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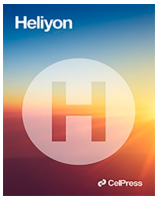

Research article

Validation of a newly developed immunoassay for TDP-43 in human plasma

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ABSTRACT

The level of TAR DNA-binding protein 43 (TDP-43) in human blood was reported to have potential for use as a specific fluid biomarker, which represents disease-specific pathologies, for TDP-43 proteinopathies, including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), which involves the aggregation and deposition of TDP-43 in the nervous system. However, at present, no reliable immunoassay can precisely quantify TDP-43 in human plasma and detect the difference in plasma TDP-43 levels between patients with ALS and controls. We recently developed a novel ultrasensitive immunoassay to quantify TDP-43 in human plasma, and in this study, we analytically validated this assay for application as a diagnostic biomarker for TDP-43 proteinopathies. The novel TDP-43 assay was assessed for the limit of detection, lower limit of quantification, intra- and interassay variation, linearity, parallelism, and analytical spike recoveries. Additionally, 17 pilot plasma samples obtained from patients with ALS and age-matched controls were analyzed using the assay. Our novel TDP-43 assay showed sufficient analytical performance to quantify TDP-43 in human plasma, with high sensitivity (LOD and LLOQ of 0.109 and 0.759 pg/mL, respectively) and high intra- and interassay precision (%CV) below 15 %. The experimental results for spike recovery, parallelism, and dilution linearity were also acceptable. In addition, despite a small sample size, significant differences in the plasma levels of TDP-43 were found between patients with ALS and controls (ALS, 66.63 ± 20.52 pg/mL; control, 42.70 ± 23.06 pg/mL, $p = 0.0330$). These results support that our novel TDP-43 assay is a reliable and innovative method for the quantification of TDP-43 in human plasma and can be a potential blood-based biomarker for the diagnosis of TDP-43 proteinopathies. Further large-scale studies are warranted to validate its usefulness.

1. Introduction

TAR DNA-binding protein 43 (TDP-43) is a 414-amino acid nuclear protein belonging to the heterogeneous ribonucleoprotein (hnRNP) family and has been identified as a major component of ubiquitin-positive inclusions found in degenerating neurons and glial cells of patients with frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) [1,2]. Since the systemic degeneration of motor neurons in ALS causes progressive muscle atrophy, paralysis, and eventual death within 2–5 years, early and accurate diagnosis would be valuable for patients, their families, and doctors. However, the early diagnosis of ALS is challenging

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because differentiating it from other diseases that mimic ALS based only on clinical symptoms and signs is difficult. To address this problem, molecular biomarkers, especially fluid biomarkers that are safe, inexpensive, and widely accessible, are necessary. Thus far, neurofilament light chain (NfL) is the most studied fluid biomarker for ALS, and its levels in the cerebrospinal fluid (CSF) and serum/plasma have been reported to increase in patients with ALS compared with those in controls. Moreover, they were associated with poor outcomes [3]. Despite these promising results, changes in NfL levels in the CSF and blood are not specific to ALS; therefore, other fluid biomarkers that reflect pathognomonic pathologies of ALS, such as TDP-43 accumulation in neurons, are still needed.

Thus far, some studies have used conventional enzyme-linked immunosorbent assay (ELISA) techniques that reported the potential usefulness of TDP-43 levels in the CSF and plasma as a biochemical biomarker to support ALS diagnosis [4–11]. Although these studies have identified high TDP-43 levels in the CSF or plasma from patients with ALS compared with controls, the absolute concentrations of TDP-43 immunoassays are inconsistent for measuring this protein in biofluids [12]. This is primarily caused by those studies that used ELISA techniques that did not have sufficient sensitivity to correctly measure small amounts of TDP-43 in human biofluids. To overcome this insufficient sensitivity, we previously reported using the Simoa assay (Quanterix, Billerica, MA, USA), an ultrasensitive digital immunoassay technique that can measure CSF levels of TDP-43; thus, the Simoa assay can be a useful diagnostic biomarker for ALS [13]. However, the assay kit that we used in that study is no longer available.

In this study, we report the development and validation of a novel in-house Simoa assay for TDP-43, which is highly sensitive, can quantify TDP-43 levels in human plasma and is useful in the diagnosis of ALS.

2. Material and methods

2.1. Immunoassay protocols for the measurement of plasma TDP-43

Plasma TDP-43 levels were analyzed using a novel ultrasensitive immunoassay specific for TDP-43 on the Simoa HD-X analyzer (Quanterix). A mouse monoclonal antibody that recognizes the mid-portion (aa 192–220, within the RNA recognition motif 2 domain) of human TDP-43 (Proteintec, Rosemont, IL, USA) was coupled to paramagnetic beads (#103207; Quanterix) and used for capture. As a detector, a commercially available anti-TDP-43 rabbit polyclonal antibody (Proteintec) was used, which binds the C-terminus of human TDP-43. The detection antibody was conjugated to biotin following the manufacturer's instructions. The TARDBP human recombinant protein (#H00023435-P02, Abnova) was used as the calibrator for the TDP-43 assay. The standard curve for our TDP-43 assay with duplicate measurements was carried out using TARDBP human recombinant protein at different concentrations, i.e., 0, 1.646, 4.938, 14.815, 44.444, 133.333, 400, and 1200 pg/mL, diluted in a sample diluent (Quanterix). All plasma samples stored at −80 °C were thawed, vortexed, and diluted four times with the Homebrew sample diluent contained in the Simoa Homebrew kit (#101351, Quanterix) before the assays to minimize matrix effects. In this assay, 100 μL of the samples or calibrator was added to the capture beads and incubated for 30 min. After washing the beads, the biotinylated detection antibody was added and incubated, followed by further bead washes. Thereafter, 100 μL of streptavidin-conjugated β-galactosidase (SGB, #100439, Quanterix) was added and incubated. After another bead wash, resorufin β−D-galactopyranoside (RGP, #103159, Quanterix) was added, and the resuspended beads were transferred into a multiwell disc array for imaging. Fluorescent signals were converted to the average enzyme per bead number as described [14], and relative concentration estimates of plasma TDP-43 were calculated from four-parametric logistic curves (standard curves) generated with known calibrator concentrations.

2.2. Procedures for method validation

Method validation was conducted according to the guiding principles of a previous study [15].

1. Determination of the limit of detection (LOD) and lower limit of quantification (LLOQ)

Six aliquots of the blank sample (Homebrew sample diluent, contained in Simoa Homebrew kit) were prepared, and the "background" signals of our novel plasma TDP-43 immunoassay on the Simoa HD-X analyzer (Quanterix) were measured. In Simoa, the measured signals were quantified by a common unit, namely, the average number of enzyme labels per bead (AEB). Then, the LOD of the assay was determined as an interpolated TDP-43 concentration derived from the mean plus 2.5 standard deviation (SD) value of AEBs for blank samples. The LLOQ of the assay was determined as an interpolated TDP-43 concentration derived from the mean plus 10 SD value of AEBs for blank samples.

2. Intra-assay precision

Twenty-four samples with different concentrations (1 low, 1 medium, and 2 high levels) of TDP-43 were prepared for the analysis of the intra-assay precision, and the levels of TDP-43 were measured in one experiment. The intra-assay precision was determined by calculating the within-run coefficient of variation of the measured concentrations for those samples.

3. Interassay precision

The interassay precision was determined by calculating the coefficient of variation (CV) of the concentrations derived from standard solutions used for making standard curves with four concentrations (i.e., 0, 0.039, 0.15, and 0.625 pg/mL) of the TDP-43

standard (TARDBP Human Recombinant Protein, Abnova). The TDP-43 levels in those samples were measured four times on different days. The interassay precision was determined by calculating the CV of the concentrations between the runs for those samples.

4. Spike recovery tests, parallelism, and experiments for diffusion linearity

For the spike recovery tests, four aliquots of each two plasma samples with different TDP-43 concentrations were prepared and spiked with 0, 1, 5, and 10 pg of TDP-43 standard in 360 μL of solution (containing patient plasma, sample buffer, and spiked peptide). These eight (4 × 2) aliquots were analyzed in duplicate in the same run. Two spike recovery curves were made, starting from the nonspiked solution to the 10 pg-spiked solution, to evaluate parallelism. Recovery rates (%Recovery) were calculated by subtracting the endogenous TDP-43 concentration from the measured concentration using the following formula [15]:

$$\%\ \text{Recovery} = \frac{\text{Concentration of the spiked sample} - \text{Concentration of the neat sample}}{\text{Theoretical value of the spiked concentration}} \times 100$$

Then, to explore dilution linearity, the high-concentration spiked sample prepared in the abovementioned spike recovery tests was used, and twofold serial dilutions (× 2, × 4, × 8, × 16, and × 32) of the spiked samples were made with the sample diluent until the theoretical concentration reached the LLOQ over the linear range of the standard curve. Serially diluted samples were analyzed in duplicate and compensated for the dilution factor. The %Recovery for the calculated concentration at each dilution was computed using the following formula:

$$\%\ \text{Recovery} = \frac{\text{Measured concentration} \times \text{dilution factor}}{\text{Theoretical concentration}} \times 100$$

Theoretical concentration.

5. Quantification of TDP-43 in human plasma obtained from patients with ALS and controls

Pilot plasma samples of patients with ALS (n = 10, mean age [range]: 62.5 [48–75], 6 males and 4 females) and age-matched normal controls (n = 7, mean age [range]: 60.2 [45–74], 4 males and 3 females) were purchased from KAC Co. Ltd. (Tokyo, Japan). Informed consent was obtained from blood transfusion donors for the use of excess blood samples for research purposes. ALS patients were clinically diagnosed according to the revised El Escorial criteria. The plasma samples were collected in EDTA-containing tubes, stored in polypropylene vials at–80 °C, and then transferred to the National Institute for Quantum Science and Technology (QST, Chiba, Japan). Plasma TDP-43 levels were measured using the above-described assay on the Simoa HD-X analyzer (Quanterix) at the Department of Functional Brain Imaging, QST. On the day of the analysis, the samples were thawed at room temperature, vortexed at 2000 rpm for 30 s, and then centrifuged at 4000×*g* for 10 min. Thereafter, fourfold-fold dilutions of the plasma samples were prepared with the Homebrew sample diluent before the analysis. All plasma samples were analyzed on the same day using the same lots of several reagents for the assay to avoid assay variability caused by lot differences.

2.3. Statistical analysis

Differences between patients with ALS and controls were compared using the Mann–Whitney *U* test. All analyses were performed using GraphPad Prism version 6.0 (GraphPad, San Diego, USA). The level of significance was set at $p < 0.05$.

3. Results

1. Standard curve for the novel TDP-43 immunoassay and determination of the assay's LOD and LLOQ

A representative standard curve for our novel TDP-43 immunoassay is shown in Fig. 1, exhibiting the AEB signal on the vertical axis and known concentrations of the calibrator (TARDBP human recombinant protein) on the horizontal axis. Dose–response analysis was

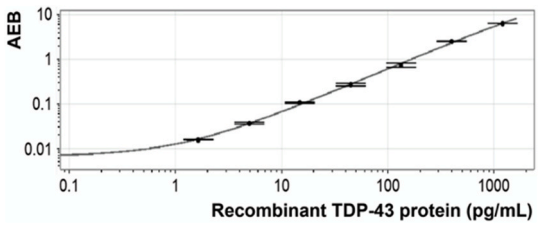

Fig. 1. Standard curve
The plot shows a representative calibration curve for the TDP-43 assay, with the average enzyme per bead (AEB) signal on the vertical axis and known concentrations of the calibrator on the horizontal axis. The curve was generated by analyzing twofold serial dilutions of the calibrator in duplicate measures and fitting the digital signals on a four-parameter logistic curve (goodness-of-fit = 0.9988).

performed by fitting the data with a four-parameter logistic model. By using our novel TDP-43 assay, the standard curve demonstrated that TDP-43 was not only detected with high sensitivity but also successfully with a wide dynamic range of approximately 1–1000 pg/mL. The goodness of fit of the standard curve was 0.9988, and the CV was 7.57–9.70 % (<10 %) within the standard range.

The LOD of the assay, which requires 50 μL of plasma, was 0.109 pg/mL (determined as an interpolated TDP-43 concentration derived from the mean plus 2.5 SD of AEBs for blank samples). The LLOQ of the assay was 0.759 pg/mL (determined as an interpolated TDP-43 concentration derived from the mean plus 10 SD of AEBs for blank samples).

2. Intra-assay precision for the new TDP-43 assay

The intra-assay precision was determined with 24 samples using different concentrations (1 low, 1 medium, and 2 high levels) of TDP-43, and it was robust, with CVs between 3.4 % and 12.9 % (Table 1).

3. Interassay precision

The interassay precision was determined by calculating the %CV of AEBs derived from four concentrations (0, 0.039, 0.15, and 0.625 pg/mL) of the TDP-43 standard solution used for making standard curves. The TDP-43 levels in those samples were measured four times on different days. The %CV of plasma TDP-43 at different concentrations was 10.1, 10.9, 8.0, and 5.0 % for standard TDP-43 solutions of 0, 1.646, 4.938, and 14.815 pg/mL, respectively (Table 2). These results indicate that our novel TDP-43 assay shows permissible interassay precision (<15 %).

4. Spike recovery tests, parallelism, and experiments for dilution linearity

In the spike recovery and parallelism experiments (Fig. 2), the recovery rate (%recovery) of each sample was 95.6%–118.4 % (Table 3). The parallelism of two spike recovery curves starting from the nonspiked solution to the 10 pg-spiked solution is also shown in Fig. 2, which demonstrated that plasma samples spiked with 0, 1, 5, and 10 pg of the TDP-43 standard gave reliable results.

Dilution linearity experiments demonstrated that a sample with a spiked concentration can be diluted to a concentration within the working range and still give a reliable result. Fig. 3 shows the results of those experiments providing reliable quantification after dilution within the standard curve range (Table 4).

5. Confirmation of the usefulness of the developed assay to quantify TDP-43 in plasma samples obtained from patients with ALS and controls

To determine the usefulness of our novel assay to quantify TDP-43 in human plasma, we measured the TDP-43 levels in pilot plasma samples obtained from patients with ALS (n = 10) and age-matched controls (n = 7) on the same day using the same lots of several reagents for the assay. All the measured TDP-43 levels in those plasma samples, even from those obtained from controls, fell into the working range of this assay over the linear range of the standard curve. The plasma TDP-43 levels measured with the developed assay were significantly higher in the ALS group (mean ± SD 66.63 ± 20.52 pg/mL, 95 % confidence interval (95 % CI) of the mean 52.44–92.62, n = 10) than in the age-matched control group (42.70 ± 23.06 pg/mL, 95 % CI of the mean 10.76–82.69, n = 7; p = 0.0330, Mann–Whitney *U* test; Fig. 4).

4. Discussion

In this study, we developed a novel assay that can reliably quantify TDP-43 in human plasma samples and has potential for use as a blood biomarker for the diagnosis of ALS. Our novel TDP-43 assay showed sufficient analytical performance to quantify TDP-43 in human plasma, with high sensitivity (LOD and LLOQ of 0.109 and 0.759 pg/mL, respectively), and the intra- and interassay precision (%CV) values were below 15 %, which is below the 20 % recommended acceptance criterion for single-laboratory accuracy of a biomarker [16]. The results of the experiments for spike recovery, parallelism, and dilution linearity were also acceptable. Additionally, despite the small number of plasma samples, significant differences in the plasma TDP-43 levels were found between the ALS group and the control group. We used an ultrasensitive digital array technology (Simoa system, Quanterix) that can be applied to the development of unprecedented biomarkers for neurological diseases because of its ultrahigh sensitivity. Even in the control group, plasma TDP-43 concentrations were measured because of the ultrahigh sensitivity of this assay to detect minute amounts of TDP-43 in human plasma. These results warrant further studies to validate the usefulness of our novel TDP-43 assay in the diagnosis of ALS and related disorders in a large number of patients with ALS, those with ALS mimics, and controls.

Table 1
Intra-assay precision (n = 12).

	Sample 1	Sample 2	Sample 3	Sample 4
Mean(pg/mL)	19.28	16.45	10.24	3.73
SD	2.50	0.57	1.03	0.24
CV (%)	12.9	3.4	10.0	6.4

Table 2
Inter-assay precision for quality control samples (n = 16).

	Sample 1	Sample 2	Sample 3	Sample 4
Mean(pg/mL)	6.89	22.55	59.77	167.85
SD	0.70	2.46	4.75	8.39
CV (%)	10.1	10.9	8.0	5.0

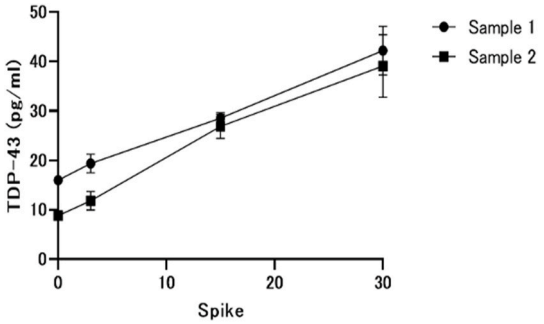

Fig. 2. Spike recovery and parallelism
Four aliquots of each two plasma samples with different TDP-43 concentrations were spiked with 0, 1, 5, and 10 pg of TDP-43 standard in 360 μL of solution. These 8 (4 × 2) aliquots were analyzed in duplicate. We made two spike recovery curves starting from nonspiked solution to 10 pg-spiked solution to evaluate parallelism. Recovery rates (%Recovery) were calculated at each concentration.

Table 3
Recovery rate (%recovery) for each plasma sample in spike recovery tests.

	Treatment	Measured concentration (pg/mL)	CV (%)	Theoretical concentration of the spiked sample (pg/mL)	Recovery rate (%)
Sample 1	Neat (0 pg spike)	16.03	5.8		
	1 pg spike	19.39	9.7	18.80	103.1
	5 pg spike	28.55	3.9	29.88	95.6
	10 pg spike	42.24	11.6	43.73	96.6
Sample 2	Neat (0 pg spike)	8.86	1.9		
	1 pg spike	11.87	15.9	11.63	102.1
	5 pg spike	26.88	9.0	22.71	118.4
	10 pg spike	39.10	16.2	36.56	106.9

CV = coefficient of variation.

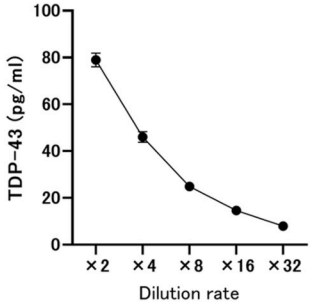

Fig. 3. Dilution linearity
The high-concentration spiked sample prepared in the spike recovery tests (Fig. 2) was serially (× 2, × 4, × 8, × 16, and × 32) diluted with the sample diluent until the theoretical concentration reached the LLOQ. The TDP-43 levels in the serially diluted samples were measured in duplicate and compensated for the dilution factor. The %Recovery for the dilution-corrected concentration was calculated at each dilution.

To our knowledge, no study has presented an immunoassay that could precisely quantify TDP-43 in human plasma and detect the difference in the plasma levels of TDP-43 between patients with ALS and controls. Our novel immunoassay for TDP-43 can quantify TDP-43 levels in human plasma and present quantitative data on the plasma levels of TDP-43 in patients with ALS and controls due to the ultrahigh sensitivity of this assay.

Some previous studies, including ours, reported the potential usefulness of TDP-43 levels in the CSF and plasma as a biochemical

Table 4
Dilution linearity for a plasma sample analyzed with the new TDP-43 assay.

	Fold dilution	Mean measured concentration (pg/mL)	CV (%)	Dilution corrected concentration (pg/mL)	Recovery rate (%)
Sample	2	79.00	3.7	79.0	100.0
	4	46.01	4.9	92.02	116.5
	8	24.87	7.6	99.48	125.9
	16	14.63	7.5	117.04	148.2
	32	8.00	1.2	128.0	162.0

CV = coefficient of variation.

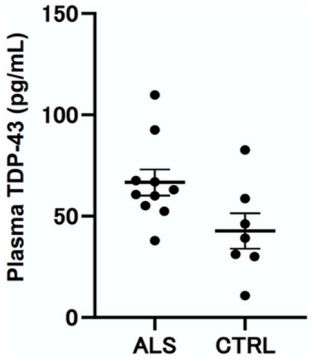

Fig. 4. Quantification of TDP-43 in human plasma obtained from patients with ALS and controls Plots for the plasma TDP-43 concentrations in clinically diagnosed patients with ALS (n = 10) and healthy controls (CTRL, n = 7). The solid lines represent the mean value ± standard error of the concentrations of each group. The plasma TDP-43 concentration in the ALS group was significantly higher than that in the age-matched control group (p = 0.0330, Mann–Whitney *U* test).

biomarker to support the diagnosis of ALS [4–11]. However, the absolute concentrations of TDP-43 immunoassays are inconsistent for measuring this protein in biofluids [12], mainly because those studies used ELISAs that do not have sufficient sensitivity to correctly quantify small amounts of TDP-43 in human biofluids. To overcome this insufficient sensitivity, we previously reported using the Simoa assay (Quanterix, Billerica, MA, USA), an ultrasensitive digital immunoassay technique that can measure CSF and plasma levels of TDP-43; thus, the Simoa assay can be useful as a fluid biomarker for the diagnosis of ALS [13]. However, the assay kit that we used in that study is no longer manufactured; accordingly, no reliable immunoassays that can quantify the exact levels of TDP-43 in human plasma samples currently exist internationally. The novel assay we have developed and presented herein could aid in the diagnosis of ALS if the usefulness and robustness of the assay are confirmed in future large-scale studies of patients with ALS and related disorders and age-matched controls.

In addition, our novel TDP-43 assay would be potentially useful in the diagnostic work-up for dementia disorders if it could evaluate the extent of brain TDP-43 deposition through the quantification of plasma TDP-43 levels. If that is the case, then our novel TDP-43 assay could be the only blood-based biomarker for detecting brain TDP-43 deposition that is not yet available at present. If our novel assay could evaluate brain TDP-43, it could possibly be used for the differential diagnosis between FTLD-tau and FTLD-TDP [17, 18] as well as the diagnosis of limbic predominant age-related TDP-43 encephalopathy neuropathological change (LATE-NC) [19]. The identification of LATE-NC in the aged population is important because the clinical manifestation of TDP-43 pathologic lesions, which exists in nearly 50 % of the brains of persons aged over 80 years [20], can recapitulate the clinical and neuroimaging picture of Alzheimer's dementia [21–23], presenting a challenge for clinical diagnosis. LATE-NC is currently not a diagnosable antemortem using clinical information or conventional biomarkers. Therefore, we now expect that our novel TDP-43 assay might be potentially applicable to the clinical diagnosis of LATE-NC. If our novel TDP-43 assay could be used for the diagnosis and evaluation of brain TDP-43 deposition, it would also be useful in clinical trials of disease-modifying therapies for AD, in which comorbid brain diseases caused by pathologies other than AD should be correctly diagnosed and evaluated.

5. Conclusions

In this study, we analytically assessed the performance of a novel in-house assay to quantify TDP-43 in human plasma and validated that our TDP-43 assay is a reliable method for the quantification of plasma TDP-43 and, accordingly, can be a potential blood-based biomarker for the diagnosis of TDP-43 proteinopathies. Further large-scale studies are warranted to validate its usefulness.

Ethics statement

This study was approved by the National Institutes for Quantum Science and Technology Certified Review Board (the ethics

approval number: 20-007). Written informed consent was obtained from all participants.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Sayo Matsuura: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Harutsugu Tatebe:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing. **Makoto Higuchi:** Funding acquisition, Supervision, Writing – review & editing. **Takahiko Tokuda:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Takahiko Tokuda reports financial support was provided by the Japan Agency for Medical Research and Development (AMED). Takahiko Tokuda has patent #PCT/JP2022/004776 pending to National Institute for Quantum Science and Technology.

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