

ORIGINAL ARTICLE

Detecting the TNF- α -308G/A Polymorphism by Quantitative PCR and its Correlation with Therapeutic Efficacy in Ankylosing Spondylitis

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ABSTRACT

Background: The tumor necrosis factor-alpha (TNF- α) -308GA polymorphism (rs1800629) may affect TNF- α expression and the effectiveness of TNF- α inhibitors in autoimmune conditions such as ankylosing spondylitis (AS). For clinical use, it is very important to have fast and accurate genotyping methods. This study aimed to comprehensively evaluate a fluorescence quantitative polymerase chain reaction (PCR) method based on TaqMan minor groove binder (MGB) probe technology in detecting the TNF- α rs1800629 polymorphism and investigate the correlation between this SNP and the efficacy of TNF- α inhibitor therapy in patients with AS.

Methods: A TaqMan-MGB FQ-PCR detection system was constructed for the detection of the TNF- α single nucleotide polymorphism (SNP) (-308G>A). The limit of detection, accuracy, precision, specificity, sensitivity, and stability were assessed. A multicenter clinical trial was conducted to assess the detection system using a double-blind method involving 1,045 patients with immune diseases. The genotypes of TNF- α -308G/A from 20 patients with hyperlipidemia or jaundice were tested using the detection systems to evaluate the influence of interference. The correlation between the clinical response to TNF- α inhibitors and TNF- α -308G/A SNP was investigated using the 32 patients with ankylosing spondylitis.

Results: The detection range limit was 151.4 ng/ μ L - 0.5 ng/ μ L. The genotype detected using the research detection system and Sanger sequencing was 100%, with a Kappa 1.00 > 0.75, $p = 0 < 0.001$. The coefficient of variation of precision was less than 8%. Interfering substances did not affect the detection system. The follow-up trial indicated that the patients with the GA genotype, three out of the four cases, had no response for TNF- α inhibitors. In contrast, all patients with the GG genotypes experienced better treatment efficacy with TNF- α inhibitors.

Conclusions: The detection system utilizing the TaqMan-MGB probe for TNF- α polymorphism - 308G/A demonstrated high reliability. TNF- α -308G/A polymorphisms are potential biomarkers for predicting the treatment efficacy of TNF- α inhibitors for patients with ankylosing spondylitis.

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KEYWORDS

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INTRODUCTION

TNF- α is a homotrimer intercellular signaling protein consisting of 157 amino acids and is in the class III region of the major histocompatibility complex (mapped

on 6p21.31), containing 4 exons and 3 introns [1]. TNF- α (tumor necrosis factor α), as one of the most typical pro-inflammatory cytokines, is secreted by various types of immune cells. TNF- α is essential for the proper proliferation and function of several immune cells, such as NK cells and macrophage, which mediate tumor destruction. Additionally, TNF- α plays a crucial role in the NF- κ B and MAP kinase signaling pathways, which are involved in proliferation, differentiation, and growth [2,3]. Dysregulation of TNF- α can lead to various immune-mediated inflammatory diseases such as rheumatoid arthritis, Crohn's disease, pathogenesis, and progression of cancer [4,5].

TNF- α is a crucial component for a normal immune response. Inappropriate or excessive production can be harmful and can lead to diseases. Serum levels of TNF- α have been reported to be significantly higher in patients with rheumatoid arthritis (RA), ankylosing spondylitis (AS) [6,7]. TNF- α inhibitors have been developed and many novel TNF- α inhibitors including etanercept, infliximab, adalimumab, golimumab, and certolizumab have been approved for clinical treatment of autoimmune diseases. However, about 40% - 60% of patients do not respond to TNF- α inhibitors [8]. Single nucleotide polymorphisms (SNPs) in the TNF- α gene significantly effect the regulation of TNF- α production. There are several TNF- α polymorphisms that have been identified; the most extensively investigated polymorphisms are at positions -308 and -238, which are in the promoter region of the TNF- α gene. The research reported -308 A allele with a higher transcriptional activity than -308 G allele. The SNPs at rs1800629 (-308G>A) are associated with the susceptibility to systemic lupus erythematosus (SLE) and ankylosing spondylitis (AS) [9,10]. The TNF- α -308G/A polymorphism has also been associated with susceptibility to stroke, pre-eclampsia, AR, and AS. TNF- α rs1800629 (308G>A) can be an excellent biomarker of the predictor for TNF- α inhibitors efficacy [11-13].

Ankylosing spondylitis (AS), rheumatoid arthritis (RA), and psoriasis arthritis (PsA) are common autoimmune-mediated inflammatory diseases. RA and AS affect the joints and typically result in redness, swelling, and arthralgia in the early stages of the disease. These diseases may limit the range of motion at a serious phase. RA affects nearly 1% of the population worldwide, and the prevalence of AS in China is approximately 0.3% [14, 15]. Additionally, individuals with RA have an increased mortality due to cardiovascular disease and infection. The TNF- α inhibitors are major therapy drugs for patients with AS or RA. However, there is no consensus regarding the association between the TNF- α -308G/A polymorphism and the therapeutic efficacy of TNF- α inhibitors in patients with AS and RA. The quick, accurate method for detection of the SNP of rs1800629 (-308G>A) of TNF- α and elucidation of the association of SNP at the TNF- α -308G/A and TNF- α antagonist efficacy is essential to facilitate accurate diagnosis and predict prognosis.

In this study, the fluorescence quantitative PCR based on TaqMan-MGB was established, and the detection system was evaluated. The application of the detection system was also validated in a multicenter clinical study. Additionally, the association between the TNF- α -308G/A polymorphism and the therapeutic efficacy of TNF- α inhibitors was investigated in a clinical study to identify potential biomarkers for predicting treatment response.

MATERIALS AND METHODS

Reagents and instruments

TIANamp Genomic DNA Kit (Tiangen Biotech (Beijing) Co., Ltd., 200 pieces/box), ABI 7500FQ-PCR (Thermo Fisher Scientific), and ABI 3730XL DNA analyzer (Thermo Fisher Scientific) were used.

Target gene sequence, primers, and probes

The DNA sequences of the TNF- α gene (rs1800629) were obtained from GenBank, National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/Genbank/), and aligned using the UCSC Genome Browser (<http://genome.ucsc.edu/>). Twelve pairs of primers and twenty probes, including ten for the -308G allele and ten for the -308A allele, were designed using Primer Express Software 2.0. Each pair of TaqMan-MGB probes for the G/A alleles was labeled at their 5' ends with fluorescein VIC and FAM, respectively, while the NFQ sequestering molecule was labeled at the 3' end. The probe for β -actin, used as an internal control, was labeled at both its 5' and 3' ends with ROX and BHQ, respectively.

Positive control sequence selection and construction

The 1,001 bp sequences surrounding rs1800629 and the 240 bp sequence of β -actin were selected as positive and internal controls, respectively. The homology of the genotypes was compared to ensure accuracy. A recombinant plasmid containing the DNA sequences of TNF- α 308G/A was constructed. The positive control was prepared by diluting a 1:1 mixture of the TNF- α 308G/A plasmid and the β -actin plasmid in TE buffer to a concentration of 1.0×10^4 copies/ μ L. Both the positive control and internal control were verified using Sanger sequencing and PCR to confirm the accuracy of the plasmid sequences. All plasmids used in this study were constructed by Tianyi Huiyuan Biotech Co.

Optimization of TaqMan-MGB FQ-PCR detection system

The artificial syntheses primers for TNF- α 308G/A were diluted in TE buffer to concentrations of 200 nM, 300 nM, 400 nM, and 500 nM. The amplification efficacy of the primers was evaluated using plasmids including -308G, -308A, and their mixture at rs1800629. The synthesized probes for -308G and -308A were diluted in TE buffer to concentrations of 80 nM, 100 nM,

120 nM, and 140 nM. The detection efficacy of the probes was evaluated using plasmids composed of the corresponding genotypes and their mixture. As an internal control, the primers and probes for β -actin were diluted in TE buffer to the concentration of 100 nM, 150 nM, 200 nM, and 250 nM and 60 nM, 80 nM, 100 nM, and 120 nM respectively. The optimal concentration for internal control primers and probes was determined using plasmids for 308G/A and their mixture at rs1800629, along with the β -actin plasmid.

Verification of the positive value at TNF- α (-308G>A)

The genotypes of 276 clinical anticoagulated whole blood samples, comprising 233 samples with the GG genotype, 38 with the GA genotype, and 5 with the AA genotype, were analyzed using Sanger sequencing. Additionally, the genotypes of these 276 samples for the rs1800629 (-308G>A) locus of TNF- α were also examined using the TaqMan-MGB PCR detection system. Cycle threshold (Ct) values were collected, with diagnostic points established at Ct values of 15.00, 16.00, 17.00, 18.00, 19.00, and 20.00. The genotype results were compared with those obtained from Sanger sequencing, and a receiver operating characteristic (ROC) curve was generated.

Limit of detection for TaqMan-MGB FQ-PCR detection system

The clinical DNA samples, for which the genotypes were already known, were diluted to concentrations of 0.5 ng/ μ L, 1.0 ng/ μ L, 2 ng/ μ L, and 5 ng/ μ L. Each sample was tested 20 times using TaqMan-MGB FQ-PCR and Sanger sequencing to determine the minimum limit of detection. Ten clinical samples with relatively high DNA concentrations were diluted to 78.2 ng/ μ L, 100.4 ng/ μ L, 106.0 ng/ μ L, 116.5 ng/ μ L, 119.4 ng/ μ L, 123.2 ng/ μ L, 149.0 ng/ μ L, 151.4 ng/ μ L, 160.1 ng/ μ L, and 180.4 ng/ μ L. All samples were tested using TaqMan-MGB FQ-PCR and Sanger sequencing to investigate the maximum limit of detection.

Accuracy and precision of the FQ-PCR detection system

The three clinical samples with the GG genotype and three clinical samples with the GA genotype at rs1800629 were diluted to concentrations of 20 ng/ μ L and 5 ng/ μ L, respectively. The -308A locus of the TNF- α gene and the β -Actin recombinant plasmid were mixed in equal proportions, resulting in final concentrations of 1.0×10^5 copies/ μ L and 1.0×10^6 copies/ μ L. All clinical samples and plasmids with different genotypes were tested using TaqMan-MGB FQ-PCR and Sanger sequencing to evaluate the accuracy of the system.

The plasmid samples with GG and GA genotypes at rs1800629 were diluted to concentrations of 5 ng/ μ L, 20 ng/ μ L, and 100 ng/ μ L. The -308A locus of TNF- α and β -Actin recombinant plasmids were mixed in equal proportions. Subsequently, the mixture was prepared to

achieve concentrations of 5.0×10^6 copies/ μ L, 1.0×10^6 copies/ μ L, and 1.0×10^5 copies/ μ L. All samples were analyzed using TaqMan-MGB FQ-PCR and Sanger sequencing twice a day for 20 consecutive days to evaluate system precision.

Specificity of TaqMan-MGB FQ-PCR detection system

The two types of plasmids were constructed with highly similar amplified region sequences at rs1800629 (308G/A). The sample mixture was prepared using the recombinant plasmid and β -Actin at a concentration of 1.0×10^5 copies/ μ L. All samples were analyzed using TaqMan-MGB FQ-PCR to investigate the impact of the similar sequences of rs1800629 on the specificity of the detection system.

The rs361525 (G>A) site of TNF- α was selected for analysis. The recombinant plasmids contained sequences of both the wild type and the mutant type at the rs361525 (G>A) site, along with an internal control. All DNA samples were tested at a concentration of 1.0×10^5 copies/ μ L using TaqMan-MGB FQ-PCR detection to investigate the influence of other SNPs on the specificity of the detection system.

Sensitivity of TaqMan-MGB FQ-PCR detection system

Each of the six clinical samples, for which the genotypes were already known, was divided into nine parts for the sensitivity investigation. The only part without any interfering substances was the control sample.

The remaining eight parts were added with the following substances: hemoglobin (final concentration: 12 mg/mL), triglyceride (final concentration: 7.5 mM), heme (final concentration: unspecified mg/mL), bilirubin (final concentration: 3 mM), EDTA (final concentration: 6 mg/mL), sodium citrate (final concentration: 6 mg/mL), recombinant human TNF-II receptor-antibody fusion protein (final concentration: 2.5 μ g/mL), and etanercept (final concentration: 2.6 mg/L).

Stability of TaqMan-MGB FQ-PCR detection system

The TaqMan-MGB FQ-PCR detection system was exposed to 2 - 8°C for 8 hours. Every 2 hours, positive, negative, and minimum limit reference samples were tested to verify the stability of the detection system.

The detection system was frozen and stored at $-20 \pm 5^\circ\text{C}$, then removed for storage and thawed at 2 - 8°C, a process that was repeated 22 times. The detection system was utilized for positive and negative control detection on the 10th, 14th, 18th, 20th, and 22nd freeze-thaw cycles. The minimum limit reference was assessed using the detection system from the 22nd freeze-thaw cycle.

Pre-examination before clinical trial

To further verify the accuracy and precision of the research detection system and to investigate the relation-

ship between the genotype at the TNF- α gene rs1800-629 (-308G>A) site and the clinical efficacy of the antagonist, a multicenter clinical trial was conducted.

First, preliminary experiments were conducted. Clinical samples of TNF- α were collected from three hospitals, with 20 cases of anticoagulated venous whole blood from each hospital. After extracting genomic DNA, the detection of TNF- α gene polymorphism at the rs1800-629 (-308 G>A) site was performed using the TaqMan-MGB FQ-PCR detection system and the Sanger sequencing method.

Verification of TaqMan-MGB FQ-PCR through clinical samples

The multicenter clinical trial was conducted using a double-blind method. From August 2019 through November 2019, 1,045 qualified EDTA-anticoagulated whole blood samples were enrolled from three hospitals. Among the patients, the male ratio was 49.09% and age < 51 years made up 46.99%.

Among the enrolled patients, there were 199 patients (19.04%) with ankylosing spondylitis (AS) and 264 (25.26%) with rheumatoid arthritis (RA). The DNA was extracted from 1,045 clinical samples, and the genotypes for rs1800629 of all samples were tested using both TaqMan-MGB FQ-PCR and Sanger sequencing. The results of the two methods were compared.

The study and clinical protocol were approved by the Ethics Committee of the three clinical hospitals, including Chinese Academy of Medical Sciences and Peking Union Medical College, Peking University People's Hospital, and Zhongnan Hospital of Wuhan University. All procedures were performed following the clinical protocol. The informed consent waivers were approved by the Ethics Committee of the three clinical hospitals.

Statistical analysis

The accuracy, sensitivity, and specificity of the detection system were analyzed using standard deviation (SD). Clinical samples were tested by both TaqMan-MGB FQ-PCR and Sanger sequencing. The group test results were analyzed using the chi-squared (χ^2) test and the estimation of relative risk with a 95% confidence interval (95% CI). A p-value < 0.05 was considered significant. All statistical analyses were conducted using SPSS 22.0.

RESULTS

Optimized primers and probes and validation

The details of the optimized primers and probes are presented in Table 1. All primers and probes used in this study were custom-made by Sangon Biotech (Shanghai) Co., Ltd.

The ratio of OD260 nm to OD280 nm for the forward and reverse primers in the TE buffer, which included TNF- α rs1800629 and β -Actin, ranged from 1.8 to 2.0. The probe buffer was scanned from 200 through 900

nm. The VIC probe exhibited two UV absorption peaks at 250 - 270 nm and 520 - 540 nm. The FAM probe and ROX probe showed UV absorption ranges of 220 - 250 nm, 460 - 510 nm, and 250 - 270 nm and 560 - 580 nm, respectively. All probes were successfully labeled. The results are illustrated in Figure 1.

Validation of TaqMan-MGB FQ-PCR detection system

Through investigation using TaqMan-MGB FQ-PCR, the optimized detection system included 400 nM for each primer of rs1800629 -308 G/A, 120 nM probe of -308G, 100 nM probe of -308A, 100 nM of each primer for β -Actin, and 200 nM probe for β -actin. The total volume of detection system was 30 μ L.

The optimized TaqMan-MGB FQ-PCR process was as follows: pre-denaturation for 1 cycle at 95°C for 1 minute, followed by 15 cycles of 95°C for 5 seconds and 61°C for 32 seconds, for a total of 30 cycles. Fluorescence signals were collected at 61°C.

The DNA concentrations from 50 patients ranged from 35.74 to 156.25 ng/ μ L, with optical density (OD) values from 1.81 to 1.99. The genotypes obtained from the detection system were consistent with those determined by Sanger sequencing.

The ROC curve was plotted using 43 positive samples and 233 negative samples of allele A at the rs1800629 locus of the TNF- α gene, as shown in Figure 3. Ct value ≤ 17 was considered the positive reference.

Maximum and minimum detection limitation

The results indicate when the sample concentration ≥ 0.5 ng/ μ L TaqMan-MGB FQ-PCR detection system is consistent with the Sanger sequencing. When the sample concentration is ≤ 151.4 ng/ μ L, the amplification curve exhibits a smooth "S" type and the results from TaqMan-MGB FQ-PCR detection system remain consistent with the Sanger sequencing results.

When the concentration of the sample is above 50 ng/ μ L, some PCR curves do not achieve a smooth "S" type. The detection range of the detection system is 0.5 ng/ μ L to 150 ng/ μ L.

Accuracy and precision of detection system

The genotype, which included two different concentration samples comprising 3 GA, 3 GG, and β -Actin with the -308A recombinant plasmid, was tested to verify the accuracy of the detection system. The test results were 100% consistent with Sanger sequencing.

High, medium, and low concentrations samples, including 1GA, 1GG, and β -Actin with the -308A recombinant plasmid, were tested to verify the precision of the detection system. The test results were 100% consistent with Sanger sequencing.

Specificity and sensitivity of the detection system

The two highly-similar sequences of target genotype sites and rs361525 (G>A) sites were tested using TaqMan-MGB FQ-PCR, with Sanger sequencing employed for verification of the detection system. No fluorescent

Table 1. Optimized primers and TaqMan-MGB probes for detection of TNF- α SNP rs1800629.

Primer/Probe	Sequence (5'→3')	Base number
Forward	CCTGCATCCTGTCTGGAAGTTAGAAG	26
Reverse	GGACACACAAGCATCAAGGATACCC	25
308G-Probe	VIC-CCCGTCCCCATGC-MGB-NFQ	13
308A-Probe	FAM-CCCGTCCCTCATGC-MGB-NFQ	13
β -Actin Forward	TGAAGATCCTCACCGAGCG	19
β -Actin Reverse	GGCAGCTCGTAGCTCTTCTC	20
β -Actin Probe	ROX-ACCACCACGGCCGAGCGG-BHQ	18

Table 2. The accuracy of the detection system.

Genotype	Times	FAM(-A)	VIC (-G)	ROX (control)	Genotype	Times	FAM(-A)	VIC (-G)	ROX (control)
Sample 1/GG	1	UN	8.00	7.22	Sample 2/GG	1	UN	8.26	7.43
	2	UN	8.01	7.02		2	UN	8.23	7.21
	3	UN	7.92	6.97		3	UN	8.08	6.97
Sample 3/GG	1	UN	8.30	7.44	Sample 4/GA	1	8.50	9.12	7.97
	1	UN	8.31	7.25		2	9.57	10.54	7.85
	3	UN	8.36	7.27		3	9.33	10.18	7.53
Sample 5/GA	1	8.07	8.48	7.62	Sample 6/GA	1	8.65	9.20	7.94
	2	8.86	9.62	7.15		2	8.76	9.39	6.97
	3	8.62	9.21	6.93		3	8.52	9.09	6.78
	1	5.90	UN	7.15					
Plasmid-1/AA	2	7.94	UN	7.15					
	3	7.90	UN	7.07					
Sample 8/GG	1	UN	11.97	11.03	Sample 9/GG	1	29.23	11.96	11.02
	2	UN	10.11	9.03		2	UN	10.25	9.14
	3	UN	10.01	8.97	9/GG	3	UN	9.94	8.94
Sample 10/GG	1	UN	12.05	11.04	Sample 11/GA	1	12.30	13.03	11.71
	2	UN	10.08	8.95		2	10.86	11.60	9.16
	3	UN	10.12	9.00	11/GA	3	10.53	11.17	8.93
Sample 12/GA	1	11.93	12.62	11.27	Sample 13/GA	1	12.32	13.18	11.53
	2	10.52	11.24	8.92		2	10.98	11.65	9.19
	3	10.77	11.37	8.96		3	10.80	11.43	9.00
	1	11.11	UN	10.61					
Plasmid-2/AA	2	9.64	UN	8.78					
	3	9.91	UN	9.03					

Samples 1 - 6: 20 ng/ μ L, samples 8 - 13: 5 ng/ μ L, Plasmid-1/AA: 1.0 x 10⁶ copies/ μ L, internal-control, Plasmid-2/AA: 1.0 x 10⁵ copies/ μ L, internal-control.

signal was detected in the detection system. The results suggest that similar sequences and other mutant sites do not affect the test results of the detection system.

The results of stability

The positive controls, negative controls, and limitation of detection were analyzed using the TaqMan-MGB FQ-PCR detection system, which was continuously ex-

Table 3. Comparison of detection results of two methods for the TNF- α gene rs1800629 (-308G>A) locus for 1,045 patients.

Genetic site	Group	SNP	Frequency	Percent (%)
		GG	946	90.53%
	TaqMan-MGB FQ-PCR			
		GA	98	9.38%
TNF- α	detection system			
rs1800629 (-308 G>A)		AA	1	0.09%
		GG	946	90.53%
	Sanger sequencing			
		GA	98	9.38%
		AA	1	0.09%

Table 4. The genotype distribution of different diseases for 1,045 patients.

SNP	Group	Sample size	Genotype		
			GG	GA	AA
rs1800629 (-308G>A)	AS	199	187/93.97%	12/6.03%	0/0%
	RA	264	250/94.70%	14/5.30%	0/0%
	Other	582	509/87.46%	72/12.37%	1/0.17%

AS: ankylosing spondylitis, RA: rheumatoid arthritis.

Table 5. The evaluation results on interference influence of the research detection system.

No.	Age	Gender	Clinical diagnosis	Sanger sequencing	PCR detection system
1	66	M	obstructive jaundice with infection	GG	GG
2	62	M	obstructive jaundice	GG	GG
3	69	M	obstructive jaundice	GG	GG
4	60	M	obstructive jaundice	GG	GG
5	65	M	obstructive jaundice	GG	GG
6	76	F	obstructive jaundice with infection	GG	GG
7	72	M	jaundice	GG	GG
8	90	F	obstructive jaundice	GG	GG
9	51	F	obstructive jaundice with lymphoma	GG	GG
10	67	M	obstructive jaundice	GG	GG
11	53	F	hyperlipidemia	GG	GG
12	68	M	renal osteodystrophy with hyperlipidemia	GG	GG
13	71	M	hyperlipidemia	GG	GG
14	44	M	hypertension with hyperlipidemia	GG	GG
15	58	F	hyperlipidemia	GG	GG
16	68	M	cerebral infarction with hyperlipidemia	GG	GG
17	37	M	hyperlipidemia	GG	GG
18	38	M	hyperlipidemia with renal hypertension	GG	GG
19	32	M	hyperlipidemia with arteriosclerosis	GG	GG
20	33	M	hyperlipidemia	GG	GG

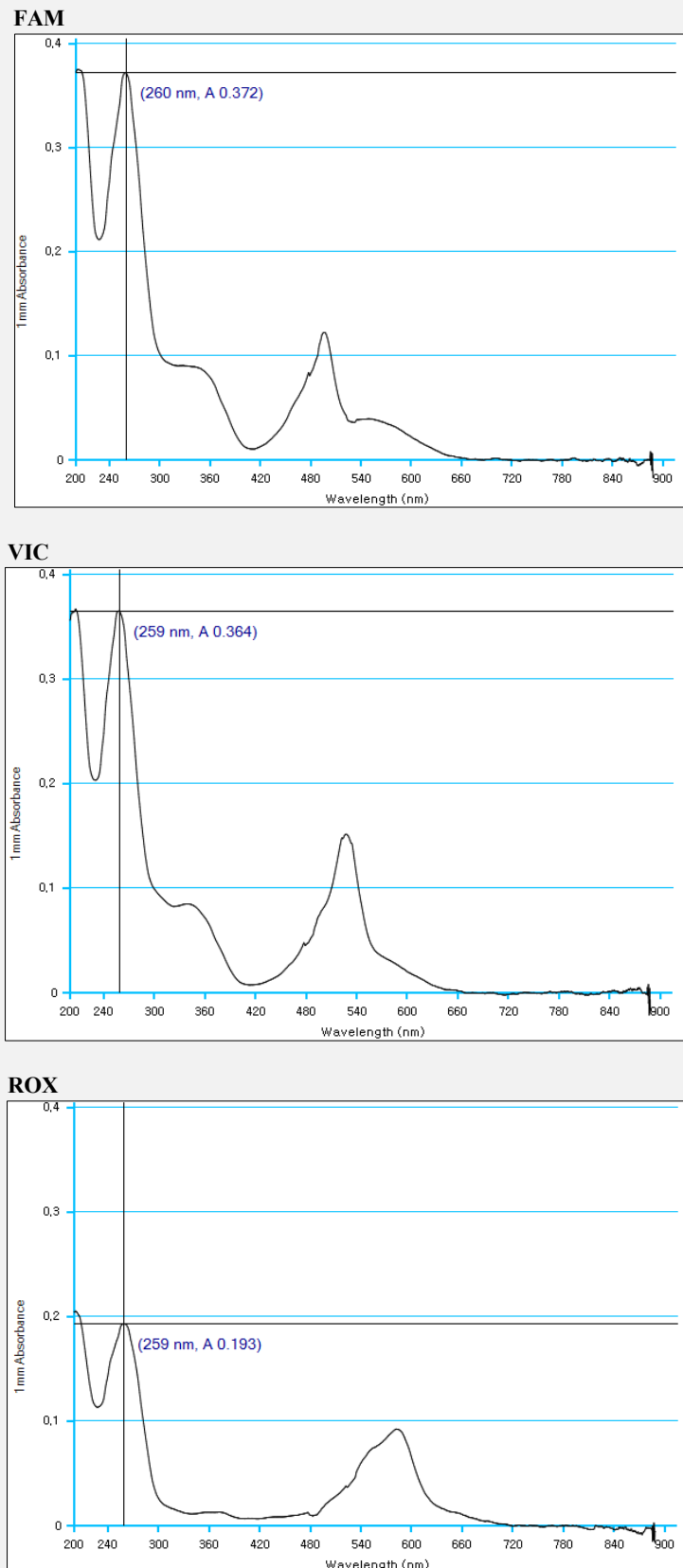
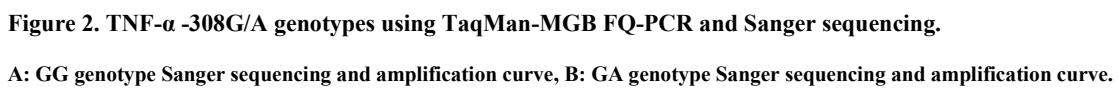


Figure 1. UV absorption of labeled probes.



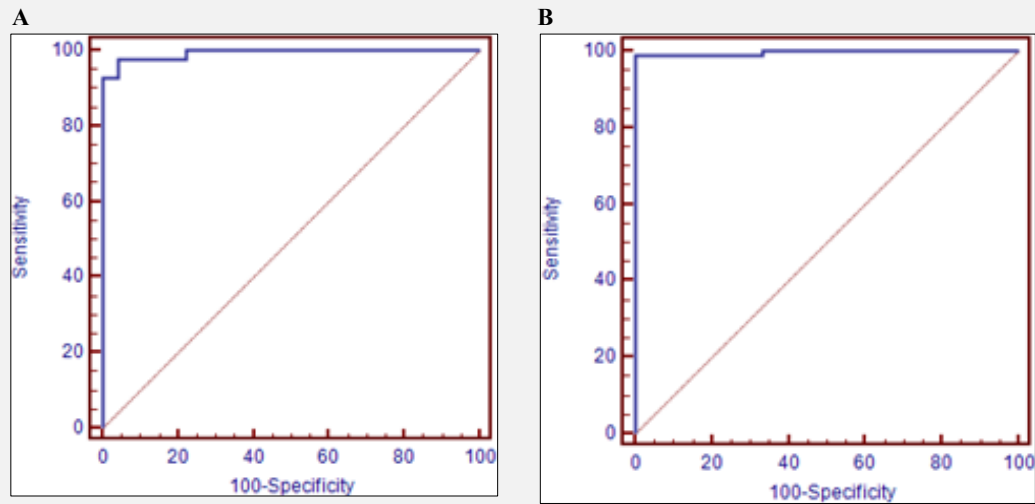


Figure 3. The ROC curve of alleles.

A: A allele of rs1800629 (AUC = 0.941, Youden's index = 0.964), **B:** G allele of rs1800629 (AUC = 0.996; Youden's index = 0.989).

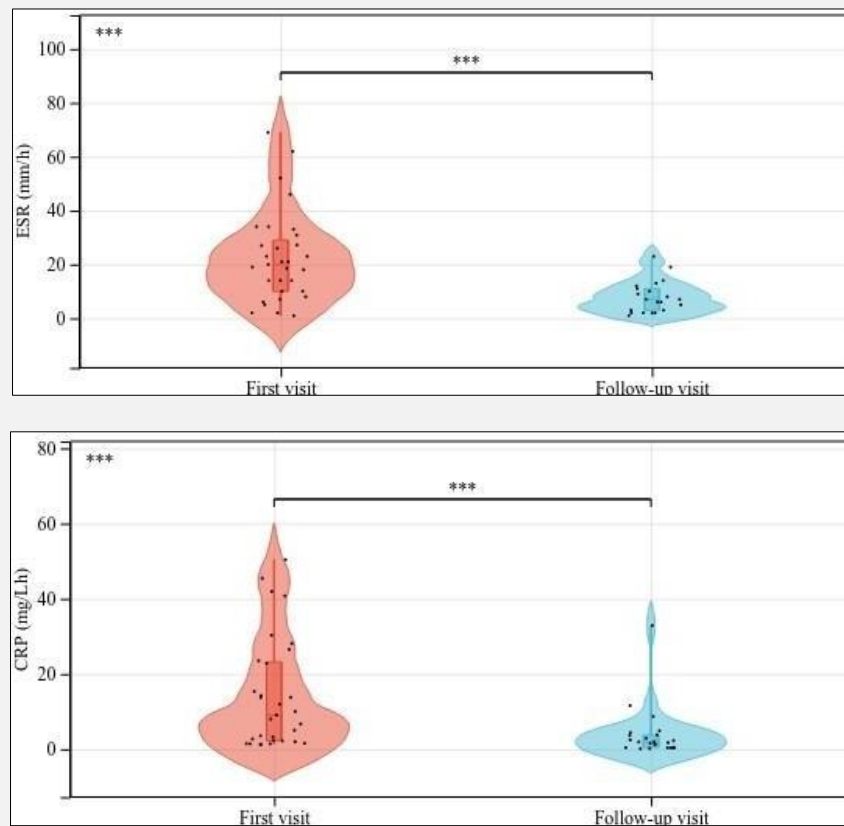


Figure 4. ESR and CRP values of patients with AS (*) $p < 0.001$.**

posed at 2 - 8°C for 8 hours or frozen and stored at -20 ± 5°C and freeze-thawed 22 times. The compliance rate for the negative control, positive control, and limitation of detection was 100%. The exposure and repeated freeze-thaw did not influence the TaqMan-MGB FQ-PCR detection system. The coefficient of variation of the investigation on the limitation of detection was less than 5%.

Verification of detection system by clinical trial

The pre-examination of clinical results indicated that the genotypes of the clinical samples at the TNF- α rs1800629 site, tested using the TaqMan-MGB FQ-PCR detection system, were consistent with the results obtained from Sanger sequencing.

A total of 1,045 clinical samples that met the eligibility criteria were tested using the TaqMan-MGB FQ-PCR detection system and Sanger sequencing. The detection results for the SNP at rs1800629 (-308 G>A) are presented in Table 3.

The statistical analysis between the gold standard and research system results indicate that the 95% confidence interval for the consistency rate is [100%, 100%], Kappa = 1.00 > 0.75, $p = 0 < 0.001$. In addition, the distribution of genotypes among different diseases for 1,045 patients is shown in Table 4.

The SNPs of 20 EDTA-anticoagulated venous whole blood samples, including 10 samples from patients with hyperlipidemia and 10 samples from patients with jaundice, were detected using the research detection system and the gold standard. The results of these detections were utilized to evaluate the interference effects on the research detection system.

From Table 5, the comparison results indicate that the interfering substances involved in the clinical sample will not affect the detection of the research detection system.

The relationship between the genotype and TNF- α antagonist efficacy

Thirty-two patients with ankylosing spondylitis were enrolled in the study. EDTA or sodium citrate anticoagulated venous whole blood was collected from these patients. The genotypes were analyzed using the research detection system and Sanger sequencing. They were treated with TNF- α inhibitors including recombinant human TNF- α receptor: IgG Fc fusion protein, golimumab, adalimumab. The patients had regular follow-up visits to monitor clinical conditions. Then CRP, ESR, and clinical symptoms were taken as response to TNF- α inhibitor criteria. The comparison of ESR, CRP between first visit and follow-up visit is shown in Figure 4. The prediction of responsiveness to TNF- α Inhibitors based on TNF- α -308G/A polymorphisms and actual clinical efficacy of TNF- α inhibitors was compared.

Among 28 patients with the GG genotype, most of them showed a superior response to TNF- α inhibitors. Although ESR or CRP of a few patients failed to normalize, their clinical conditions significantly relieved.

Among 4 patients with the GA genotype, there were three cases that demonstrated poor response to TNF- α inhibitors, the clinical symptoms showed no relief.

DISCUSSION

Tumor necrosis factor alpha (TNF- α) is a cytokine with pleiotropic effects on various cell types. It can be released by multiple immune cell types, including macrophages, T lymphocytes, and natural killer cells, in response to cellular damage, stress, or infection. TNF- α is essential for immune surveillance and serves as a pathological component in autoimmune diseases. TNF- α inhibitors are utilized as therapeutic drugs for these conditions. Several TNF- α polymorphisms have been identified. The rs1800629 variant, in the promoter region of the TNF- α gene, is being extensively studied. Polymorphisms at position -308 significantly influence TNF- α expression. Research has demonstrated that the -308A allele exhibits higher transcriptional activity compared to the -308G allele [16]. The accumulation of TNF- α can lead to chronic inflammation and tissue destruction. The -308G/A genotype significantly enhances inflammatory responses, and patients with the mutant 308A allele demonstrate reduced efficacy compared to those with the wild-type -308G allele when undergoing antagonist therapy [12,17].

In this study, fluorescence quantitative PCR detection system based on the TaqMan-MGB probe technique was established. To evaluate the accuracy of the detection system, Sanger sequencing was employed as the gold standard. Through systematic evaluation, the detection system demonstrated an extremely high level of consistency between the two methods. Therefore, the TaqMan-MGB FQ-PCR detection system is stable and reliable in detecting the TNF- α -308G/A gene polymorphism.

The studies on correlation between TNF- α -308G/A polymorphism and responsiveness of TNF- α inhibitors in patients with AS are controversial [18,19]. In our study, the frequency of the GG genotype was 90.53% among all patients, while the frequencies of the GA and AA genotypes were 9.38% and 0.09%, respectively. Among all samples from AS patients, the frequencies of the GG and GA genotypes were 93.97% and 6.03%. In all samples from RA patients, the frequencies of the GG and GA genotypes were 94.70% and 5.30%, respectively. The AA genotype was not detected in either AS or RA patients. The distribution of the three genotypes was consistent with findings from other studies conducted in China [17,20,21].

For patients with autoimmune diseases who do not achieve satisfactory efficacy with conventional drugs, TNF- α inhibitors are a better choice. However, the high cost and significant side effects of TNF- α inhibitors, along with their potential failure to achieve the expected therapeutic outcomes, should be of concern. Exploring biomarkers based on TNF- α -308G/A polymorphisms to

predict treatment response to TNF inhibitors in patients with RA or AS patients is crucial for promoting rational drug use and minimizing adverse effects. Among 32 patients with AS, 28 exhibited the GG genotype, while 4 had the GA genotype. All patients were treated with TNF- α inhibitors, and most demonstrated a favorable outcome. Among the patients with the GA genotype, three out of the four cases had no response for TNF- α inhibitors, while one case showed slight improvement. In contrast, all patients with the GG genotypes experienced better treatment efficacy with TNF- α inhibitors. These research findings suggest that TNF- α -308G/A polymorphisms are reliable biomarkers in predicting the treatment efficacy of TNF- α inhibitors. The patients with the GG genotype had a favorable response to TNF- α inhibitors in treating AS. In contrast, the patients with the GA genotype showed minimal response to TNF- α inhibitors for the same condition. This finding aligns with the report of Ma et al. [22].

CONCLUSION

The research has established TaqMan-MGB fluorescence quantitative PCR for detection of human TNF- α polymorphism. The detection system has high accuracy, precision, sensitivity, and stability. The interference in clinical samples has not influenced the detection. The research preliminarily indicates that TNF- α -308G/A polymorphisms may be used as a potential biomarker to predict the responsiveness to TNF- α inhibitors in AS. The GG genotype implies the patients have a good response to TNF- α inhibitors.

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Declaration of Generative AI in Scientific Writing:

The authors declare that they did not use generative artificial intelligence (AI) or AI-assisted technologies in the writing process.

Declaration of Interest:

The authors declare that they have no conflict of interest.

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