Identification of BANK1 polymorphisms by unlabelled probe high resolution melting: association with systemic lupus erythematosus susceptibility and autoantibody production in Han Chinese

Ming Guan1,2,*, Bo Yu3,4,*, Jun Wan4,5,*, Xinju Zhang1, Zhiyuan Wu1, Qili Zhong3, Wei Zhang5,6 and Hejian Zou7

Abstract

Objectives. The three functional SNPs of BANK1 (rs10516487, rs17266594 and rs3733197) have been shown to be associated with SLE in Caucasian populations. The aim of this study was to investigate whether the association of BANK1 polymorphisms with SLE could be replicated in a Chinese population and whether the autoantibody production is relevant to BANK1 polymorphisms.

Methods. Genotyping of three variants in BANK1 was carried out by unlabelled probe high resolution melting (HRM) assay in 264 SLE cases and 268 controls in a Chinese Han population living in Shanghai region. The genotype frequencies of the detected polymorphisms were analysed in relation to the production of autoantibodies (ANA, anti-dsDNA, anti-RNP, anti-SSA, anti-SSB and anti-Smith) in SLE patients.

Results. Samples with the target genotypes were accurately detected and easily distinguishable by unlabelled probe HRM assay. The frequencies of the rs10516487 C allele and the rs17266594 T allele were significantly increased compared with the controls (C allele: 88.6 vs 83.2%, P = 0.011; T allele: 88.3 vs 83.2%, P = 0.019). However, the frequencies of the rs3733197 G allele were not associated with SLE (G allele: 79.9 vs 79.1%, P = 0.741). The rs10516487 and rs17266594 polymorphisms were significantly associated with high-titre ANA (≥ 1:320) and production of anti-SSA antibodies in SLE patients compared with the control subjects.

Conclusions. Genotyping using unlabelled probes is a rapid, accurate and cost-effective closed-tube method. This study implies that rs10516487 and rs17266594 polymorphisms might contribute to individual susceptibility to SLE and influence the ANA/SSA autoantibody response in SLE patients in Chinese population.

Key words: BANK1, SLE, Single nucleotide polymorphism, High resolution melting.

Introduction

SLE is considered to be the prototypic systemic autoimmune disease, with multisystem organ involvement, characterized by heterogeneous systemic involvement, production of a wide spectrum of autoantibodies and involvement of complex genetic and environmental components [1]. Worldwide, the prevalence of SLE appears to vary, with racial and geographical differences [2]. The prevalence of SLE ranges from 31 to 70 cases per 100,000 persons among Chinese populations [3] and from 7 to 71 cases per 100,000 persons in European populations [4]. There is considerable evidence that the
development of SLE has a strong genetic basis [5–13]. The genetics of SLE have been studied since the early 1970s, when the first histocompatibility (HLA) allele association with lupus was found [14]. In particular, recent genome-wide association studies (GWASs) of SLE have identified dozens of robust susceptibility genes and/or loci [10, 13, 15].

BANK1 (B-cell scaffold protein with ankyrin repeats 1) is a B-cell adaptor protein that is highly expressed in B cells [16, 17], and functions in B-cell receptor-induced calcium mobilization from intracellular stores. This protein can also promote Lyn-mediated tyrosine phosphorylation of inositol 1, 4, 5-trisphosphate receptors. Increased interaction of BANK1 to downstream targets may lead to a steady state marked by B-cell hyperresponsiveness or deregulated B-cell activation [18]. BANK1-deficient mice had higher levels of mature B cells and spontaneous germinal centre B cells, particularly in response to T-dependent antigens, as well as higher serum IgG2a [19]. Thus, variations in BANK1 expression and function could have profound effects on the modulation of B-cell activity and immune phenotypes.

Kozyrev et al. [10] reported that the non-synonymous SNP rs10516487 (R61H), branch point-site SNP rs17266594 and a third associated variant rs3733197 (A383T) in the ankyrin domain in BANK1 are functional disease-associated variants that contribute to the SLE susceptibility in several European populations. The genetic heterogeneity between ethnic populations has been suggested to be important in SLE risk [20], showing the need for replication in different populations or geographical regions.

Recently, unlabelled probe high resolution melting (HRM) assay for genotyping has been described [21, 22]. The method combines both saturation dyes and unlabelled oligonucleotide probes in an asymmetric PCR, leading to simultaneous production of probe–target and whole-amplicon dsDNA duplexes that can be analysed from the same HRM run and would be suitable for genotyping.

In this study, we developed a sensitive, clinically useful HRM assay with unlabelled probe to examine the BANK1 variants in a clinically well-defined SLE patients and unaffected controls in a Chinese population living in Shanghai region. Given the plausible link between B-cell hyperactivity and autoantibody production, analyses were performed to assess whether BANK1 polymorphisms were associated with the production of common lupus autoantibodies.

### Materials and methods

#### Patients

The subjects in the SLE study group comprised 264 Chinese Han patients with SLE (mean age 46.2 years, 92.3% females) and 268 unrelated control subjects (mean age 47.7 years, 88.7% females) from Huashan Hospital, Fudan University. All patients with SLE fulfilled the 1997 ACR revised criteria for SLE [23].

<table>
<thead>
<tr>
<th>Table 1 Clinical characteristics of the study subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical profiles</strong></td>
</tr>
<tr>
<td>No. of subjects</td>
</tr>
<tr>
<td>Age, mean (s.d.), year</td>
</tr>
<tr>
<td>Sex, female, %</td>
</tr>
<tr>
<td>Disease duration, mean (s.d.), years</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disease manifestation</th>
<th><strong>SLE</strong></th>
<th><strong>Controls</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosensitivity</td>
<td>126 (47.7)</td>
<td>–</td>
</tr>
<tr>
<td>Malar rash</td>
<td>162 (61.4)</td>
<td>–</td>
</tr>
<tr>
<td>Discoid rash</td>
<td>32 (12.1)</td>
<td>–</td>
</tr>
<tr>
<td>Oral ulcers</td>
<td>96 (36.4)</td>
<td>–</td>
</tr>
<tr>
<td>Arthritis</td>
<td>134 (50.8)</td>
<td>–</td>
</tr>
<tr>
<td>Serositis</td>
<td>37 (14.0)</td>
<td>–</td>
</tr>
<tr>
<td>Renal involvement</td>
<td>110 (41.7)</td>
<td>–</td>
</tr>
<tr>
<td>Neurological disorder</td>
<td>14 (5.3)</td>
<td>–</td>
</tr>
<tr>
<td>Haematological disorder</td>
<td>230 (87.1)</td>
<td>–</td>
</tr>
<tr>
<td>Leucopenia</td>
<td>142 (53.8)</td>
<td>–</td>
</tr>
<tr>
<td>Lymphopenia</td>
<td>213 (80.7)</td>
<td>–</td>
</tr>
</tbody>
</table>

Patients in family relationship were excluded to avoid a bias effect. Patient characteristics are shown in Table 1. The study was approved by Institutional Ethical Committees of Huashan Hospital and was conducted according to the Declaration of Helsinki principles.

#### Genotyping with unlabelled probe

Genomic DNA was isolated from peripheral blood lymphocytes. DNA was extracted with a QIamp DNA Blood Kit (Qiagen, Valencia, CA). All oligonucleotide primers and probes were obtained from Sango Biotech Co. (Shanghai, China). To prevent the extension of the probe during PCR, a C3 blockage was introduced.

We selected three functional BANK1 SNPs: rs10516487 (C>T), rs17266594 (T>C) and rs3733197 (G>A). Genotyping of BANK1 SNPs was carried out by unlabelled probe HRM assay. Sequences of primers and unlabelled probes are shown in Table 2. Unlabelled probe HRM analysis was developed from asymmetric PCR. After asymmetric PCR, a large number of superfluous single strands will combine with unlabelled probes. When the temperature drops, therefore, it will produce two types of melting curves. The part of curve representing low melting temperatures represents the region of probe and product.

Asymmetric PCR was performed on a Rotor-Gene Q (Qiagen) in 20 μl of reaction volume. The master mix contained: 1 × reaction buffer (TaKaRa Bio, Shiga, Japan), 1 × SYTO9 (Invitrogen, Carlsbad, CA, USA), 200 μM dNTP, 0.05 μM forward primer, 0.5 μM reverse primer, 0.5 μM probe, 2 mM MgCl2, 1 U TaKaRa Ex Taq DNA polymerase (TaKaRa Bio) and 1 μl (15–25 ng/μl) of genomic DNA. All amplifications were initiated with a 5-min hold at 95°C, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 58°C (for rs10516487 and rs17266594) or 56°C (for rs3733197) for 30 s, and an extension at 72°C during 30 s.

The PCR products were subjected to denaturation step at 98°C for 2 min, followed by cooling to 40°C for 2 min to facilitate the heteroduplex formation and then heating slowly at 0.2°C/s from 50°C to 95°C. HRM analysis was
performed using Rotor-Gene Q 1.7 software (Qiagen). For each assay, we always include positive controls (homozygous wild-type, heterozygous and homozygous variants).

Measurement of autoantibodies
Serum was obtained from 5 ml of the peripheral blood by standard procedures and was stored frozen at −70°C until used for antibodies detection. ANA was evaluated by indirect immunofluorescence on Hep-2 cells (Euroimmun, Lübeck, Germany). The results were considered ANA positive, when positive reactions were seen at a serum dilution of at least 1:100. Detection of anti-dsDNA was performed by ELISA (Organtec Diagnostika GmbH, Mainz, Germany). All sera had been previously tested for anti-SSA, anti-SSB, anti-Smith and anti-nRNP by immunoblotting using EUROBLOT (Euroimmun).

Sequencing
We reamplified 20 samples with different genotypes of each SNP as determined by unlabelled probe melting using same primers for sequencing analysis. Amplicons were gel purified using the QIAquick gel purification kit (Qiagen). DNA sequencing analysis was performed in ABI PRISM 310 genetic analyser (Applied Biosystems, Foster City, CA, USA).

Statistical analysis
Chi-square distribution was used to analyse categorical data and detect associations between autoantibodies and genotypes. \( P < 0.05 \) was considered as statistically significant. Odds ratios (ORs) and 95% CIs were calculated.

Results
Unlabelled probe HRM
Genotyping with unlabelled probes is a homogeneous, end-point assay. The probe is included in the PCR mix, but is not consumed during amplification, since it is blocked at the 3’ end. PCR is performed in the presence of a saturating fluorescent dye. There are two melting transitions, one for the unlabelled probe at a lower temperature and another for the amplicon at a higher temperature. Genotyping is accomplished by monitoring the melting of probe–target duplexes post-PCR. Since the double-stranded region can only be as long as the probe, a high sensitivity rate can be obtained. After asymmetric PCR, use of one primer in excess leads to the overproduction of the single target strand and the probes anneal to single-stranded product. Melting curves show regions of both probe/product and product/product melting. Different alleles result in different probe/product melting transitions based on the stability of the mismatches present. It is easiest to see the transitions by plotting the negative derivative \( (dF/dT) \) of fluorescence \( (F) \) vs temperature \( (T) \).

All genotypes are distinct and easily resolvable without interference from the no-template control. Homozygous alleles are represented by a single probe melting peak, whereas heterozygous samples have two peaks, corresponding to both alleles separated by ~2–4°C. Probe–target melting for rs10516487 C > T polymorphism was observed between 55 and 88°C. A perfectly matched probe–target hybrid has a characteristic melting temperature that is higher than a mismatched hybrid. A closer examination of the region of probe melting showed that samples with the C allele had a derivative melting peak at 66.4°C, whereas samples harbouring the T allele showed a melting peak at 63.0°C (Fig. 1A). The heterozygous samples showed two peaks, one at each temperature representing the combination of both alleles. Therefore, a single probe was able to recognize all three genotypes within the given sample set.

Probe–target melting for rs3733197 G > A polymorphism was observed between 60 and 88°C. A probe was designed for ancestral allele G. Samples with the G allele had a derivative melting peak at 74.7°C, whereas samples with the A allele showed a melting peak at 69.4°C (Fig. 1B). Probe–target melting for rs17266594 T > C polymorphism was observed between 50 and 88°C. A probe was designed for the ancestral allele T. Samples with the T allele had a derivative melting peak at 65.7°C, whereas samples with the C allele showed a melting peak at 63.5°C (Fig. 1C).

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**Table 2** Sequences of primers and unlabelled probes

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer/probe</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10516487</td>
<td>Forward</td>
<td>ACATTGTAAGACGTTAAGTTCAAGCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGAATATGAAGAAGATGAGAGA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>GAAAAGAAGATCTTCGACGAGCATATATACAGATG</td>
</tr>
<tr>
<td>rs3733197</td>
<td>Forward</td>
<td>GCTTCAATGTTCAGGAGCAAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGCTGTCTTCTTCAATATCCAGGAA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CAGACCCCCAGCATATTGTCCAGGGACATGGTCA</td>
</tr>
<tr>
<td>rs17266594</td>
<td>Forward</td>
<td>CATGCTGCTGATGTCATTTCCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGTCTCTTCTACAATATAACAGAA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TAAATAATTAAACTCGTTGATGACTTGCAATAATATAT</td>
</tr>
</tbody>
</table>

Underlined nucleotides denote the locations of the SNP(s) that is (are) genotyped in the assay.
To determine whether the association of BANK1 polymorphisms with SLE could be replicated in a Chinese population, we genotyped three SNPs: rs10516487, rs17266594 and rs3733197. The genotype and allele frequencies of the three SNPs in SLE patients and healthy controls were shown in Table 3. Genotype frequencies were in Hardy–Weinberg equilibrium in patients and controls for all the polymorphisms analysed. For the BANK1 rs10516487 SNP, the T allele was found on 11.4% of chromosomes from the SLE patients as compared with 16.8% of the chromosomes from the controls (OR 0.64; 95% CI 0.45, 0.90; \( P = 0.011 \); Table 3). With regard to the rs17266594 SNP, the C allele was found on 11.7% of chromosomes from the SLE patients as compared with 16.8% of chromosomes from the controls (OR 0.66; 95% CI 0.47, 0.93; \( P = 0.019 \)). SNPs rs17266594 and rs10516487 were found to be in strong linkage disequilibrium (LD) \( (D' = 0.99; r^2 = 0.96) \), which is consistent with previous reports [10].

Association between BANK1 polymorphisms and the presence of ANA/anti-SSA

Sera from 160 patients with primary SLE were analysed for the presence of autoantibodies. The genotype frequencies of the detected polymorphisms were first analysed in relation to the production of autoantibodies (ANA, anti-dsDNA, anti-RNP, anti-SSA, anti-SSB and anti-Smith) in SLE patients. Case-only analysis indicated that the genotype frequencies of the three SNPs were not significantly associated with the presence with ANA (high titre), anti-dsDNA, anti-RNP, anti-SSA, anti-SSB and anti-Smith antibodies in SLE patients. Then we performed subphenotype-control analysis to examine the risk conferred by the three SNPs in the presence of different types of autoantibodies of SLE. C/C genotype of rs10516487 was significantly more prevalent among patients with high-titre ANA (\( \geq 1:320 \); 82.1%) than among healthy controls (69.0%; \( P = 0.0367 \); Table 4). The linked polymorphisms, rs10516487 and rs17266594, gave \( P \)-values of 0.0229 and 0.0431, respectively, when anti-SSA-positive patients were compared with the controls. The frequency of the C/C at rs10516487 was 85.1% in anti-SSA-positive patients, 74.4% in anti-SSA-negative patients and 69.0% in controls, whereas the T/T genotype at rs17266594 was present in similar frequencies: 83.8, 74.4 and 69.0%, respectively (Table 5). No other polymorphisms reached statistical significance.

Haplotype analysis

We performed a haplotype analysis using two SNPs, rs10516487 and rs17266594. According to the results found for SLE, the major CT haplotype was found at a higher frequency among SLE patients, and the minor TC haplotype was more frequent among the control subjects. There were statistically significant differences between the patients and controls. When a three-SNP haplotype analysis (in the order rs10516487, rs3733197 and rs17266594) was performed, we found that the TGC haplotype was being protective against SLE (\( P = 0.018 \)).

Discussion

This study is the first to examine the possible role of BANK1 variants, which were previously found to be
associated with SLE, in genetic susceptibility to SLE in Han population in Mainland China. We found that rs10516487 and rs17266594 were associated with SLE. A statistically significant association between both of these polymorphisms and the presence of anti-SSA antibodies was found. In addition, rs10516487 is significantly associated with an increased production of ANA. Taken together, these results not only suggested that the BANK1 gene was associated with SLE in Chinese Han population, but also implied that BANK1 is involved in the production of anti-antibodies in SLE.

Consistent with the genetic susceptibility identified in SLE patients, European Caucasian populations and a cohort of Hong Kong Chinese, we observed that the rare allele of both the rs10516487 and rs17266594 SNPs of BANK1 acts with a protective effect on susceptibility to SLE. Both allele and genotype frequencies were much lower than the frequencies previously reported in European Caucasian populations; however, they are similar with a recent report in Hong Kong Chinese [24]. With regard to rs10516487, we observed a homozygous T allele frequency of 11.4%, whereas Kozyrev et al. [10] and Chang et al. [24] reported respective frequencies of 29.2 and 13.9% in their analyses. With regard to rs17266594, we found a similar trend. This highly supports the accuracy of these genotyping data, and therefore a similar genetic trait in Cantonese population living in Guangdong Province and Han population in Shanghai, with the rare alleles being protective.

In contrast, our study did not reveal statistically significant difference in genotypic frequencies for rs3733197, between patients and controls, which may reflect the slight genetic differences between the two ethnic groups in China. Recently, a genome-wide survey of SNP genotyping from a large number of Han Chinese samples from 10 provinces in China revealed the proportion of genetic

### Table 3 - Distribution of BANK1 SNP genotypic and allelic frequencies in SLE cases and controls

<table>
<thead>
<tr>
<th>SNP/group</th>
<th>Genotype allele</th>
<th>SLE patients, (n = 264), (n (%))</th>
<th>Controls, (n = 268), (n (%))</th>
<th>(\chi^2)</th>
<th>(P)-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10516487</td>
<td>TT</td>
<td>5 (1.9)</td>
<td>7 (2.6)</td>
<td>0.3110</td>
<td>0.5771</td>
<td>0.72 (0.24, 2.18)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>50 (18.9)</td>
<td>76 (28.4)</td>
<td>6.5275</td>
<td>0.0106</td>
<td>0.59 (0.39, 0.90)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>209 (79.2)</td>
<td>185 (69.0)</td>
<td>7.1134</td>
<td>0.0077</td>
<td>1.70 (1.15, 2.53)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>60 (11.4)</td>
<td>90 (16.8)</td>
<td>6.4698</td>
<td>0.0110</td>
<td>0.64 (0.45, 0.90)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>468 (88.6)</td>
<td>446 (83.2)</td>
<td>6.4698</td>
<td>0.0110</td>
<td>1.57 (1.11, 2.23)</td>
</tr>
<tr>
<td>rs3733197</td>
<td>AA</td>
<td>11 (4.2)</td>
<td>16 (6.0)</td>
<td>0.8979</td>
<td>0.3434</td>
<td>0.68 (0.32, 1.48)</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>84 (31.8)</td>
<td>80 (29.8)</td>
<td>0.2414</td>
<td>0.6232</td>
<td>1.10 (0.76, 1.58)</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>169 (64.0)</td>
<td>172 (64.2)</td>
<td>0.0016</td>
<td>0.9686</td>
<td>0.99 (0.70, 1.41)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>106 (20.1)</td>
<td>112 (20.9)</td>
<td>0.1097</td>
<td>0.7405</td>
<td>0.97 (0.71, 1.28)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>422 (79.9)</td>
<td>424 (79.1)</td>
<td>0.1097</td>
<td>0.7405</td>
<td>1.05 (0.78, 1.42)</td>
</tr>
<tr>
<td>rs17266594</td>
<td>CC</td>
<td>5 (1.9)</td>
<td>7 (2.6)</td>
<td>0.3110</td>
<td>0.5771</td>
<td>0.72 (0.24, 2.18)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>52 (19.7)</td>
<td>76 (28.4)</td>
<td>5.4603</td>
<td>0.0195</td>
<td>0.62 (0.41, 0.93)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>207 (78.4)</td>
<td>185 (69.0)</td>
<td>4.4995</td>
<td>0.0339</td>
<td>1.53 (1.03, 2.27)</td>
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<tr>
<td></td>
<td>C</td>
<td>62 (21.7)</td>
<td>90 (16.8)</td>
<td>5.5367</td>
<td>0.0186</td>
<td>0.66 (0.47, 0.93)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>466 (88.3)</td>
<td>446 (83.2)</td>
<td>5.5367</td>
<td>0.0186</td>
<td>1.52 (1.07, 2.15)</td>
</tr>
</tbody>
</table>

### Table 4 - Distribution of genotypes among the ANA-positive patients with SLE and the control subjects

<table>
<thead>
<tr>
<th>Location (position)</th>
<th>Nucleotide change</th>
<th>GENOTYPIC FREQUENCY, (n (%))</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANA ((&lt;1:320)), (n = 85)</td>
<td>ANA ((\geq 1:320)), (n = 67)</td>
<td>SLE patients ((n = 160)) vs Control subjects</td>
</tr>
<tr>
<td>rs10516487</td>
<td>C/C</td>
<td>72 (77.4)</td>
<td>55 (82.1)</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>20 (21.5)</td>
<td>9 (13.4)</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>1 (1.1)</td>
<td>3 (4.5)</td>
</tr>
<tr>
<td>rs3733197</td>
<td>G/G</td>
<td>59 (63.4)</td>
<td>40 (59.7)</td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>33 (35.5)</td>
<td>25 (37.3)</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>1 (1.1)</td>
<td>2 (3.0)</td>
</tr>
<tr>
<td>rs17266594</td>
<td>C/C</td>
<td>1 (1.1)</td>
<td>3 (4.5)</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>20 (21.5)</td>
<td>10 (14.9)</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>72 (77.4)</td>
<td>54 (80.6)</td>
</tr>
</tbody>
</table>
variance responsible for the north–south stratification of the Han Chinese [25]. Metropolitan Shanghai showed a similar pattern, with the majority of individuals coming from the northern and central provinces, whereas the Cantonese group represents the southernmost cluster of the Han Chinese. Their findings highlight the importance of understanding these differences in the design and interpretation of studies to identify genes that confer susceptibility to such common diseases as SLE in ethnic Chinese individuals.

The LD and the results of the haplotype analyses suggested that both BANK1 SNPs in LD, either individually or as haplotypes, confer susceptibility to SLE. The strong association of both the protective TC haplotype and the risk CT haplotype, which was superior to that observed for both SNPs tested independently, suggests an additive effect of both BANK1 variants.

Autoantibody formation against self-antigens is one of the core features of autoimmune diseases such as SLE. Our examination of the associations between the three polymorphisms and the presence of autoantibodies revealed that SNP rs10516487 was significantly associated with high-titre ANA (Table 4). The T/T genotype frequency of rs17266594 was also higher among patients with high-titre ANA (>1:320; 80.6%) than among healthy controls (69.0%), although it did not reach statistical significance (P = 0.0672). The P-value is very close to achieving significance and likely would have done so if the sample size had been larger given the rs17266594 in almost complete LD with rs10516487. ANAs are hallmarks of SLE; these antibodies are found in 95% of the patients. Previous study showed that the serum levels of ANA-EIA were significantly higher in SLE patients with active disease compared with those with inactive or mildly active disease [26]. Higher serum levels of ANA-EIA also correlated with some clinical and laboratory manifestations of the disease such as: high anti-dsDNA antibodies, low C3 and/or C4, pyuria, arthritis and new rash. We also observed a correlation between the risk alleles of rs10516487 and rs17266594 in LD and a higher frequency of antibodies against SSA among SLE patients, which suggests that polymorphisms in BANK1 contribute to the underlying autoimmune process in SLE. This effect could be due to the many different effects of these two functional variants of BANK1 in the immune system. SNP rs10516487 leads to a non-synonymous substitution of arginine by histidine at position 61 (R61H) of the BANK-1 protein, which lies within the region essential for the binding of IP3R. SNP rs17266594 is located in a branch-point site and affects the relative splicing efficiency of BANK1, which in turn, could lead to a more active protein in at-risk individuals [10]. In the case of individuals carrying BANK1 functional mutations (R61H), altered B-cell activation through the B-cell receptor leads to BANK1 phosphorylation and signalling and could contribute to the production of autoantibodies as well.

The important role of functional BANK1 variants in the development of a variety of autoantibodies in autoimmune diseases has been supported by several studies [27, 28]. Thus, variations in BANK1 function could have profound effects on the modulation of B-cell activity and immune phenotypes. However, further functional studies are necessary to elucidate the exact molecular mechanisms by which BANK1 is implicated in the B-cell activation process and, more precisely, how it can lead to the development of SLE.

A recent GWAS in Chinese Han population [29] revealed that the BANK1 gene did not confer a significant risk of SLE (OR = 1.21; P = 0.0893). The potential causes for this discrepancy include heterogeneity in clinical subphenotypes and/or environment risk factors between two cohorts. In addition, multiple testing, the possibility of genotyping error and the genetic/geographical variation of the Han Chinese might also explain the disparity. It is therefore important to evaluate these results further, either by studying its potential biological relevance to the disease or by systematically attempting to replicate them in further studies.

In this study, we developed a new genotyping method involving an unlabelled probe HRM on the Roter-Gene Q instrument. It displayed 100% accuracy compared with

### TABLE 5 Distribution of genotypes among the anti-SSA-positive patients with SLE and the control subjects

<table>
<thead>
<tr>
<th>Location (position)</th>
<th>Nucleotide change</th>
<th>SSA+ (n = 160)</th>
<th>SSA- (n = 268)</th>
<th>SSA+ vs SSA-</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10516487</td>
<td>C/C</td>
<td>63 (85.1)</td>
<td>64 (74.4)</td>
<td>185 (69.0)</td>
<td>0.2322</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>10 (13.5)</td>
<td>19 (22.1)</td>
<td>76 (28.4)</td>
<td>0.0229</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>1 (1.4)</td>
<td>3 (3.5)</td>
<td>7 (2.6)</td>
<td>0.4966</td>
</tr>
<tr>
<td>rs3733197</td>
<td>G/G</td>
<td>48 (64.9)</td>
<td>50 (58.2)</td>
<td>172 (64.2)</td>
<td>0.4795</td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>23 (31.1)</td>
<td>34 (39.5)</td>
<td>80 (29.8)</td>
<td>0.1369</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>3 (4.0)</td>
<td>2 (2.3)</td>
<td>16 (6.0)</td>
<td>0.1369</td>
</tr>
<tr>
<td>rs17266594</td>
<td>C/C</td>
<td>1 (1.3)</td>
<td>3 (3.5)</td>
<td>7 (2.6)</td>
<td>0.0431</td>
</tr>
<tr>
<td></td>
<td>T/C</td>
<td>11 (14.9)</td>
<td>19 (22.1)</td>
<td>76 (28.4)</td>
<td>0.4666</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>62 (83.8)</td>
<td>64 (74.4)</td>
<td>185 (69.0)</td>
<td>0.4666</td>
</tr>
</tbody>
</table>
sequencing results. No real-time PCR or allele-specific amplification is needed. Compared with TaqMan-based SNP genotyping, the HRM approach is much more cost-effective. However, differentiating between the two possible homozygotes can be problematic when probes are not used. Unlabelled probe HRM is superior to conventional HRM in the identification of many small insertions or deletions and some Class 3 and 4 SNPs (~4% of human SNPs) [30, 31]. Unlabelled probes are usually approximately 30–40 bp in length and are blocked at their 3’ end to prevent extension [21, 22], so a high sensitivity rate can be obtained. By introducing an unlabelled probe covering the SNP, the different genotypes can be clearly distinguished.

Genotyping with unlabelled probes and PCR product melting have been successfully applied for the detection of disease-related SNPs such as factor V Leiden [32] or hot-spot mutations in cystic fibrosis [33] and even detection and typing of herpes simplex virus [34] and peroxisome proliferator-activated receptor (PPAR) [35]. Unlabelled probe HRM is high-throughput, inexpensive, and typing of herpes simplex virus [34] and peroxisome proliferator-activated receptor (PPAR) [35]. Unlabelled probe HRM is high-throughput, inexpensive, and typing of herpes simplex virus [34] and peroxisome proliferator-activated receptor (PPAR) [35].

The rs10516487 polymorphism is associated with SLE is replicated in Han population in Mainland China.

The $BANK1$ gene polymorphism is associated with the incidence of SLE and production of autoantibodies in a Chinese population.

**Rheumatology key messages**

- Unlabelled probes are inexpensive and allow unambiguous identification of multiple alleles by melting analysis.
- Association of $BANK1$ with SLE is replicated in Han population in Mainland China.
- The rs10516487 polymorphism is associated with ANA/SSA autoantibody response in SLE patients.

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**References**


