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Downregulation of ITM2A Gene Expression in Macrophages of Patients with Ankylosing Spondylitis

Lari, Arezou

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Evaluation of the Ankylosing Spondylitis Transcriptome for oxidative phosphorylation pathway: the shared pathway with neurodegenerative disease

ABSTRACT:

Ankylosing spondylitis (AS) is a systemic inflammatory disease of joints and enthes. Recent studies reported an increased prevalence of dementia in AS patients. However, data for exploring the association between dementia and AS remain lacking. The aim of this study was specifically to focused on oxidative phosphorylation pathway that enriched in AS dataset and neurodegenerative disease.

First, we explored critical pathways that might potentially be involved in the pathogenesis of AS in GSE25101 expression dataset obtained from GEO. The four pathway of Oxidative phosphorylation, Alzheimer's, Parkinson's and Huntington's disease were significantly enriched and have the same trend. Using limma method, we found that 22 common genes involved in oxidative phosphorylation pathway have shown increased trend in AS. Five significant genes were selected for further experimental exploration. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy and AS patients (each 20), and then RNA contents of PBMC were extracted. The expression level of interest genes were measured through real-time PCR assay using SYBR Green Master Mix. The results indicated that COX7B and ATP5J were significantly increased in AS group compared to the control group. Based on our result, we propose the common pathway of Oxidative phosphorylation as a possible cause of comorbidity between AS and dementia.

INTRODUCTION:

Ankylosing spondylitis (AS) is an autoimmune disorder that primarily affects the spine, sacroiliac joints and, occasionally, peripheral joints. It is an inflammatory rheumatologic disease that can progress to significant functional disability (1; 2; 3). The prevalence of the disease is between 0.1 and 2% in various populations and men are more affected than women (4; 5; 3).

AS patients have several comorbidities, including cardiovascular diseases and psychiatric disorders (6; 7). According to a recent report, AS patients develop a significantly higher prevalence of overall dementia and Alzheimer's dementia than the general population. Although the numerous hypotheses have attempted to explain the relation between AS and dementia, like increased level of serum amyloid (6; 8; 9) or chronic inflammation (10), the exact cause is still unclear.

In recent years, a large amount of transcription data has been produced and deposited in publically available data repositories (11). These repositories allow researchers to discover genetic and diagnostic signatures by bioinformatics analysis, which can provide insight into the involved genes and pathway in AS.

In the current study, we initially downloaded the whole blood transcription data of AS patients in Gene Expression Omnibus (GEO). Then by using the Gene set enrichment analysis (GSEA) and

limma method, enriched pathways and significant genes were identified. Finally, the expression of interest genes was experimentally measured in AS and healthy groups.

2-METHODS AND DONORS:

2-1 Microarray data analysis

The publicly available microarray data set GSE4290 and its GPL6947 Illumina HumanHT-12 V3.0 expression beadchip platform were obtained from the National Centre for Biotechnology Information Gene Expression Omnibus (GEO). The dataset included 16 AS patients and 16 healthy control and the platform contains 49576 probes (12).

2-1-1 GSEA

Gene set enrichment analysis (GSEA) by using C2- KEGG from the Molecular Signatures Database (MSigDB) was conducted on the normalized expression data with 1,000 gene set permutations to identify significant pathways that may involve in AS (<http://www.broad.mit.edu/gsea/>). It evaluates how genes in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways are distributed in the fold change (As versus Control) ordered list generated by our data. This is quantified by using the Enrichment Score (ES), a weighted Kolmogorov-Smirnov-like statistic that evaluates if the members of the pathway are randomly distributed or found at the extremes (top or bottom) of the list (13).

2-1-2 LIMMA

The analysis of this section were undertaken using the R (version 3.5.2) (<http://CRAN.R-project.org/bin/windows/base>) statistical computing environment and BioConductor (version 3.9). The limma package (14) with empirical Bayes method was used to assess the differentially expressed enriched genes of GSEA method, between AS and controls in normalized and log2 transformed of series matrix file. Then the probe IDs basing on the annotation files, which were downloaded from GEO, were converted into gene symbols. the genes which met our criterion (adjust p value < 0.05) were deemed to be differentially expressed genes.

2-2 EXPERIMENTAL SECTION:

2-2-1 AS patients and healthy control subjects

For this study, 20 AS patients (5 females and 15 males) and 20 age and gender matched healthy controls (5 females and 15 males) were recruited by the Rheumatology Research Center (RRC), Shariati Hospital. Patients were diagnosed according to modified New York Criteria (MNYC) for AS by a rheumatologist. The mean disease duration of AS cases was 8.7 years. The patient group had the mean age of 35 ± 11.3 years and the mean age of healthy controls was 36 ± 7.4 years. The healthy group had neither clinical evidence nor family history of any type of autoimmune disorders (more demographic features are shown in Table 1). All participants signed an informed consent form for participation in this study and The Human Research Ethics Committee of Tehran University of Medical Sciences approved this study.

Table 1. Demographic feature of AS patients and healthy controls		
<i>Property</i>	<i>AS patients (n = 20)</i>	<i>Healthy controls (n = 20)</i>
<i>Male</i>	15 (75%)	15 (75%)
<i>Female</i>	5 (25%)	5 (25%)
<i>Age</i>	35 ± 11.3	36 ± 7.4
<i>BASDAI</i>	6.4 ± 2	-
<i>BASFI</i>	4.5 ± 2.6	-
<i>BASMI</i>	3.2 ± 1.2	-
<i>Disease duration</i>	8.7	-
Abbreviations: AS, ankylosing spondylitis; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index.		

2-2-2 PBMC isolation and RNA extraction

PBMCs were isolated from whole blood samples using Ficoll-Hypaque gradient (inno-train Diagnostik GmbH, Kronberg im Taunus, Germany). Total cellular RNA was extracted from PBMCs using High Pure RNA Isolation Kit (Roche, Mannheim, Germany, 11828665001) according to the manufacturer's protocol. The quantity and quality of RNA was confirmed by spectrophotometry (NanoDrop ND-2000C, Thermo Fisher Scientific, Waltham, MA, USA).

2-2-3 cDNA synthesis and quantitative real time PCR

Isolated RNA was used to synthesize complementary DNA (cDNA) by Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacturer's instructions. SYBR Green (Ampliqon, Odense, Denmark) and an StepOnePlus real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA) were utilized for quantitative PCR. A sequence of primers used in this study is shown in Table 2.

Table 2. Sequence of primers used in qPCR in PBMC.

Primer name	Forward primer	Reverse primer	Product size(bp)
<i>COX7B</i>	AGCGCACTAAATCGTCTCCA	TGTACGTTTCTGGTGGCTCT	72
<i>ATP5J/ATP5PF</i>	TCAGCCGTCTCAGTCCATT	ACTAGCATCAACAGGTCCTCC	150
<i>UQCRB</i>	ATCAGCCCATTGAGTGTCCC	TGCAGTTCAAGGGGTGAGAG	73
<i>NDUFS4</i>	TGCTCGCAATAACATGCAGTC	GATCAGCCGTTGATGCCCAA	113
<i>NDUFB3</i>	GCTGGCTGCAAAGGGCTA	CTCCTACAGCTACCACAAATGC	146
<i>HPRT1</i>	GGTGAAAAGGACCCACGAA	AGTCAAGGGCATATCCTACAACA	92

2-2-4 Statistical analysis

Comparative CT method was used for the relative gene expression analysis (15). The relative amount of target mRNA in each test sample was calculated and normalized to the corresponding

hypoxanthine phosphoribosyl transferase 1 (HPRT1) mRNA transcript level as a housekeeping gene. The Kolmogorov-Smirnov test was used to assess whether the variables distribution were normal or not and consequently, the independent sample t-test and Mann-Whitney test were used respectively. In order to draw the graphs, GraphPad Prism 8.0 (GraphPad Software Inc, CA, USA). was used. The result with P value <0.05 was considered statistically significant.

3-RESULT:

GSEA showed that genes differentially expressed in AS were significantly enriched in the eight biological pathway. We used normalized enrichment score (NES) > 1.5, false discovery rate (FDR) < 0.1 and p value < 0.01 as the cutoff point to determine whether KEGG pathways were significantly enriched. Fig.1 shows 22 shared genes involved in Oxidative phosphorylation, Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) pathway were significantly enriched and showed the same trend (Fig. 1).

Figure 1. a) Enrichment plot. Genes expression in four pathways including oxidative phosphorylation and PD, HD, and AD are positively correlated in AS group compared to control group. b) Network of enriched genes of the four

mentioned pathway. Colorless circle contained shared genes in GSEA methods and the circles in gray, red, blue and green included the exclusive genes of oxidative phosphorylation, PD, HD and AD pathways, respectively. Size of circles indicate absolute LogFc and thicken border related with adjust p.value < 0.05 in limma method. The genes connections were made by stringApp in cytoscape (16). Abbreviations: PD, Parkinson's disease; HD, Huntington's disease; AD, Alzheimer's disease; GSEA, Gene set enrichment analysis

In limma result between the common genes, nine genes were significantly up-regulated with adj p.value < 0.05 in AS patient compared to control group. As it is shown in heatmap plot (Fig. 2a) 5 genes COX7B, NDUF3, ATP5J, UQCRB and NDUF4 were expressed at significantly higher levels in AS than control group (with fold change logarithm of 1.05, 0.93, 0.78, 0.70 and 0.6 respectively). Principal component analysis (PCA) was applied for the selected gene between all 16 AS and 16 control samples from microarray datasets. PCA (Fig. 2b) showed that the AS group (indicated by orange color) was clustered relatively from the control group (indicated by blue color). Since these 5 genes were significantly different between two group (adj p.value < 0.05) and have the biggest |logFC| in oxidative phosphorylation pathway, they were selected for further investigation using real time PCR method.

Figure 2. Heatmap and PCA plots. a) revealed that the expression of COX7B, NDUF3, ATP5J, UQCRB and NDUF4 were expressed at higher level in AS than control group. b) PCA plot shows that the expression of 5 interest genes could relatively clustered between two groups. AS and control group indicated by orange and blue colors, respectively.

mRNA expression level of selected gene in PBMCs

Expression level of COX7B mRNA in AS patients was significantly higher than healthy controls (fold change = 1.73 and 1.36, $P < 0.0001$; Fig. 3a). Furthermore, AS patients expressed the ATP5J mRNA higher than healthy controls too (fold change = 1.36, $P = 0.0001$; Fig. 3b). The NDUF3,

NDUFS4 and UQCRB mRNA expression level were not different between two groups (Figs. 3c, 3d, 3e).

Figure 3. Bar graphs demonstrate the relative mRNA expression of (a) COX7B; (b) ATP5J; (c) NDUFB3; (d) NDUFS4; and (e) UQCRB, in 20 AS patients vs 20 healthy controls.

4-Discussion:

Ankylosing spondylitis is a common inflammatory rheumatic disease that affects the axial skeleton and causing characteristic inflammatory back pain, which can lead to structural and functional impairments and a decrease in quality of life (17).

AS have many comorbidities with other diseases. There is accumulating evidence that patients with AS suffer from an increased cardiovascular (CV) risk (7). BASDAI, BASFI and BASMI scores in AS patients correlated significantly with anxiety, depression and internal validity scores (18). Moreover half of the patients with AS are reported to have sleep disturbances (19). Based on a recent cohort study the prevalence of overall dementia (1.37%) and Alzheimer's dementia (AD) (0.99%) in the AS group was significantly higher than in the control group (6). The inflammation hypothesis of common mental disorders, proposes that chronic inflammation in AS patients plays an important role in the pathophysiology of these conditions. Because of a lack of studies on the effects of chronically elevated inflammation, it has remained unclear whether the association between inflammation and common mental disorder is the consequence of acute or chronic

inflammation (10). In this study, we propose a probably associations between AS and neurodegenerative diseases.

Mitochondrial oxidative phosphorylation is one of the most important cellular processes, efficiently providing ATP to all life activities (20). It consists of respiratory enzyme complexes I, II, III, IV and V (21). Related disease of the processes, is a significant cause of human disease with a population prevalence of approximately 1 in 5000 in adults and children (22).

In previous studies it shows that, mitochondrial dysfunctions and particularly of electron transport chain are supposed to be responsible for many neurodegenerative diseases dominating in AD, PD, and HD (23; 11). Zhang et al., showed that the most significant term in Alzheimer's disease was oxidative phosphorylation ($P\text{-value} = 2.05 \times 10^{-5}$) (11).

In current study by utilizing GSEA method, it was shown that 22 enriched genes were common in the four pathways of oxidative phosphorylation, PD, HD, and AD, that is accessible in pathway of homo sapiens- electron transport chain in wikipathways database [Fig. 4]. The figure 2 was constructed by using a plug-in for the Cytoscape software(version 3.6.1), wikipathways (24; 25).

Figure 4. Electron transport chain pathway. The red rectangles contain the common enriched genes in oxidative phosphorylation, PD, HD, and AD pathways by GSEA method. Abbreviations: PD, Parkinson's disease; HD, Huntington's disease; AD, Alzheimer's disease; GSEA, Gene set enrichment analysis.

The expression level of five interest gene of this pathway, including COX7B, ATP5J, NDUFV3, NDUFV4 and UQCRB were measured by quantitative real-time PCR method using StepOnePlus real-time PCR system.

It was interesting that in a previous study, COX7B, NDUFB3 and ATP5J2 genes were considered differentially expressed in an Alzheimer dataset (11). The result of real-time PCR method revealed that the expression of COX7B and ATP5J were significantly increased in AS patients compared to healthy subjects.

Cytochrome c oxidase (CcO) is a crucial cellular enzyme with a central role in oxidative metabolism. Defective CcO biogenesis is frequently related to severe mitochondrial diseases that often involve tissues with a high energy demand (26; 27; 28). The mammalian CcO complex is composed of 13 different polypeptide subunits (29; 30). Cox7b is a nuclear-encoded protein that develops a complex with three mitochondrial-encoded proteins (COX1, COX2, and COX3). It is localized to the Xq21.1 region, comprises three coding exons, and encodes an 80 amino acid mitochondrial protein. The role of this gene in Microphthalmia with linear skin lesions (MLS), an X-linked dominant male-lethal disorder, is well demonstrated (31).

ATP synthase-coupling factor 6, mitochondrial (ATP5J also known as CF6) is a protein connecting F0 and F1, which are two components of ATP synthetase (32). It is required for the interactions of the catalytic and proton-translocating segments (33). The ATP5J association with many diseases was reported; it plays a crucial role in the development of insulin resistance and hypertension (34). On the other hand, an increased ATP5J level may be responsible for the decreased prostacyclin level observed in patients with coronary heart disease. Therefore, as a potential risk factor for coronary heart disease, ATP5J might have important clinical significance (35). ATP5J is a novel risk factor for ischemic heart disease in end-stage renal disease (36). and over-expression of this gene correlates with cell migration in colorectal cancer (37).

On the other hand, AS was found to be associated with an increased risk of cancer, Insulin dependent diabetes mellitus and Cardiac complications (38; 39; 40). Findings in this study might advance our understanding of the mechanisms for variety of comorbidities and extra articular affections of ankylosing spondylitis.

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