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ORIGINAL ARTICLE

Genetic association of miRNA-146a with systemic lupus erythematosus in Europeans through decreased expression of the gene

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A recent genome-wide association study revealed a variant (rs2431697) in an intergenic region, between the pituitary tumor-transforming 1 (*PTTG1*) and microRNA (*miR-146a*) genes, associated with systemic lupus erythematosus (SLE) susceptibility. Here, we analyzed with a case–control design this variant and other candidate polymorphisms in this region together with expression analysis in order to clarify to which gene this association is related. The single-nucleotide polymorphisms (SNPs) rs2431697, rs2910164 and rs2277920 were genotyped by TaqMan assays in 1324 SLE patients and 1453 healthy controls of European ancestry. Genetic association was statistically analyzed using Unphased. Gene expression of *PTTG1*, the miRNAs *miR-3142* and primary and mature forms of *miR-146a* in peripheral blood mononuclear cells (PBMCs) were assessed by quantitative real-time PCR. Of the three variants analyzed, only rs2431697 was genetically associated with SLE in Europeans. Gene expression analysis revealed that this SNP was not associated with *PTTG1* expression levels, but with the microRNA-146a, where the risk allele correlates with lower expression of the miRNA. We replicated the genetic association of rs2341697 with SLE in a case–control study in Europeans and demonstrated that the risk allele of this SNP correlates with a downregulation of the miRNA 146a, potentially important in SLE etiology.

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INTRODUCTION

The genome-wide association studies (GWAS) that have been carried out in the last years have revealed a great number of genetic variants predisposing to different complex diseases, including systemic lupus erythematosus (SLE). SLE is a complex autoimmune disease characterized by the production of autoantibodies, formation of deleterious immune complexes and systemic inflammation in multiple organs. Aside from dysregulation of adaptive immunity, many studies have shown the association of innate immune responses with SLE pathogenesis, such as the involvement of the complement system, activation of Toll-like and Fcγ receptor-mediated signaling.[1] In addition, the importance of the type I interferon (IFN) system for disease development was shown.[2–4] Many SLE patients have elevated serum levels of IFN-α, a key regulator of innate immunity, and increased expression of IFN-α-inducible genes in the white blood cells, termed the 'interferon signature', which could be correlated with both disease activity and severity, and has also been observed in several other autoimmune diseases.[2–4]

In the majority of genetic studies, the associations target regions that are far away from known genes and the biological importance of such variants is rather unknown. In the case of SLE, a recent GWAS revealed an association signal for a single-nucleotide polymorphism (SNP) rs2431697 located in an intergenic region, between the pituitary tumor-transforming 1 (*PTTG1*) and the microRNA-146a (*miR-146a*) genes.[5] *PTTG1* gene codes for a protein called securin, primarily important in the regulation of sister chromatid separation during cell division, and that has been consistently related to several types of cancer.[6] miR-146a, on the other hand, is a microRNA (miRNA) recently described as an important player in the regulation of innate and adaptive immune system as well as in regulating tumor progression.[7]

miRNAs represent a recently discovered class of small, non-coding RNAs, present in virtually all species. miRNAs have revealed a new mechanism of gene expression regulation, playing a crucial role in 'fine-tuning' negative regulation in many physiological and pathophysiological processes.[8–11] A growing number of miRNAs like miR-125b, miR-150, miR-155, miR-181a/b, miR-223, miR-101,

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miR-16 and miR-146a have been associated with regulation of diverse immune functions including T-cell selection, B-cell maturation and selection, and development of regulatory T cells (Tregs), suggesting that the miRNA regulatory mechanism may be central in immunological tolerance (reviewed in Pauley *et al.*[11] and Tsitsiou and Lindsay[12]).

miR-146a was first described in 2006 by Taganov *et al.*[13] in human acute monocytic leukemia cell line, showing also its upregulation in response to stimulation with lipopolysaccharides and its role as a negative feedback regulator of the Toll-like receptor signaling by targeting *TRAF6* (tumor necrosis factor receptor-associated factor 6) and *IRAK-1* (interleukin-1 receptor-associated kinase 1), suggesting for the first time its important role as a negative regulator of inflammation.

To date, neither the functional role of the SNP rs2431697 nor the gene to which this association could be related to has been investigated. In this study, we replicated a strong genetic association of this variant with susceptibility to SLE in Europeans and demonstrated a correlation of the risk allele of this SNP with lower expression of the *miR-146a*, relevant in SLE pathology.

RESULTS

Association of the intergenic variant rs2431697 with SLE

We investigated the genetic association of three candidate SNPs in the *PTTG1-miR146a* region (rs2431697, rs2277920 and rs2910164) in an European multicenter collection from seven European countries. Genotyping call rates were $>95\%$, and there was no evidence of deviation from Hardy–Weinberg Equilibrium of any SNP ($P>0.6$). By likelihood-based association analysis (Unphased), there was no association of rs2910164 or rs2277920 (proxy for rs57095329) with SLE (Table 1). To note, the frequency of the risk allele of rs2277920/rs57095329 found associated with SLE in Chinese (frequency of 0.16–0.29) was very low in our European cohort (~ 0.027), showing that this risk variant is barely present in Europeans.[14]

rs2910164 is an interesting SNP since it has been shown to affect the efficiency of the premature miR-146a processing, which results in differential expression levels of the mature miRNA.[15] However, no association was found for this SNP with susceptibility to SLE in our European cohort, which is in concordance to the recent study showing no association of this variant in Han Chinese either.[14]

Our meta-analysis replicated, on the other hand, a robust association of rs2431697 with SLE ($P=0.00028$, odds ratio $=1.23$ (1.10–1.38)) with the homozygosity for the risk allele having an odds ratio of 1.49.

The linkage disequilibrium (LD) between those three polymorphisms was proven to be extremely low (Figures 1 and 2); and thus, these variants can be considered as completely independent markers in this region. Also, considering the whole *PTTG1-miR146a* region in HapMap, rs2431697 was found to be rather independent of any major LD block in this region (Figure 1).

Association of rs2431697 with miR146a expression

After confirming the genetic association of rs2431697 with SLE susceptibility in our cohort, we aimed to analyze if this SNP would have an effect on expression levels on either *PTTG1* or *miR146a* genes. Since this polymorphism is located approximately in the middle between the two genes (24.23 kb downstream of the *PTTG1* gene and 15.3 kb upstream of *miR-146a* exon 1), it was unclear to which gene the association of this SNP was related to. We first attempted to investigate *PTTG1* expression levels in peripheral blood mononuclear cell (PBMC) samples with different genotypes for rs2431697. *PTTG1* expression proved to be very low in these cells; however, no difference was seen between the genotypes (Figure 3a). We next analyzed if the SNP was associated with the expression levels of the mature form of *miR-146a*, and we found a decrease of ~ 1.6-fold in the individuals' homozygotes for the risk allele ($P=0.008$; Figure 3b). The expression levels of mat-*miR-146a* are known to be dependent on the SNP rs2910164, which we could corroborate (Figure 3c); however, since both variants are not in LD with each other, this effect is rather independent of rs2910164. To validate that rs2431697 exerts an independent of rs2910164 effect, we analyzed the expression of the full-length primary *miR-146a* transcript consisting of two exons. We found that there was indeed a correlation of rs2431697 with a downregulation of primary *miR-146a* transcript of ~ 2.2-fold ($P=0.009$) in 'risk' homozygotes (Figures 3d and e).

Table 1. Genetic association of the variants in *PTTG1*-miRNA-146a region

Allelic association			*Risk allele counts and frequencies*			
	Case	Control	Ca-Freq	Co-Freq	*P*-value	OR (95% CI)
rs2431697	1370	1586	0.620	0.569	0.00028	1.23 (1.10–1.38)
rs2277920	66	72	0.029	0.026	0.360	1.17 (0.84–1.64)
rs2910164	550	687	0.248	0.240	0.541	1.04 (0.92–1.18)
Genotypic association						
rs2431697	$P=0.0006$					
C/C	157	240	0.142	0.172	0.040	1
C/T	524	718	0.475	0.516	0.041	1.12 (0.89–1.41)
T/T	423	434	0.383	0.312	0.00019	1.49 (1.17–1.90)
rs2277920	$P=0.166$					
A/A	1044	1338	0.942	0.949	0.461	1
G/A	62	72	0.056	0.051	0.587	1.10 (0.78–1.57)
G/G	2	0	0.002	0	0.111	8.2×10^{9}[a]
rs2910164	$P=0.553$					
G/G	623	819	0.562	0.574	0.553	1
C/G	422	531	0.381	0.372	0.655	1.05 (0.89–1.23)
C/C	64	78	0.058	0.055	0.737	1.08 (0.76–1.53)

Abbreviations: CI, confidence interval; miRNA, microRNA; OR, odds ratio; *PTTG1*, pituitary tumor-transforming 1.
Risk alleles: rs2431697-T, rs2277920-G and rs2910164-C.
[a]Since the frequency of this genotype is very rare and present only in the case group the OR for this genotype was disproportionally high.

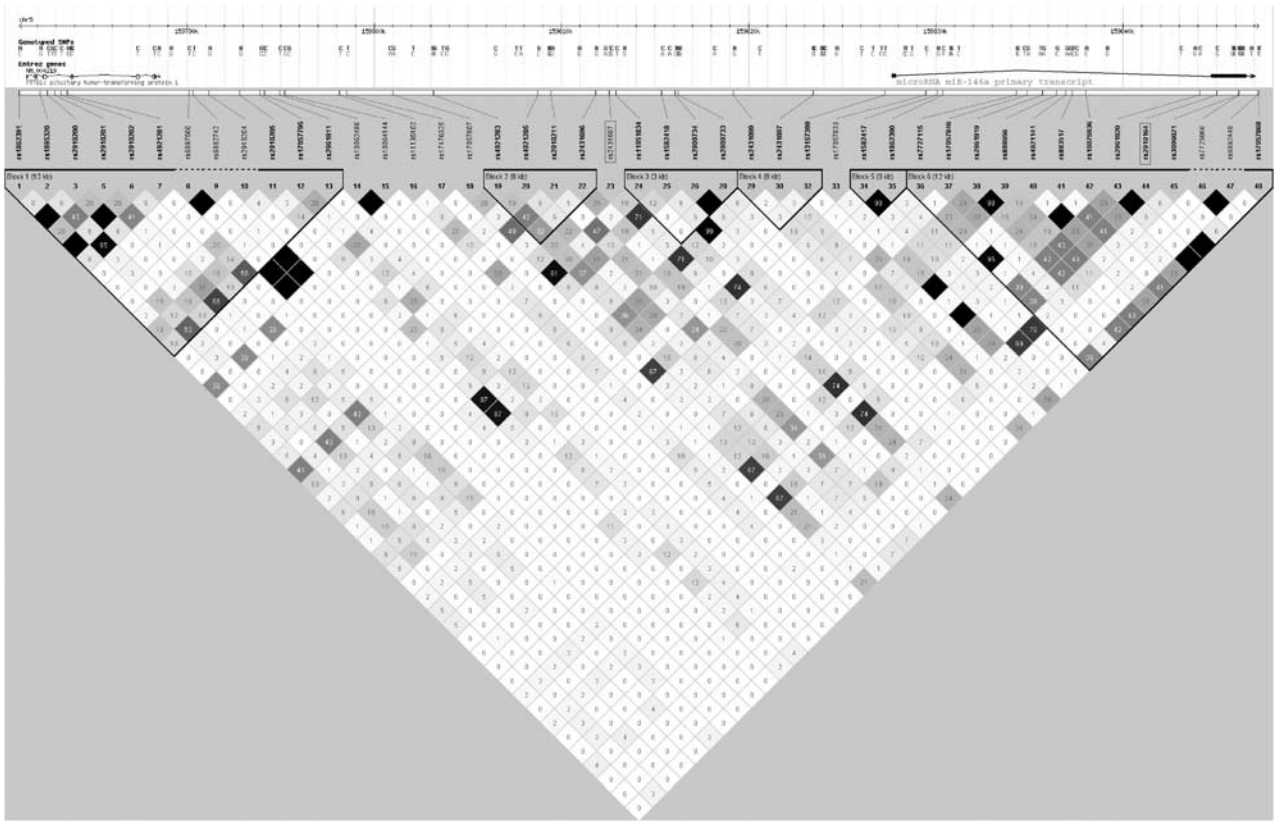

Figure 1. LD structure of the SNPs in the *PTTG1-miR-146a* region generated from HapMap (release 28) with Haploview version 4.2. LD blocks (r^2) were defined using the solid spine method.

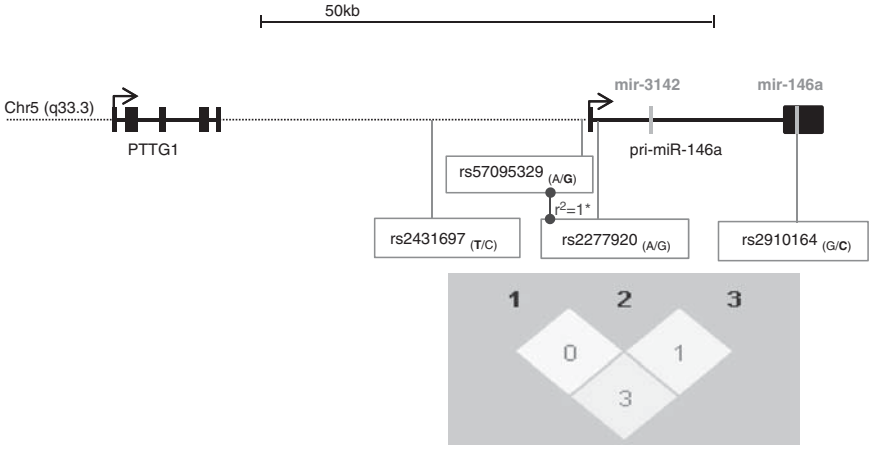

Figure 2. Schematic representation of the *PTTG1-miR146a* genomic region and the position of the SNPs analyzed. Exons are shown as black bars and dotted line represents the intergenic region. The location of the two mature miRNA annotated in this region, *miR-3142* and miR-*146a*, is indicated in the pri-*miR-146a* transcript. The positions of the four SNPs included in this study are indicated, together with the LD plot showing the linkage (r^2) between them (generated with Haploview from our genotyping data, except for the LD between rs2277920 and rs57095329 (*) that was extracted from 1000 genomes pilots for Utah residents with ancestry from northern and western Europe (CEU) and Han Chinese in Beijing, China (CHB) + Japanese in Tokyo, Japan (JPT).

We further analyzed the expression of the *miR-146a* transcripts in lymphoblastoid cell lines (LCLs) with different genotypes. In these cells, the expression of *PTTG1* and mature *miR-146a* was not significantly different between the rs2431697 genotypes ($P = 0.663$ and $P = 0.057$, respectively), although a trend of miR-146a toward decreased levels was seen in the cells homozygous for the risk allele (Figure 3f). However, when investigating the primary transcript we could detect a significant difference in expression between genotypes ($P = 0.047$; Figure 3g).

In the latest version of the human genome assembly (hg19) available, a new microRNA (*miR-3142*) was annotated in the intronic region of the *miR-146a* gene (Figure 2). This miRNA was identified by deep sequencing in a pigment cell and melanoma library[16] and was included in the miRBase (www.mirbase.org, v.17,

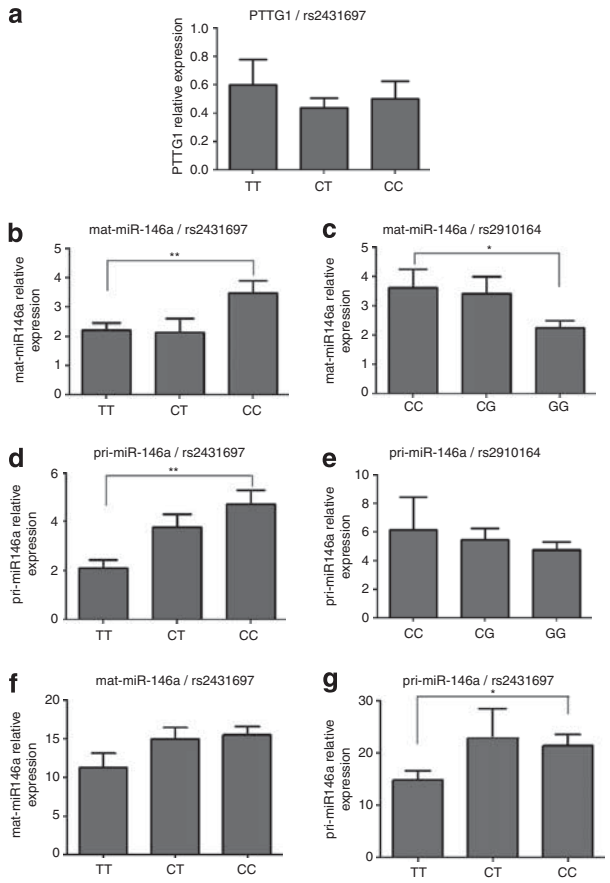

Figure 3. *PTTG1* and *miR-146a* gene expression analysis in PBMCs (**a**–**e**) and LCLs (**f**, **g**) from healthy donors, stratified by genotypes. No association was seen with *PTTG1* expression (**a**) neither for rs2910164 with the primary transcript of *miR-146a* (**e**). As for corroboration of previous findings, rs2910164 correlated with *miR-146a* expression levels (**c**). Both mature and primary forms of miR146a were significantly downregulated in individuals' homozygotes for the risk allele of rs2431697 (**b**, **d**). *miR-146a* expression analysis in LCLs also showed a trend of downregulation of mat-*miR146a* (**f**) and statistically lower levels of pri-*miR-146a* (**g**) in cells from individuals homozygotes for the rs2431697 risk allele. Values correspond to means ± s.e.m. error bars. Expression levels were normalized to 18S rRNA.

April 2011). Since we observed significantly altered levels of *miR-146a* primary transcript, we also wondered if it could correlate with *miR-3142* levels as well. Thus, we used a TaqMan assay for the mature form of this miRNA to test the expression in both PBMCs and LCLs. However, we could not detect any expression of *miR-3142* in our samples, although the control snRNA (*RNU66*) was expressed at high levels (data not shown).

DISCUSSION

To date, the importance of miRNAs in regulation of many immune processes has been widely shown and includes examples of regulation of granulopoiesis, T- and B-cell development and maturation, antigen presentation, Toll-like receptor signaling and cytokine production, immunoglobulin class-switch recombination in B cells, and T-cell receptor signaling.[17] The crucial role of miRNAs in regulating in a 'fine-tuning' manner the immune response can be enlighten by the need of the immune system to avoid uncontrolled and overreacted immune responses, which potentially leads to substantial self-damage. In fact, the importance of this regulation is demonstrated by the severe impairment in the development and/or function of immune cells when miRNA expression is experimentally altered or by the fact that they can be correlated with many different immune-related diseases.[11,18] Thus, the ablation of miR-146a, the subject of the present study, in Treg cells in a knockout mouse model led to the development of autoimmune disease with severe break-down of immunological tolerance, leading to fatal IFN-γ-dependent immune-mediated lesions in many different organs.[7]

Changes in *miR-146a* expression have been observed in a number of human diseases, such as inflammatory and autoimmune diseases, viral infections, sepsis, multiorgan failure, and cancer (reviewed in Li *et al*.[19]). In the case of autoimmune diseases, numerous studies have shown that *miR-146a* is upregulated in synovial tissue and PBMCs of rheumatoid arthritis (RA) patients[18,20,21] as well as in skin lesions of psoriasis patients.[22] On the other hand, *miR-146a* expression levels were significantly decreased in PBMCs from patients with type 2 diabetes.[23] The controversial observations on the expression levels of *miR-146a* in various autoimmune diseases may be attributed to the differences in the pathogenic pathways important for different diseases as well as the stage at which the samples have been taken during disease development.

Tang *et al*.[24] showed a threefold downregulation of miR-146a, which negatively correlated with disease activity and IFN scores, in SLE patients. The authors also reported that beside TRAF6 and IRAK1, miR-146 also regulates the levels of the signal transducer and activator of transcription 1, and IFN regulatory factor 5, all of which are key players in the type I IFN pathway. The overexpression of *miR-146a* in normal PBMC greatly reduced the induction of type I IFN, while on the other hand the inhibition of endogenous *miR-146a* increased its production in response to activation of Toll-like receptor-7. Thus, decreased expression of *miR-146a* in PBMC may contribute to the enhanced type I IFN production observed in SLE.

The analysis of serum miR-146a also showed the reduced levels in the serum supernatants from SLE patients, and that the levels could be correlated with renal function, proteinuria and disease activity in patients.[25]

Polymorphisms affecting miRNA expression, maturation or mRNA recognition may represent an important risk determinant in disease susceptibility. In *miR-146a*, a variant was described in the passenger strand of the pre-miRNA (rs2910164) that was associated with susceptibility to papillary thyroid cancer and shown to affect *miR-146a* processing. This led to reduced levels of pre-miR-146a as well as mat-miR-146a and eventually to an increased expression of the target genes, including *TRAF6* and *IRAK1*.[15] This SNP has been further controversially associated with some forms of cancer, such as cervical cancer,[26] but failed to correlate with others[27–29] as well as atrial fibrillation.[26] Interestingly, it was not associated with psoriatic arthritis,[30] neither with RA nor with SLE in Asians,[31,32] but to our knowledge this is the first study to investigate the role of this variant in SLE susceptibility in Europeans. As in other related diseases, this variant, despite its interesting functional consequences, is apparently not relevant to SLE susceptibility.

A very recent study also described a variant, rs57095329, in the promoter region of *miR-146a* primary transcript associated with SLE in Chinese and with downregulation of *miR-146a* expression through reduced binding affinity of the transcription factor Ets-1.[14] According to HapMap data, the frequency of the risk allele identified in this study is very low in the (Utah residents with ancestry from northern and western Europe (CEU)) population (0.017), while in Asian and Africans this variant is considerably more frequent (0.20–0.24). In our study, we included an SNP rs2277920 located 257 bp downstream of exon 1 donor splice site of the *miR-146a* primary transcript that could be of significance to splicing of the full-length transcript. This variant is in complete LD with rs57095329 found associated with SLE in Chinese and is

therefore a proxy of this polymorphism. We determined that rs2277920 is not significant in conferring susceptibility to SLE in Europeans and by that also highlight the ethnical difference in the genetic background of SLE susceptibility and the importance of genetic studies in distinct populations.

The upstream SNP rs2431697, on the other hand, was first identified as a susceptibility variant for SLE in a GWAS in Europeans[5] and has later also been genetically associated with psoriasis in a Chinese GWAS,[33] but not with RA in an European case–control study.[34] Considering the opposite expression levels of *miR-146a* found in SLE and RA patients,[19,24] it seems coherent the fact that this SNP may not be genetically associated with RA. The same study that identified the functional SNP rs57095329 also detected a strong genetic association of rs2431697 as an independent associated locus,[14] suggesting that this variant is most likely a common risk variant among distinct populations.

The molecular mechanism of how the T allele of rs2431697 leads to the downregulation of *miR-146a* requires a more detailed analysis. A bioinformatic examination of the region with rs2431697 was carried out in an attempt to elucidate its potential regulatory role on *miR-146a* expression. The SNP is located in a region with high regulatory potential–it overlaps with the DNase I hypersensitivity cluster and H3K4me3 trimethylation marks as well as with ChIP-seq signals for Pol II and transcription factors NF-κB and PAX5 (Encode transcription factor ChIP-seq and Encode digital DNase I hypersensitivity clusters databases, available at UCSC genome browser http://www.genome.ucsc.edu/cgi-bin/hgGateway). Thus, either this variant itself or some others in LD with it may have a role in the regulation of *miR-146a* transcription. Further analysis by targeted resequencing or fine mapping of this region in European SLE patients and controls may possibly reveal the genetic architecture of the locus and help identify causative variant. It is important to note, that contrary to the results presented here, in a study by Luo *et al.*[14] this variant was not found associated with expression of *mir146a*, but rather its expression was dependent on the alleles of rs57095329. Given the extremely low frequency of the minor allele of the latter SNP in Europeans and lack of LD between rs2431697 and rs57095329, we find it difficult to explain the differential expression of *mir146a* in Europeans by rs57095329 only. It might be that the associated region shared between the Europeans and Asians and represented by the SNP rs2431697 has a bigger role in *miR-146a* transcriptional regulation; and further the gene expression is modulated by ethnic-specific variants. As we showed here, the regulation takes place at the level of transcription rather than maturation of miRNA, since it affects the levels of the full-length primary RNA transcript.

It will be of great interest to further investigate the effect of this variant on expression of miR-146a in specific cell populations of leukocytes. Cell type-specific regulatory mechanisms may not only affect the expression of the miRNA but also lead to the more dramatic changes in the pattern of genes regulated by miR-146a.

We also do not exclude the possibility that expression of the recently discovered miRNA *miR-3142* located in intron 1 of the *miR-146a* gene could be dependent on the SNP rs2431697 as well. Although, according to our data it is not expressed in peripheral mononuclear blood cells, it still may be expressed in some other tissues.

In summary, we found that rs2431697 is associated with SLE in Europeans and that the T allele of this SNP correlates with lower expression of the *miR-146a* gene which could lead to enhanced function of the target genes involved in type I IFN pathway important for SLE.

MATERIALS AND METHODS

Study subjects

The study group comprised a total of 1324 SLE patients and 1453 ethnicity-matched healthy controls, including 62 patients and 97 controls from Argentina, 63 patients and 70 controls from Belgium, 391 patients and 190 controls from Germany, 75 patients and 57 controls from Hungary, 357 patients and 382 controls from Italy, 175 patients and 176 controls from Portugal and 201 patients and 481 controls from Sweden. All SLE patients fulfilled the American College of Rheumatology classification criteria for SLE and all individuals were tested for European ethnicity by principal component analysis.

SNP selection and genotyping

Three SNPs in the *PTTG1-miR146a* region, rs2431697, rs2277920 and rs2910164 were chosen to be genotyped in a large case–control European cohort. The SNP rs2431697 is situated in the intergenic region between the *PTTG1* and *miR146a* genes and has previously been associated with SLE in a GWAS study in Europeans.[5] The second intronic SNP rs2277920 is located 257 bp downstream of exon 1 donor splice site of the *miR-146a* primary transcript and was chosen for genotyping because of its location in the region with the potential to affect splicing of exon 1. Given that the SNP rs2277920 is a proxy ($r^2 = 1$ in 1000 genomes pilots for Utah residents with ancestry from northern and western Europe (CEU) and Han Chinese in Beijing, China (CHB) + Japanese in Tokyo, Japan (JPT)) of rs57095329, the SNP recently found associated with SLE in Chinese,[14] the results obtained for rs2277920 in our European cohort could be attributed to rs57095329. The third SNP rs2910164 is located in the *miR-146a* passenger strand and has been shown to influence the expression levels of the mature *miR-146a*,[15] but its association with SLE or any other autoimmune diseases in Europeans had not been investigated yet. All SNPs were genotyped by TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA, USA). All genotyping was performed at Uppsala University using ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems).

Statistical analysis of genetic association

Allelic and genotypic association of the polymorphisms with SLE was evaluated using Unphased version 3.1.5 (University of Cambridge, Cambridge, UK), including the country of origin as a stratification variable. Pairwise LD analysis of the *PTTG1-miR-146a* region and Hardy–Weinberg equilibrium tests were performed with Haploview version 4.2 (Broad Institute of Harvard and MIT, Cambridge, MA, USA).

GraphPad Prism software (www.graphpad.com) was used for statistical analysis and calculation of mean ± s.e.m. values of the gene expression data. Comparisons were performed using two-tailed unpaired *t*-test.

Gene expression analysis

For the analysis of expression levels of *PTTG1*, *miR-146a* and *miR-3142*, total RNA was extracted with TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) from 87 PBMC samples collected from healthy Swedish individuals at Uppsala University Hospital. In addition, we analyzed expression of these genes in 44 LCLs with different genotypes. cDNA was synthesized from 2 μg of total RNA at 42 °C for 80 min followed by heat inactivation at 95 °C for 5 min. The reaction contained 1.25 μM random hexamers, MuLV transcriptase, RNase inhibitor and buffer supplemented with 5 mM $MgCl_2$ and 1 mM dNTPs. Quantitative PCR for *PTTG1*, the primary transcript of *miR-146a* (pri-miR-146a) and 18S rRNA were performed using 15 ng cDNA, PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 U Platinum Taq polymerase (Invitrogen), 0.25 μM of each primer and SYBR Green (Molecular Probes, Eugene, OR, USA) for amplification detection. The PCR conditions were as follows: denaturation at 95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C, 30 s at 72 °C and final extension at 72 °C for 3 min. Expression of *PTTG1* and *pri-miR-146a* was normalized by that of 18S rRNA. The primers used were as follows: *PTTG1* forward: GCGGCTGTTAAGACCTGCAATAATCCA, *PTTG1* reverse: GTTC GATGCCCCACCAGCCT; *pri-miR-146a* forward: TTAGGAGCTCGCTGGCTGGG ACA, *pri-miR-146a* reverse: CAGGATCTACTCTCTCCAGGTCCTCA. Mature miRNAs (*miR146a*, *miR3142*) and the snRNA-control *RNU66* were reverse transcribed and quantified with TaqMan microRNA expression assays (ABI assay IDs 000468, 244120-mat and 001002, respectively), according to

the manufacturer's instructions. Briefly, cDNA was produced from 10 ng of total RNA with the commercial miRNA-specific stem-loop primers. Quantitative PCR was performed using the specific microRNA TaqMan assay.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDIX

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