Transthyretin as a potential serological marker for the diagnosis of patients with early rheumatoid arthritis

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Abstract
Objective
To investigate the serum levels and modifications of transthyretin (TTR) in patients with rheumatoid arthritis (RA) by mass spectrometry, and the potential role of TTR in early RA.

Methods
Serum samples were collected from early RA (ERA), middle and late RA (LRA), osteoarthritis (OA) patients, and healthy controls (HC). Levels of TTR were measured by ELISA, and serum TTR was further detected by Western blot. A subsequent MALDI-TOF-MS was performed to analyse the modified TTR.

Results
Serum TTR levels in ERA (502.46±108.15 mg/l) was significantly higher than that of healthy controls (424.98±117.63 mg/l) (p<0.05). TTR levels in LRA was higher than that of HC but without statistical significance (p>0.05), and no statistical significance between OA (363.90±105.21mg/l) and HC (p>0.05). Two protein bands were identified corresponding to monomer and dimmer TTR by western blot. The proportion of TTR monomer was similar in each group. However, the proportion of TTR dimer in RA was lower than that in HC, which was decreased more in LRA (p<0.05). By MALDI-TOF-MS, four major peaks were observed in sera corresponding to native TTR (13749.86±1.48 m/z), Sul-TTR (13829.63±2.76 m/z), Cys-TTR (13870.70±2.70 m/z), and Cysgly-TTR (13927±5.77 m/z). The proportion of modified TTR varied with different disease stages.

Conclusion
TTR levels in sera of patients with early RA were significantly increased. Four modified TTR were identified by MALDI-TOF-MS, and the proportion of modified TTR varied with different disease stages. Thus serum TTR could be considered as a potential serological marker for early diagnosis of RA.

Key words
rheumatoid arthritis, transthyretin, MALDI-TOF-MS, osteoarthritis
Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease as the main manifestation of systemic autoimmune disease, affecting 0.5%–1% of the world population. RA is characterised by synovialitis and destruction of synovial joints, which leads to destruction of cartilage and bone, and eventually to severe disability and premature mortality (1-3). As RA severely affects the quality of patient’s life and also has major economic consequences for the society, attempts should be made to prevent the erosive processes in joints. To achieve the ultimate goal of RA patients before joint damage occurs. The optimal strategy to manage RA is currently to start an early and intensive treatment (4-5). Therefore, early identification and diagnosis of RA is of major importance. Currently, the diagnosis and classification of RA relies mainly on the criteria described by the American College of Rheumatology (ACR) (6) formulated 50 years ago. RA criteria were based mainly on several clinical parameters, which is not appropriate for the early diagnosis of RA (7). RA criteria, last adjusted in 2010, was undertaken to develop new classification (8). In patients with RA, apart from rheumatoid factor (RF), several serological markers and RA-specific auto-antibodies were added, such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and anti-citrullinated protein antibodies (ACPA). Even so, information obtained from these markers is not enough to provide a complete picture of the disease and treatment efficacy. It is still a big challenge to determine the diagnosis and to choose the appropriate therapy in early patients of RA. Additional markers to benefit early diagnosis of RA are still needed.

Nowadays, a range of proteomic approaches have been used in biomarker discovery, such as mass spectrometry (MS) and two-dimensional electrophoresis (2-DE). Proteomics has emerged as the most powerful tools in biomarker discovery, especially in identification of specific post-translational modifications (9). Recently, those proteomic approaches have been employed in the study of pathogenesis of RA. Those studies investigated the presence of potential biomarkers of RA in fibroblast-like synoviocytes (FLS) (10), human whole saliva (11), and serum (12). Moreover, it reported that transthyretin (TTR) might be a biomarker to distinguish patients with RA from healthy control (HC) using surface enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF MS) (13). Recently, other researchers have reported higher levels of TTR in early patients with RA (ERA) using 2-DE (14).

TTR, a 13.8-kDa protein, is synthesised predominantly in the liver and secreted into plasma. And the choroid plexus and the eye are also sites of production of TTR (15). In plasma, tetramer TTR transports the hormone thyroxine and the retinol-binding protein-retinal complex (16). Several pathologic and non-pathologic TTR variations or forms have already described including their specific gene mutations that are associated with familial amyloidosis (17, 18). Many studies had also indicated the potential value of serum TTR in cancer diagnosis (19, 20).

The aim of the present study was to investigate the serum levels and post-translational modifications of TTR in RA patients. A MS-based approach, matrix-assisted laser desorption/ionisation mass spectrometry (MALDI) mass spectrometry (MS), was employed to characterise variant forms of TTR (21). The results from MS had provided valuable information in post-translational modifications of TTR.

Patients and methods

Patients and samples

We studied a total of 159 patients, including 79 RA, 40 OA patients, and 40 healthy people. All the participants were from the outpatient or inpatient department of the General Hospital of Tianjin Medical University, aged from 29 to 65 years, between November 2008 and December 2010 (Table 1). Patients who suffered from other chronic diseases or any acute infections within 3 months were excluded from this study. RA patients all met the ACR 1987 criteria for RA (6) at the time of enrolment. RA patients assigned to two subgroups:

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1) the early stage group (ERA, n=36), who had a course less than 6 months and not received disease-modifying anti-rheumatic drugs and/or steroid therapy before enrolled; 2) the middle and late stage group (LRA, n=43), who had course of disease longer than 2 years. All patients with OA fulfilled the criteria for OA. None of the healthy controls (HC) had previous history of arthralgia, any other chronic diseases or acute infections during 3 months before the enrolment.

All participants signed written informed consent before their enrolment. The study was approved by the Medical Ethics and Human Clinical Trial Committee of Tianjin Medical University. The informed consent was obtained from all patients and the control subjects.

Sample preparation
All serum samples were collected and kept at 4°C for 1 h for clotting, then centrifuged at 4000 rpm/min for 5 min, immediately aliquot and stored at -80°C. All serum samples were only allowed to thaw once.

Antibodies and reagents
Rabbit polyclonal antibodies against human TTR were purchased from Dako (Glostrup, Denmark). HRP-conjugated goat anti-rabbit antibodies were obtained from Bioword (St. Louis Park, MN, USA). The enzyme-linked immunosorbent assay (ELISA) system of human transthyretin was purchased from Yue Yan (Shanghai, China).

Determination of TTR levels in sera
Serum concentrations of TTR from different groups were determined by ELISA. Human TTR ELISA kit (Yue Yan, Shanghai) was used according to the manufacturer’s instruction. The tests were carried out in individual serum samples. Briefly, serum sample was diluted (1:2000), and 50μl was added to a 96-well the wells and were incubated for 30 min at room temperature. Secondary antibody was added and incubated. Optical densities (OD) were measured at 450 nm by microplate reader (Multiskan MK3, Thermo, USA). The primary concentrations of each test ample were calculated from the linear regression equation based on OD value of the standards.

Western blot analysis of TTR in sera
Serum TTR was denatured at 95°C for 10 minutes after addition of SDS. The serum proteins were separated by SDS-PAGE and subsequently were transferred to the polyvinylidene difluoride (PVDF) for 15-30 min at a constant current of 250 mA. The membrane was blocked for 1 h at room temperature in 5% skim milk/TBST for 1 h: 2500 in 5% skim milk/TBST). After washing proteins were detected with an enhanced chemiluminescence system (Solarbio, Shanghai, China).

Immunoprecipitation of TTR in sera
Rabbit anti-human TTR polyclonal antibody (Dakopatts, Glostrup, Denmark) was used to immunoprecipitate TTR in the sera as described previous (22, 23). Briefly, 150 μl of serum samples were treated with an equal amount of a polyclonal rabbit anti-human antibody (Dakopatts, Glostrup). The mixture was incubated for 2 h at 37°C and then centrifuged at 13000 rpm/min for 20 min at room temperature. The supernatant was removed and the immunoprecipitated complex was washed. Then samples were incubated overnight at 4°C. The next day, samples were centrifuged at 13000rpm/ min for 20 min, then careful removal of supernatant. The deposit was washed with high performance liquid chromatography grade water and the eluate was diluted with 100 μl icy acetic acid for analysis of mass spectrum.

Detection and identification of TTR by MALDI-TOF-MS
All experiments were performed with a MALDI-TOF-MS (Shimadzu/Kratos, Manchester, UK) operated at a wave-length of 337nm. The best spectra of TTR were obtained at an ion accelerating voltage of 27.5 kV and a reflection voltage of 30 kV. The spectra were calculated by using external calibration with [M + H] ions produced from horse cytochrome c (m/z 12,361.96) and horse myoglobin (m/z 16,952.27). The matrix was a saturated solution of sinapinic acid in acetonitrile plus water (1:2, v/v) containing 0.1% trifluoroacetic acid. The samples were deposited onto the sample probe assembly. MALDI-TOF-MS data was analysed using Launchpad software Version 2.4 (Kratos Analytical, Manchester, UK) (24).

Statistical analysis
Data were expressed as mean ± standard deviation (SD). Data were processed with SPSS software 13.0. (SPSS Inc., USA). Differences among four groups were analysed with one-way ANOVA. Student-Newman-Keuls test was used for a comparison between two groups. A p-value of less than 0.05 was considered statistically significant.

Results
Clinical characters of the participants
A total of seventy-nine participants were included in this study, including of 79 RA (ERA n=36; LRA n=43), 40 OA and 40 healthy people as control. In RA patients, levels of CRP and ESR were tested at the same time of serum samples collect. The detailed clinical status of these participants was shown in (Table I).

There was no statistical difference in gender, age constitute among all groups (p>0.05). Compared with OA and HC groups, the levels of CRP, ESR were significantly higher in the RA group (p<0.05). However, baseline levels of ESR and CRP showed no significant difference between the ERA and LRA patients (p>0.05).

Increased levels of TTR in sera from patients with early RA
Serum TTR levels were measured by ELISA. Data of TTR levels from four groups was analysed by one-way ANOVA. The difference was statistically significant (F=8.331, p<0.001). Further, pairwise
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Comparisons were performed using Student-Newman-Keuls test. Our results shown that TTR levels in ERA [(502.46 ± 108.15) mg/l] were significantly higher than that in HC, and which was decreased more in LRA group (p<0.05) (Fig. 2).

Four major TTR peaks were detected in RA by MALDI-TOF-MS

Modified TTR isoforms were detected by MALDI-TOF-MS. As depicted in Figure 2, four major peaks were observed in the mass spectrum of serum, including native TTR (13749.86 ± 1.48 m/z), Sul-TTR (13829.63 ± 2.76 m/z), Cys-TTR (13870.70 ± 2.70 m/z), and Cysgly-TTR (13927 ± 5.77 m/z).

The proportion of TTR isoforms in RA group was further analysed. The proportion of Cys-TTR in ERA significantly decreased relative to healthy controls (p=0.034), and which tended to increase in LRA. Meanwhile, an increase was found in the proportion of Sul-TTR in ERA group. Our results indicated that the proportion of TTR isoforms varied between ERA and LRA.

Discussion

Early diagnosis to enable early treatment of RA is essential for the clinical management. Traditional imaging techniques used in the evaluation of RA, especially x-ray and computed tomography (CT), are restricted to anatomical changes in the joints, which fail to provide an early diagnosis of RA. In the past years, a few biomarker candidates, such as RF and ACPA, have been widely used in the diagnosis of RA. However, many investigators have pointed to what they perceive to be a dried-up blood-borne biomarkers for RA detection since recent searches for a single, specific marker have not proved fruitful. Therefore, identification of new biomarkers specific for RA tends to be an important challenge for clinical rheumatologists.

In response to this challenge, scientists have shifted their focus in an effort to utilise experimental methods such as mass spectrometry (MS). These MS-based methods offer new approaches in identifying potential biomarkers for RA diagnosis (10-12). Detection of TTR isoforms by MS would be great of help to analyse the pathogenesis of RA.
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TTR is a 127-amino acid residue protein synthesised mainly in the liver. The level of TTR in healthy population ranges from 20 to 40 mg/dl. In clinical, TTR was found to decline in the sera of patients with ovarian cancer, advanced cervical and endometrial carcinomas, which is still unknown involved in the mechanisms (19). In the study of Liu LY et al., TTR was found to be decreased in sera of lung cancer compared with benign lung disease and normal sera (20). In addition, increased levels of TTR were found in aqueous humour between primary open-angle glaucoma patients (25). 

In the present study, serum TTR levels were detected in RA, OA patients, and HC. Our results showed that TTR levels were statistically difference among these groups, which was consistent with previous reports. A recent study of An Y et al. indicated that higher levels of TTR were found in early RA compared with HC (14). Moreover, we found that TTR levels in ERA were significantly higher than that in LRA patients, which suggested that serum TTR might be a potential serological marker for early diagnosis of RA. The lower levels of TTR in LRA may be attributed to immune dysfunction, nutritional status (26) and therapeutic drugs (27).

Typically, native TTR accounts for only 5~15% of total TTR circulating in blood. The other 85~95% is modified post-translationally in the form of S-sulfonation and S-thiolation (28). In its native state, TTR is a tetramer, four single chain TTR monomers form a tetrameric complex (29). In our study, however, two protein bands were identified corresponding to TTR monomer and dimer. The proportion of monomer and dimer TTR was calculated. The results showed the proportion of TTR monomer was similar in each group. Nevertheless, the proportion of TTR dimer in RA was lower than that in OA and HC groups, which was decreased more in LRA compared with ERA. Kingsbury JS (30) reported that the stability of both the tetramer and dimer of TTR were related to post-translational modifications. Our results in the present study suggested that post-translational modifications of TTR might be involved in RA.
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A pioneering work from Biroccio A et al. demonstrated that differential post-translational modifications of TTR were found in the cerebral spinal fluid of Alzheimer’s disease (31). Recently, Ueda M et al. (32) reported SELDI-TOF MS as a reliable tool for quantitative evaluation of TTR variants for the potential diagnosis of familial amyloidotic polyneuropathy (FAP). We previously reported that immunoprecipitation and MALDI-TOF-MS were employed for characterising serum TTR isoforms in patients with FAP. The MS results revealed that post-translational modifications of TTR were different from those of lipoprotein, which indicated metabolic status was associated with chemical modifications (33).

In order to identify TTR isoforms and further explore their role in RA, MALDI-TOF-MS was used to identify the relative abundance, types and proportion of TTR modification in serum of each group. In the present study, four major peaks were observed, including native TTR, Sul-TTR, Cys-TTR, and Cysgly-TTR. Moreover, proportion of Cys-TTR decreased in 5/6 patients of Cysgly-TTR. Moreover, proportion of native TTR, Sul-TTR, Cys-TTR, and major peaks were observed, including detection of TTR modification in serum of relative abundance, types and proportion of TTR isoforms in patients with FAP. The MS results revealed that post-translational modifications of TTR were different from those of lipoprotein, which indicated metabolic status was associated with chemical modifications (33).

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