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Isotypes of autoantibodies against differentially expressed novel malondialdehyde-modified peptide adducts in serum of Taiwanese women with rheumatoid arthritis

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ABSTRACT

This study identified and validated four differentially expressed novel malondialdehyde (MDA)-modified peptide adducts and evaluated autoantibodies against native and MDA-modified peptides among Taiwanese women patients with rheumatoid arthritis (RA), osteoarthritis (OA) and healthy controls (HCs). Ig kappa chain C region$^{76–99}$, alpha-1-antitrypsin$^{284–298}$, alpha-2-macroglobulin$^{824–841}$, and apolipoprotein B-100$^{4022–4040}$ exhibiting 2-fold differences in relative modification ratios were identified by concanavalin A (Con A) affinity chromatography, 1D SDS-PAGE, in-gel digestion, nano-LC/MS/MS and nano-LC/MS using pooled serum-derived Con A-captured proteins from 9 RA and 9 age-matched HCs. Furthermore, the levels of proteins, serum MDA, and MDA-modified protein adducts were further validated against individual serum from 20 RA and 20 HCs, and autoantibodies against native and their MDA-modified peptides used 45 RA, 30 OA and 45 HCs. Levels of serum MDA and MDA-modified protein adducts were significantly higher in RA than HCs but protein levels were not significantly different. Serum Igs G and M against MDA-modified peptides showed better diagnostic performance in differentiating among patients with RA, OA and HCs, with an area under the receiver operating characteristic curve of 0.96–0.98, sensitivity of 88.9%–97.8%, and specificity of 88.9%–100%. Autoantibodies against MDA-modified epitopes become useful clinical biomarkers for RA.

Biological significance: By using a label-free relative quantitative proteomic analysis of concanavalin A (Con A)-bound serum samples, the current study discovered and validated malondialdehyde (MDA)-modified peptide adducts as novel biomarkers for differentiating between rheumatoid arthritis (RA) patients and healthy controls (HCs). In addition, the serum levels of MDA, proteins, and MDA-modified protein adducts as well as the MDA modification of proteins were determined. Isotypes of autoantibodies against MDA-modified peptide adducts can be used as serological biomarkers for further discriminating among RA patients, osteoarthritis patients and HCs. This strategy can become the basis for identifying potential diagnostic and pathological biomarkers for RA.

Abbreviations: MDA, malondialdehyde; 1-D SDS-PAGE, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis; nano-LC–MS/MS, nano-liquid chromatography–tandem mass spectrometry; HC, healthy control; Ig, immunoglobulin; RF, rheumatoid factor; anti-CCP, anticyclic citrullinated peptide; LDL, low-density lipoprotein; Con A, concanavalin A; ACR, American College of Rheumatology; XIC, extracted-ion chromatogram; IP, immunoprecipitation; IGKC, Ig kappa chain C region; A1AT, alpha-1-antitrypsin; α2M, alpha-2-macroglobulin; ApoB-100, apolipoprotein B-100; TBARS, thiobarbituric acid reactive substance; RSD, relative standard deviation; SD, standard deviation; ROC, receiver operating characteristic; AUC, area under the ROC curve; CBB, Coomassie brilliant blue; anti-Ro (SSA), anti-Sjögren's-syndrome-related antigen A; anti-La (SSB), anti-Sjögren's-syndrome-related antigen B

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1. Introduction

In 2002–2007, the annual incidence of rheumatoid arthritis (RA) was 15.8 (men, 10.1; women, 41.0) cases per 100,000 persons and the crude mortality rate of RA was 14.7 (men, 25.0; women, 11.8) deaths per 1000 person-years in Taiwan [1]. RA is a chronic autoimmune rheumatic disease, characterized by progressive and systemic inflammation of synovial joints [1]. Oxidative stress-generated free radicals within an inflamed joint can induce autoimmunity, which damages connective tissue in rheumatoid synovitis; oxidative stress is critical in RA pathogenesis [2,3]. Epidemiological studies have indicated that the risk factors for RA include age, sex, ethnicity, rheumatoid factors [RFs; anti-immunoglobulin (Ig) G-advanced glycation end-product (AGE) antibodies] or antinuclear antibodies, genetic heterogeneity, environmental factors, and smoking [1,4,5].

During the pathogenesis of inflammatory arthritis, free radicals easily depolymerize polyunsaturated fatty acids in lipids through a lipid peroxidation process [2]. Malondialdehyde (MDA), a byproduct of this process, can trigger secondary protein modifications [6]. MDA modifies a protein to form a Schiff base complex, predominantly with the primary amino groups of lysine, histidine, arginine, glutamine, and asparagine, as well as the N-termini of peptide chains [7]. MDA levels can be significantly 1.37–3.80-fold higher in the plasma or serum of RA patients than in that of healthy controls (HCs); therefore, the degree of MDA formation may be a biomarker for RA [2,8–10].

Several isotypes of autoantibodies against neoepitopes, including those glycated, citrullinated, carbamylated, and oxidized, have been detected in RA patients' serum or plasma. Thus far, both RFs and anticyclic citrullinated peptide (anti-CCP) antibodies have been used as RA biomarkers in clinical practice [11]. In RA patients, MDA-modified low-density lipoproteins (MDA-LDLs) are involved in the progression of atherosclerosis [12,13]. MDA-modified epitopes may possess immunogenicity and elicit specific autoantibody formation in connective tissue diseases, vasculitides, cardiovascular diseases, and diabetes mellitus [14–16]. However, few studies have investigated autoantibodies against MDA-modified epitopes in RA [12,13]. Importantly, MDA is going to break down and form acetaldehyde (AA) spontaneously. Further, malondialdehyde-acetaldehyde (MAA) is produced through MDA and AA interaction and then MMA will modified proteins to generate MMA-protein adducts and anti-MMA autoantibodies in RA [17].

In this study, we identified and quantified novel native and MDA-modified peptides through concanavalin (Con) A affinity chromatography, one-dimensional (1D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in-gel digestion, and label-free nano-liquid chromatography tandem mass spectrometry (nano-LC–MS/MS) and mass spectrometry (nano-LC–MS) in RA patients and HCs. On verifying the MDA modification of proteins, we determined the levels of proteins, serum MDA, and MDA-modified protein adducts. In addition, we assessed whether the isotypes of autoantibodies against MDA-modified peptide adducts can be biomarkers for discriminating among patients with RA, osteoarthritis (OA) and HCs.

2. Materials and methods

2.1. Patient samples

Serum samples from 120 female patients [45 with RA (54.7 ± 10.44 years old), 30 with OA (60.06 ± 10.36 years old) and 45 HCs (54.27 ± 8.54 years old)] were obtained from the Division of Allergy, Immunology, and Rheumatology, Department of Internal Medicine and the Department of Laboratory Medicine, Shuang-Ho Hospital, New Taipei City, Taiwan. The RA patients had received diagnoses from a rheumatologist and fulfilled the appropriate classification criteria—either the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism classification criteria [18] or 1987 ACR classification criteria [19]. The OA patients were diagnosed according to the clinical symptoms and assisting with osteoarthritis criteria by American College of Rheumatology [20,21]. MDA-modified peptide adducts were relatively quantified through 1D SDS-PAGE, in-gel digestion, nano-LC–MS/MS, and nano-LC–MS by using pooled serum-derived Con A-captured protein samples randomly selected from nine RA patients and nine age-matched HCs. The protein levels of MDA-modified peptide adducts were examined through Western blotting by using individual serum randomly selected from another 20 RA patients and 20 age-matched HCs. The MDA modification of proteins was evaluated through immunoprecipitation (IP) and Western blotting using the aforementioned 20 pairs of pooled Con A-captured serum samples. Next, the serum levels of MDA and MDA-modified protein adducts were determined from individual serum samples of all 45 RA patients and 45 HCs, and autoantibody isotypes against native and their MDA-modified peptides were assessed among 45 RA patients, 30 OA patients and 45 HCs. This study was approved by the institutional review board of the study hospital, and all volunteers provided informed consent to participate. Serum was stored at − 20 °C until analysis. The clinical and demographic characteristics of the RA patients, OA patients and HCs are presented in Supplementary Table 1. However, age was significantly higher in OA patients compared to RA patients and HCs, respectively (Supplementary Table 1).

2.2. Con A affinity chromatography, 1D SDS-PAGE, and in-gel digestion

Enrichment of Con A-captured serum proteins was performed according to the protocol of Uen et al. [22,23]. Serum-derived Con A-captured proteins (50 μg) were subjected to 10% SDS-PAGE, followed by in-gel digestion in triplicate (Supplementary Fig. 1). Details are provided in the Supplementary information.

2.3. Relative quantitative analysis of differentially expressed MDA-modified peptide adducts through nano-LC–MS/MS and nano-LC–MS

Tryptic peptides of individual gel slices were pooled and used for nano-LC–MS/MS and nano-LC–MS (Supplementary Fig. 1). In brief, 50-μg serum-derived Con A-captured proteins (9 RA and 9 HC pooled samples) were run on 10% SDS-PAGE three times and stained with Coomassie brilliant blue (CBB) staining solution (Bio-Rad Laboratories, Hercules, California, USA). The gel lanes were cut into 10 slices based on the molecular weight and labeled from 1 to 10, from high molecular weight to low molecular weight, respectively. In-gel digestion was conducted for each gel slice. Two independent tryptic digests were pooled as one sample according to labels (1,2), (3,4), (5,6), (7,8), and (9,10). Each intact gel lane could pool five samples. In total, there were 15 samples each for patients with RA and HCs. The native and their MDA-modified peptide sequences were established using MS/MS spectra from nano-LC–MS/MS and our in-house PTM finder program [23]. Furthermore, 10 independent tryptic digests were pooled as one sample and labeled 1–10. In total, there were three samples each for patients with RA and HCs. Subsequently, the native and their MDA-modified peptides were subjected to a relative quantitative analysis by integrating peak areas on extracted-ion chromatograms (XICs) from the MS scans according to the established peptide sequence lists using nano-LC–MS and the in-house PTM Q program in triplicate [23]. For quantitative analyses, the relative modification ratio (%) was defined as follows: (XIC peak area of MDA-modified peptide)/[(XIC peak area of native peptide) + (XIC peak area of MDA-modified peptide)] × 100%. Data are available through ProteomeXchange with the identifier PXD004546. Minor details of modifications are provided in the Supplementary information.

2.4. IP–Western blotting

MDA-modified proteins were verified through IP–Western blotting