A Human Glucocorticoid Receptor Gene Variant That Increases the Stability of the Glucocorticoid Receptor ß-isoform mRNA Is Associated with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To study the occurrence and function of polymorphism in the human glucocorticoid receptor (hGR) gene in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).

Methods. We used single stranded conformation polymorphism (SSCP) and direct sequencing to study the hGR gene in 30 patients with RA, 40 with SLE, and 24 controls. A newly identified polymorphism was transfected in COS-1 cells and the stability of the mRNA containing the polymorphism was tested using real-time PCR.

Results. A polymorphism in the hGR gene in exon9ß, in an "ATTTA" motif, was found to be significantly associated with RA. Introduction of this polymorphism in the hGRb mRNA was found to significantly increase stability *in vitro* compared to the wild-type sequence.

Conclusion. Our findings show an association between RA and a previously unreported polymorphism in the hGR gene. This polymorphism increased stability of hGRß mRNA, which could contribute to an altered glucocorticoid sensitivity since the hGRß is thought to function as an inhibitor of hGRα activity. (J Rheumatol 2001;28:2383–8)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
GLUCOCORTICOID RECEPTOR
SYSTEMIC LUPUS ERYTHEMATOSUS
ATTTA MOTIF

Glucocorticoids are widely used in the management of chronic inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)[1,2]. However, in clinical practice pharmacological dosages are needed, suggesting glucocorticoid resistance of the target tissue[3,4]. In

addition, several studies have suggested that this glucocorticoid resistance could play an important role in the clinical management of RA and SLE[4-9].

Glucocorticoids exert their effect by binding to an intracellular receptor, the glucocorticoid receptor-α (GRα). The GR-ligand complex translocates to the nucleus where it interacts with glucocorticoid responsive elements (GRE) in promoter regions of target genes, altering their expression. GRα has been shown to interact with other transcription factors, such as AP-1 and NF-κB, thereby inhibiting their activity. The interaction with NF-κB appears to play a central role in the immunosuppressive action of glucocorticoids[10]. The human GR gene (hGR) consists of 10 exons, of which exons 1–9α are transcribed into hGRα mRNA, which is translated into a functional receptor, hGRα. Alternative splicing of the primary transcript results in an mRNA containing exons 1–9ß, which results in the expression of the alternate product, the hGRß[11-13]. The hGRß does not bind hormone and is transcriptionally inactive. *In vitro* studies have shown that hGRß can act as a dominant negative inhibitor of hGRα activity, by a mechanism that is largely unknown[14,15]. However, since several laboratories have not been able to reproduce this result[16,17], this inhibiting activity is still under debate[18].

Changes in expression of GR have been reported[5,8],

while in SLE the amount of hGR was found to correlate with glucocorticoid sensitivity and therapy efficacy[6]. Several polymorphisms in the hGR gene have been shown to be associated with overt glucocorticoid resistance[19-22]. However, no studies have been conducted to investigate whether any GR variant confers genetic risk for rheumatic diseases.

To determine whether hGR gene variants contribute to susceptibility to RA or SLE, we screened all exons and splice boundaries for variants in a panel of patients with RA and SLE and conducted association tests using a case-control paradigm. We report a possible association with RA and a hGR gene variant that confers stability to the transcript encoding the hGRß isoform.

MATERIALS AND METHODS

Patient and control DNA samples. Thirty RA patients and 40 SLE patients were recruited from the outpatient department of Sellyoak Hospital and Queen Elizabeth Hospital in the United Kingdom by Dr. Emery. The 40 patients with SLE consisted of 39 women and one man. Patients with RA or SLE according to American College of Rheumatology criteria for moderate to severe disease were selected randomly from those attending a routine secondary care clinic. Blood samples were drawn with informed consent and genomic DNA was isolated using peripheral blood mononuclear cells, which were separated by density gradient centrifugation, and total polymerase chain reaction grade cellular DNA was prepared by sodium dodecyl sulfate-Nonidet-Tween 20 treatment followed by proteinase K digestion, boiling, and phenol-chloroform extraction. DNA was stored in aliquots at –20°C at an approximate concentration of 20 ng/ml.

Control DNA was obtained from donor blood from a group of 24 healthy volunteers from an anonymous DNA collection of North British Caucasians with no personal or family history of chronic autoimmune, metabolic, or infectious disease. Due to the source of blood donor groups as controls there is a bias towards male sex (about two-thirds male). For ethical reasons no other identifier information was available on these subjects.

Polymerase chain reaction-single stranded conformation polymorphism (PCR-SSCP). To screen the hGR gene for variants we employed SSCP analysis followed by sequencing[23]. Primer pairs were designed to span the hGR exons and intron sequences flanking the splicing sites presented previously[12]. In general, the size of the PCR products was kept to a maximum of 250 bp. Below are the primers associated with the polymorphism in exon 9ß.

> 9ßN5: 5′ AGT GTC TTT TTA CCT ACG CA 3′
> 9ßO3: 5′ ATG TTT CTC CAT ATT TGG CA 3′

The oligomers were purchased from BioServe Biotechnologies (Gaithersburg, MD, USA). Between 30 and 40 ng of genomic DNA was used as template for each PCR. PCR I buffer from Perkin-Elmer-Cetus and a mixture of 4 dNTP in a final concentration of 200 μM each were used. PCR amplification was done in a Perkin-Elmer PE GeneAmp 9600 thermocycler using the following conditions: initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extensions at 72°C for 1 min followed by a final extension at 72°C for 10 min. An aliquot of each amplimer was used as a template in the second PCR that was performed in the same manner except for the presence of α^{32}p-dCTP (ICI Biochemicals) and a lower concentration of dCTP (0.2 μM). Sequence loading buffer was added to each PCR product and denaturation was conducted at 95°C for 5 min. After cooling on ice, aliquots of the denatured amplimers were applied to 6% nondenaturing MDE gels in 0.5 × TBE buffer. Electrophoresis was performed either in the cold room (4°C) or at room temperature. Autoradiography was done for a minimum of roughly 1 hour.

Cycle sequencing. Prior to cycle sequencing, the PCR amplicons were purified using Sephadex G-25 spin columns. Cycle sequencing reactions were done either in the presence of the α^{32}p-dATP (ICI Biochemicals) for manual electrophoresis or by using the dye terminator method for automated sequencing with either the ABI 373 or 377 sequencer. PCR conditions were as described in the previous section.

Cell culture, transfection, and actinomycin-D treatment. COS-1 cells were grown as described[24]. Subconfluent monolayers were transfected, using TransIt reagent (PanVera, USA). After 5 h incubation with the TransIt reagent/DNA mixture, cells were re-fed with supplemented Dulbecco's modified Eagle's medium.

Cells were transfected with either the hGRß expression vector pCMVhGRß or the mutated version of this vector, pCMVhGRßmut. This mutated vector was obtained by site-directed mutagenesis of pCMVhGRß, using the QuikChange kit (Stratagene, Palo Alto, CA, USA) according to the manufacturer's instructions. At position 3736 in the hGRß cDNA, an A was replaced by a G (primers used: GTGTGGAAATGTTTAACTTT-TATTTTTTCCTTTAAATTTGCTGTTCTGG and CCAGAACAGC-AAATTTAAAGGAAAAAATAAAAGTTAAACATTTCCACAC).

Twenty-four hours after the transfection, cells from each transfection were transferred and distributed over 3 smaller wells (one well for each time point to be taken). Twenty-four hours later, actinomycin-D was added to the medium (1 μg/ml). At 0, 3, and 6 h after addition of actinomycin-D, total RNA was isolated using Trizol reagent (Life Technologies Gibco BRL), according to the manufacturer's instructions. RNA was DNAse-treated (DNAse purchased from Promega) and after 2 phenol/chloroform extractions stored at –20°C.

Real-time PCR. To quantitate hGRß mRNA levels, real-time RT-PCR was performed, using the TaqMan Gold RT-PCR kit (PE Applied Biosystems). About 50 ng of total RNA was added to 49 μl of 1 × TaqMan buffer A, 10 mM MgCl$_2$, 300 μM dATP, dCTP, dGTP, 600 μM dUTP, 10 μM GRß forward primer GCT-GGA-TAA-TTA-GCA-TGG-GAT-G, 10 μM GRß reverse primer AAT-TGC-TCC-CTG-CCT-CTG-A, 5 μM GRß probe ATG-AAG-GAA-AGC-CAC-GCT-CCC-T, 0.05 U/μl AmpliTaq Gold DNA polymerase, 0.25 U/μl MultiScribe Reverse Transcriptase, and 0.4 U/μl RNase inhibitor. Reactions were run on an ABI Prism 7700 sequence detection system (PE Applied Biosystems). Reverse transcription was performed for 30 min at 48°C, then the sample was incubated 10 min at 95°C and 40 cycles of PCR were performed (15 s at 95°C and 1 min at 60°C). As a standard, the ribosomal RNA (rRNA) content was measured for each sample in a separate reaction, using the rRNA TaqMan PCR kit according to the manufacturer's instructions. For both hGRß mRNA and rRNA, relative standard curves were obtained by making serial dilutions of a control sample. Using these curves, the relative amount of hGRß mRNA was measured in each sample, and divided by the relative amount of rRNA measured in that sample. Subsequently, levels were normalized to the t = 0 level. Experiments were performed in duplicate. Data shown are means of 3 separate experiments (n = 3).

Statistical analysis. Statistical significance of the occurrence of a certain polymorphism was tested using chi-square statistics provided by the SPSS+ statistical package. Compared were: RA versus controls, SLE versus controls, and RA versus SLE patients. The Hardy-Weinberg equilibrium was also tested with chi-square statistics.

hGRß mRNA levels at 3 and 6 h after actinomycin-D addition were analyzed by analysis of variance (ANOVA). Post-hoc comparisons were done by Tukey's t test. Statistical significance was accepted at p < 0.05.

RESULTS

Variants in hGR exon 9ß and association studies with RA and SLE. A previously unreported polymorphism in the 3′ untranslated region (UTR) located at the end of exon 9ß was detected at position 3669. Figure 1 shows the SSCP pattern

for unrelated patients with either RA or SLE, and Table 1 indicates the allele frequencies of this polymorphism.

As indicated there are 4 possible bands (1–4, Figure 1). For example, SLE Patient 22 (Figure 1B), showed 3 bands (1–3). Some SLE patients, for example Patient 9, displayed band 4, the fastest migrating band. Samples 8 and 26 did not display band 2. Sequencing of the PCR amplimers of SLE Patients 8, 9, 22, and 30 indicated a nucleotide transition "A" to "G" in the "ATTTA" sequence, giving rise to a "GTTTA" sequence in Patients 8, 9, and 30 (Figure 1C). SLE Patient 22 displayed the wild-type "A" and SLE Patient 8 was homozygous for the "G" allele, while Patients 9 and 30 were heterozygotes (note the faint band 2 in Figure 1B). We found 12 out of 40 patients with SLE were carriers of the polymorphism (Patients 2, 3, 8, 9, 13, 15, 17, 26, 30, 32, 37, and 39). Moreover, 2 SLE patients (8 and 26) were homozygotes.

We scored the genotypes of the RA patients based on knowledge of the sequences that correspond to specific SSCP banding patterns (Figure 1A). For example, RA Patient 15 displayed a banding pattern that was identical to SLE Patient 22; therefore it should have the wild-type sequence. RA Patient 27 had the identical banding pattern as SLE Patient 9, i.e., heterozygote. RA Patient 30 gave the same banding pattern as SLE Patients 8 and 26. We concluded that 10 RA patients (3, 4, 5, 8, 9, 12, 19, 20, 27, 29) were heterozygotes. Only one RA patient (30), missing the second band (Figure 1B), was designated homozygote for this polymorphism. When tested separately, controls, and patients with SLE and RA are, for this mutation, in Hardy-Weinberg equilibrium.

Direct sequencing of DNA samples from 24 healthy control individuals, using the PE-ABI377 system, revealed that only 3 of these 24 controls were heterozygote carriers of the polymorphism (data not shown).

The allele frequencies in controls and RA and SLE patients are shown in Table 1. Subsequent chi-square statistics indicate significant differences in the frequency of occurrence of this polymorphism in RA patients and controls. Thus, the Pearson chi-square of 4.2, p < 0.035, between RA patients versus controls was significant, while between SLE patients and controls the Pearson chi-square of 3.3, p = 0.057, was not significant. There was no significant difference in allele frequencies between patients with RA and SLE.

Effect of polymorphism on hGRβ mRNA stability. To determine whether the nucleotide substitution exerted an effect on the stability of the hGRß transcript, a mutated version of an hGRß expression vector was prepared by site-directed mutagenesis. The mutated version and the wild-type hGRß expression plasmids were transfected into COS-1 cells. Actinomycin-D was added and 0, 3, and 6 h later, cells were harvested and total RNA was isolated. Quantitation of mRNA levels was by real-time PCR (Figure 2). ANOVA revealed a stabilizing effect of the mutation at 3 and 6 h [$F(1,1) = 5.32$, p < 0.05] on the mRNA, as revealed by the increased half-life. Post-hoc comparison showed a significant difference between the wild-type hGRß and the mutant at 3 h (p < 0.05).

DISCUSSION

Based on the importance of glucocorticoid therapy and the frequent occurrence of glucocorticoid resistance in rheumatoid diseases, we investigated the association between polymorphisms in the hGR gene and RA and SLE. We found an association between a previously unreported A to G transition in exon9ß of the hGR gene in patients with RA. The transition was observed in an "ATTTA motif" that was located in a region encoding the 3′ UTR of hGRß mRNA, and was found to confer stability to the mRNA.

The pathogenesis of both RA and SLE is not well understood, but a pronounced difference in concordance rates between monozygotic and dizygotic twins, as well as linkage and segregation animal studies, provides strong evidence for the role of genetic factors[25,26]. Among the many loci identified to have a possible association with SLE or RA[25], one report described a susceptibility locus for RA in the 5q32-33 region[27], which is in close proximity to the hGR gene thought to be located at 5q31[28]. The association of the A to G transition in exon 9ß of the hGR gene with RA would be consistent with the presence of a susceptibility locus at or near the hGR gene region. Our results showing no significant association of the A to G transition in SLE are also consistent with the lack of linkage in SLE with the hGR gene[29].

In addition to the polymorphism in exon 9ß, a silent mutation [AAT (Asn) to AAC (Asn)] in the coding region of exon 9α was found to occur in about 25% of the seemingly healthy population[30]. We detected this polymorphism at a similar frequency in RA and SLE patients and in controls (range 27.5–36%, data not shown), and no statistically significant differences between groups were found. Thus

Table 1. Frequencies of polymorphism in the "ATTTA motif" at position 3736 in exon 9β. The table shows the gene type and allele frequencies of the hGRβ polymorphism in SLE and RA patients and controls. Two gene types exist, the wild-type A and the G polymorphism at the first position in the ATTTA sequence. Accordingly, wild-types (AA), heterozygotes (AG), and homozygotes (GG) were identified using SSCP and sequencing, (Figure 1). In addition, allele frequencies were calculated and, using chi-square testing, we observed a significant difference in allele frequencies between RA patients and controls (p = 0.035), but not between SLE patients and controls (p = 0.057) or between RA and SLE patients.

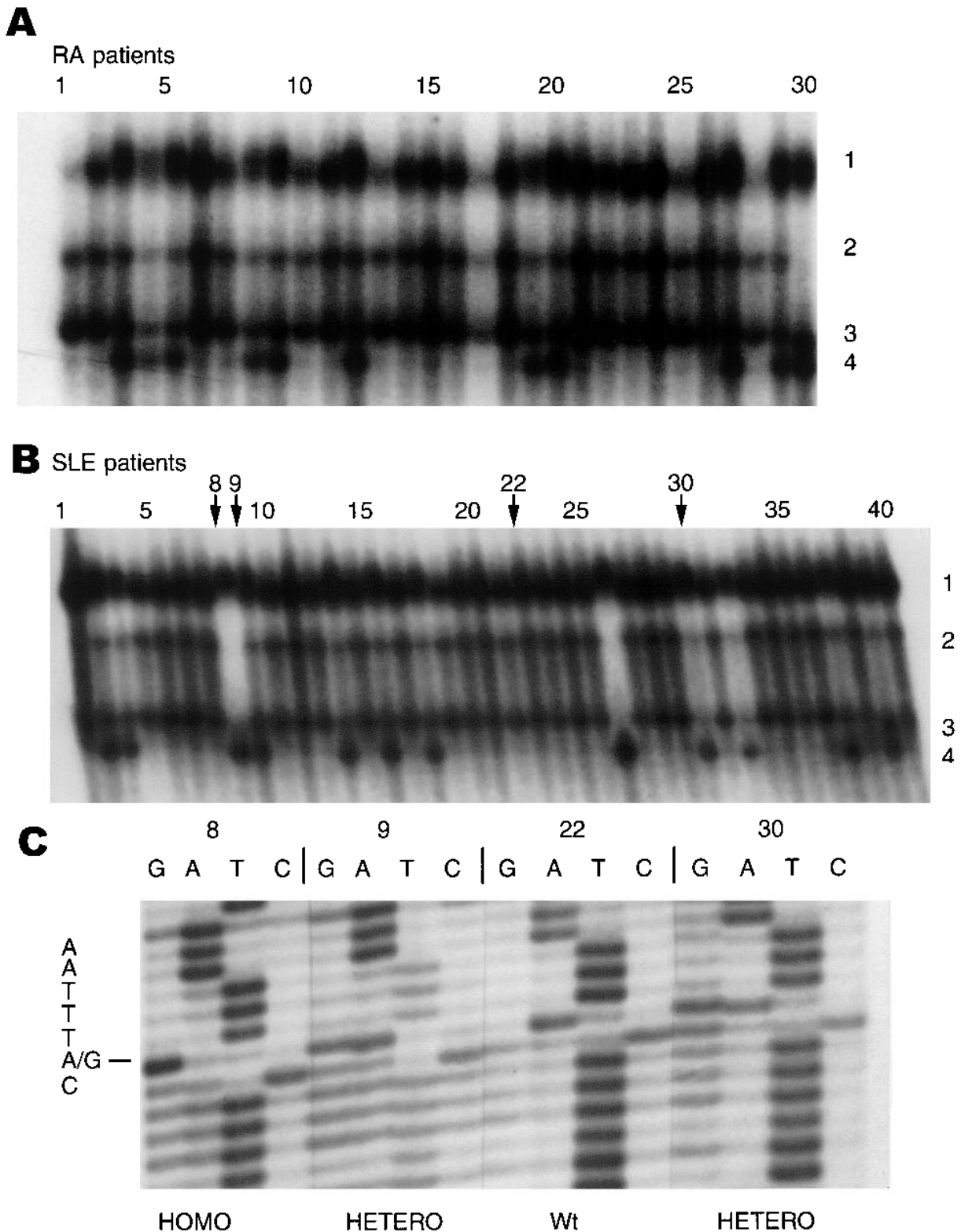| Patients	Gene Type			Allele Frequencies		
	AA	AG	GG	A	G	p
SLE (n = 40)	28	10	2	0.82	0.18	0.057
RA (n = 30)	19	10	1	0.8	0.2	0.035
Control (n = 24)	21	3	0	0.94	0.06	

Figure 1. Polymorphism in the "ATTTA motif" in exon 9ß revealed by SSCP gels and sequencing. PCR products of 30 patients with RA and 40 with SLE were run on SSCP gels, which revealed a shift in bands in 11 RA patients (panel A) and 12 SLE patients (panel B). Four SLE patients (Patients 8, 9, 22, 30) were further analyzed by direct sequencing (panel C). Direct sequencing showed that SLE Patient 8 is homozygous for the polymorphism and showed a transition from "ATTTA" to "GTTTA," as indicated. SLE Patients 9 and 30 have both bands (Figure 1C), an "A" and "G" at this position and are therefore characterized as heterozygotes. In contrast, SLE Patient 22 shows the wild-type sequence. Based on these data, we characterized 12 patients with SLE as carriers of the polymorphism, while Patients 8 and 26 are probably homozygotes. Since the SSCP pattern of patients with RA was almost completely identical to the SLE SSCP gel, we designated 11 of the 30 patients with RA (Patients 3, 4, 5, 8, 9, 12, 19, 20, 27, 29, 30) as carriers of the GTTTA polymorphism. Only RA Patient 30 seems to be a homozygote (band 2 is missing).
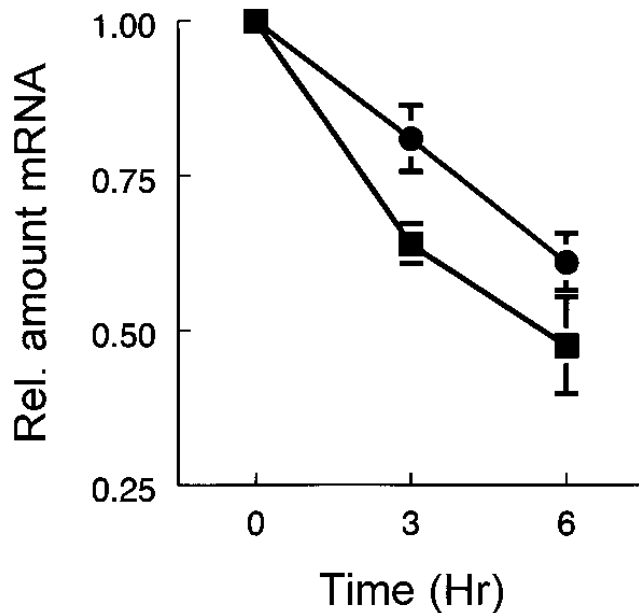
Figure 2. GRß mRNA levels after actinomycin-D treatment. COS-1 cells were transfected with the expression plasmid pCMVGRß (■), or vector pCMßmut, which contains a mutated "ATTTA motif" (to "GTTTA") in the 3′ UTR (●). At 3 and 6 h after actinomycin-D treatment, the cells tranfected with pCMVGRß show a significantly (p < 0.05) larger decrease in GRß mRNA levels than the cells transfected with the mutated vector. Data are expressed as mean (n = 3) ± SEM.

while this second polymorphism seems to occur at high frequency in several different populations, the transition polymorphism appears to be more selectively expressed in patients with RA. Confirmation of this finding in larger samples would be warranted.

AUUUA pentamers are often found in A/U-rich elements (ARE) in the 3′ UTR of mRNA that code for protooncogenes, nuclear transcription factors, and cytokines and can function as regulators of mRNA stability. In most cases, its presence destabilizes mRNA[31,32], but under certain conditions stabilization has been observed[33]. Most likely it represents a binding site for specific proteins. A mutation of an AUUUA sequence has been shown to increase the half-life of c-fos[34] and interleukin 3[35] mRNA. The 3′ UTR of hGRß mRNA contains 2 tandems, at positions 2561 and 2597 and at 3669 and 3736[11], which are both located in an ARE. The ARE does not contain overlapping UUAUUUAUU nonamers, which means they are classified as class I AUUUA containing ARE[32], like the ARE that are found in the 3′ UTR of c-fos, c-jun, and c-myc mRNA[36]. Mutation of the pentamer at position 3669 (the mutation associated with RA, this study) resulted in an increased stability of the hGRß mRNA *in vitro*. Therefore, we conclude that turnover of hGRß mRNA is dependent on the presence of this AUUUA motif in the 3′ UTR, and that the described transition results in an increased stability of this messenger *in vivo*.

While the functional implications of this polymorphism

in vivo in RA are not known and require further study, previous functional investigations have indicated abnormalities in hypothalamic-pituitary-adrenal activity in which alterations in glucocorticoid sensitivity or feedback could potentially play a role[37-39].

In asthma, another chronic inflammatory disease, glucocorticoid resistance was found to be associated with elevated expression of the hGRß[40,41]. In addition, recently it has been shown that glucocorticoid treatment of asthma patients decreased expression of hGRα but not hGRß in skin biopsies, as revealed by immunocytochemistry, thereby decreasing the GRα/GRß ratio[40]. It has been demonstrated *in vitro* that such a decrease in the GRα/GRß ratio results in decreased hGRα induced transactivation on a GRE driven promoter[13,14]. Therefore it has been suggested that a shift in the GRα/GRß ratio could be responsible for the often observed glucocorticoid resistance that develops in asthma after prolonged treatment[40]. The data we report here, showing a mutation in RA patients of an "AUUUA motif" that *in vitro* is associated with enhanced stability of the hGRß, could result in increased expression of hGRß and consequently in glucocorticoid resistance in RA patients carrying this polymorphism.

In summary, we describe for the first time a possible association in RA patients with a previously unknown polymorphism in the hGR gene. This "ATTTA" to "GTTTA" transition was found to result in increased stability of hGRß mRNA when tested *in vitro*. We suggest that this polymorphism could contribute to changes in glucocorticoid resistance by means of attenuation of hGR efficacy. It remains to be shown if this polymorphism can be viewed as a susceptibility factor in the pathogenesis of RA.

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