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Urine levels of the polyglutamine ataxin-3 protein are elevated in patients with spinocerebellar ataxia type 3

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2021.07.018.

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Abstract

Introduction: Accumulation of polyglutamine (polyQ) ataxin-3 (ATXN3) contributes to the pathobiology of spinocerebellar ataxia type 3 (SCA3). Recently, we showed that polyQ ATXN3 is elevated in the plasma and cerebrospinal fluid (CSF) of SCA3 patients, and has the potential to serve as a biological marker for this disease [1]. Based on these findings, we investigated whether polyQ ATXN3 can also be detected in urine samples from SCA3 patients.

Methods: We analyzed urine samples from 30 SCA3 subjects (including one pre-symptomatic subject), 35 subjects with other forms of ataxia, and 37 healthy controls. To quantify polyQ ATXN3 protein levels, we used our previously developed immunoassay.

Results: PolyQ ATXN3 can be detected in the urine of SCA3 patients, but not in urine samples from healthy controls or other forms of ataxia. There was a significant statistical association between polyQ ATXN3 levels in urine samples and those in plasma. Further, the levels of polyQ ATXN3 urine associated with an earlier age of SCA3 disease onset.

Conclusion: As clinical trials for SCA3 advance, urine polyQ ATXN3 protein has potential to be a useful, noninvasive and inexpensive biomarker for SCA3.

Keywords

Urine; Biomarker; Polyglutamine (PolyQ); Ataxin-3 (ATXN3); Spinocerebellar ataxia type 3 (SCA3)/machado-josephs disease (MJD)

1. Introduction

Spinocerebellar ataxia type 3 (SCA3) is an autosomal dominant genetic disorder resulting in progressive ataxia. SCA3 is characterized by the accumulation of aggregates composed of polyglutamine (polyQ) ataxin-3 (ATXN3) proteins translated from expanded CAG trinucleotide repeats in the *ATXN3* gene [2]. Although SCA3 is defined as a single disorder caused by the repeat expansion mutation in *ATXN3*, multiple phenotypes may exist within one family sparking the study of genotype-phenotype correlations [3]. The exact underlying pathogenic mechanisms of SCA3 and fundamental treatment for preventing the disease remain active topics of investigation. However, many therapeutic approaches to target and reduce this abnormal polyQ ATXN3 protein have been developed [2]. In order to assess therapeutic efficacy in clinical trials, reliable neuroimaging [4] and disease biomarkers are important. Recently, we demonstrated that polyQ ATXN3 proteins can be efficiently detected in human biological fluids [plasma and cerebrospinal fluid (CSF)], and may serve as a pharmacodynamic marker for mutant ATXN3 targeting therapies [1].

Although plasma and CSF are very useful biofluids, patients do not always consent to their collection. Further, lumbar puncture for collecting CSF are sometimes hampered by technical difficulties or medical risk [5]. Thus, urine has the potential to become the most comfortably provided biofluid by patients during clinical trials, and may facilitate patient

participation during longitudinal studies. Here, we considered the possibility that polyQ ATXN3 proteins could function as a biomarker in urine. We investigated: 1) whether polyQ ATXN3 proteins in urine can distinguish SCA3 patients from healthy controls and patients with other form of ataxia, and 2) whether polyQ ATXN3 protein levels in urine correlate with those in plasma, and with SCA3 clinical information.

2. Methods

2.1. Sample collection

All protocols were approved by the Mayo Clinic Institutional Review Board (IRB) and Ethics Committee, and the Ethical review boards responsible for the sites where patients were included. All collaborative sites were also approved by the Mayo Clinic IRB Committee and material transfer agreements were developed. Urine and plasma samples from patients with SCA3, and urine samples from patients with other forms of ataxia and healthy control individuals were obtained following standardized protocols at Mayo Clinic in Florida and Nagoya University in Japan. All samples were placed on ice upon collection from the patients and transferred to the laboratory. Plasma samples collected from blood EDTA vacutainers were centrifuged within 30 min of collection and processed as previously described [1]. All samples were aliquoted and stored at −80 °C until immunoassay analysis. Plasma samples were collected at the same time as urine samples from the same participants. Inclusion of subjects in the study was based solely on the availability of urine samples. As such, our cohort was racially diverse and included White [11 SCA3 (36.7%), 29 other ataxia (82.8%), 27 healthy controls (73.0%)], Black or African American [16 SCA3 (53.5%), 3 other ataxia (8.6%), 9 healthy controls (24.3%)], Asian [3 SCA3 (10%), 2 other ataxia (5.7%)], and Native Hawaiian or Other Pacific Islander [1 other ataxia (2.9%), 1 healthy control (2.7%)]. Both males and females where included in the study and there was a slight overrepresentation of females over males in each study cohort [17 SCA3 (56.6%), 20 other ataxia (57.1%), 25 healthy controls (67.6%)].

2.2. PolyQ ATXN3 immunoassay

To detect and quantify polyQ ATXN3 proteins, we used a sandwich immunoassay using the Meso Scale Discovery (MSD) electro-chemiluminescence detection technology [6,7]. To quantify polyQ ATXN3 proteins in human samples, we first coated MSD Multi-array 96-well plates (Cat#L15XA-6, MSD) with a mono-clonal anti-ATXN3 antibody (clone 1H9, Cat# MAB5360 from Millipore) at 1:2000 dilution as capture antibody (diluted in TBS: Tris buffered saline) overnight at 4 °C. Wells were washed with TBS-T (0.2% Tween 20 in TBS) and blocked with 3% MSD Blocker A (Cat#R93AA-1, MSD) in TBS-T at room temperature for 1 h. After wells were washed 3 times with TBS-T, a total of 150 μl of urine (no dilution) or 10 μl of plasma (total 50 μl per well, 1:5 dilution in diluent 12, Cat#R50JA-3, MSD) were added to each well (each sample was ran in duplicate), incubated on the plates for 2 h at room temperature at 600 rpm, and washed 3 times with TBS-T. For detection, an anti-polyQ detection antibody (clone 3B5H10, Cat#P1874, Sigma) was conjugated to SULFO-TAG NHS-Ester group according to the manufacturer's recommendation (Cat#R91AO-1, MSD), and used at 1.25 μg/ml. The plates were incubated 1 h at room temperature and 600 rpm, after which time the plates were washed 3 times again with TBS-T, and read with 1X MSD

Read Buffer T with Surfactant (Cat#R92TC-2, MSD) on the MSD Meso QuikPlex SQ 120 Plate Reader. Note two independent assays, carried out by two different technicians, were performed blinded and each time using a new sample aliquot. Data presented is the average of data obtained from both assays.

2.3. Urinary creatinine measurement assay

To correct for renal function, we measured the levels of creatinine in urine. To quantify urine creatinine, the urine samples were diluted 1:20 with distilled water, and the Creatinine Urinary Detection Kit (Cat# EIACUN, Invitrogen) was used according to the manufacturer's protocol. Note this assay was performed blinded, in duplicates.

2.4. Statistical analyses

Comparisons of polyQ ATXN3 protein levels in urine samples (both with and without corrected by urine creatinine levels) among patients with SCA3, which did include the pre-symptomatic SCA3 case, patients with other forms of ataxia, and healthy controls, were evaluated by One-way ANOVA and Tukey's multiple comparison test. Associations of Log transformed polyQ ATXN3 protein levels in urine samples with those in plasma, and with clinical information were assessed using Pearson's correlation test. Note we were not able to detect polyQ ATXN3 in one SCA3 urine sample and thus, was excluded from the correlation studies. All statistical tests were performed using GraphPad Prism (version 8.3.0). GraphPad Prism was also utilized to originate the graphs in Fig. 1. $P < 0.05$ is considered statistically significant.

3. Results

Our study population included 30 patients with SCA3 including one pre-symptomatic case (Median age: 58.27, range: 20.47–73.47 years), 35 patients with other forms of ataxia (Median age: 57.42, range: 22.97–78.08 years), and 37 healthy controls (Median age: 51.02, range: 22.88–81.95 years). The presence/absence of the CAG-repeat expansion in *ATNX3* was verified for all the cases in the study (SCA3: median 66, range: 51–74; other ataxia: median 24, range: 14–37; healthy controls: median 23, range: 15–33). The other forms ataxia cohort was composed of non-SCA3 genetic forms of ataxia: 2 SCA1, 4 SCA2, 2 SCA5, 1 SCA6, 2 SCA7, 2 SCA8, 1 SCA12, 1 SCA28, 1 case associated with *SYNE1* mutation, 3 cases associated with a mutation in *TRIO* gene [8] as well as cases with not known pathogenic mutations (n = 16). For the symptomatic SCA3 patients, the median age of onset was 60 (range: 19–64 years), SARA total median score was 11 (range: 1–34), and median gait mobility score was 3 (range:1–4). We measured polyQ ATXN3 protein levels in urine using our established immunoassay [1], and showed that polyQ ATXN3 protein levels were significantly higher in urine samples derived from symptomatic SCA3 patients compared to healthy controls ($p < 0.0001$) and other forms of ataxia cases ($p < 0.0001$) [Fig. 1A]. Of note, one pre-symptomatic SCA3 patient also showed an increase in polyQ ATXN3 levels (filled red circle in graph) [Fig. 1A]. We also measured the levels of urine creatinine in order to correct for potential differences in renal function among patient samples. We observed no differences in creatinine levels among our cohorts of patients [Supplementary Fig. 1A]. Further, when correcting urine polyQ ATXN3 levels with urine creatinine levels,

the values of polyQ ATXN3 in symptomatic SCA3 patients were still significantly higher than those in the other forms of ataxia ($p < 0.0001$) and healthy controls ($p < 0.0001$) [Supplementary Fig. 1B].

Next, we investigated whether the levels of polyQ ATXN3 protein in urine correlate with those in matching plasma. As a result, a mild correlation was found between polyQ ATXN3 protein levels in urine and those in plasma ($p = 0.0449$) [Table 1].

Further, we also evaluated the relationship between polyQ ATXN3 levels in urine with age at sample collection, age at disease onset, disease duration (defined as time elapsed from disease onset to sample collection), *ATXN3* CAG repeat length, total SARA scores and gait mobility scores. Interestingly, we found a significant association between urine polyQ ATXN3 levels and an earlier age of SCA3 onset ($p = 0.0370$) [Fig. 1B, Table 1]. Of note, we also observed a trend with the age at sample collection, but it did not reach statistically significance ($p = 0.0599$) [Table 1]. In addition, we found no significant associations with disease duration, *ATXN3* CAG repeat length, SARA scores or gait mobility scores [Table 1].

4. Discussion

In this study, we demonstrated the following: 1) polyQ ATXN3 proteins can be detected in the urine samples from all SCA3 patients, but not in healthy controls or other forms of ataxia cases; 2) polyQ ATXN3 levels in urine samples correlated with those in plasma samples; 3) urine polyQ ATXN3 levels associated with an earlier age of disease onset.

The most important point of our study is that polyQ ATXN3 levels were elevated in urine derived from patients with SCA3, and could distinguish SCA3 patients from healthy controls and other forms of ataxia patients (negative for mutations on *ATXN3*). Therefore, we believe that changes in polyQ ATXN3 resulting from treatments targeting the mutant gene may be reflected in urine. Urine is the most noninvasive and easily accessible biofluid, representing the least difficult biomaterial for most patients to provide. This would be of great benefit to investigators, as longitudinal data from a large number of subjects in clinical trials and during clinical follow-up visits could be easily collected. Moreover, this finding of polyQ ATXN3 accumulating in urine of SCA3 cases may encourage investigators to determine whether other polyQ proteins associated with other polyglutamine diseases, such as Huntington's disease [9], can be also detected in urine.

In this study, a significant but mild correlation was found between polyQ ATXN3 levels in urine and those in plasma. We assume that the small number of cases might have influenced this result. The lack of a strong association between both biofluids may be explained by differences in renal function among SCA3 patients. However, when normalizing urine polyQ ATXN3 levels to creatinine, the association with plasma polyQ ATXN3 levels was no longer significant ($p = 0.2566$). Further, creatinine levels did not differ between SCA3 cases and other ataxia cases or healthy controls, and urine polyQ ATXN3 levels remained significantly elevated in SCA3 patients after normalizing to creatinine (Supplementary Fig. 1). Thus, it does not appear that changes renal function affects our ability to detect polyQ ATXN3 in urine. Nonetheless, future studies should more carefully evaluate renal function in SCA3

patients and investigate what may lead to the accumulation of polyQ ATXN3 proteins in urine.

As we are able to collect urine from additional SCA3 cases, we could discuss the possibility that urine samples, which is easiest to collect from patients, as an alternative fluid to plasma or CSF. Interestingly, polyQ ATXN3 was elevated in one pre-symptomatic SCA3 case, although we cannot discuss anything beyond the fact. The usefulness of urine polyQ ATXN3 as a prognostic marker should be investigated in larger cohorts of pre-symptomatic SCA3 cases, as well as in longitudinal studies in the future.

The evaluation of urinary proteins is just emerging as potential biomarkers for neurodegenerative diseases. Indeed, Shepheard et al. observed that urinary p75ECD changes longitudinally in individual patients with amyotrophic lateral sclerosis, and showed that urinary p75ECD has the potential to serve as a prognostic, disease progression, and pharmacodynamic biomarker in this neurodegenerative disease [10]. Of interest, we found a significant correlation between urine polyQ ATXN3 levels and an earlier age of SCA3 onset. However, urine polyQ ATXN3 levels did not associate with *ATXN3* CAG repeat length, given that this is a strong predictor of disease onset [11] and, as expected, repeat length strongly associated with age of SCA3 onset in our cohort (Spearman r: −0.7008, $p < 0.0001$). Thus, the association between urine polyQ ATXN3 with earlier age of onset may be independent from the effect of the *ATXN3* CAG repeats.

Strengths of our study include our analyses of a potential biomarker for SCA3 that can be detected in urine. There are also several limitations in this study: First, it was a cross-sectional study that collected urine at only a single time point for each patient; and second, the sample size of each group was small and included only one pre-symptomatic SCA3 case. In order to elucidate the usefulness of urine polyQ ATXN3 as a biomarker for early diagnosis and therapeutic effects, and further validate its association with an earlier age of SCA3 onset, additional studies are warranted. As such, collection of urine samples from larger cohort of cases, including enough pre-symptomatic SCA3 cases, and assessing polyQ ATXN3 longitudinally, are essential. Despite these limitations, our study highlights the potential benefit of measuring polyQ ATXN3 in urine from SCA3 cases and may encourage investigators to include urine biomarkers in their SCA3 studies and in clinical trials.

In conclusion, our study demonstrates that polyQ ATXN3 proteins accumulate in the urine derived from SCA3 patients with SCA3, and measuring polyQ ATXN3 in urine may have the potential to serve as a useful biomarker in SCA3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Author disclosures

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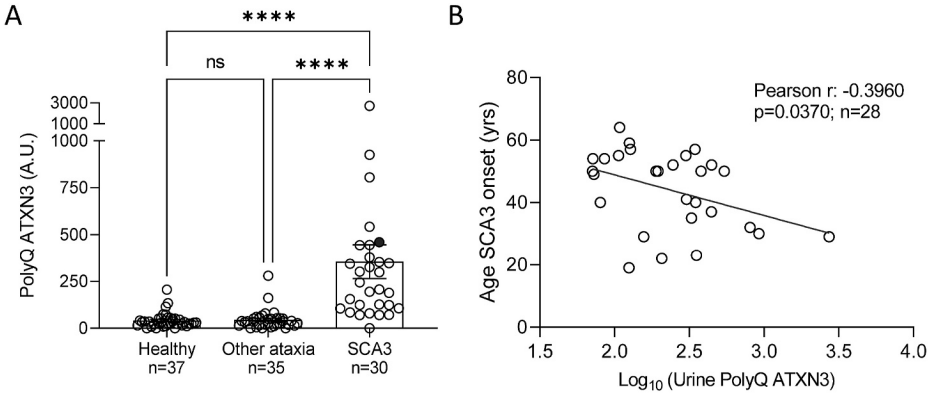

Fig. 1. Urine polyQ ATXN3 protein levels are elevated in patients with SCA3, and correlate with an earlier age of disease onset.

(A) Urine polyQ ATXN3 levels were measured by immunoassay as described (see Methods) in SCA3 cases (including one pre-symptomatic case, filled red circle), other forms of ataxia cases [pathogenic mutation positive cases: one *ATXN1* (SCA1); four *ATXN2* (SCA2); three *ATXN7* (SCA7); two *ATXN8* (SCA8); one *CACNA1A* (SCA6); two *SPTBN2* (SCA5); one *SYNE1*; and three *TRIO*], and healthy controls. Graph represents mean ± SEM. Statistical differences were assessed by One-way ANOVA followed by Tukey's multiple comparison test. ****$p < 0.0001$, n.s.: non-significant differences. (**B**) Visualization of the correlation between urine polyQ ATXN3 levels and age of SCA3 onset assessed by Log transforming the urine polyQ ATXN3 values and performing Pearson's correlation analyses.

Table 1

Association of polyQ ATXN3 protein levels in urine samples with those in plasma and with clinical information.

Association of polyQ ATXN3 in urine and:	n	Pearson's r (95% CI)	p-value
PolyQ ATXN3 in plasma	30[a]	0.3688 (0.009819–0.6436)	0.0449
Age at urine collection	29[a]	−0.3536 (−0.6375 to 0.01481)	0.0599
Age at disease onset	28	−0.3960 (−0.6701 to 0.02688)	0.0370
Disease duration[b]	28	0.06938 (−0.3118 to 0.4313)	0.7257
ATXN3 CAG repeat length	29[a]	0.1673 (−0.2122 to 0.50308)	0.3857
SARA total score	29	0.1641 (−0.2154 to 0.5005)	0.3951
Gait mobility score	29	0.1145 (−0.2631 to 0.4616)	0.5544

[a]includes one pre-symptomatic case.

[b]Defined as time elapsed from onset to sample collection.