

# Sex-specific association of X-linked Toll-like receptor 7 (TLR7) with male systemic lupus erythematosus

Nan Shen<sup>a,1,2</sup>, Qiong Fu<sup>a,b,1</sup>, Yun Deng<sup>a,b,1</sup>, Xiaoxia Qian<sup>a</sup>, Jian Zhao<sup>b</sup>, Kenneth M. Kaufman<sup>c</sup>, Yee Ling Wu<sup>d</sup>, C. Yung Yu<sup>d</sup>, Yuanjia Tang<sup>a</sup>, Ji-Yih Chen<sup>e</sup>, Wanling Yang<sup>f</sup>, Maida Wong<sup>b</sup>, Aya Kawasaki<sup>g</sup>, Naoyuki Tsuchiya<sup>g</sup>, Takayuki Sumida<sup>g</sup>, Yasushi Kawaguchi<sup>h</sup>, Hwee Siew Howe<sup>i</sup>, Mo Yin Mok<sup>j</sup>, So-Young Bang<sup>k</sup>, Fei-Lan Liu<sup>l</sup>, Deh-Ming Chang<sup>m</sup>, Yoshinari Takasaki<sup>n</sup>, Hiroshi Hashimoto<sup>n</sup>, John B. Harley<sup>c</sup>, Joel M. Guthridge<sup>o</sup>, Jennifer M. Grossman<sup>b</sup>, Rita M. Cantor<sup>p</sup>, Yeong Wook Song<sup>q</sup>, Sang-Cheol Bae<sup>k</sup>, Shunle Chen<sup>a</sup>, Bevra H. Hahn<sup>b</sup>, Yu Lung Lau<sup>f</sup>, and Betty P. Tsao<sup>b,2</sup>

<sup>a</sup>Joint Molecular Rheumatology Laboratory of Institute of Health Sciences and Shanghai Renji Hospital, Shanghai JiaoTong University School of Medicine and Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200001, China; <sup>b</sup>Division of Rheumatology, University of California, Los Angeles, CA 90095; <sup>c</sup>Arthritis and Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104; <sup>d</sup>Center for Molecular and Human Genetics, Research Institute at Nationwide Children's Hospital and The Ohio State University, Columbus, OH 43205; <sup>e</sup>Division of Allergy, Immunology and Rheumatology, Chang Gung Memorial Hospital, Chang Gung University, Tao-Yuan, Taiwan 333, Republic of China; <sup>f</sup>Department of Pediatrics and Adolescent Medicine, University of Hong Kong, Hong Kong 10000, China; <sup>g</sup>University of Tsukuba, Ibaraki 305-8577, Japan; <sup>h</sup>Institute of Rheumatology, Tokyo Women's Medical University, Tokyo 162-8666, Japan; <sup>i</sup>Department of Rheumatology, Allergy and Immunology, Tan Tock Seng Hospital, Singapore 308433; <sup>j</sup>Division of Rheumatology and Clinical Immunology, University of Hong Kong, Hong Kong 10000, China; <sup>k</sup>Department of Rheumatology, Hospital for Rheumatic Diseases, Hanyang University, Seoul 133-792, South Korea; <sup>l</sup>Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei City 114, Taiwan; <sup>m</sup>Rheumatology/Immunology/Allergy, Tri-Service General Hospital, National Defense Medical Center, Taipei City 114, Taiwan; <sup>n</sup>Juntendo University, Tokyo 113-8421, Japan; <sup>o</sup>Clinical Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104; <sup>p</sup>Department of Human Genetics, University of California, Los Angeles, CA 90024; and <sup>q</sup>Division of Rheumatology, Seoul National University, Seoul 151-742, South Korea

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**Systemic lupus erythematosus (SLE) is a multisystem, autoimmune disease that predominantly affects women. Previous findings that duplicated Toll-like receptor 7 (*TLR7*) promotes lupus-like disease in male BXSB mice prompted us to evaluate *TLR7* in human SLE. By using a candidate gene approach, we identified and replicated association of a *TLR7* 3'UTR SNP, rs3853839 (G/C), with SLE in 9,274 Eastern Asians ( $P_{\text{combined}} = 6.5 \times 10^{-10}$ ), with a stronger effect in male than female subjects [odds ratio, male vs. female = 2.33 (95% CI = 1.64–3.30) vs. 1.24 (95% CI = 1.14–1.34);  $P = 4.1 \times 10^{-4}$ ]. G-allele carriers had increased *TLR7* transcripts and more pronounced IFN signature than C-allele carriers; heterozygotes had 2.7-fold higher transcripts of G-allele than C-allele. These data established a functional polymorphism in type I IFN pathway gene *TLR7* predisposing to SLE, especially in Chinese and Japanese male subjects.**

functional polymorphism | disease susceptibility | autoimmunity | type I interferon

Systemic lupus erythematosus [SLE; Online Mendelian Inheritance in Man (OMIM) no. 152700] is a multisystem, autoimmune disease with strong genetic and environmental components (1). SLE predominantly affects women, with a female-to-male ratio of approximately 9:1. Male patients with SLE, although rare, tend to have more severe disease and poorer outcome (2), suggesting potential sex dimorphism in the disease development. Although the sex effect has often been attributed to sex hormones, the fact that XXY male subjects have approximately a 14-fold higher risk of developing SLE than 46 XY men indicates that X-linked genes may be risk factors for human SLE (3).

Located at Xp22.2, Toll-like receptor 7 (*TLR7*; OMIM no. 300365) and its functionally related gene *TLR8* (OMIM no. 300366) encode proteins that play critical roles in pathogen recognition and activation of innate immunity (4). They recognize endogenous RNA-containing autoantigens and induce the expression of type I IFN, a pivotal cytokine in the pathogenesis of SLE (5). In lupus-prone BXSB mice, the translocation of a segmental duplication of X chromosome to Y chromosome creates the Y-linked autoimmune accelerator (*Yaa*) locus, which was associated with autoreactive B cell responses to RNA-related antigens and exacerbation of glomerulonephritis in male mice (6). Although translocated X chromosome segment in *Yaa* may contain as many as 16 genes, the major gene for causation of the autoimmune phenotypes was identified to be *TLR7* (7), making it

a potential susceptibility gene for SLE. By using a candidate gene approach, we report herein that a functional polymorphism in 3'UTR of *TLR7* is associated with SLE in Chinese and Japanese populations, with a stronger effect in male than female subjects.

## Results

**Discovery and Replication of the Association of a *TLR7* 3'UTR SNP with SLE in Eastern Asian Population.** We genotyped 27 SNPs from the *TLR7*–*TLR8* region (12 in *TLR7* and 15 in *TLR8*) in 1,434 SLE cases and 1,591 control subjects of Eastern Asian ancestry using the Beadstation Infinium II platform (Illumina). Eleven SNPs in *TLR7* and 12 SNPs in *TLR8* that showed a minor allele frequency greater than 0.01 were included in association analysis (Fig. 1*B* and Table S1). We observed evidence of association with SLE in 2 *TLR7* SNPs (rs5935436 and rs3853839) and 2 *TLR8* SNPs (rs3764880 and rs4830805; Fig. 1*B*). Only rs5935436 and rs3853839 in *TLR7* remained significant after Bonferroni correction ( $P = 0.041$  and  $P = 0.016$ , respectively). The strongest association signal was detected at rs3853839 among Chinese subjects [cases vs. controls, 563 vs. 522;  $P = 6.3 \times 10^{-6}$ ; odds ratio (OR) = 1.67 (95% CI = 1.33–2.08)], but not in Korean subjects [ $P = 0.32$ ; OR = 0.92 (95% CI = 0.79–1.08); Fig. 1*B*], suggesting potential genetic heterogeneity of SLE between the two populations.

We next performed a haplotype-based association test using Haploview 4.03 software. The “GAACAC” haplotype formed by rs2897827, rs5935436, rs2302267, rs179019, rs5743740, and rs179016 had a frequency of 3.1% in SLE cases and 4.8% in con-

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<sup>1</sup>N.S., Q.F., and Y.D. contributed equally to this work.

<sup>2</sup>To whom correspondence may be addressed. E-mail: nanshensibs@gmail.com or btsao@mednet.ucla.edu.

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**Table 1. Association of rs3853839 with SLE in Eastern Asian populations**

Ethnicity and panel	Case/control	G risk allele frequency		P value	OR (95% CI)
		Case	Control		
Eastern Asian*:					
Discovery					
Male	126/229	0.83	0.73	0.038	1.79 (1.03–3.13)
Female	1,308/1,362	0.79	0.76	3.00E-03	1.22 (1.06–1.39)
All	1,434/1,591	0.80	0.76	6.70E-04	1.24 (1.1–1.41)
Chinese: Replication 1					
Male	196/931	0.92	0.81	2.31E-04	2.73 (1.57–4.74)
Female	2,144/1,505	0.83	0.80	4.24E-03	1.19 (1.06–1.34)
All	2,340/2,436	0.83	0.80	9.02E-04	1.21 (1.08–1.35)
Japanese: Replication 2					
Male	36/390	0.89	0.69	0.014	3.51 (1.22–10.2)
Female	524/523	0.75	0.71	0.037	1.23 (1.01–1.49)
All	560/913	0.75	0.70	7.00E-03	1.28 (1.07–1.53)
Eastern Asian*: Combined					
Male	358/1,550	0.89	0.77	1.33E-06	2.33 (1.64–3.30)
Female	3,976/3,390	0.80	0.77	1.19E-07	1.24 (1.14–1.34)
All	4,334/4,940	0.81	0.77	6.50E-10	1.27 (1.17–1.36)

\*Includes subjects of Chinese, Korean, or Japanese descent.

showing a strong association with SLE ( $P = 1.33 \times 10^{-6}$ ) and significantly higher OR in male versus female subjects [2.33 (95% CI = 1.64–3.30) vs. 1.24 (95% CI = 1.14–1.34);  $P = 4.1 \times 10^{-4}$ ]. The significant male sex effect of rs3853839 in SLE was confirmed by a higher G allele frequency in male cases than female cases ( $P = 1.1 \times 10^{-4}$ ; OR = 1.90 (95% CI = 1.36–2.64)) and by a higher OR in male subjects than the OR calculated by using only female homozygotes [GG vs. CC, OR = 1.45 (95% CI = 1.17–1.79);  $P = 0.02$ ].

**TLR7 SNP rs3853839 Confers Elevated Expression of TLR7 in Vivo.** The consistent association of rs3853839 with SLE led us to investigate the potential functional consequences of the G risk allele. We first examined whether the 3'UTR polymorphism affected the expression level of TLR7 transcripts. We isolated peripheral blood mononuclear cells (PBMCs) from selected Chinese individuals carrying various genotypes and measured the mRNA expression levels of TLR7 by using real-time PCR. As shown in Fig. 2A, male controls carrying G allele had significantly higher TLR7 transcripts than those carrying C allele ( $P = 0.02$ ). Similarly, female cases and controls homozygous for G allele showed notably higher TLR7 mRNA expression than those carrying homozygous C allele ( $P = 7.4 \times 10^{-6}$  and  $P = 0.03$ , respectively). No sex differences in TLR7 mRNA expression levels were observed between normal controls carrying the same allelic genotype of rs3853839 ( $P = 0.07$  or  $P = 0.55$  for GG females vs. G males or CC females vs. C males, respectively; Fig. 2A). The sex comparison was not replicated in patients with SLE because of insufficient sample size of blood collected from male patients with SLE.

To exclude potential bias between individuals, we next assessed the allelic specific transcriptional expression of TLR7 in Chinese female G/C heterozygotes by pyrosequencing. We extracted genomic DNA (gDNA) and cDNA from PBMCs of heterozygous SLE patients and controls and determined the exact ratios of G/C in gDNA and cDNA for each individual. As shown in Fig. 2B, the mean G/C ratio in gDNA of all individuals was 1.05, which was consistent with the theoretical value. However, SLE and control female heterozygotes showed a 2.7-fold higher G/C ratio in TLR7 transcripts than that in gDNA ( $P = 2 \times 10^{-4}$  and  $P = 2 \times 10^{-3}$ , respectively;  $P_{\text{overall}} = 1.2 \times 10^{-6}$ ), meaning that there was significantly higher level of G allele-containing TLR7 transcripts than C allele-containing transcripts in PBMCs, even though there was same amount of gDNA that contains either allele.

To investigate whether the disparity observed between transcript and gDNA was a result of imbalanced X chromosome inactivation (XCI) in female subjects, we analyzed the XCI pattern in these individuals by measuring the degree of methylated CAG alleles in androgen receptor (*AR*) gene as previously described (9). Fig. 2C showed no significant skewed XCI in either SLE or controls ( $P = 0.8$ ), suggesting that skewed XCI or somatic selection might not account for the current finding. Taken together, these data suggested rs3853839 polymorphism could play a functional role in the regulation of TLR7 transcript levels in vivo.

To investigate whether allelic differences of rs3853839 corresponded to TLR7 protein levels, we isolated proteins from PBMCs of healthy Chinese male subjects carrying the G or C allele for Western blot analysis. Fig. S1 showed that five of the six male G allele carriers had higher expression levels of TLR7 protein than the two C allele carriers after normalization of the amount of protein loading using  $\beta$ -actin. Although the result was not statistically significant ( $P = 0.21$ , probably because of the very small sample size), the finding of higher TLR7 protein expression of the G risk allele carriers was consistent with what we observed at mRNA levels of TLR7.

We next investigated that whether the functional SNP rs3853839 might influence the IFN reaction downstream of TLR7. A prominent feature of patients with SLE is an increased expression of type I IFN-regulated genes (i.e., IFN signature) in PBMCs compared with unaffected controls (10, 11). We measured expression levels of four type I IFN-regulated genes, including lymphocyte antigen 6 complex, locus E (*LY6E*), myxovirus resistance 1 (*MX1*), IFN-inducible protein with tetratricopeptide repeats 1 (*IFIT1*), and *IFIT3* by real-time PCR in PBMCs from clinically inactive female SLE patients carrying various rs3853839 genotypes and calculated an IFN score for each individual. As shown in Fig. 2D, female SLE patients carrying GG genotype showed a more pronounced IFN signature in PBMCs compared with those carrying GC and CC genotypes, indicating rs3853839 might modulate expression levels of TLR7, affecting IFN response in vivo.

## Discussion

There is compelling evidence supporting the contribution of TLR7 to the development of autoimmunity. Transgenic mice with a twofold overexpression of TLR7 would have increased production of RNA-related autoantibodies and develop spontaneous



line chosen or lack of suitable condition or agonists applied (14). Further bioinformatic analysis based on current databases did not provide a clue on how the SNP regulates mRNA expression: rs3853839 is not located in the binding site of any transcriptional factors or miRNAs; neither is it in the AU-rich sequence that is usually involved in mRNA decay. The exact mechanism is therefore yet to be elucidated.

It is perplexing that the X-linked *TLR7* association does not explain the female dominance of SLE. The observed stronger male effect of rs3853839 supports the notion that unique genetic predispositions might exist for male lupus. Given that both *Tlr7* duplication in *Yaa* mice and *TLR7* SNP rs3853839 in humans were preferably associated with male lupus, there might be potential epistasis between *TLR7* and male sex background. This is supported by previous observation of substantially lower IFN response in male versus female subjects upon *TLR7* agonist stimulation (15, 16). The sex difference in *TLR7*-mediated response has been used to account for the finding of higher viral loads in men than women in early HIV infection (16) and might explain, at least in part, the increased severity of several infectious diseases in male subjects (17). The sex-specific effect demonstrated by *TLR7* SNP therefore might have implications that go beyond SLE. It would be interesting to investigate the contribution of *TLR7* polymorphism to other diseases with the *TLR7* pathway involved, especially in those with sex-specific incidence and manifestations.

To help exclude the possibility that the low LD of rs3853839 with neighboring SNPs is caused by CNV in this region, we used three independent methods including SYBR Green quantitative real-time PCR assay, PmeI pulsed-field gel electrophoresis (PFGE) and TaqI genomic Southern blot analyses to detect the existence of CNV at *TLR7-8* locus. Samples from 223 patients with SLE and 139 normal controls were tested by real-time PCR assay (Fig. S2 and *SI Materials and Methods*), and *TLR7-TLR8* CNVs were detected only in patients with SLE, at a frequency of less than 2% (one male patient had a two-copy variant of both *TLR7* and *TLR8*, one female patient had a one-copy variant of *TLR7*, and another female patient had a one-copy variant of *TLR8*; Fig. S3). The PmeI-PFGE and TaqI genomic Southern blot analyses performed among 250 subjects, including 203 female and 47 male subjects, with a total of 453 X chromosomes did not show physical evidence of structural variations at *TLR7-TLR8* locus (frequency less than 0.2%; Fig. S4 and *SI Materials and Methods*). Our results were consistent with two recent studies that used customized CGH platforms, which have reported no evidence of greater than a 5% frequency of *TLR7-TLR8* CNVs greater than approximately 500 bp in 19 HapMap trios of individuals of European descent from Utah, 20 HapMap trios from Yoruba individuals in Nigeria, 10 HapMap trios of Han Chinese individuals from Beijing, 10 HapMap trios of Japanese individuals from Tokyo, and 10 Korean female individuals, with an estimated statistical power of 95% (18, 19). Additionally, no structural variants at *TLR7-TLR8* locus have been described in the current Database of Genomic Variants (<http://projects.tcag.ca/variation>; RCh37, February 2009). Taken together, it is unlikely that the low LD between rs3853839 and neighboring SNPs is attributed to CNVs of this locus.

Currently, four European and two Asian genome-wide association studies (GWASs) in SLE have been published and more than 20 risk loci have been identified and replicated, which do not include the SLE-associated *TLR7* SNP described here (20–25). There are various factors that may influence the association detection in a GWAS, including sample size, study design, cohort demographics (ethnicity and sex composition, for example), analytical strategies, and in the case of rs3853839, SNP selection. Rs3853839, located in a region with poor LD structure, has not been included in commercial predesigned genotyping arrays, which could explain why the previous GWAS failed to capture this risk SNP. To estimate power of these GWAS to identify *TLR7* SNP association, we separately examined the male and female samples,

set the level of significance (*P* value) for the subsequent replication study in each GWAS, and assumed the associated allele G with an odds ratio of 1.79 in male subjects and 1.22 in female subjects (OR was identified in our discovery panel study). The power estimate for male samples is less than 1% in Asian GWASs and ranges between 2% and 7% in European GWASs, whereas for female samples it ranges between 1% and 3.4% in Asian GWASs and 3% and 24% in European GWASs. Thus, these studies have inadequate power to identify this *TLR7* association in their primary analyses. Our study was focused on the X chromosome; therefore, our correction for multiple testing was less stringent than the others that were conducted genome-wide, providing us greater power. In addition, fairly large sample size of male subjects with SLE and male controls (358 and 1,550, respectively, currently the largest male lupus study to our knowledge) facilitated our ability to detect the stronger effect of rs3853839 in male subjects. However, our male sample size is still not large enough to be comparable to female SLE genetic studies, and large-scale independent studies are needed to verify the effect of *TLR7* in men.

In summary, we identified a functional polymorphism in rs3853839 that may confer elevated expression of *TLR7* and predisposes to the development of SLE, especially in Chinese and Japanese male individuals. These data provide compelling evidence supporting a causal role for the type I IFN pathway genes in human autoimmunity and highlight the importance to explore sex-specific genetic contribution in a sex-biased disease such as SLE.

## Materials and Methods

**Clinical Samples.** In the discovery panel, we genotyped 27 SNPs spanning the *TLR7* and *TLR8* gene region in 1,434 SLE cases and 1,591 controls of Eastern Asian ancestry. These samples were obtained from multiple centers in the United States and Eastern Asia, of which 563 cases and 522 controls were Chinese, 845 cases and 1,022 controls were Korean, and 26 cases and 47 controls were Japanese. The Chinese replication panel included 2,340 independent Chinese SLE cases and 2,436 controls, collected by collaborators in Shanghai (case vs. control: 687 vs. 849), Hong Kong (881 vs. 840), and Taiwan (772 vs. 747). The Japanese replication panel included 560 SLE cases and 913 controls that were recruited from two centers in Japan. All SLE cases fulfilled the 1997 revised American College of Rheumatology criteria for the classification of SLE (26). Informed consent was obtained from all subjects. The study was approved by the human subject institutional review boards or ethical committees at each of the participating locations.

**SNP Selection and Genotyping.** In the discovery panel, DNA from each subject was genotyped using custom-designed Beadstation Infinium II platform (Illumina). Genotyping was performed at the Lupus Genetics Studies Unit of the Oklahoma Medical Research Foundation. Twelve SNPs within *TLR7* and 15 SNPs within *TLR8* that either tagged major haplotypes in the HapMap database (January 2006) or were potentially functional were selected for genotyping. The average call rate for all samples was 98.3%. In the replication panels, rs3853839 and rs5935436 were genotyped by predesigned TaqMan SNP genotyping assay (Applied Biosystems) according to the manufacturer's instructions.

**DNA Sequencing.** We sequenced 5' promoter region (up to 2 kb upstream) as well as three exons (including 3' UTR in exon 3) of *TLR7* in 48 Chinese female subjects. Briefly, consecutive overlapping amplicons were amplified from gDNA extracted from peripheral blood leukocytes. Primer pairs used are shown in Table S2.

**Allelic Expression of *TLR7* in PBMCs.** The mRNA expression level of *TLR7* was measured in PBMCs from Chinese individuals carrying different genotypes of rs3853839. Briefly, total RNA were extracted from PBMCs using TRIzol (Invitrogen). RNA samples were then treated with DNase to eliminate gDNA contamination before being reverse-transcribed into cDNA. SYBR Green real-time PCR was used to measure the relative expression of *TLR7*. RPLP13A was used as internal control. We performed allelic-specific transcription quantification assay to measure the allelic expression of rs3853839 by using pyrosequencing as previously described (14). The allelic ratio for each cDNA and gDNA from each individual was calculated by using PSQMA software (version 2.1; Biotage) and compared by paired Student *t* test.

**IFN Signature in Patients with SLE.** Expression levels of four type I IFN-regulated genes including *LY6E*, *MX1*, *IFIT1*, and *IFIT3* were measured by real-time PCR in PBMCs from female patients with SLE carrying various rs3853839 genotypes and normal controls. RPL13A was used as an internal control. The patients with SLE recruited in this part of study were in clinical remission defined by SLE Disease Activity Index 2000 (27) lower than 4 and prednisone dosage less than 15 mg/d. IFN scores were calculated as described in previous studies (10, 11).

**XCI Analysis in Female Participants.** We performed XCI analysis in female GC heterozygotes by measuring the degree of methylated CAG repeats in *AR* gene as previously described (9). Briefly, methylation-sensitive restriction enzyme HpaII was used to digest unmethylated (active X chromosome) DNA, which is thereby unable to amplify during the following PCR. Postdigestion PCR products therefore represent methylated (inactive X chromosome) DNA sequence. PCR products were sized using 5% denaturing polyacrylamide gel electrophoresis in an ABI Prism 3730 DNA automated sequencer, and analyzed by using Genescan Analysis 3.1 software (Applied Biosystems) as described previously (28).

**Western Blot Analysis.** Proteins were extracted from PBMCs of Chinese male controls carrying rs3853839 G and C allele ( $n = 6$  and  $n = 2$ , respectively) to conduct Western blot analysis (*SI Materials and Methods*).

**CNV Discovery Experiment.** CNV discovery experiments are described in *SI Materials and Methods*. Study cohorts for CNV using real-time PCR assay and Southern blot analyses are shown in *Tables S3* and *S4*, respectively.

**Statistical Analysis.** For single SNP analysis, we used gPLINK 1.062 software (<http://pungu.mgh.harvard.edu/~purcell/plink/gplink.shtml>) to evaluate the significance of differences in allelic frequencies of each SNP in the case-control samples. Bonferroni correction was used for multiple testing corrections in the discovery panel. For haplotype-based analysis, we used Haploview version 4.03 software (<http://www.broad.mit.edu/mpg/haploview/index.php>) to calculate pairwise LD indices  $R^2$  and  $D'$ , define LD blocks, infer haplotype frequencies, and analyze the significance of associations. We used the Q statistic (weighted sum of squares) to test the heterogeneity of odds ratio between male and female subsets. We used the Mann-Whitney test to compare the mRNA expression level of TLR7 in PBMCs from individuals carrying different genotypes. A paired Student *t* test was used to compare the G/C ratio between DNA and cDNA. A P value lower than 0.05 was considered to be statistically significant.

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