Clinical and Experimental Rheumatology 2004; 22: 219-222.

Association between urokinase gene 3'-UTR T/C polymorphism and Chinese patients with rheumatoid arthritis in Taiwan

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Received on March 25, 2003; accepted in revised form on October 14, 2003.

Key words: Urokinase, rheumatoid arthritis, single nucleotide polymorphism.

ABSTRACT

Objectives. *The purpose of this study was to investigate whether the urokinase gene 3'-UTR C/Tpolymorphism is a marker of susceptibility to or severity of rheumatoid arthritis (RA) in Chinese patients.*

Methods. *A total of 145 RA patients and 134 healthy control subjects were enrolled in this study. We identified the C/T polymorphism of the urokinase gene, which is mapped on the 3'-untranslated region (3'-UTR) on chromosome 10 by polymerase chain reaction (PCR).*

Results. *There were significant differences in the distribution of the urokinase gene 3'-UTR C/T polymorphism frequency between RA patients and subjects in the control group. However, we did not detect any association between the urokinase gene 3'-UTR C/T polymorphism and rheumatoid factor (RF), extraarticular involvement or bone erosion in RA patients.*

Conclusion. *The urokinase gene 3'-UTR "T" allele was associated with RA in Chinese patients in Taiwan.*

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease affecting about 1% of the world's population. Both environmental and genetic factors are thought to be involved in the onset of the disease. However, the genetic basis of RA is largely unknown. The major histocompatibility (MHC) class II region is an important susceptibility factor and the human leukocyte antigen (HLA)-DR4 has been associated with serious disease courses (1,2). However, HLA-DR associations probably account for only a third of the overall genetic contribution. This suggests that other genetic factors are present in the susceptibility to RA.

Two types of plasminogen activators (PA), tissue type PA (tPA) and urokinase PA (uPA), which are structurally, immunologically, and genetically distinct, have been identified in mammals. The primary role of tPA is thought to be in fibrin dissolution and thrombolysis, while uPA is mainly involved in pericellular matrix degradation during tissue remodeling (3,4). The effect of uPA is intensified and localized through binding to a specific cell bound receptor (uPAR) that is expressed on a variety of cell types, including neutrophils, monocytes/macrophages and malignant cells (4). Plasminogen activators, such as uPA, may modulate synovial inflammation, angiogenesis, and joint destruction in a number of ways. Urokinase PA may be involved in the recruitment of inflammatory leukocytes into the synovial tissue, as it is chemotactic for neutrophils (5) and it stimulates cytokine dependent monocyte adhesions (6).

Increased levels of uPA and uPAR were detected in synovial tissue extracts from patients with RAcompared to patients with osteoarthritis (OA) (7). In addition, increased uPA and decreased tPA antigenic levels measured in the synovial fluids of RApatients are associated with the clinical severity of the disease (8). However, there is a lack of genetic evidence to support the hypothesis that urokinase is associated with RA.

We have used single nucleotide polymorphisms (SNPs) as a tool to search for the genetic makers of SLE (9, 10). We therefore focused on SNPs to determine whether urokinase is associated with RA. The urokinase gene is located at chromosome 10q24 (11). A C/T polymorphism at +4065 nucleotide was previously reported (STS Accession number: G27040) by Tripputi *et al.* (11). The polymorphic site is located at 3'-UTR (untranslated region) of the urokinase gene. Thus, we used PCR to investigate whether this polymorphism is associated with RA patients. We compared the genotype distributions and allelic frequencies between 145 patients with RA and 134 healthy individuals.

Patients and methods

Patients selection

We enrolled 145 patients (122 females and 23 males) with definite RAaccording to the 1987 revised American College of Rheumatology criteria (12). The median age of the patients was 55 years (range 29-70). We also recruited 134 unrelated healthy individuals (110 females and 24 males) living in central

Taiwan to serve as control subjects. The median age was 52 years (range 26-66). Informed written consent was obtained from both groups that participated in this study.

Rheumatoid factor (RF) was detected by nephelometry. Values ≥ 30 Iu/ml were classified as positive. Extra-articular RA involvement (13) was considered to be present when at least one of the following clinical manifestations was found: (1) subcutaneous rheumatoid nodules; (2) cutaneous vasculitis; (3) eosinophilia; (4) lymphadenopathy; (5) pulmonary disease (pleurisy, interstitial fibrosis, nodular lung or pulmonary hypertension); (6) cardiac disease (pericarditis or conduction defect); (7) non-compressive neuropathy; (8) Raynaud's phenomenon; or (9) sicca syndrome. Radiographs of the hands, wrists, and feet of patients with RA were taken. Bone erosion was evaluated by a radiologist and a rheumatologist. We examined only the presence or absence of erosion and did not calculate a radiological score.

Polymerase chain reaction

The genomic DNA was prepared from peripheral blood by a Genomaker DNA Extractor kit (Bloosm, Taiwan). Polymerase chain reactions (PCRs) were carried out to a total volume of 50 µl, containing genomic DNA, 2-6 pmole of each primer, 1X Taq polymerase buffer (1.5 mM $MgCl_2$), and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, U.S.A.). The primer for the urokinase gene 4065 polymorphism C/T was designed as (5'-CCGCAGTCACACCA AGGAAGAG-3') and (5'-GCCTGAGGGTAAAGC-TA TTGTCGTGCAC-3') according to published data (STS Accession: G27040). Polymerase chain reaction amplification was performed by a programmable thermal cycler GeneAmp PCR System 2400 (Perkin Elmer). The cycling condition for the urokinase gene 3'-UTR C/T polymorphism was set as follows: one cycle at 94°C for 5 min, 35 cycles at 94° C for 30 sec, 58°C for 30 sec, and 72°C for 40 sec. In addition, one final cycle of extension was performed at 72°C for 7 min.

The PCR product of 210-bp was mixed with 2 units *ApaL* I (New England Biolabs, Beverly, USA) and the reaction buffer according to the manufacturer's instructions. The restriction site was designed to be located at the allele of 3'-UTR (T) to form a digestible site. Two fragments of 185-bp and 25-bp are present when the product is digestible. The reaction was incubated for 3 hours at 37°C. Then, 10 µl of the product was loaded into 3% agarose gel containing ethidium bromide for electrophoresis. The polymorphism was divided into three groups: digestible (TT homozygote), indigestible (CC homozygote) and C/T heterozygote.

Statistics

The carriage rate of an allele is the number of individuals carrying at least one copy of the allele relative to the total number of individuals. Allelic frequency was expressed as a percentage of the total number of alleles. Differences between genotypes and allele frequencies and deviations from the Hardy-Weinberg equilibrium were analyzed using the χ^2 test and Fisher's exact test, depending on the minimum expected values. A p value less than 0.05 was considered statistically significant. The odds ratios (OR) were calculated from the allelic frequency with a 95% confidence interval (95% CI) for the polymorphism of the urokinase gene.

Results

The frequencies of the genotypes in the RA patients and control subjects are shown in Table I. The distribution of the genotypes in patients and controls was in Hardy-Weinberg equilibrium. The genotype distributions of C/T polymorphisms at the 3'-UTR of the urokinase gene in the healthy subjects showed that 127 (94.8%) had the genotype CC and 7 (5.2%) had C/T. Among the 145 RA patients, the genotype CC was found in 123 (84.8%) and C/T in 22 (15.2%). There were no TT homozygotes in either group. Several randomly selected samples were sequenced to further confirm the accuracy of the PCR analysis (data not shown). There were significant statistical differences between the RA patients and healthy subjects (chi-square test, p = 0.007).

The data were further subdivided into C and T groups according to the allelic frequencies in each group (Table II). The distribution of genotypes between patients and controls was in Hardy-Weinberg equilibrium. The allelic distribution of C/T polymorphisms at the 3'-UTR of the urokinase gene in healthy subjects was 0.974 (C allele) and 0.026 (Tallele). The RApatients had C allele: 0.924 and T allele: 0.076. There were statistically significant differences in allelic frequencies between the RA patients and control subjects (p =

Table I. Frequency distribution of the urokinase gene 3'-UTR T/C polymorphism in patients with RAand healthy individuals.

	CC (%)	C/T (%)	Total	p-value
Control	127 (94.8)	7 (5.2)	134	0.007
RA	123 (84.8)	22 (15.2)	145	

Table II. Comparison of allelic frequency and carriage rate between rheumatoid arthritis (RA) patients and control subjects.

	Allelic frequency			Carriage rate		
	RA	Controls	P	RA	Controls	P
C	268 (92.4)	261 (97.4)	0.008*	145(86.8)	134(95.0)	0.01#
T	22 (7.6)	7 (2.6)		22(13.2)	7 (5.0)	

*χ^2 = 6.99, OR for Tallele in RA= 3.06, 95% CI = 1.65 ~ 5.66
#χ^2 = 6.04, OR for Tallele in RA= 2.90, 95% CI= 1.54 ~ 5.49

0.008); the odds ratio for the risk of the T allele in RA patients was 3.06 with a 95% CI ranging from 1.65 to 5.66. Furthermore, there were statistically significant differences in the carriage rate between the RA patients and control subjects (p=0.01), meaning that the odds ratio for the risk of the T allele in RA patients was 2.90 with a 95% CI ranging from 1.54 to 5.49.

Table III presents the relationship between 3'-UTR C/T genotype and clinical signs and findings in patients with RA. There were no statistically significant differences between the RF positive and RF negative patients (p=0.07). Similarly, we did not detect any association between the 3'-UTR C/T polymorphism and extra-articular involvement or RA bone erosion in Chinese patients (p = 0.64, and 0.30, respectively).

Discussion

Two types of plasminogen activators (PA) have been characterized into tissue type plasminogen activator (tPA) and urokinase plasminogen activator (uPA) (14, 15). Both tPA and uPA were found in cartilage and cleave plasminogen to plasmin. The uPA molecule was first purified from urine as a proenzyme of 54 kD (14). It was then converted to the active form of two chains of 30 kD and 24 kD linked by a disulfide bond. Urokinase PA activates a cascade of proteolysis, leading to extracellular matrix degradation, and thus it is involved in the cell adhesion, migration, and proteolysis underlying tumor invasion, inflammation, angiogenesis, and tissue remodeling (16, 17). Pro-uPA and two-chain uPA bind to a specific uPA receptor (uPAR), which is a single-chain glycoprotein with a glycosyl-phosphatidylinositol moiety expressed on fibroblasts, macrophages, and tumor cells.

Rheumatoid arthritis is a progressive destructive disease characterized by inflammation, abnormal immune responses, and synovial hyperplasia. Clinical and experimental evidence suggest that the PA system is involved in the pathogenesis of RA. Increased levels of uPA and uPAR were detected in synovial tissue extracts from patients with RA compared to patients with OA (7). In addition, increased uPA and decreased tPA antigenic levels measured in RA synovial fluids are associated with the clinical severity of the disease (8). The availability of animals genetically deficient in uPA offers us the means to clarify the uPA roles in patients with RA (18). Ultimately, it may help researchers design novel therapeutic strategies for these debilitating disorders.

There was a lack of genetic evidence to support the hypothesis that urokinase is associated with RA. To our knowledge, this study is the first report of the urokinase gene polymorphism in patients with RA. We were able to demonstrate that the urokinase gene 3'-UTR "T" allele was associated with Chinese patients with RA in Taiwan. Individuals possessing the "T" allele in the 3'-UTR of the urokinase gene had higher incidence of RA(3.06-fold) than those who did not. The evidence indicates that the urokinase gene 3'-UTR "T" allele may be a genetic marker for RA. However, the clinical signs and findings (RF, extra-articular involvement and bone erosion) in RA patients were not associated with urokinase gene 3'-UTR C/T polymorphism.

There is strong epidemiological evidence that genes contribute to the risk of developing many common diseases (19). Genes susceptible to rheumatic diseases are being sought through serial association studies by screening DNA polymorphisms for IL-1β, IL-1 receptor antagonist (20) and vitamin D receptor gene (9, 10) and others. Evidence has shown that multiple genes contribute to disease susceptibility and that each may confer a small increase in risk (19). The association between genes and RA will help identify additional risks, improve preventive medicine, and lead to a choice of unique treatment strategies for some subtypes of the disease.

In conclusion, we found that the "T" allele of 3'-UTR region of the urokinase gene was associated with Chinese RApatients in Taiwan.

Table III. Relationship between 3'-UTR T/C genotype and clinical signs and findings in patients with RA.

	CC		C/T		Total	p-value
	n=124	(%)	n=21	(%)	n=145	
RF (+)	79	(63.7)	9	(42.9)	88	0.07
Extra-articular involvement	60	(48.4)	9	(42.9)	69	0.64
Bone erosion	50	(40.3)	11	(52.4)	61	0.30

RF: rheumatoid factor

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