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# Vitreous and serum levels of transthyretin (TTR) in high myopia patients are correlated with ocular pathologies

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ABSTRACT

**Purpose:** To detect serum and vitreous transthyretin (TTR) in high myopia patients and to evaluate potential associations between TTR and clinical parameters and ocular pathologies, including different ocular pathologies.

**Design and methods:** Serum samples from 16 high myopia patients and 4 controls were analyzed by LTQ-MASS. Serum samples from 116 high myopia patients and 86 healthy controls were tested by Western blots and ELISA. Eight healthy and 40 pathologic vitreous samples were analyzed by ELISA. And corresponding serum samples were also analyzed by ELISA.

**Results:** Significant increased TTR serum levels were detected in high myopia patients compared to healthy controls. The high levels of serum TTR were associated with ocular pathologies, long axial length, and low visual acuity. TTR in high myopia patients with macular hole and macular detachment was upregulated in both vitreous and the corresponding serum samples. TTR levels in serum samples of high myopia patients with long axial lengths were higher than in the vitreous.

**Conclusions:** Serum TTR may be a biomarker for high myopia patients with ocular pathologies.



## Introduction

High myopia is usually defined as eyes with -6 dioptres (D) of myopia, or>26.0 mm in axial length [1]. Progressive and excessive elongation of the eyeball leads to secondary ocular diseases such as retinal detachment [2] and macular degeneration [3]. These complications can cause irreversible visual disturbances. Although the diagnosis and therapies for high myopia are improving, a high percentage of cases still result in blindness. In the Beijing Eye Study of 4409 Chinese individuals under 40 years old, high myopia was the second most frequent cause of low-vision and blindness [4]. The aging population is growing disproportionately in China, so the visual acuity of the elderly population is of great social concern [5]. To reduce the complications and improve the effectiveness of diagnosis and therapy, it is important to detect the protein biomarkers that are associated with high myopia progression as these may be useful for diagnosis or as therapeutic targets.

Transthyretin (TTR) is a homotetrameric protein of 55 kDa synthesized mainly in liver, choroid plexus, retinal pigment epithelium, and pancreas [6–8]. In serum, it functions as a carrier for thyroxin and retinol-binding protein (RBP). In ophthalmology research, vitreous amyloid fibrils were the result of local synthesis of mutated TTR. Nuclear cataract was associated with low protein intake and low serum levels of TTR [9]. Immunohistochemical analysis of drusen in patients with age-related macular degeneration showed the presence of TTR [10]. So far, there are few reports of TTR levels in clinical high myopia samples. Duan et al [11] reported TTR levels were elevated in the aqueous humor of five high myopia samples, but it is still uncertain if this phenotype is common in high myopia patients.

In this paper, serum samples of 16 patients and 4 controls were analyzed by LTQ-MASS. We also determined TTR levels in the vitreous and corresponding serum from high myopia and normal samples by ELISA, and tested for TTR concentrations in serum samples from patients with different ocular pathologies by Western blot and ELISA. The aim of the present study was to evaluate potential associations between levels of TTR in the vitreous and serum samples of high myopia patients with clinical parameters such as age, sex, axial length, and ocular pathology.

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## Materials and methods

### Serum samples

The serums of high myopia patients were obtained from ophthalmology outpatients of Shanghai Jiao Tong University Affiliated First People's Hospital. High myopia patients with axial lengths (ALs) of 26.0 mm or more were designated as study cases ($n=116$), while emmetropia subjects with ALs ranging from 21.0 to 23.99 mm constituted the control cases ($n=86$). Eight-six normal serum samples were obtained from healthy volunteers. Subjects with a history of intraocular surgery, ocular trauma, raised intraocular pressure, uveitis, pseudoexfoliation, diabetes mellitus, LASIK/PRK, prophylactic laser photocoagulation, and systemic diseases such as Alzheimer's disease, Parkinson's disease, schizophrenia, depression and several types of cancer, rheumatoid arthritis, glomerular disease, hepatitis, tissue injury, and inflammation were excluded from the study. The mean age of high myopia subjects was $49.7\pm12.3$ years, while the mean age of the emmetropia group was $48.5\pm10.5$ years. All study participants underwent a complete ophthalmic examination. After all the exams, patients were diagnosed with fundus manifestation of macular detachment ($n=17$), macular hole ($n=14$), choroidal neovascularization ($n=20$), macular epimacular membrane ($n=11$), atrophy ($n=28$), or no significant pathology ($n=26$). The mean AL of high myopia patients was $29.35\pm2.47$ mm, significantly longer ($p<0.01$) than that of the normal group ($24.12\pm1.38$ mm). In the high myopia group, the ALs of 48 patients ranged from 26.0 mm to 27.99 mm, the ALs of 37 patients ranged between 28 mm and 29.99 mm, and 31 patients had ALs of 30 mm or more. All patients and control subjects involved in this study were similar in social background and were from the local ethnic Han Chinese population, with no ethnic subdivision.

A total amount of 5 mL of blood was collected in 10 mL glass tubes and allowed to clot for 1.5 h at room temperature. The clotted material was removed by centrifugation at 3000 rpm for 10 min. 200 μL of each serum sample was diluted with 600 μL of 20 mM PBS, separated into 4 tubes (200 μL/tube) and stored at −20 °C for further analysis.

### Vitreous samples

The high myopia undiluted vitreous humor samples ($n=40$, 0.3 to 1.0 mL) were obtained during pars plana vitrectomy under visual control by aspirating liquefied vitreous from the center of the vitreous cavity with a syringe before the vitrectomy infusion. The corresponding serum samples were obtained before surgery. The control vitreous samples from normal human eyes with no known ocular diseases ($n=8$) were obtained from eyes donated for corneal transplant (in accordance with the Standardized Rules for Development and Applications of Organ Transplants) from the Eye Bank of Shanghai in China. The normal vitreous samples (0.8 to 1.0 mL volume) were all aspirated with a syringe at pars plana. The normal serum samples were obtained from 8 healthy volunteers without any known ocular and systemic diseases. Harvested vitreous humor samples were collected in Eppendorf tubes, placed immediately on ice, centrifuged for 15 min at 12,000 rpm to separate the cellular contents; 200 μL of each sample was diluted with 600 μL of 20 mM PBS, separated into 4 tubes (200 μL/tube) and stored at −20 °C for further analysis.

### Materials

A TTR ELISA Kit (catalog number T771-50) was purchased from Groundwork Biotechnology Diagnosticate Ltd. (USA). A polyclonal antibody to TTR (catalog number A0002) was purchased from Dako Co. Ltd. (Denmark); other chemicals were of analytical grade and from local companies.

### LTQ-MASS analysis

Total serum proteins from healthy individuals were used to immunize rabbits, and then the antibodies for human serum background proteins were coupled with CNBr-activated sepharose; serum samples from four healthy and 16 high myopia individuals were applied onto the medium, and then the 20 flow-through fractions were analyzed by LTQ-MASS. Briefly, 25 μg trypsin was dissolved in 2.5 mL tosylphenylalanylchloromethane (TCPK) mixed with 250 μL of 0.1% redistilled acetonitrile. A 15 μL sample of this trypsin solution was activated in 100 μL 50 mM $NH_4HCO_3$. A 5 μL sample from one healthy individual and the concentrated flow-through proteins were reduced with 100 μL of reducing buffer (containing 200 μL of TCEP in 2 mL digestion buffer) and incubated at 60 °C for 10 min. Following incubation, 100 μL of alkylation buffer (containing 60 mg of iodoacetamide in 3 mL digestion buffer) was added to the tube and incubated in the dark at room temperature for 1 h for carboxymethylation and oxidation of cysteine and methionine residues. Then, 20 μL of activated trypsin solution was added to the tube, and incubated at 37 °C for 1 h and then at 25 °C overnight with gentle mixing. This peptide mixture was injected onto a Zorbax 300 SB-C18 peptide trap (Agilent Technologies, Wilmington, DE) to desalt, and separation was performed on a Zorbax 300SB-C18 reverse phase capillary column (300 μm inner diameter × 15 cm, Agilent Technologies). The mobile phases were 0.1% formic acid (A) and 84% $CH_3CN$ and 0.1% formic acid (B). The flow rate was 500 nl/min with a linear gradient of 4–50% B over 50 min, a step up to 100% B over 4 min, then 100% B for 10 min. The peak was injected online into a Finnigan LTQ (single linear quadrupole ion trap) mass spectrometer for peptide identification. Mass spectrometry was performed on a Finnigan LTQ linear ion trap. The MS method consisted of a cycle combining one full MS scan with two MS/MS events (25% collision energy). Dynamic exclusion duration was set to 30 s.

### Western blot analysis

Serum samples (1 μL) from the subjects with high myopia and normal volunteers were analyzed by Western blotting. The samples were electrophoresed on 10% SDS-PAGE gels, and then electrophoretically transferred to NC membranes (Hybond-C; Amersham Biosciences UK limited, Arlington Heights, IL) at 60 mA for 0.5 h. Membranes were blocked for 2 h at room temperature with blocking buffer (2% BSA in PBS-T) and incubated for 1–2 h at 37 °C with rabbit anti-TTR antibody (1:2,000).The membrane was washed four times for 5 min each with PBS containing 0.1% Tween-20 and then incubated with the secondary antibody (goat anti-rabbit antibody labeled with HRP) for an additional 30 min. The membrane was then washed several times and scanned using an Odyssey infrared imaging system (LI-COR, Lincoln, NE) at 700 to 800 nm. Western blots were repeated 3–5 times and qualitatively similar results were obtained each time.

### ELISA

The concentration of TTR in serum samples from high myopia patients was determined using an enzyme linked immunosorbent assay (ELISA, Groundwork Biotechnology Diagnosticate Ltd.) following the manufacturer's instructions. and then the samples (50 μL) were added into wells pre-coated with antibody, and 100 μL of 5 % BSA was added to each well. The wells were covered and incubated for 1 h at 37 °C. All wells were then washed five times with distilled or de-ionized water. The HRP-coupled antibody was added and the wells were recovered and incubated for 1 h at 37 °C. All wells were then washed five times with distilled or de-ionized water. Then, 50 μL of substrate (0.1 % tetramethylbenzidine) was added to each well. The wells were covered and incubated for 15 min at 20–25 °C, followed by