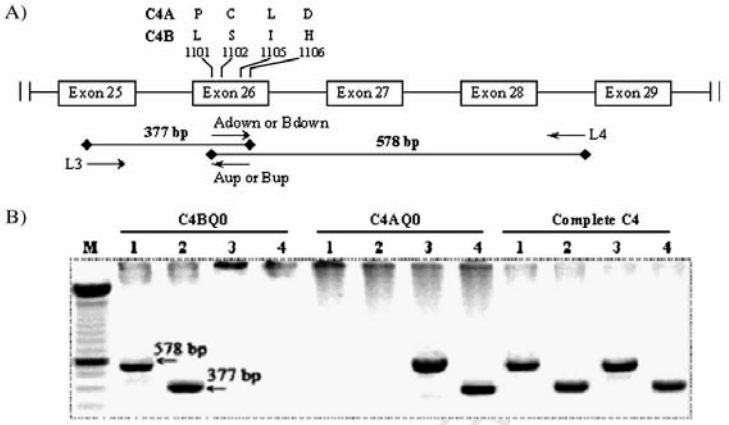

Fig. 1. Elucidating the genetic defect of the C4 genes. (A) Schematic representation of primer positions for touchdown PCR amplification. Exons are shown as solid boxes. PCR primers are shown in horizontal arrows, and the size of the amplicon is indicated. (B) The results of touchdown PCR for C4AQ0 and C4BQ0 genotyping. Lanes 1–4 are the products from a sample by 4 pairs of primers: Adown/L4, Aup/L3, Bdown/L4 and Bup/L3, respectively. M is the DNA marker. All amplicons were analyzed on 2% agarose gel electrophoresis.

non-related healthy controls was genotyped by "touchdown" PCR for C4AQ0 and C4BQ0 using 4 primer pairs: Aup or Bup/L3 and Adown or Bdown/L4, which amplified the different fragments: 377 bp and 578 bp in non-C4 deficiency (Fig. 1). No PCR product from C4AQ0 and C4BQ0 were amplified by these primers. The results revealed that 3 of 118 SLE patients (2.54%) were C4AQ0 indicated by showing only fragments of PCR products at 377 bp and 578 bp from Bup/L3 and Bdown/L4 amplifications, respectively with no PCR product from Aup/L3 and Adown/L4 amplification. In addition, 4 of 118 SLE patients (3.39%) were C4BQ0 indicated by giving 377 bp and 578 bp fragments from Aup/L3 and Adown/L4 amplifications, respectively with no PCR product from the other primer pairs. The rest of SLE patients and 145 normal controls gave 4 fragments at 377 bp and 578 bp from Aup/L3, Bup/L3, Adown/L4 and Bdown/L4, respectively. It is concluded that 7 of

Table 3
The oligonucleotide sequences (5′–3′) of primers used to screen C4 null gene and their common mutations by PCR

Gene	Code	Sequences (5′–3′)	Length of PCR product (bp)
C4A/C4AQ0	Aup	cgca tgc tcc tgt cta aca ctg ga	377/null
	L3	agc cca tcc agc agt ttc gga ag	
	Adown	agg acc cct gtc cagt tgt tag ac	578/null
	L4	ata gga tcc taa ggt ccc ctg ggc ct	
C4B/C4BQ0	Bup	tgc tcc tat gta tca ctg gag aga	377/null
	L3	agc cca tcc agc agt ttc gga ag	
	Bdown	agg acc tct ctc cag tga tac at	578/null
	L4	ata gga tcc taa ggt ccc ctg ggc ct	
C4 exon 26–29 for 2-bp insertion	C4E26.5	agg aac cca ggg gtc cag	860
	C4ins29	gct ctg aga acc agt gac tag ag	
C4 exon 13 for 1-bp deletion	C413delF	cat cac ctg gca ccc tcc ttt a	400
	C413delR	ctt gcc cat gtt gag ggg ct	
21-Hydroxylase	CYP21A	tgt ggc cat tga gga gga a	757
	CYP21B	tgc cac cga tca gga ggt c	
C4 exon 29/2 bp insertion in exon 29	C4ins29F	cac ttt cag ata acc tgt act g	59/61
	C4ins29R	cga cac ggc att gct ctg ag	
Specific fragment for sequencing	Adown or Bdown	as above	~2800/null
	C4 exon 32	gca cgt ggc cttt gac tgt ca	

118 (5.93%) SLE patients showed homozygous C4 null alleles: either C4AQ0 (3/118) or C4BQ0 (4/118). Interestingly, there was no homozygous C4AQ0 or C4BQ0 in normal control.

3.2. Mutation of C4 gene by SSP-PCR

The screening of two common mutations by multiplex PCR resulted in a flame-shift stop codon of the 2-bp insertion in exon 29 that caused a defective C4AQ0 or C4BQ0 and a novel 1-bp deletion in exon 13 of C4B gene. Both genotypes were analyzed by 2 pairs of primers that flanked the mutation sites C4E26.5/29ins29 and C413delF/C413delR which produced the mutant fragments at 860 and 400 bp, respectively. A primer pair of CYP21A/CYP21B was used as the internal control at 757 bp. The results showed that 117 from 118 SLE patients and all normal controls gave a band at 757 bp fragment (CYP21 gene; PCR positive control). There was only one patient who also had C4A deletion indicated by touchdown PCR above showed two fragments at 860 bp and 757 bp by multiplex PCR corresponding to 2-bp insertion allele in exon 29 of the C4B gene (Fig. 2).

To confirm the 2-bp insertion in exon 29, we performed other SSP-PCR by using C4ins29F/

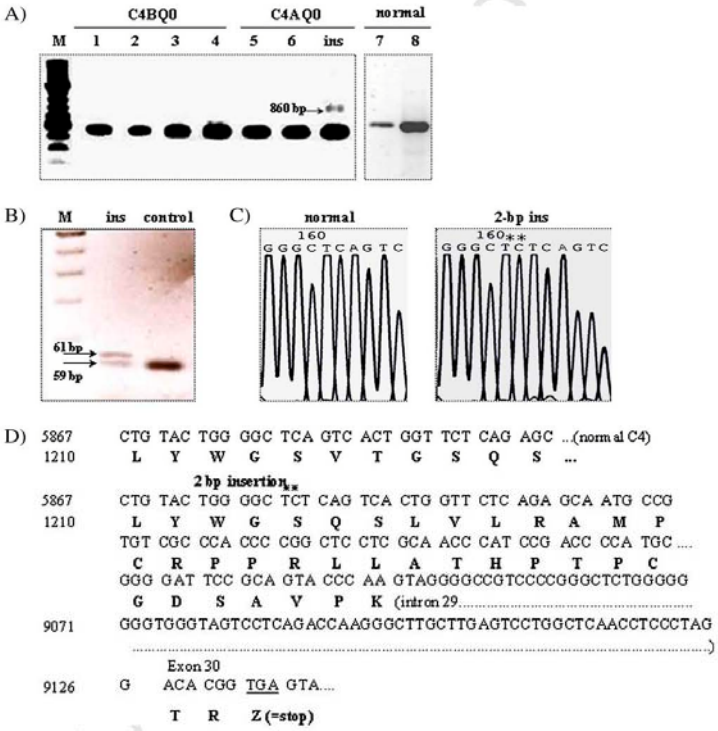

Fig. 2. Elucidating the molecular defect of the C4B mutant gene with C4AQ0. (A) Multiplex PCR to detect mutations in exons 13 and 29 of human C4 genes. The 860-bp band represents the 2-bp insertion in exon 29; the 757 bp band represents the CYP21 gene and serves as a positive control of the PCR. Lanes 1–6 showed only positive control band at 757 bp from individual patient with C4BQ0 (4 cases) and C4AQ0 (2 cases) genes, respectively. Lane "ins" showed the positive band at 757 bp and 2-bp insertion in exon 29 with a band at 860 bp from C4AQ0 patient. Lanes 7 and 8 are the examples of the band at 757 bp which are amplified from normal controls. M lane indicates the DNA marker. (B) The confirmation of mutation of C4B gene in C4AQ0 by containing the 2-bp insertion in exon 29 by SSP-PCR. The amplicon at 59 bp represent the normal control with no insertion sequence. A sample from C4AQ0 with 2-bp insertion in exon 29 on mutant C4B gene (lane "ins") revealed 2 bands at 59 bp and 61 bp. M is the DNA marker. (C) Sequence electropherograms from C4 exon 29 (non-coding strand) obtained by PCR amplification with ~2.8 Kb isotype specific fragment and sequence primer probed on intron 28. The normal sequence is shown at the left side and the mutationally altered sequence with the 2-bp insertion is shown at the right side (** is the nucleotide position of the insertion). (D) DNA and translated amino acid sequences of exon 29 around the insertion site and location of the stop codon in exon 30 (underlined) in comparison to the respective sequence of exon 29 of a normal C4 gene.

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C4ins29R primers that flanked the insertion site. Normal allele showed a DNA fragment of 59 bp whereas insertion allele showed a fragment of 61 bp. We found a DNA fragment of 59 bp from all of normal control (145 samples) and 117 of 118 SLE patients. Two fragments at 59 bp and 61 bp were amplified from the same SLE patient who showed C4AQ0 by touchdown PCR and 2-bp insertion by multiplex PCR (Fig. 2).

3.3. Two base pair insertion in codon 1213 screening by direct sequencing

The genomic DNA from both groups: SLE patients and normal control were studied by direct sequencing of ~2.8 Kb isotype specific fragment which was amplified by using primer pairs: A down or B down and C4 exon 32 primer revealed the 2-bp insertion in exon 29 of mutant C4B fragment in one SLE patient carrying C4AQ0 (Fig. 2). We concluded that this patient carried both C4AQ0 and heterozygous 2-bp insertion of C4B gene. There was no 2-bp insertion in exon 29 of neither C4A nor C4B gene in normal individual and the rest of SLE patients.

4. Discussion

We suggest that both C4AQ0 and C4BQ0 be the risk factors of SLE in Thai population. We found surprisingly no homozygous genetic deficiency of both isotypes in healthy population. C4Q0 was not found in the 145 control samples which was different from most intensive studies in Europeans and American Caucasians that showed the C4AQ0 frequencies in 0.5–2.93% among different normal ethnic groups [31]. However, we confirmed the studies in Europeans, Anglo-Saxons, Caucasians, African Americans, Chinese, Koreans, French and Japanese. The data from those populations showed higher prevalence of C4AQ0 in SLE patients than in normal controls [37,38]. The relative risk of C4AQ0 in SLE varied between 2.3 and 5.3 among different populations.

Moreover, we confirmed the studies in Spanish, Mexican and Australian Aborigine SLE patients [30,31]. The increased prevalence of C4B deficiency in comparison to C4A deficiency has been observed. Such phenomenon would suggest a delicate shift of physiologic roles of C4A and C4B among different ethnic groups or genetic backgrounds, or a difference in the genetic milieu, such as racial backgrounds [39,40]. It could change the dependence of C4A or C4B in the process of autoimmunity. Nevertheless, the importance of both C4A and C4B is clear because homozygous deficiency of both isotype is one of the strongest genetic risk factor for SLE. To date, the prevalence of SLE in Thai population has not been extensively investigated. However, our record between years 2002 and 2004 revealed that one of the 200 patients who attended the outpatient clinic at Ramathibodi Hospital, a university hospital in Bangkok was affected with SLE (Totemchokchyakarn, personal communications).

On the grounds of the studies in Northern and Central Europeans and in African Americans, the presence of mono-S, a RCCX haplotype with single short C4 gene is the significant cause of the C4AQ0 phenotype. The mono-S structures in the Europeans SLE patients and controls are present in a special haplotype, A1 Cw7 B8 DR3 (DRB1*0301) C4AQ0 B1. While in African Americans, it is frequently associated with haplotypes such as HLA B44 DRB1*1503 (DR2) instead of B8 DR3 [19–22,41,42]. Besides, the C4AQ0 in Orientals is not associated with HLA DR3.

A small proportion of C4A null alleles is a 2-bp (TC) insertion into the sequence for codon 1213 in the Caucasians and Africans (Caucasian SLE: 0.034, control: 0.004; African American SLE: 0.018, control: 0) [37]. Nonetheless, the 2-bp insertion is not present in Chinese SLE or normal population [23]. In contrast, mono-S RCCX module in C4AQ0 in Orientals is almost absent. Mono-L RCCX structure of C4B is present instead. At present the frequency of mono-L RCCX structures coding for C4B protein and the molecular basis leading to the non-expression of C4A have not been elucidated in the Oriental SLE patients [31].

From our finding, the 2-bp insertion in exon 29 of mutant C4B gene in one C4AQ0 by multiplex PCR was displayed. Moreover, we had confirmed this mutation by SSP-PCR and direct sequencing. This mutation was unlikely the major cause for C4A null alleles in Thais. Moreover, we did not find a 1-bp deletion in exon 13 commonly found in the mutation of C4B null alleles in C4BQ0 case. We concluded that this deletion was not the cause for C4B null alleles in our population either. It is strongly suggested that this mutation was not the result from mono modular of RCCX structure of C4B gene. The results from SSP-PCR gave the bands at 59 bp (normal) and 61 bp (insertion) in a C4AQ0 patient. It may be the results of duplicate or more RCCX modules with either long or short structure of C4B gene with one or more modules showing normal sequence associated with mutant sequence (2-bp insertion). Thus, the frequency of RCCX structures coded for C4 proteins level including the genetic basis resulting in C4Q0 in these populations would be studied further.

From our clinical observations, there was neither difference in clinical manifestation nor severity of SLE patients among those who were normal C4, C4AQ0 and C4BQ0 (Tables 1 and 2). Basically, C4A is more efficient in processing immune complexes and C4B has a greater hemolytic activity. Deficiency of C4A results in an impairment in the clearance of immune complexes and hence, their relative overproduction. These complexes