Efficacy research of salazosulfamide in ankylosing spondylitis and NAT1 gene polymorphism

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Received August 31, 2016; Accepted April 3, 2017

DOI: 10.3892/etm.2017.4844

Abstract. The aim of this study was to explore the correlation of salazosulfamide efficacy on ankylosing spondylitis and N-acetyltransferase 1 (*NAT1*) gene polymorphism. Thirty-two patients with ankylosing spondylitis were recruited in the experimental group and 36 normal individuals were recruited to the control group. The experimental group received 8.0 mg of salazosulfamide (MTX) per week and the control group received isodose of normal saline. Twenty-six patients in the experimental group responded to the salazosulfamide treatment and 6 did not show response. Morning stiffness time of patients in the experimental group who responded to salazosulfamide was significantly lower than that of patients with no reaction to salazosulfamide, and similar to patients in the control group. The average tender joint count of patients in the experimental group that responded to salazosulfamide was lower than in patients with no response to treatment, and similar to patients in the control group. *NAT1* gene sequencing determined that the patients sensitive to salazosulfamide treatment manifested as AA/AG at 263 locus, whereas patients not sensitive to salazosulfamide were GG. *NAT1* expression was comparable between the different genotypes at the mRNA level. However, there was a significant difference of NAT1 protein between groups. Overall, salazosulfamide demonstrates curative activity for ankylosing spondylitis and we believe that *NAT1* AA/GG genotype at 263 locus can promote salazosulfamide effectiveness on ankylosing spondylitis.

Introduction

Ankylosing spondylitis (AS) is a spinal disease condition that manifests as sacroiliac joint and spine attachment point inflammation that can cause spine fibrosis and poker spine, leading to damage of muscle, skeleton, and lung function (1). The morbidity rates of ankylosing spondylitis in Asia China are 0.89 and 1.83%, respectively (2). Ankylosing spondylitis affects mainly young adults aged 24-43 years, and is a cause of major loss for both family and society (3). Although ankylosing spondylitis research has drawn attention in recent years (4), the pathogenesis of ankylosing spondylitis is not clear so far. The morbidity of ankylosing spondylitis is proposed to be related to environmental and genetic factors (5). For instance, the incidence of ankylosing spondylitis in people who do manual work or stand for a long period of time is higher than that for the general population. As an important enzyme of acetylated metabolism in humans, N-acetyltransferase 1 (NAT1) provides nutrients for normal growth and development of organism osteoblast (6), and can also degrade carcinogens and teratogens, to maintain a steady state within the organism (7).

In this study, we observed the therapeutic effect of salazosulfamide on patients with ankylosing spondylitis and sequenced *NAT1* gene. We found a strong correlation between salazosulfamide efficacy in ankylosing spondylitis and *NAT1* polymorphism, which provides theoretical and experimental basis for the treatment of ankylosing spondylitis.

Materials and methods

Subject data. We recruited 32 patients with ankylosing spondylitis who were admitted to our hospital from February 2014 to February 2015 as the experimental group. Of the 32 patients, 18 were male and 14 female, with an average age of 32.4±12.6 years. We also recruited 36 normal individuals as the control group, which contained 20 males and 16 females, with an average age of 33.7±11.8 years.

Methods. The experimental group received 7.5 mg of salazosulfamide (MTX) per week and the control group received an isodose of normal saline. We measured morning stiffness time and tender joint count measured at weeks 4, 8, 12, 16, 20, 24 and 26 post-treatment. Five milliliters of elbow venous blood was collected, centrifuged at 2,650 x g for 5 min, cryopreserved solution was added, and the supernatant was stored at -80˚C for follow-up tests.

Genomic extraction. DNA was extracted from the blood of the two groups using the DNA extraction kit of Qiagen

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Key words: salazosulfamide, ankylosing spondylitis, N-acetyltransferase 1, gene polymorphism correlation

Table I. Primer sequences.

Primer	Sequence
nat-F	GTCGATGCTAGCTACGGCTAG
nat-R	GTCGATCGGCTAGCTAGAAGC

F, forward; R, reverse.

Table II. PCR fluorescent quantitation primer.

Primer	Sequence
nat-F	AGTCGATGCTAGCTGATCGC
nat-R	CGTAGCTGCTAGCTAGCTAG
GAPDH-F	TGACTTCAACAGCGACACCCA
GAPDH-R	CACCCTGTTGCTGTAGCCAAA

F, forward; R, reverse.

(Hilden, Germany) and DNA concentration was measured by micro-ultraviolet-visible spectrophotometer.

Genotype. PCR-SSP kit was used to determine the *NAT1* genotypes of the patients. The primers were obtained from Shanghai Bioengineering (Shanghai, China) (Table I). The genotype of the common *NAT1* polymorphisms was obtained by different primers, then PCR products were heated to 95˚C in a GeneAmp PCR for denaturation, and added to the nylon membrane that was marked by specificity nucleotide fragment probe in advance. Through complementation, single-strand DNA and specific fragments hybridized, then horseradish peroxidase (HRP)-labeled with streptomycin was added for 2 min with mixed tetramethyl benzidine and H_2O_2 for chromogenic reaction. Then, we developed coloration in the nylon membrane by Vilber Lourmat (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and NAT1 polymorphisms were distinguished by hybridizing bands.

RNA extraction and quantitative PCR (8). Samples (0.25 g) were placed on ice. Then, 0.4 ml of RNA Plus (Takara Bio) was added and samples were grinded quickly in precooled mortar. RNase inhibitor was added to the 1.5 ml tubes, and 0.15 ml of RNA Plus was used to rewash the mortar, which was transferred to a centrifuge tube after washed. Three hundred fifty mircoliters of chloroform was added to the tube, with quick shaking for 15 sec, incubated for 15 min and centrifuged at 10,050 x g at 4˚C for 15 min (Bio-Rad Laboratories, Inc.). The supernatant was transferred to a tube without RNase, and equivalent isopropanol volume was added, rapidly inverted to mix, incubated for 10 min and centrifuged at 10,050 x g at 4˚C for 10 min. The supernatant was removed and 1,000 μl of 75% ethanol was added, with gentle mixing and centrifuging at 10,050 x g at 4˚C for 10 min. The supernatant was collected and ethanol was cleared. RNase water was added to determine RNA quality.

Fluorescent quantitation inverse transcription and quantitative experiment were conducted following Takara Bio (Dalian, China) instructions using the fluorescent quantitative reaction system: 5 μl SYBR Premix Ex Taq II (2X), 0.5 μl PCR forward primer (10 μM), 0.5 μl PCR reverse primer (10 μM), 1 μl cDNA and 3 μl dH_2O. (The primer sequence shown in Tables II and III).

Enzyme-linked immunosorbent assay (ELISA) (9). Total protein (10-20 μg) was extracted from serum from all the subjects to measure NAT1 protein expression following the instructions of the ELISA kit (Qiagen). The standard protein sample for the ELISA standard curve was diluted at 1:100 by elution buffer, and the standard curve was created according to the instruction. After dilution at 1:200 in sterilized PBS (pH 7.2), 100 μl solution was added to 96-well plates, and 50 μl detection solution was added to each plate. After incubation at room temperature for 2 h, TMB chromogenic substrate was added. Light absorption was measured at 495 nm and NAT1 protein concentration in the samples was calculated according to the standard curve.

Western blotting. For western blotting, we followed the instructions in Molecular Cloning, third version. Mouse monoclonal HLA primary antibody (cat. no. MA5-11723; Thermo Fisher Scientific, Waltham, MA, USA), and SO-HLA genotype kit (Dynal Biotech Ltd., Wirral, UK) were also used.

Measurement of morning stiffness time. To measure morning stiffness, we followed the procedures described by Jansen *et al* (10).

Measurement of tender joint count. To measure tender joint count, we followed the procedures described by Arends *et al* (11).

Statistical analysis. We used software SPSS 20.0 (IBM SPSS, Armonk, NY, USA) to analyze the data. Experiment data was presented by mean ± standard deviation (mean ± SD). Single factor method was conducted for statistical analysis of different groups. $P<0.05$ meant that the difference had statistical significance.

Results

Morning stiffness time. We analyzed morning stiffness time for the control and the experimental groups. A fraction of the patients in the experimental group demonstrated response to salazosulfamide (Fig. 1). These responsive patients demonstrated low morning stiffness time (4.03 min) that was comparable to the control group (2.83 min). However, the salazosulfamide non-responsive patients in the experimental group exhibited high morning stiffness time (36.7 min) that was significantly higher than the salazosulfamide responsive and control patients (Fig. 1).

Tender joint count. We next measured tender joint count in both groups. The salazosulfamide responsive patients from the experimental group showed a tender joint count of 0.3, which was similar to the result for the control group (0.24) (Fig. 2). However, the salazosulfamide non-responsive patients had a tender joint count of 2.41, which was significantly higher than that of the control group and the salazosulfamide responsive patients (Fig. 2).

Table III. Statistics of HLA-A allelomorph and gene frequency.

	Observation (32 patients)			Control (36 patients)			
	Sensitive to salazosulfamide			Insensitive to salazosulfamide			
Nat	Cases	Frequency (%)	Cases	Frequency (%)	Cases	Frequency (%)	χ^2
AA	15	57.7	0	0	1	2.8	6.04
AG	11	42.3	0	0	3	8.3	0.12
GG	0	0	6	100	32	88.9	0.18

P<0.05 means that the difference has statistical significance.

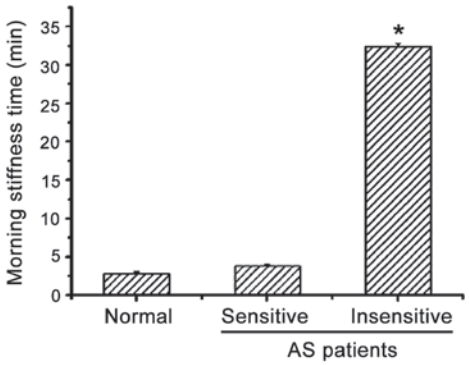

Figure 1. Morning stiffness time. Asterisk indicates statistically significant differences between groups.

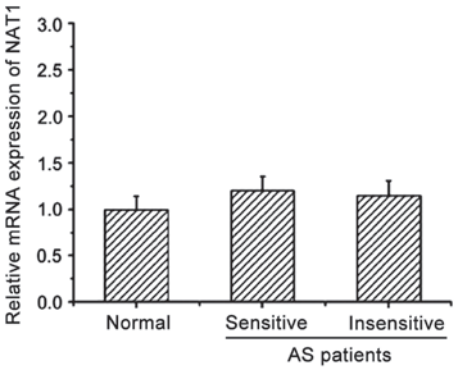

Figure 3. *NAT1* mRNA expression in different groups. NAT1, N-acetyltransferase 1.

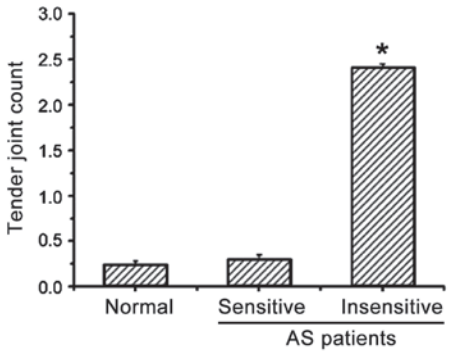

Figure 2. Tender joint count. Asterisk indicates statistically significant differences between groups.

NAT1 gene polymorphisms. To determine the role of *NAT1* in the response to salazosulfamide, we sequenced the *NAT1* gene sequencing to determine the distribution of known polymorphisms. Twenty-six patients sensitive to salazosulfamide contained AA (57%) and AG (42.3%) at locus 263. Six patients insensitive to salazosulfamide were GG genotype (88.9%) at locus 263, with a small frequency of AA (2.8%) and AG (8.3%). These results suggested that *NAT1* polymorphism distribution strongly correlated with salazosulfamide sensitivity in ankylosing spondylitis.

NAT1 mRNA expression. To determine whether the *NAT1* polymorphisms affect mRNA expression, we quantified mRNA from our patients. We found that the expression of *NAT1* mRNA was comparable between the control group, and salazosulfamide responsive and non-responsive in the experimental group (Fig. 3).

NAT1 protein expression: ELISA. We then determined the levels of NAT1 protein expression from all the groups by ELISA to determine if the polymorphisms could affect protein expression or stability. The expression of NAT1 protein in salazosulfamide responsive patients (1.63±0.12 μg/l) was comparable to the expression in the control group (1.4±0.1 μg/l) (Fig. 4). However, the expression of NAT1 in the salazosulfamide non-responsive patients was much lower (0.26±0.04 μg/l) than in the salazosulfamide responsive and the control patients (Fig. 4).

NAT1 protein expression: Western blotting. To confirm the ELISA results with a different technique, we measured NAT1 expression by western blotting. The expression of NAT1 protein in the salazosulfamide responsive patients in the experimental group was comparable to the levels in control patients (Fig. 5). However, NAT1 protein expression in salazosulfamide non-responsive patients decreased significantly (Fig. 5). These results are consistent with the ELISA results described above.

Discussion

The incidence of ankylosing spondylitis is closely associated with environmental factors, such as living style, dietary habits, and genetic factors (12). As a systemic autoimmune disease,

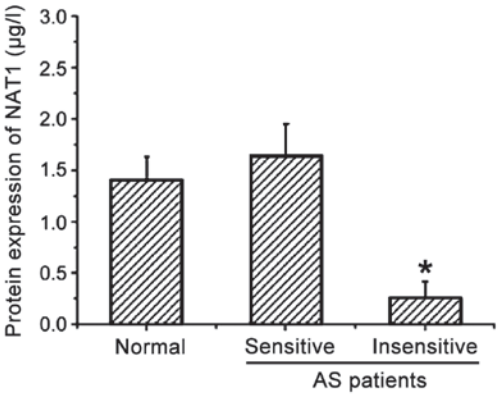

Figure 4. NAT1 protein expression in different groups by ELISA. Asterisk indicates statistically significant differences between groups. NAT1, N-acetyltransferase 1; ELISA, enzyme-linked immunosorbent assay.

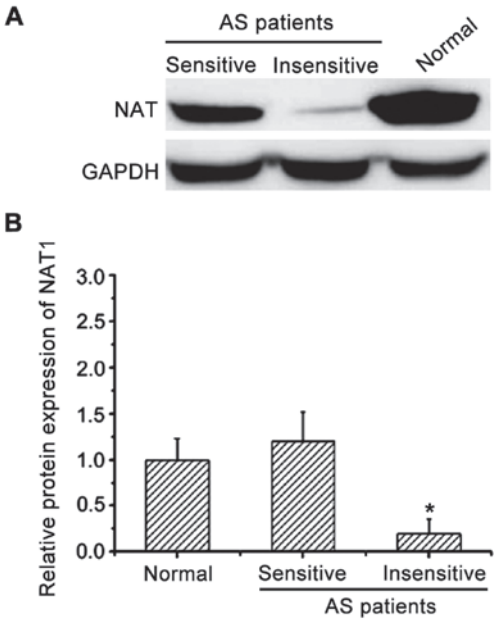

Figure 5. NAT1 protein expression in different groups by western blotting. (A) Western blotting qualitative result. (B) Western blotting quantitative result. Asterisk indicates statistically significant differences between groups. NAT1, N-acetyltransferase 1.

ankylosing spondylitis can cause infection due to decreasing immunocompetence. Salazosulfamide can improve pain and the development of ankylosing spondylitis, and decrease IgA level in serum (13), which is appropriate for improving peripheral articular synovial inflammation of patients with ankylosing spondylitis. However, the clinical efficacy of salazosulfamide is not obvious in different populations. In recent years, it was found that ankylosing spondylitis is associated with imbalance of immune function (14). For example, the critical immunoregulator Th17 cells plays a significant regulating role in the mediation of inflammatory reaction and autoimmune disease (15). The number of Th17 cells in patients with ankylosing spondylitis was significantly decreased compared with normal individuals, but the reason was not clear (16). Salazosulfamide is widely used in the treatment of ankylosing spondylitis (17); however, clinical data showed that salazosulfamide is not effective in all patients (2). Previous findings showed that the *NAT1* gene is highly polymorphic (18), and over 26 NAT1 polymorphisms have been described. NAT1 is a xenobiotic metabolizing enzyme mainly involved in substrate acetylation, a main route of modifying gap-associated proteins, and the function of the protein modified by acetylation changes correspondingly (19).

In the present study, we found that salazosulfamide efficacy for the treatment of ankylosing spondylitis is associated with *NAT1* polymorphisms. However, we did not study the mechanisms mediating the role of *NAT1* polymorphism on salazosulfamide effectiveness in ankylosing spondylitis. Future studies should be conducted to investigate these issues.

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