plasmid. Actb (actin) taken as internal control (n = 3). 72 h post transfection samples were analyzed. (C (Inset)) TCF reporter activity in terms of relative luciferase activity in Q7 cell transfected with empty U61 vector and miR-214-U61 plasmid cells shown here (n = 3). 72 h post transfection samples were assayed. (D) Western blot shows beta catenin expression in empty U61 vector and miR-214-U61 plasmid transfected Q111 cell. 72 h post transfection samples were assayed. Intensity analysis is normalized to actin and displayed in bar diagram (n = 3) (E). (F) Normalized fold change of Myc, Ccnd1, Jun, Fosl1 (Fra 1) RNA levels in Q111 cells transfected with empty U61 vector and miR-214-U61 plasmid. Actb (actin) taken as internal control (n = 3). 72 h post transfection samples were analyzed. (F (Inset)) TCF reporter activity in terms of relative luciferase activity in Q111 cell transfected with empty U61 vector and miR-214-U61 plasmid shown here (n = 3). 72 h post transfection samples were assayed. Error bar represents standard deviation. Statistical significance, *P ≤ 0.05, **P < 0.01, ***P < 0.001.

We tested our hypothesis whether upregulation of a beta catenin targeting miRNA was responsible for the observed downregulation of beta catenin in Q111 cell. A previous study on Q7 and Q111 cell reported alteration of some miRNAs in Q111 cell [26]. Among the miRNAs that were upregulated in Q111 cell compared to Q7 cell, miR-214 was increased more than tenfold in Q111 cell which was highest among the upregulated miRNAs in Q111 in that study [26]. Moreover online miRNA validated target database 'mirtarbase' (http://mirtarbase.mbc.nctu.edu.tw/) lists miR-214 as one of the experimentally validated miRNAs to target beta catenin. MiR-214 was previously documented to decrease beta catenin protein level in hepatocellular carcinoma cell lines [27,28]. For these reasons, we selected miR-214 in this study. Since miR-214 expression is low in Q7 than that of Q111 and beta catenin level is much higher in Q7 than that of Q111 cell, we first overexpressed miR-214 in Q7 cell to check whether miR-214 can reduce beta catenin protein level. Transient transfection with pRNA-U61-pre-miR-214 (designated as miR-214-U61 in figures) significantly decreased beta catenin protein level 72 h post transfection and also significantly reduced wnt/beta catenin signaling downstream transcriptional activity as determined in TCF-reporter luciferase assay (Fig. 4A–C (inset). Similar results were obtained in Q111 cell (Fig. 4D–F (inset). MiR-214 overexpression also reduced the expression of wnt/beta catenin responsive genes as observed in this study, viz.; Myc, Ccnd1, Jun and Fosl1 (Fra 1) in both Q7 cell (Fig. 4C (excluding the inset)) and Q111 cell (Fig. 4F (excluding the inset)). But, unlike beta catenin protein, the Ctnnb1 RNA level was not significantly changed upon miR-214 overexpression in Q7 and Q111 cell (Supplementary Figure S2), and this data is similar to the data which showed that Ctnnb1 RNA level in Q111 cell is no less than that of Q7 cell (Fig. 1E). So miR-214 upregulation is playing a significant role. Our data suggest that miR-214 has a significant contribution to the observed post transcriptional downregulation of beta catenin in Q111 cell, probably through translational inhibition.

In this study we reported that in our experimental setup, beta catenin was extremely downregulated in a well established Huntington's disease cell model Q111 that reduced transcriptional level of wnt responsive genes mediated by beta catenin/TCF. We showed that this extreme downregulation of beta-catenin level was purely a post-transcriptional phenomenon and was not mediated by Gsk3beta dependent proteasomal degradation of beta catenin. We further showed that miR-214 has a significant contribution to this observed downregulation of beta catenin in Q111 cell.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.137.

Transparency document

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