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Beta catenin is regulated by its subcellular distribution and mutant huntingtin status in Huntington's disease cell STHdhQ111/HdhQ111

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ABSTRACT

Dysregulation of gene expression at RNA and protein level is a hallmark of Huntington's disease (HD). Altered levels of microRNAs and beta catenin in HD were studied earlier; however, any direct involvement of full length, basally-expressing mutant huntingtin (Htt) remained to be elusive. Here we reported that the gain-of-function mutation of full-length basally-expressing Htt in HD cell Q111 (STHdhQ111/HdhQ111) upregulated microRNA-214 and decreased beta catenin & its transcriptional activity in an aggregate-independent manner. The result was quite opposite of the function of aggregate-forming mutant Htt fragment 83Q-DsRed. Here, we also reported an elevated level of beta catenin phosphorylation in Q111 cell compared to Q7 cell (SThdhQ7/HdhQ7). We showed that in Q111 cell (compared to Q7), beta catenin was more localized in the cytosol than that of the plasma membrane. This is significant as Gsk3beta phosphorylates beta catenin in the cytosol. Hence, for the first time, our study identified beta catenin localization and mutant Htt status as two key factors of beta catenin regulation in HD.

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1. Introduction

Huntington's Disease (HD) is an autosomal, dominant, neurodegenerative disorder characterized by involuntary muscle movements, behavioral changes and cognitive decline, which is initiated by huntingtin (*HTT*) gene mutation [1].

Beta catenin is a moonlighting protein and is involved in two different cellular functions: cell-cell adhesion and canonical Wnt signaling pathway [2]. Beta catenin is indirectly involved in cellular proliferation, division, cell fate and have proven role in cancer progression [3]. However, its role and regulation in HD are not clear as of now.

Previously, we reported proteasome-independent beta catenin downregulation in mouse HD cell model STHdhQ111/HdhQ111 (Q111) mediated by translational repression of Ctnnb1 (beta catenin mRNA) upon micro (mi)RNA-214 overexpression [4]. Since Q111 cell harbors basally-expressing full-length mutant Htt, this indicates a possible role of mutant Htt in beta catenin regulation in Q111 cell that remains to be elucidated.

Gsk3beta is a key enzyme that sequentially phosphorylates beta catenin on threonine 41, serine 37 and serine 33 residues [5]. However, Gsk3beta can only phosphorylate the free cytosolic beta catenin monomers. In the present work, we studied beta catenin phosphorylation in Q7 (harbors wild-type Htt) and Q111 cell. Since Gsk3beta levels and activities are unaltered in Q7 and Q111 cell [4], the study of subcellular distribution of beta catenin protein is important.

In this study, we aimed to find out the exact role of Htt expression pattern and Htt mutation in terms of its functionality and aggregate-formation in the regulation of beta catenin. We also studied whether altered subcellular localization of beta catenin could alter its phosphorylation status.

2. Materials and methods

2.1. Cell culture and transfection

The culture methods for Q7 and Q111 cells were described earlier [4]. Transfection method for plasmid construct was done as reported previously [4]. Briefly, 3ug empty DsRed vector or mutant Htt exon-1 construct 83Q-DsRed (contains 83 CAG repeats; CAG codes for glutamine (Q)) was transfected in Q7 and Q111 cells and results were analyzed after 48 hr. For siRNA oligo transfection,

Abbreviations: BDNF, Brain Derived neurotrophic factor; HD, Huntington's Disease; Htt, Huntingtin (mouse); HYPK, Huntingtin Yeast Partner K; miR-214, MicroRNA-214; snRNA, Small Nuclear RNA.

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control siRNA (Cell Signaling Technology) or Htt siRNA (Qiagen) complexed with 5ul lipofectamine 2000 (Invitrogen) was added to 50% confluent cells in 35 mm dish (final siRNA concentration in the medium was 100 nM). Transfected cells were incubated for 48 hr.

2.2. Luciferase assay

Luciferase assay with TOPFLASH TCF-reporter plasmid (Millipore) to assess the transcriptional activity of beta catenin was done as described [4]. Briefly, 200 ng TOPFLASH was transfected along with 100 nM (final concentration) control siRNA or Htt siRNA in Q7 and Q111 cells. Results were analyzed after 48hr.

2.3. Western blotting

Western blotting was done with the standard method. Briefly, boiled samples were resolved in 10% SDS-polyacrylamide gel, transferred onto PVDF membrane, blocked with BSA and probed with 1:4000 diluted anti-beta catenin antibody (Abcam) or 1:1000 diluted anti-phospho-beta catenin (serine 33) antibody (Cell Signaling Technology) whenever required.

2.4. RNA isolation, cDNA preparation, and real-time PCR

Total RNA was isolated from cells using Trizol (Invitrogen) reagent using manufacturer's protocol. cDNA was prepared from total RNA as described [4]. Real-time analysis of gene amplification was done using SYBR-Green (KAPA Biosystems) chemistry as described [4]. Actin (Actb) used as reference gene for Htt mRNA expression. For miRNA specific cDNA preparation, 100 ng total RNA was reverse transcribed with M-MuLV reverse transcriptase (Thermo scientific), stem-loop primer mix of miR-214 (1:200 diluted) and U6 snRNA (1:200; U6 snRNA used as reference). Amplification of miR-214 was carried out using 1:80 diluted miR-214 forward primer and 1:80 diluted universal reverse primer and amplification of U6 snRNA was carried out using 1:80 diluted U6 snRNA forward primer and 1:80 diluted universal reverse primer using SYBR-Green chemistry. $2^{-\Delta\Delta Ct}$ method was applied for analysis of gene expression. Supplementary Table 1 provides the sequences of the primers used.

2.5. Subcellular fractionation

Isolations of cytosolic and nuclear fractions were done as described [6]. Membrane fractionation was done using Membrane Extraction Buffer (MEB) (Thermo Scientific). The required amount of MEB was added to the residual pellet after cytosolic extraction. The suspended pellet was vortexed at top speed for 5sec and incubated on ice for 10min followed by centrifugation at 3000 x g for 5min. The supernatant was taken in a pre-chilled vial as membrane extract and kept at −20 °C until use.

2.6. Immunofluorescence

Untransfected cells grown on coverslips were fixed with 3.7–4% formaldehyde, permeabilized with Triton-X100 (0.25%) in PBS on ice followed by blocking with 1% BSA in PBS-Tween 20 (0.2%). Then the cells were incubated with 1:200 diluted anti-beta catenin antibody (Abcam) for 2 hr at 37 °C followed by incubation with 1:1000 diluted Alexa-488 conjugated secondary antibody for 1 h at 37 °C. After counterstaining with DAPI the cells were mounted on the glass slide. Cells were visualized under 100x (oil immersion) objective of Zeiss Axiovert fluorescence microscope (Carl Zeiss). 83Q-DsRed transfected cells growing on coverslips were fixed as above and aggregates of 83Q-DsRed were visualized under 100x (oil immersion) objective of the same microscope.

2.7. Statistical analysis

Statistical analyses were performed using student's t-test as described [4].

3. Results and discussion

3.1. Effect of Htt mutation on beta catenin and miR-214

When Htt is mutated, some of its wild-type functions are lost and some novel functions are gained. Htt mutation causes Htt to form aggregates [7] and impair proteasomal function [8]. On the other hand, the mutation costs Htt to lose the ability to regulate the expression of some genes, such as BDNF [9]. We went to find out whether the downregulation of beta catenin in Q111 cell [4] was due to the loss-of-function of wild-type Htt or Gain-of-function of mutant Htt. The first possibility implies that wild-type Htt is necessary to maintain the cellular abundance of beta catenin and upon Htt mutation, this functionality is lost, and beta catenin level is altered. The second possibility indicates that Htt mutation enables the mutant Htt to alter beta catenin level. In order to verify this, we knocked down basally-expressing full-length endogenous wild-type Htt in Q7 normal cell and mutant Htt in Q111 HD cell with 100 nM Htt siRNA. Transfection with Htt siRNA significantly reduced the wild-type and mutant Htt expression by 2-fold in Q7 and Q111 cells respectively (Fig. 1 A). Subsequent experiments followed this standardized Htt siRNA transfection. Htt siRNA transfection in Q7 normal cell could not alter beta catenin protein level (Fig. 1 B), also it could not alter the transcriptional activity of beta catenin in TOPFLASH luciferase assay experiment (Fig. 1 C). However, Htt siRNA transfection in Q111 HD cell increased beta catenin protein level as well as its transcriptional activity (Fig. 1 D, E).

As we earlier reported that miR-214 overexpression in Q111 cell reduced beta catenin [4], we wanted to observe miR-214 expression upon wild-type and mutant Htt knockdown. Htt siRNA transfection in Q7 cell could not alter miR-214 expression; however, Htt siRNA transfection in Q111 cell significantly reduced miR-214 level more than 2-fold (Fig. 1 F). All these data indicated that wild-type Htt had no role in miR-214 and beta catenin regulation, rather, the gain-of-function of mutant Htt lead to beta catenin downregulation by upregulating miR-214.

3.2. Effect of expression and aggregate formation of mutant Htt on beta catenin and miR-214

Several phenomena in HD, like toxicity and cell death, were studied in the light of mutant Htt aggregate formation [10,11]. Despite HD has some degree of striatal cell specificity, aggregate formation was reported to be very low (1–4%) in the human striatum [12,13]. Moreover, it was earlier reported that the basally-expressing mutant Htt in Q111 HD cell formed no aggregate [14]. This implied that the mutant Htt in Q111 cell altered beta catenin and miR-214 levels in an aggregate-independent manner. For this reason, we observed no change in beta catenin level in Q111 cell (Supplementary Figure S1) upon HYPK overexpression (HYPK was earlier shown to reduce mutant huntingtin aggregate [15]).

Since the effect of basally-expressing aggregate-independent mutant Htt was clear to us, we further studied the effect of the overexpression of aggregate-forming mutant Htt. The overexpression of 83Q-DsRed (Exon-1 of mutant HTT cloned in pDsRed vector; having CAG repeats coding 83 glutamine (Q) repeats in the protein) resulted in mutant Htt cellular aggregate formation (Fig. 2 A). 83Q-DsRed predominantly formed aggregates inside cytosol. We found that 83Q-DsRed overexpression in Q111 cell had no effect on beta catenin level (Fig. 2 B, C) unlike an earlier study that

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