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ORIGINAL ARTICLE

D-Tyr-tRNA^{Tyr} Deacylase, a New Role in Alzheimer's-associated Disease in SAMP8 Mice[△]

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Key words: D-Tyr-tRNA^{Tyr} deacylase; D-amino acid; SAMP8 mice; neurodegenerative disease

Objective To assess the expression level of D-Tyr-tRNA^{Tyr} deacylase (DTD) in SAMP8 mice and speculate the function of DTD in disorders associated with Alzheimer's disease (AD).

Methods Altogether 12 SAMP8 mice and 12 SAMR1 mice were used in this study. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blot were performed to detect the mRNA and protein levels of DTD in the mice. Purified DTD protein was injected into lateral ventricle to investigate the function of DTD in SAMP mice. The behavior of the mice was tested by using a Step-through Test System.

Results Both mRNA and protein levels of DTD were found to be significantly lower in SAMP8 mice compared with those in SAMR1 mice ($P < 0.05$). *In vivo* injection of DTD protein did not lead to an obvious change in behavior of SAM mice.

Conclusions DTD might function in the process of AD-associated pathology and could possibly participate in physiology process in a long-term manner to orchestrate with other regulators in order to maintain the balance of organism.

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D-AMINO acids have recently been detected in various living organisms from bacteria to mammals, and especially in the central nervous system (CNS) of vertebrates with high concentration. Many works focused on the physiological

and pathophysiological roles of D-amino acids in CNS and peripheral tissues. For instance, there is high concentration of D-serine (D-Ser) and D-aspartate (D-Asp) in mammal brain.^{1,2} D-Ser is an internal ligand of N-methyl-D-aspartate (NMDA) receptor, which is a new target in some acute and chronic neurological diseases. D-Asp acts as a "neurotransmitter", transmitting information between the neuron and the glia.^{3,4} But D-amino acids may cause misfolding of proteins once they participate in the protein synthesis. As a solution to this problem,

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D-Tyr-tRNA^{Tyr} deacylase (DTD) motif can deacylate mis-amino acylated D-aminoacyl-tRNAs.⁵⁻⁸ DTD has been found to exist in pyramidal neurons in cortex, pyramidal neurons in hippocampus, dopamine neurons in midbrain, and Purkinje cells in cerebellum.⁸ We assume that the presence of enriched DTD in hippocampus may indicate that DTD is associated with learning and memory performance. To test this assumption, we introduced a classic Alzheimer's Disease (AD)-associated aging mouse model, the SAMP8 strain, which has defects in age-related learning and memory; the SAMR1 strain was used as a normal control.^{9,10}

MATERIALS AND METHODS

Animals

The 4-month-old SAMP8 mice ($n=6$), 8-month-old SAMP8 mice ($n=12$), 4-month-old SAMR1 mice ($n=6$), and 8-month-old SAMR1 mice ($n=12$) were purchased from the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine. The mice were maintained at the Animal Centre of Peking Union Medical College at $25\pm1^{\circ}\text{C}$ under a light-dark cycle, each lasting for 12 hours. All the protocols of the animal experiments were examined and approved by the college Animal Care and Use Committee according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from mouse hippocampal tissue samples with Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and 2 μg of the extracted RNA were reverse-transcribed to cDNA using reverse transcriptase (New England Biolabs, Ipswich, MA, USA). Both steps were conducted following the manufacturers' protocols. For amplification, 2.5% of the cDNA pool was used, and PCR was run for 25-30 cycles in each analysis.

The primer sequences used in the semi-quantitative RT-PCR for detection of mouse DTD mRNA were 5'-GTGCTGTGTGTCAGCCAGTT-3' (forward primer) and 5'-TCTCTGCTGCTGTTTCTCCA-3' (reverse primer). The primer sequences of GAPDH were 5'-ACCACAGTCCATGCCATCAC-3' (forward primer) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse primer).

Protein extraction and Western blot

Total protein of hippocampal tissue samples was extracted with protein lysis buffer (150 mmol/L NaCl, 1% NP-40, and 50 mmol/L Tris-HCl, pH 8.0) supplemented with protease

inhibitor cocktail [2 $\mu\text{g}/\text{mL}$ phenylmethanesulfonyl fluoride (PMSF), 2 $\mu\text{g}/\text{mL}$ pepstatin, 2 $\mu\text{g}/\text{mL}$ aprotinin, and 2 $\mu\text{g}/\text{mL}$ leupeptin, produced by Roche, Basel, Switzerland]. After lysis on ice for 30 minutes, the lysates were centrifuged at 12 000 rpm ($r=6\text{ cm}$) for 20 minutes, and the supernatant was collected. The Bradford method was used to mensurate protein concentration. The lysates were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was transferred onto nitrocellulose membrane. The protein was detected with our lab-made polyclonal DTD antibody or mouse monoclonal β -actin antibody (Cat.A5441, Sigma-Aldrich, St. Louis, MO, USA). β -actin was used as loading control.

DTD protein expression and purification

The open reading frame (ORF) of DTD was first cloned into pET28a+ vector and expressed in *Escherichia coli*. We applied affinity chromatography to purify the collected DTD proteins, and Ni^{2+} charged sepharose beads to fish His-tagged protein from the sonicated cell lysates. Then, the purified DTD protein was introduced to gel filtration chromatography system ($\text{\AA KTA}$, GE Healthcare, Pittsburgh, PA, USA) using sephacryl S-200. Silver staining was used to detect DTD protein.

In vivo injection of DTD protein

Twelve 8-month-old SAM mice (6 SAMP8 and 6 SAMR1) were infused with 3 μL DTD protein (315 $\mu\text{g}/\text{mL}$, experimental group) or solvent (1 $\times$ phosphate buffered saline, control group) in the lateral ventricle through a dental drill in order to observe whether increasing exogenous DTD protein could affect learning and memory ability. The stereotaxic instrument of the brain (RWD Life Science, Shenzhen, China) was used to access the exact location of the lateral ventricle.^{11,12}

Step-through Test

The injected 8-month-old mice were introduced to a Step-through Test System 2 days after injection to evaluate their memory. The design of the test was as follows. The test cage was divided by a guillotine door into a light compartment and a dark one. A mouse was placed in the light compartment and allowed to remain there for a preset period of time so as to get familiar with the surroundings. Then the guillotine door was opened and it tried to get into the dark room because of its natural preference to dark place. As soon as it got into the dark compartment, an electrical shock was given manually. After a training procedure, the 12 mice, all assessed as suitable for the test, were introduced into the system, and the frequency of

entry into the dark compartment was recorded within 180 seconds.

Statistical analysis

Student's *t*-test was used to compare the numeric data between the two groups of mice. Statistical significance was defined as $P < 0.05$.

RESULTS

To speculate the function of DTD in neurodegenerative disorders, we tested DTD expression pattern at transcriptional level in 4- and 8-month old SAMP8 and SAMR1 mice with semi-quantitative RT-PCR. DTD mRNA level was found to be significantly lower in SAMP8 mice than in SAMR1 mice in both 4- and 8-month-old groups ($P < 0.05$). In the following Western blot, we noticed that DTD protein was expressed less in SAMP8 mice than in SAMR1 mice as presumed ($P < 0.05$) (Fig 1). These findings suggested that DTD might be one of the regulators involved in senescence-associated neurodegenerative diseases.

The abnormal expression of DTD in AD mice led us to investigate the function of DTD *in vivo*. We expressed and purified DTD protein using gel filtration system (Fig. 2) and attained a concentration of 315 $\mu\text{g/mL}$ for *in vivo* injection. The Step-through Test results showed that neither experimental group nor control group in SAMR1 mice entered the dark compartment within the preset 180-second period, whereas all SAMP8 mice, both the experimental and control groups, entered the dark compartment with electric shocks within the 180 seconds (Fig. 3). There seemed to be no change in behavior after the injection of purified DTD when compared with control.

DISCUSSION

DTD is a conserved protein able to hydrolyse D-Tyr-tRNA^{Tyr} into free tRNA^{Tyr} and D-Tyr. It has been reported that inactivation of DTD increased toxicity of D-Tyr in *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae*.^{6,13,14} It was also demonstrated that D-Tyr was toxic in *E. coli* by charging of tRNA^{Tyr}, and by depletion of free

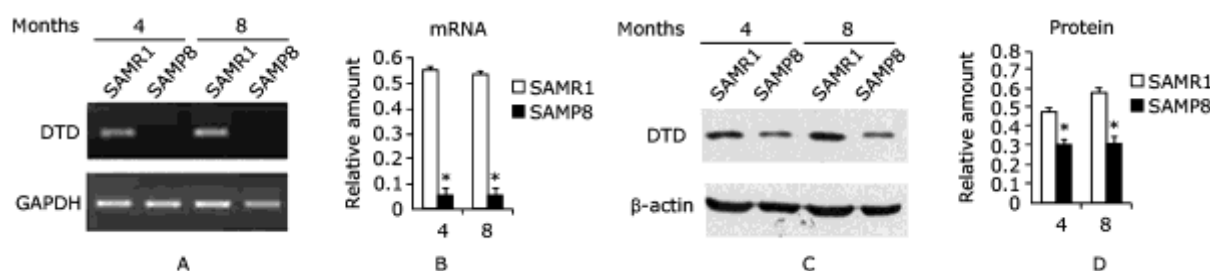


Figure 1. Expression profile of mRNA and protein of D-Tyr-tRNA^{Tyr} deacylase (DTD) in 4- and 8-month-old SAMP8 and SAMR1 mice.

A. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) results of DTD mRNA;

B. Quantitative analysis of RT-PCR results;

C. Western blot results of DTD protein expression;

D. Quantitative analysis of Western blot results.

GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

* $P < 0.05$ compared with the results in SAMR1 mice.

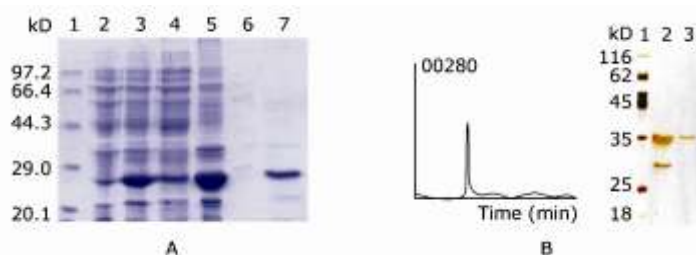


Figure 2. Expression and purification of DTD protein.

A. Expression and affinity purification of DTD protein in *Escherichia coli*;

1. marker; 2. before inducement; 3. after inducement; 4. deposition; 5. supernate; 6. effluent of washing; 7. effluent of elution.

B. Gel filtration chromatography of DTD protein;

Left: chromatogram of DTD protein; right: silver staining results after gel filtration.

1. maker; 2. Ni column purification fraction of DTD protein; 3. gel filtration fraction of DTD protein.

tRNA^{Tyr} and L-Tyr-tRNA^{Tyr} in DTD-deficient *E. coli*.¹⁵ De-

AD-associated pathological mechanisms.

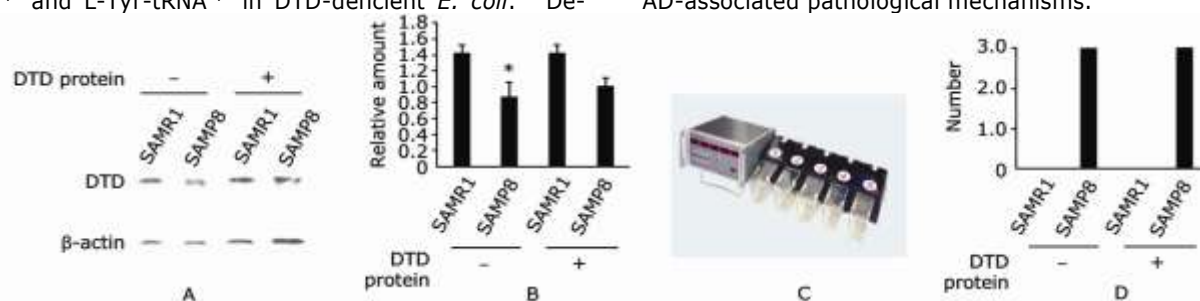


Figure 3. *In vivo* injection of DTD protein and Step-through Test of SAMP8 and SAMR1 mice.

- A. Western blot results in 8-month-old SAMP8 ($n=3$) and SAMR1 mice ($n=3$) injected with DTD protein (+) or control solvent (-);
 B. Quantitative analysis of Western blot results;
 C. Step-through Test System;
 D. Step-through Test results. Injection of DTD protein did not show to bring any change in behavior. Number means the number of mice entered the dark compartment.

* $P < 0.05$ compared with the results in SAMP8 mice injected with DTD.

positions of D-Asp and D-Ser in β -amyloid in neurons during aging are possibly related to AD, and the ratio of D- to L-amino acids in proteins may be used as a marker of aging.^{2,16} It has recently been reported that an editing-defective tRNA synthetase caused protein misincorporation, misfolding, neuronal loss, and finally neurodegeneration.¹⁷

In a previous study, we found DTD in mouse cortical pyramidal neurons, hippocampal pyramidal neurons, cerebellar Purkinje cells, and dopaminergic neurons, which all play important roles in the CNS. Results of that study suggest that human DTD may be a significant component in cellular resistance against D-amino acids by deacylating D-aminoacyl-tRNAs at the nuclear pore. It was also found that mouse DTD was specifically enriched in CNS neurons. Its localization on the nuclear envelop indicates that D-aminoacyl-tRNA editing may be vital for the survival of neurons under high concentration of D-amino acids.⁸

The accumulation of DTD in CNS, especially in hippocampal area implies that DTD could be a regulator involved in AD and other neurodegenerative diseases. The SAM mice were derived from an AKR/J breeding colony by Professor Takeda. Selective breeding led to the development of a number of rapid aging sublines, including SAMP8 mice. The SAMP8 mice subline has attracted much interest in gerontological research on dementia because of their learning and memory defects characteristic in old age.¹⁹ The SAMP8 mice show early difficulties in learning and memory related to abnormalities in septohippocampal function, with a decrease in serotonin level leading to an increase in gamma-aminobutyric acid (GABA) level and a decrease in acetylcholine level.¹⁸ The naturally developed SAMP8 mice are used as a model to investigate AD and

Using the SAM strains of mice as a model of AD-associated disease, we detected DTD expression in SAMP8 and SAMR1 mice at different stages. Experimental results showed that the expression of DTD was lower in the hippocampus of SAMP8 mice than that in SAMR1 mice at both transcriptional and translational levels, which might explain the cognitive defects in SAMP8 mice. It is reasonable to conclude that the decline of DTD would affect the normal process of tRNA aminoacylation in SAMP8 mice, thereby enhance the risk of mismatch aminoacylation of tRNAs, and thus lead to protein misfolding. Although *in vivo* injection of DTD protein did not show to induce any significant changes in behavior, it might be due to the short time of injection. The pathology process is a long-term consequence; therefore the instant change at molecular level cannot transfer into a huge change in learning and memory. The significant reduction of DTD in SAMP8 mice and the known function of DTD suggest that DTD could be an important regulator in AD-associated pathology, providing another clue to the cure of AD-associated diseases.

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