A Possible Role for Long Interspersed Nuclear Elements-1 (LINE-1) in Huntington’s Disease Progression

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Background: Recent studies have shown that increased mobilization of Long Interspersed Nuclear Elements-1 (L1) can promote the pathophysiology of multiple neurological diseases. However, its role in Huntington’s disease (HD) remains unknown.

Material/Methods: R6/2 mice – a common mouse model of HD – were used to evaluate changes in L1 mobilization. Pyrosequencing was used to evaluate methylation content changes. L1-ORF1 and L1-ORF2 expression analysis were evaluated by RT-PCR and immunoblotting. Changes in pro-survival signaling were evaluated by L1-ORF overexpression studies and validated in the mouse model by immunohistochemistry and immunoblotting.

Results: We found an increased mobilization of L1 elements in the caudate genome of R6/2 mice (p<0.05) – a common mouse model of HD – but not in wild-type mice. Subsequent pyrosequencing and expression analysis showed that the L1 elements were hypomethylated and their respective ORFs were overexpressed in the affected tissues. In addition, a significant decrease in the pro-survival proteins such as the phosphoproteins of AKT target proteins, mTORC1 activity, and AMPK alpha levels was observed with the increase in the expression L1-ORF2.

Conclusions: These findings indicate that hyperactive retrotransposition of L1 triggers a downstream signaling pathway affecting the neuronal survival pathways via downregulation of mTORC1 activity and AMPK alpha and increasing apoptosis in neurons.

MeSH Keywords: AMP-Activated Protein Kinases • Cell Survival • Huntington’s Disease • Long Interspersed Nuclear Elements-1

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Background

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disease characterized by complex cognitive and psychiatric-related problems [1–3]. The symptoms are usually manifested as a mid-life onset and progress inexorably over 15–20 years [1,2,4]. The disease pathology is usually observed in the caudate nucleus and the putamen in the striatum, at least in early stages, and progress to other regions of the brain [1]. At the molecular level, the disease is caused due to the expansion of CAG repeat (Polyglin) in the HTT gene on chromosome 4, which leads to an extended glutamine tract in the encoded huntingtin protein, affecting its structure and functions, and causing misfolding and aggregation in the cells [4–6].

Multiple remarkable studies have shown that autophagosomal degradation pathway as a mechanism to clear the mutant protein in cells, and its dysregulation is often implicated in the pathogenesis of HD [7–9]. Recent studies using HD models have shown that AMPKalpha, a critical regulator of autophagy, is altered in the disease conditions and its restoration can ameliorate the disease manifestations [10,11]. While these analyses provide insights into the molecular signaling driving HD pathogenesis, it is still unclear why the pathology varies among different tissues, despite the mutant protein being expressed in all tissues [12–14]. Multiple studies have reasoned that tissue-specific modifiers might co-function or modulate the disease protein attributes and lead to such tissue-specific pathological onset and progression.

Recent studies have suggested that mobile elements in the genome such as long interspersed nuclear elements-1 (LINE-1 or L1) are modified in a tissue-specific way and are associated with the pathology of multiple neurological diseases such as schizophrenia and major depressive disorder [15,16]. In addition, alterations in the L1 content of tissues are known to affect the expression of its 2 ORFs (ORF1 and ORF2), which could affect the signaling pathways to facilitate disease progression [17]. While these studies highlight the importance of the L1 content in neurological diseases, its significance in the context of Huntington’s disease remains unexplored.

Material and Methods

Mouse models

The present study was performed in accordance with the guidelines provided in the Declaration of Helsinki. All mouse experiments were performed with prior approval from the institute’s Animal Care Committee at Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology (protocol no: 10132015). Housing and all procedures using animals were in compliance with the Animals (Scientific Procedures) Act, 1986 (UK) (amended 2013) and reported as per the ARRIVE Guidelines for reporting animal research [18]. All mice were housed in individually ventilated cages (5 per cage) under specific pathogen-free (SPF) condition, temperature-controlled, and with a 12 h/12 h light/dark cycle. Animal weights were routinely monitored every other day. The wild-type and R6/2 mice – a transgenic model expressing exon 1 of human N-mut HTT containing 150 poly-Q repeats – were purchased from Jackson Laboratory (West Grove, PA, USA). The caudate regions of mouse brain from 10–12-week-old wild-type and R6/2 mice were dissected and used for L1 retrotransposition analysis. For analysis of older mice, brain samples taken at ages 6–9 months from both male and female mice.

Evaluation of L1 content in brain tissues and liver tissues

The L1 content was evaluated as copy number content of ORF1 and ORF2 in brain and liver tissues samples of both HD transgenic and control mice. The method used to determine the L1 content was followed as described previously with minor modifications [15,19]. Briefly, 500 pg of DNA was used as a template and was amplified using m5’UTR-, mORF1-, and mORF2-specific primer sets in an SYBR green assay. 5SrRNA was used as internal control for the assay.

Pyrosequencing assays targeting L1 regions

DNA was extracted from mouse samples and evaluated for the status of methylation in Cpg dinucleotides located in the L1 region. The 5’UTR region upstream of ORF1 in the LINE-1 element was PCR amplified and analyzed for DNA methylation using the Qiagen PyroMark assay kit (Qiagen, USA) [20]. The primer sets (5’-biotin-labeled) used for PCR amplification were: Fwd: CCAGCTGGGGAGGCAGCCTA, Rev: CTGGTAATCTCTGGAGTT and the sequencing probe used was: GCCACAGCAGCAG. Briefly, the PCR products were suspended using the PyroMark Q24 kit (Qiagen) following the manufacturer’s protocol, and the methylation status was quantified with an estimated score of 0–100, with 0 being no methylation and 100 representing complete methylation for all of the Cpg dinucleotides in the region. The threshold in the assay was >5%.

Overexpression experiments

The coding DNA sequence of both L1-ORFs was amplified (GenBank: AF081144.1) and cloned into a pCMV6 vector and used for overexpression studies. For overexpression experiments, 293T cells were transfected with pCMV6–L1 ORF1 or ORF2 plasmids and analyzed for expression and other associated changes at 72 h after transfection.
Detection of apoptosis

Apoptosis was detected using a TUNEL staining kit (Promega, WI) [21]. For mouse samples, 20-micron tissue samples were processed and stained as per the manufacturer’s instructions. For cells, TUNEL staining was performed directly in cells and the absolute number of TUNEL-positive cells in randomly picked microscopy fields were noted.

Immunoblotting

The cell lysates were separated on a 4–20% gel under reducing conditions, transferred to a PVDF membrane (Bio-Rad, USA), and blotted using specific antibodies. The membrane was blocked with 3% milk and used with primary antibodies. The primary antibodies and the dilutions used were: rabbit LC3B (79D7, Cell Signaling Technology, USA, 1: 1000), rabbit pS6 (D908K, Cell Signaling Technology, USA, 1: 1000), rabbit AMPK alpha (P0056, Sigma, USA 1: 500), rabbit CXCR (UMB2, Abcam USA, 1: 500), rabbit AMP alpha (D6D9, Cell Signaling Technology, USA, 1: 1000), rabbit AKT (C67E7, Cell Signaling Technology, USA, 1: 1000), chicken L1-ORF2 (Rockland Antibodies Inc 1: 500), mouse L1-ORF1 (Millipore, 1: 500) and mouse Actin (BH10D10 Cell Signaling Technology, USA, 1: 2000), and mouse HA (C29F4 Cell Signaling Technology, USA, 1: 1000). After the primary antibody incubation, the membrane was then washed and incubated with respective secondary antibodies conjugated with horseradish peroxidase (HRP) and developed using a chemiluminescence substrate (Super Signal West Dura, Pierce Biotechnology, USA). Densitometry analysis was performed to determine the relative fold change in protein levels. All immunoblots were done at least 3 times and a representative blot was shown.

RT-PCR

Total RNA was isolated at 72 h after transfection in cultures or brain tissue samples and reverse transcribed as described previously [22]. PCR was performed with a 7500 Applied Biosystem instrument using TaqMan probes with the Universal PCR Master Mix (Life Technologies, USA). The following primer sets were used for L1-ORFs PCR amplification: ORF1-FWD: ATGGCGAAAGGTAAACGG, ORF1-REV: TTCCAGATTTCTTTTCCTAGG and ORF2-FWD: TTAACAACTAAAATAACAGGAAG, ORF2-REV: TATGGTTTCTCTGAGATGAGTTTC Gapdh (glyceraldehyde 3-phosphate dehydrogenase): Hs02758991_g1; (Applied Biosystems, USA) was used as a control. Empty vector-transfected cells or wild-type tissue samples were used as a respective reference to determine the changes in gene expression. All samples were run in PCR as triplicates.

AKT array assay

AKT array assay kit (Full Moon Biosystems) was used to evaluate the changes in the cell survival pathway-related genes. The assays were performed as per the manufacturer’s instructions in L1- ORF2-overexpressed cell lysates. All samples were analyzed in triplicate.

Immunohistochemistry

Mouse brain tissues were fixed in 4% paraformaldehyde and paraffin-embedded for sectioning. We collected 5-micron sections corresponding to caudate regions and performed anti-apoptosis retrieval using a citric acid buffer (H-3300, Vector Labs, USA). Sections were then blocked and stained for pS6 (4858, Cell Signaling Technology, USA 1: 25) or AMPK alpha (P0056, Sigma, USA 1: 50). Then, sections were processed using respective HRP conjugated secondary antibody and developed using ImmPRESS polymer detection kit (Vector Labs, USA). Sections from 5 different mice per group were analyzed.

Statistics

All statistical analyses were performed using one-way ANOVA. Statistical significance was set at p<0.05. All calculations were made using GraphPad Prism Version 6.

Results

L1 content in the brains of HD transgenic mice

To test if there are changes in the L1 content in the HD mice, the L1 content in the 10–12-week-old HD mouse caudate regions were evaluated and compared to age-matched wild-type mice. The primer sets used for the analysis are provided in Figure 1A. The results showed a significant increase in the L1-ORF2 copy number in HD mouse brain tissue as opposed to the control mouse brain tissue (Figure 1). Similar results were also observed with the copy number of L1-ORF1 and the 5’UTR regions, suggesting that L1 content was increased in the HD mice (Figure 1B). However, these changes were not observed from matched liver tissues of mice, indicating that the changes were limited to brain tissues (Figure 1C). We also assessed whether this increase is specifically associated with any of the demographic factors, such as age and sex of the mice, and the results showed there was no correlation (Figure 1D), and the increase in L1 content in HD mice is irrespective of them.
Decreased methylation content and increased expression of L1ORFs in HD transgenic mice

To study whether increased L1 content gets translated as an increased expression of its ORFs, the expression of L1 transcripts and protein in affected tissues (brain caudate) from HD mice were evaluated and compared to similar regions of wild-type mice. We observed elevated levels of both the transcripts ORF1 and ORF2 in the HD mice, but it was not detectable in control mice (Figure 2A; * p<0.01). Similar trends were observed in the protein levels of ORF1 and ORF2 from the HD mouse brain tissues (Figure 2B). In addition, as methylation of CpG sites represents a major mechanism for gene expression regulation, we probed for methylation content of the L1-regions. The results showed a decreased methylation content in HD mice (Figure 2C). This suggests that the increased L1 content in the mouse genome has an impaired remethylation process or, alternatively, an augmented de-methylation has occurred, which in turn causes the increased expression of L1 mRNAs.

Decreased mTOR activity and AMPK alpha levels in HD mice

It is well known that changes in cell survival signaling pathways facilitate the disease progression in HD [1,3]. Here, we hypothesized that L1 copy number and expression changes could modulate the survival signaling pathways. In accordance with that, mouse samples indicated that both mTORC1 activity as measured through pS6 levels and AMPK α levels were downregulated in the affected regions but not in controls regions (Figure 3A). Finally, we also tested if there is any corresponding change in cell death, and the data showed that increased L1 content regions were correlated with increased cell death (Figure 3B).
**Figure 2.** L1-ORF expression and methylation in HD mice. (A) Real-time analysis showing increased L1-ORFs expression in HD mouse brain tissues (n=10; age 10–12 weeks). (B) Immunoblot showing ORF 1 and 2 expressions in HD mice. Tissue samples from 5 different mice were analyzed independently and a representative blot is shown. (C) Methylation analysis by pyrosequencing. HD mice L1 regions are less methylated than in WT mice. Values are mean ± standard deviation (n=10; age 10–12 weeks).

**Figure 3.** mTOR activity and AMPK alpha signaling in HD mice. (A, B) WT and HD mice were evaluated for mTOR activity and AMPK alpha levels. (A) Immunostaining showed decreased pS6 and AMPK alpha levels in HD mice. Five mice were analyzed, and a representative image is shown. (B) Immunoblot showing a similar decreased pattern of mTOR activity and AMPK alpha in HD mice. Tissue samples from 5 different mice were analyzed independently, and a representative blot is shown. (C) Quantification of TUNEL analysis showing increased cell death in HD mice. Three 20-micron sections from 5 mice were analyzed. Values are reported as mean ± standard deviation.
Table 1. L1-ORF2 modulates survival signaling – AKT array. ORF2-overexpressed and control cell lysates were analyzed for changes in survival signaling using AKT array. Protein phosphorylation and total levels were noted as arbitrary absorbance units and their ratio was calculated. Proteins showing greater than the 3-fold decrease in phosphorylation levels were listed along with their site and the potential kinase associated.

| Phosphorylation site          | Ratio (phospho/total protein) | Amino acid sequence | Kinase involved | Biological effects/references |
|-------------------------------|-------------------------------|---------------------|-----------------|------------------------------|
| ORF2                          | Control                       |                     |                 |                              |
| AFX/FoxO4 (Phospho-Ser197)    | 0.27                          | RRRApSMDS            | Akt             | Cell survival [25]           |
| ASK1 (Phospho-Ser83)          | 0.16                          | RGRGspSVGG           | Akt             | Cell survival [26]           |
| ASK1 (Phospho-Ser966)         | 0.16                          | YLRSpSLPVP           | Unknown         | Cell survival [26]           |
| BAD (Phospho-Ser112)          | 0.18                          | RSRHspSYPAG          | p90RSK, PKA     | Cell survival [27]           |
| BAD (Phospho-Ser136)          | 0.07                          | RGRSpSAPPN           | Akt             | Cell survival [28]           |
| BAD (Phospho-Ser155)          | 0.04                          | ELRMpSDEFV           | PKA             | Cell survival [25,27,28]     |
| BCL-2 (Phospho-Ser70)         | 0.06                          | PVARTpSPLQT          | IL3, JNK        | Cell survival [28,29]        |
| BCL-2 (Phospho-Thr56)         | 0.66                          | SQPGHpTPHA           | Unknown         | Cell survival [26]           |
| BCL-XL (Phospho-Ser62)        | 0.18                          | WHLADpSPAVN          | JNK             | Cell survival [29]           |
| c-Jun (Phospho-Ser63)         | 0.08                          | SDLTpSPDVG           | Unknown         | Cell survival [30]           |
| FKHR/FoxO1 (Phospho-Ser319)   | 0.28                          | RPRTspSNAST          | Akt             | Cell survival [25,31]        |
| FKHR/L/FoxO3a (Phospho-Ser253)| 0.15                          | RRAVpSMDNS           | Akt             | Cell survival [32]           |
| GSK3b (Phospho-Ser9)          | 0.11                          | RPRTspESA            | Akt             | Cell survival [33,34]        |
Overexpression of L1 ORF2 modulate mTOR activity and AMPK alpha in 293T cells

To understand the significance of L1-ORFs in HD, we overexpressed L1-ORFs in 293T cells and tested whether it can induce in vivo-like changes in mTORC1 activity and AMPKα in cells. The results indicated that overexpression of L1-ORF2 down-regulates mTOR activity as measured by the expression of pS6 and AMPKα and by cell death (Figure 4A, 4B). In contrast, ORF1 expression had no significant effect in modulating these proteins or causing cell death (Figure 4C). In addition, as recent studies showed that mTORC1 and huntingtin modulate autophagy, we also tested for autophagy induction in overexpression experiments. The results showed minimal change in autophagy induction as measured by LC3BII, indicating that the autophagy induction is not part of L1ORF2-induced signaling.

Overexpression of L1-ORF2 modulates cell survival signaling pathways

To further understand the significance of L1-ORF2 in HD progression and cell death pathways, we further tested for changes in other target proteins involved in cell survival signaling. As AKT is widely indicated as pro-survival and its activity is associated with amelioration of HD disease [23], changes in AKT target proteins were evaluated as a readout for changes in cell survival. The AKT array analysis between control and ORF2-overexpressed cells showed that ORF2 expression decreased phosphorylation of at least 13 different AKT target proteins (Table 1), suggesting that ORF2 has a direct effect in modulating cell survival signaling. Similar trends were checked in the tissue samples of HD mice, at least for ASK1, c-Jun, GSK3β, and BCL-2 (Figure 5).

Discussion

Polygluton expansions (CAG repeats) as seen in HTT protein of HD are associated with multiple neurodegenerative diseases [23]. It is probable that in each case they develop a gain of function that may act through a common mechanism. R6/2 mice are an early transgenic model based on the polygluton expansion with its ubiquitous expression in all tissues, as in mutant human gene expression [23]. Also, the progressive neurological phenotype mimics many features of human HD, including choreiform-like movements, involuntary stereotypic movements, tremors, and epileptic seizures, and thus the observations made can be correlated with the human form of HD.
Growing evidence indicates that the roles of retrotransposable elements underlie progression of multiple neuronal diseases [15,16]. Here, we report that the caudate genome of the Huntington’s disease mouse model exhibits increased copies of L1 retrotransposon and are associated with the increased transcription of its transcripts, ORF1 and ORF2. In addition, we have shown that a decrease in the methylation content of the L1 promoter is associated with increased retrotransposition and transcript expression.

L1 retrotransposition has been observed at different stages, including adult neurogenesis of the hippocampus, implying that there could be a possible modulation with age [19]. In the present study, we analyzed the potential role for confounding factors such as age, symptomatic onset, and sex, and did not find any correlation with them. Also, the lack of L1 content change in other tissues indicates that L1 copy number increase is not a global change and is possibly limited to brain tissue alone. These results raise the question of whether the L1 content changes in the brain are confined to an early neurodevelopment stage. Given that abnormalities during early brain development could lead to later clinical symptoms of neurological diseases [24], future studies directed towards analyzing the L1 content in neuronal development of R6/2 mice would be of great importance to understand the significance of this change.

Multiple mechanisms have been proposed to mediate alterations in the content of L1 in tissues. While DNA methylation is the most common mechanism, other methods, such as RNA interference, have also been shown to inhibit retrotransposition post-transcriptionally [25]. In addition, the APOBEC3 family has also been suggested to mediate L1 retrotransposition [26]. Our observations, although showing that changes in DNA methylation profiles drive retrotransposition and are correlated with increased expression, it is intriguing that these effects are mediated in a tissue-dependent manner. Future work directed towards the role of other mechanisms involved would provide more detailed information about L1 content regulation in specific tissues. In any case, our study provides the initial set of data on how DNA methylation changes regulate L1 content in HD samples.

Autophagy has been identified as one of the main degradation pathways for the removal of mutant protein aggregates [7]. Defective autophagy pathways have been shown to facilitate HD progression. Changes in mTOR signaling have been used as a readout for autophagy induction [10], but mTOR involvement in diverse cellular pathways and processes makes it difficult to delineate its specific relationship with autophagy and HD pathology. Recent studies have shown that AMPK alpha is a better readout for autophagy induction, and its activation can also ameliorate HD disease conditions in cells [10,11]. We have shown that increased expression of L1-ORF transcripts decreased the AMPK alpha expression. Additionally, our in vitro overexpression experiments suggest a direct involvement of L1-ORF in regulating these genes. These results suggest that L1 transposition, AMPK alpha and autophagy, and mutant HTT protein all function in a common pathway. We expect that these findings will promote future research on genomic instability and its relationship with mutant HTT protein and other signaling pathways to better understand the etiology of HD.

Conclusions

Our results provide the first evidence that increased L1 content is observed in HD, which leads to changes in its ORF expression and subsequent downstream signaling related to cell survival processes.

Conflict of interest

None.

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