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The impact of V30A mutation on transthyretin protein structural stability and cytotoxicity against neuroblastoma cells

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ABSTRACT

Single point mutations in the *transthyretin* (*TTR*) gene may cause a hereditary neurodegenerative disease termed familial amyloidotic polyneuropathy (FAP) due to accelerated deposition of amyloid fibrils, resulting in peripheral and autonomic nervous system dysfunction. Recently, we found a Chinese FAP family involving a *TTR* V30A mutation. To understand the pathogenic mechanisms of this V30A TTR, we investigated the effects of this mutation on TTR quaternary and tertiary structural stabilities and cytotoxicities against neuroblastoma cells along with the most common variant V30M TTR and the wild-type (WT) TTR. Our results showed that the V30A mutation impaired the thermodynamic and kinetic stabilities of the TTR protein by increasing the extent and rate of tetramer dissociation and unfolded monomer and amyloid fibril formation, even to a greater extent than the V30M mutation under several experimental conditions. Further, an obviously cytotoxic effect of the V30A TTR on the human neuroblastoma cell line, IMR-32, was observed. The V30A TTR induced apoptosis and autophagy concomitant with the accumulation of reactive oxygen species (ROS) and DNA double-strand breaks, reflected in the induction of phosphor-H2A.X. These results suggest that the V30A mutation in the *TTR* gene promotes the formation of unfolded monomers and amyloid fibrils, which potentially contribute to the increased neurotoxicity and the pathology associated with this FAP family.

Introduction

Familial amyloid polyneuropathy (FAP)[2] is a hereditary neurodegenerative disease caused by mutations in the *transthyretin* (*TTR*) gene [1]. The clinical and pathologic features include systemic deposition of mutated TTR amyloid fibrils, resulting in peripheral and autonomic nervous system dysfunction [2]. Currently, more than 100 different point mutations in the *TTR* gene have been reported with most of them associated with FAP [1–4].

TTR is a 55-kDa homotetrameric protein which contains identical 127-residue subunits with a predominant β-sheet structure. TTR is present in human plasma (0.2–0.4 mg/ml) and cerebral spinal fluid (0.0002–0.02 mg/ml) and transports L-thyroxine (T_4) and retinol binding protein complex [5,6]. Extensive data support the hypothesis that the majority of amyloidogenic mutations destabilize the native TTR, favoring dissociation of tetramers into partially unfolded monomeric species which can self-assemble into either soluble high molecular weight oligomers or insoluble amyloid fibrils which subsequently cause tissue damage [7–10]. However, the thermodynamic and kinetic stabilities of TTR variants differ depending on the position of mutation and amino acid substitution [11]. A correlation between the amyloidogenicity of TTR variant *in vitro* and the severity of FAP disease has been previously reported [7,8,12,13]. Therefore, biochemical and biophysical analyses of TTR variants may partly elucidate their pathologic potentials.

Recently, we found a Chinese FAP family involving a rare TTR Val30 to Ala substitution [14]. The FAP family members with the

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[2] *Abbreviations used:* TTR, transthyretin; FAP, familial amyloidotic polyneuropathy; WT, wild type; ROS, reactive oxygen species; OD, optical density; ThT, thioflavin; DMSO, dimethyl sulfoxide; T_4, L-thyroxine; Flu, flufenamic acid; ITC, isothermal titration calorimetry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EMEM, eagle's minimum essential medium; FBS, fetal bovine serum; MTT,3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PVDF, polyvinylidene difluoride.

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V30A heterozygous mutation had symptomatic onset at about 20–35 years and complete penetrance [14]. The clinical symptoms of this V30A TTR amyloidosis have been characterized including sensorimotor neuropathy of the lower limbs, followed by the appearance of dysautonomy, severe cardiomyopathy, and chronic kidney failure [14]. This mutation was first reported in 1992 in a FAP family of German descent [15], followed by two other reports from China [16,17]. These reports mainly focused on the clinical characterization and genetic analysis of the FAP cases. However, the effects of the V30A mutation on thermodynamic and kinetic stabilities of TTR homotetramer have not been determined.

The native state of TTR is a homotetramer with eight β-strands A–H organized into a β-sandwich in each monomer (Fig. S1). The β barrel constituting by 16 strands forms two identical thyroxine-binding sites through the center of TTR tetramer [18]. V30 is in the B-strand that forms the hydrophobic core [19]. Although not located in the T_4 binding site, previous study has shown that the most common TTR variant, V30M TTR significantly lowered T_4 binding affinity compared to wild-type (WT)-TTR [20]. The substitution of V by M may force the two β-sheets of each monomer to become more separated, resulting in a distortion of the T_4-binding cavity and decreased affinity for T_4 [21]. It is conceivable that the V by A substitution may have similar effects.

In the present study, we determined the quaternary and tertiary structural stabilities of this TTR variant and explored the molecular mechanisms underlying the cytotoxic effects of this variant on neuroblastoma cells, along with the WT TTR and the V30M variant. We found that the V30A TTR variant was substantially less stable than the WT TTR, even worse than the V30M variant under some experimental conditions, accompanied by significantly increased amyloid fibril formation and cytotoxicity against neuroblastoma cells.

Materials and methods

TTR expression and purification

Expression constructs for the V30A and V30M mutants were prepared by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the pQE30-wtTTR as template. Clones containing the desired nucleotide changes were identified by automated DNA sequencing by using the pQE30 sequencing primer (Qiagen, Alameda, CA) at Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, China). The V30A, V30M, and WT TTR plasmids were transformed into the M15 competent cells (Qiagen), and proteins were expressed and purified, as previously described [22].

Amyloid fibril formation assays

Amyloid formation was detected by turbidity assays which measure the large insoluble aggregates and amyloid fibrils, as described previously [23,24]. TTR (1 mg/ml) in 10 mM phosphate buffer with 100 mM KCl and 1 mM EDTA (pH 7.0) was diluted 1:1 with a 200 mM buffer containing 100 mM KCl and 1 mM EDTA at desired pH (sodium acetate for pH 3.4–5.4, and phosphate buffer for pH > 5.4). After incubating at 37 °C for 72 h, all samples were vortexed gently and the optical density (OD) at 400 nm was measured with a Synergy™ four hybrid microplate reader (BioTek Instruments Inc., Winooski, VT).

Thioflavin T (ThT) binding was another approach to evaluate amyloid fibril formation [24]. It measures not only large insoluble aggregates and amyloid fibrils but also soluble amyloid-like small oligomers. A 400 μl aliquot of TTR samples (2 mg/ml) was mixed with 2.75 ml of 50 mM Tris buffer (100 mM KCl, 1 mM EDTA at pH 8.0) and 30 μl of ThT stock solution (2.0 mM). After incubating at 37 °C for 72 h, the samples were then excited at 440 nm, emission at 482 nm was recorded by using a Synergy™ four hybrid microplate reader (BioTek Instruments Inc.). A time course of fibril formation at pH 4.2 was also evaluated by this ThT binding assay.

The inhibition of TTR fibril formation by T_4 was investigated by ThT binding. Stock solution of T_4 was prepared by dissolving T_4 in dimethyl sulfoxide (DMSO) at 5 mM. The TTR solutions (0.25 mg/ml) at pH 4.2 were incubated with variable concentrations of T_4 at 37 °C for 72 h, and then ThT fluorescence was recorded.

Isothermal titration calorimetry (ITC)

The dissociation constants characterizing the binding of flufenamic acid (Flu) to WT TTR and its variants were determined by using a Nano-ITC-LV (TA, USA). A solution of flufenamic acid (final concentration 300 μM in 10 mM phosphate buffer, pH 7.6/100 mM KCl/1 mM EDTA/1.5% DMSO) was prepared and titrated into an ITC cell containing WT TTR or its variants (17 μM in 10 mM phosphate buffer, pH 7.6/100 mM KCl/1 mM EDTA/1.5% DMSO). Each experiment was performed by 41 injections of 1 μl at 5 min intervals at 25 °C. The data were recorded automatically and analyzed by NanoAnalyze software. Dissociation constants were obtained by curve fitting with the multiple binding sites model.

Quaternary structural changes assessed by SDS–PAGE and resveratrol binding assays

Changes of TTR quaternary structure were first evaluated by monitoring pH- or urea-induced tetramer dissociation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), as previously described [24]. The TTR solutions (0.5 mg/ml) at variable pH or in the presence of different concentrations of urea were incubated at 4 °C for 40 h. The TTR samples (4 μl) were then mixed with 1 μl of gel-loading buffer containing 0.1% SDS and 13% glycerol. Samples without boiling were separated on a 12% SDS–polyacrylamide gel and stained with coomassie blue.

The binding of resveratrol to the TTR tetramers was performed to quantitatively assess their quaternary structure stabilities as a function of urea concentration, as previously described [8,10,25]. The TTR solutions (0.5 mg/ml) in the presence of variable concentrations of urea were incubated at 4 °C for up to 40 h. Then the TTR samples (200 μl) were mixed with 1.44 μl of resveratrol from a 12.5 mM stock solution in DMSO. The fluorescence emission spectra were obtained with excitation and emission wavelengths of 320 and 390 nm, respectively.

Tertiary structural changes monitored by Trp fluorescence

The tryptophan fluorescence was used to monitor TTR tertiary structural changes, as previously described [10,23]. Upon denaturation, tryptophan residues in the TTR proteins (Trp41 and Trp79) become more solvent exposed, and the $\lambda_{max,\ emission}$ shifts from 335 nm in the native state to 355 nm in the unfolded state. Therefore the fluorescence intensities at 335 or 355 nm may reflect the relative amount of folded and unfolded protein [26]. The TTR protein solutions (0.5 mg/ml) containing various concentrations of urea were incubated at 25 °C for up to 96 h. The fluorescence intensities at 335 or 355 nm were recorded to reflect urea-induced unfolding of each TTR sample.

Molecular dynamics simulation

We performed molecular dynamics simulation analysis for the WT-TTR (PDB number: 1BMZ) and its variants. The structure of