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STUDY OF PLASMA-DERIVED MIRNAS MIMIC DIFFERENCES IN HUNTINGTON'S DISEASE BRAIN

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Abstract

Background—Biomarkers for Huntington's disease (HD) progression could accelerate therapeutic developments and improve patient care. Brain microRNAs (miRNA) relating to clinical features of HD may represent a potential HD biomarker in blood.

Objective—Examine candidate miRNAs in plasma to determine if changes observed in HD brains are detectable in peripheral samples.

Methods—Four miRNA from 26 manifest HD, 4 asymptomatic HD gene carriers and 8 controls were quantified in plasma using RT-qPCR. Linear regression was used to assess miRNA levels across control, asymptomatic gene carriers and manifest patients.

Results—miR-10b-5p ($p= 0.0068$) and miR-486-5p ($p= 0.044$) were elevated in HD plasma. miR-10b-5p was decreased in asymptomatic gene carriers as compared to HD patients ($p= 0.049$), but no difference between asymptomatic gene carriers and controls was observed ($p= 0.24$).

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Authors' Roles

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Conclusions—These findings suggest miRNA changes observed in HD brain may be detectable in plasma and have potential clinical utility.

Keywords

Huntington's disease; microRNA; miRNA; blood; biomarker

Introduction

Huntington's disease (HD) is caused by an expanded CAG trinucleotide repeat sequence in the huntingtin gene [1]. Clinical onset of the disease is defined by the emergence of abnormal, choreiform movements, often accompanied by neurophysiological, psychiatric or cognitive impairments [2], and generally occurs around middle age [3]. Neurodegeneration precedes clinical diagnosis, with as many as half of the neurons in the caudate nucleus lost before clinical onset occurs [4], and volumetric changes in the striatum as early as two decades before predicted onset age [5]. Because motor and cognitive impairments correlate with the neuroanatomical changes in the striatum [6], to prevent neuronal loss and delay disease onset, therapeutic intervention would ideally occur prior to HD manifestation.

While genetic testing can reliably detect the presence of an expanded CAG repeat, the lack of validated biomarkers for HD onset and progression limits the evaluation of preventive and early-stage disease-modifying therapies. Current measures for prodromal and early-stage disease rely on ratings of functional decline, which are susceptible to inter-rater variability and limited sensitivity [2].

Large, multicenter, longitudinal studies comparing cohorts of asymptomatic gene carriers and early-stage HD to healthy controls have used a battery of clinical and neuroimaging based assessments. Aimed at identifying robust quantitative measures [7, 8], these studies have identified a number of image-based biomarkers that may relate to HD progression including morphometric changes [7, 9], the glial cell marker myo-inositol [10], the neuronal integrity marker N-acetyl aspartate [10], and recently, the medium spiny neuron marker PDE10A [11]. Although these results are encouraging, neuroimaging is susceptible to reproducibility issues due to technical and analytical inconsistencies across centers, which must be addressed prior to clinical adoption [12].

Alternatively, disease mechanisms observed in the brain may be detectable in the blood. Studies of 8-OHdG levels [13], mutant HTT accumulation [14], inflammatory markers [15], and genome-wide RNA changes [16–18], have uncovered a number of concordant changes between brain and blood. While some are promising, the clinical utility of these measures is yet to be established[19].

Our recent evaluation of altered miRNA levels obtained through small RNA sequencing in human HD and control prefrontal cortex identified 75 miRNAs significantly altered in HD [20]. Several of these were associated with age at motor onset, or the level of neuropathology in the striatum [21], including miR-10b-5p, which associated with both [22]. Furthermore, in asymptomatic HD gene carriers, miR-10b-5p levels were distinguishable from both the low expression observed in controls and higher levels seen among

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symptomatic HD patients, suggesting a progressive relationship between cortical levels of miR-10b-5p and disease stage.

Because brain-derived miRNAs may pass through the blood-brain-barrier by exosome transport [23] and are stable in serum [24], the miRNAs identified in postmortem brain tissue may be detectable in peripheral fluids [25–27], and thus potentially provide accessible biomarkers for disease stage and rate of progression in clinical trials.

The first step in the evaluation of clinical utility is to determine whether the observed HD brain-related miRNA alterations are detectable in peripheral samples. We therefore compared the levels of four miRNAs related to HD clinical features in postmortem brains (miR-10b-5p, miR-486-5p, miR-132-3p, and miR-363-3p) in HD, asymptomatic and healthy control plasma samples.

Methods

Study participants (n=38) were recruited through the Boston University Neurological Associates (BUNA) and Tewksbury State Hospital from 2012–2014, with appropriate IRB approval and consent (BUSM Protocol Number H-31052 and Massachusetts Department of Public Health Protocol Number 328647-2) (see Table 1). No significant differences were observed in the age or sex distribution between the 3 groups (HD cases, asymptomatic HD carriers, and controls). BD Vacutainer CPT Mononuclear Cell Preparation Tubes containing 0.1 mL sodium citrate anticoagulant and 0.1 M Ficoll medium were used to isolate plasma from 8 mL of whole blood drawn by a trained phlebotomist.

One mL of plasma was used for RNA extraction. To minimize platelet contamination [28], residual platelets were removed by centrifugation, collecting supernatant after spinning for 5 min at 16,000 × g. 0.22 um filtration was used to remove heterogeneous, phospholipid membrane bound microparticles, 0.05–1.5 um in size, shed from platelets and other blood cells [29]. RNA was extracted using Qiazol and miRNeasy RNA isolation kit from Qiagen, according to manufacturer’s protocol. RNA purity and abundance was assessed by spectrophotometry.

Four miRNAs were selected based on the following criteria: (1) genome-wide significant changes in HD brain [22] (2) abundance in both brain and blood [30], and (3) nominal association in the cortical study ($p<0.05$) to clinical HD features (onset: miR-10b-5p, miR-486-5p miR-363-3p; striatal neuropathological involvement: miR-10b-5p, miR-132-3p).

Exiqon miRCURY LNA Universal RT miRNA PCR was used following the manufacturer’s protocol. UniSp6 synthetic spike-in was used to evaluate cDNA efficiency. Following cDNA synthesis, samples were diluted to 0.2 ng/ul in RNAse free water. Both SNORD44 and miR-451a were used for normalization. For quantitative PCR (qPCR), samples were assayed in triplicate across three 384-well plates, using Applied Biosystems 7900HT Real-Time PCR System. For analysis, threshold cycle (Ct) values for triplicate wells were normalized by average RNU44 and miR-451a values. Extreme outlier wells and samples (standard deviations above 10) were removed. miRNA levels were calculated using the

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Ct method [31], where positive Ct values indicated increased levels and negative Ct values indicated decreased levels as compared to controls.

Linear regression analyses predicting – Ct were used to test the association between miRNA levels and disease in HD cases and controls. One-tailed tests were used to test the a priori hypothesis of consistent direction of effects as observed in the brain study [22], thus relationships inconsistent with the cortical findings would not be identified as significant. The relationship of miR-10b-5p, miR-486-5p, miR-363-3p to age of motor onset in cases was assessed using linear regression (miR-132-3p did not show a relationship to onset in the cortical study). Bonferroni correction for four or three comparisons were applied respectively.

For miR-10b-5p, the relationship was further examined between asymptomatic HD gene carriers and HD cases and controls separately using linear regression, again using a one-tailed test of the previously observed relationships. In addition, a linear trend test was implemented in SAS (GLM Procedure) to test if an ordered relationship was observed between controls, asymptomatic gene carriers and HD cases.

Results

All four miRNAs were detected in plasma (average Ct range 25.7–33.8). After calculating – Ct levels, 26 HD patients were compared to the 8 controls to test whether miRNAs alterations in HD plasma resembled changes observed in HD brain. Increased levels of miR-10b-5p (one-sided $p= 0.0068$, $\beta= 2.39$) and miR-486-5p (one-sided $p= 0.044$, $\beta= 1.44$) were observed in manifest HD patients compared to control subjects, consistent with the changes in the brain [22] where both miRNAs were also increased. Levels of miR-132-3p, though not significant, were lower in HD plasma, consistent with the results in brain (one-sided $p = 0.92$, $\beta= -0.62$). miR-363-3p levels were not altered in blood, nor consistent with changes observed in HD brain (one-sided $p = 1.00$, $\beta= -0.079$).

Three previously observed associations to age at motor onset in the brain study were also tested. The relationship between plasma miR-10b-5p and onset age did not have the same direction as previously observed in HD brain. In fact, a positive association, opposite to that seen in brain and our tested a priori hypothesis, may exist (one-sided p=1.00, two-sided p=0.0096, beta=0.13). The nominal associations to age of onset observed in brain for miR-486-5p and miR-363-3p were not present in blood (miR-486-5p one-sided p=1, miR-363-3p one-sided p=1).

There was no statistical difference between asymptomatic HD gene carriers and controls in miR-10b-5p levels, (one-sided $p= 0.24$, $\beta= 0.53$), however miR-10b-5p levels were significantly elevated in manifest HD patients compared to asymptomatic gene carriers (one-sided $p= 0.049$, $\beta= 1.15$). A linear trend test of the ordered relationship of miRNA levels between controls, asymptomatic HD gene carriers and manifest HD patients found a significant positive association with miR-10b-5p levels (one-sided $p= 0.0012$) (see Figure 1), concordant with the direction of the effects observed in HD prefrontal cortex.

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Discussion

The results from this study identify two candidate miRNAs, miR-10b-5p and miR-486-5p, as increased in both brain and blood in HD. In our previous HD brain study, miR-10b-5p had the strongest associations to disease stage, age of onset and extent of neuropathological involvement [22]. While we did not replicate the relationship of miR-10b-5p levels to age of onset observed in brain, the levels of plasma miR-10b-5p were significantly elevated HD patients compared to asymptomatic HD gene carriers consistent with the brain study. While a consistent trend of increasing levels from controls, to asymptomatic HD to manifest HD was observed, plasma miR-10b-5p levels were not significantly different in asymptomatic subjects compared to controls, and given the small number of samples in the asymptomatic (N=4) and control (N=8) groups, caution should be taken in interpreting those findings. The significant differences between asymptomatic and manifest HD do however suggest pre-clinical HD miRNAs changes may occur.

The concordance of these changes in both HD brain and blood supports the role of miR-10b-5p in the disease and suggests potential utility as a clinically useful biomarker for HD. Longitudinal within-subject tests of miRNA levels over time, as well as tests of the relationship of miRNA levels to motor, cognitive and functional ratings may be warranted for miR-10b-5p in particular. The identification of two of the four candidate miRNA examined also suggests that a full examination of all 75 brain-identified miRNA may reveal additional miRNA with potential clinical utility.

Blood-based biochemical assays are minimally invasive and relatively simple compared to neuroimaging-based diagnostics. The strength of this study and overall approach is that the blood-based assays described here are based on miRNA alterations observed in HD cortex, providing greater probability that these alterations are representative of the disease process. This is in contrast to signals detected primarily in peripheral tissues, which are more likely to represent systemic physiological responses independent of neurodegeneration and other disease features [32]. Because of this strategy, we did not test miR-34b which has been previously reported as changed in premanifest HD plasma, but is not altered in HD brain [20, 22].

These findings are a key first step towards the evaluation of the potential utility of miRNA as biomarkers of disease progression and for use in examination of treatment efficacy in early premanifest patients.

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A patent application has been submitted.

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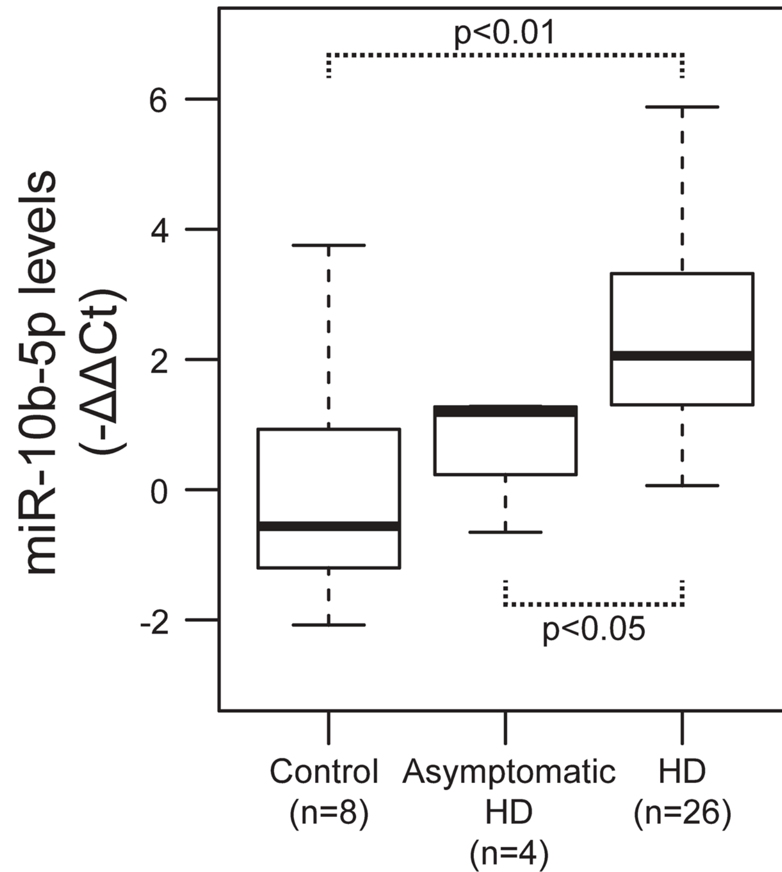

Figure 1. miR-10b-5p has an ordinal association with HD stage

Boxplot of miR-10b-5p levels (– Ct) for control, asymptomatic HD gene, and manifest HD subjects. miR-10b-5p levels had a significant linear trend from controls, to asymptomatic HD gene carriers to manifest HD patients.

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Table 1

Summary of the samples used for the study

Condition	N	Age	Onset age	Gender
Control	8	46.1 ± 13.5		3M, 5F
Asymptomatic HD	4	42.5 ± 28.7		1M, 3F
Manifest HD	26	53.0 ± 8.7	47.6 ± 9.9	11M, 15F