A simple PCR-based method for the rapid genotyping of inherited fifth complement component (C5)-deficient mice

Qingkai WANG1,#), Na WANG1,#), Xin ZHANG1), and Weiguo HU1,2)

1) Fudan University Shanghai Cancer Center and Institutes of Biomedical Sciences, Shanghai Medical College, Fudan University, Shanghai 200032, P.R.China
2) Department of Immunology, Shanghai Medical College, Fudan University, Shanghai 200032, P.R.China

Abstract: The fifth component of complement (C5) is considered to be the center of complement activation and function. However, there are no genetically engineered knockout mice for this gene, and the only commercially available inherited C5-deficient mice, in which a "TA" nucleotide deletion in the coding frame was previously identified, are in the C57BL/10Sn genetic background rather than the commonly used backgrounds C57BL/6 and BALB/c. Therefore, these mice must be backcrossed into the desired genetic background. Here, we developed an ARMS (amplification refractory mutation system) PCR method using a specific primer pair that was able to discriminate between the genotypes when the resulting product was analyzed by agarose gel electrophoresis. These results were supported by quantitative RT-PCR and semi-quantitative PCR and were consistent with the results from sequencing each backcrossed generation. Using ARMS-PCR method, we generated C5-deficient mice in the C57BL/6 background over 9 backcrossed generations and further verified the phenotype using complement-mediated hemolytic assays. In this study, we describe a simple, rapid and reliable PCR-based method for genotyping inherited C5-deficient mice that may be used to backcross C57BL/10Sn mice into other genetic backgrounds.

Key words: ARMS-PCR, C5-deficient mice, genotyping

Introduction

The complement system can be activated via the classical, alternative and lectin pathways, and the cleaved complement fragments and complexes execute versatile functions, including efficient immune surveillance and modulation of immunological and inflammatory processes. Upon activation, complement cascades converge at the C3 level using distinct C3 convertases that subsequently trigger the formation of fifth complement component (C5) convertases, thus leading to the cleavage of C5 to C5b and anaphylatoxin C5a. The C5b-7 complex inserts into the targeted cell membrane and assembles with C8 and C9, thereby inducing the polymerization of C9 to form a lytic pore, termed the membrane attack complex (MAC) [20]. C5a, the most potent anaphylatoxin, is a major pro-inflammatory mediator that involves in a wide range of diseases because it not only regulates the inflammatory process in innate immunity but also comprehensively modulates the adaptive immune response [9]. C5 can be cleaved independently of C3 convertase by serine proteases such as thrombin [6]. Therefore, C5 is considered to be at the center of complement activation and function.

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Address corresponding: W. Hu, Cancer Institute, Fudan University Shanghai Cancer Center, 270 Dong’an Road, Shanghai 200032, P.R.China
#These authors contributed equally to this work.
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A mouse with sufficient or deficient expression of a certain gene is a powerful tool to investigate the function of the gene. A genetic congenic strain usually refers to specially inbred strains of mice that differ only in restricted regions of the genome, and can be produced by repeated backcrosses to an inbred (background) strain with selection for a particular marker from the donor strain. C57BL/6 mice are the most widely used inbred strain of laboratory mice that can be genetically modified to serve as human disease models due to the availability of congenic strains, its easy breeding and robustness, clear genetic background and entire genome sequence. BALB/c mice are also inbred mice that are widely used in experimental research on immunology and cancer. This strain of mice is particularly well known for the production of monoclonal antibodies. Other stains, such as C3H/He, DBA/1 and 129/Sv, are also commonly used for different research purposes. However, the available C5-deficient mouse strains in The Jackson Laboratory are the C57BL/10Sn, DBa/2J, A/HeJ, AKR/J, NZB/BJN, and SWR/J rather than C57BL/6J background. This line was developed by Dr. George Snell during the late 1940s and early 1950s using tumor rejection to select each generation from an initial cross between C57BL/10Sn and DBa/2 mice. The DBa/2J strain, together with five other strains (A/HeJ, AKR/J, NZB/BJN, SWR/J and B10.D2/oSnJ), was later found to be inherited C5-deficient (Hc0/Hc0, homozygous mutations at the Hc gene locus on chromosome 2) due to a 2-base pair “TA” deletion at positions 661 and 662 of the C5 mRNA (NM_010406.2) coding frame [28]. The consequence of this deletion is the creation of a stop codon at positions 707–709 rather than at normal positions 5099–5101 in the mRNA, thus resulting in C5 deficiency. Until now, no one, including the strain provider, has developed a typical genotyping protocol for these mice. Although a recently reported gene-sequencing method provides an alternative approach to C5-deficient genotyping, it appears to be complicated [2]. Backcrossing into the desired genetic background for 9 generations is ideal to refine a lineage, and the method described here provides a simple and rapid approach for genotyping the inherited C5-deficient sequence.

Amplification refractory mutation system (ARMS)-PCR can rapidly analyze a known mutation in genomic DNA and provide an easy way to visually inspect a PCR amplification product in an agarose gel [15]. In this study, we designed a pair of specific primers to amplify the un-mutated DNA chain in Hc+/Hc+ and Hc+/Hc− mice using ARMS-PCR. This method resulted in a stronger band for Hc+/Hc+ than for Hc+/Hc− mice on an agarose gel, and no product was amplified for Hc−/Hc− mice. Thus, the strong, weak and absent bands in the agarose gel represent the Hc+/Hc+, Hc+/Hc− and Hc−/Hc− genotypes, respectively. This method was further verified by sequencing and by functional tests. Therefore, we provide a simple, rapid and reliable method of genotyping inherited C5-deficient mice.

Material and Methods

Animals and breeding
Parental wild-type C57BL/6 (Stock Number SLAC-0307, SLAC Laboratory Animal Co., Ltd., Shanghai, P.R. China) and C5-deficient (Stock Number 000461, background strain C57BL/10Sn, The Jackson Laboratory, Bar Harbor, Maine) mice were maintained at the animal facility of the Shanghai Medical School, Fudan University. All rodent work was performed with the consent of the Animal Ethics Committee at Shanghai Medical School, Fudan University. All the animals were fed a standard diet and maintained in a pathogen-free environment on a 12-h light/12-h dark cycle with ad libitum access to rodent chow.

Each breeding cage contained 1 male and 3 females that were approximately 8 weeks of age. The first generation of heterozygous Hc+/Hc− mice was obtained by crossing C57BL/6 and C5-deficient C57BL/10Sn mice. Then, the resulting Hc+/Hc− mice, termed the first generation, were backcrossed with C57BL/6 for 9 generations. Finally, Hc+/Hc− mice were intercrossed to obtain homozygous Hc0/Hc0 mice with a pure C57BL/6 genetic background. At each generation, we checked at least ten offspring to determine the genotype using both gene sequencing and ARMS-PCR methods.

DNA extraction from the mouse tail
At least 3 mm of the mouse tail was cut, and the bleeding was immediately stanched using mono-polar electrocoagulation. Genomic DNA was then extracted using the TailGen DNA Kit (CoWin Bioscience, Beijing, China), quantified using a NanoVue™ spectrophotometer (GE Healthcare Life Sciences, Pittsburgh, PA) and stored at −20°C until use as a PCR template.

Gene sequencing
We used a common reverse primer (R) and designed
a forward primer1 (F1), as shown in Fig. 1, to amplify the genomic fragments by PCR. The resulting fragment was 179 bp or 177 bp in length, depending on if the “TA” mutation site was present or deleted. Each PCR reaction was performed as follows: denaturation at 95°C for 3 min; then denaturation at 95°C for 15 s, annealing at 56°C for 25 s and extending at 72°C for 20 s for 30 cycles; followed by a final extension at 72°C for 1 min. The 20-µl reaction mixtures contained 2 µl of 5× buffer, 1.6 µl of 2.5 µM dNTP, 0.5 µl of 10 µM primers, 200 ng of template and 0.2 µl of rTaq Takara Taq™ (Takara Biotechnology, Dalian, China). The reaction was brought up to 20 µl with double-distilled H2O (ddH2O).

The PCR products were purified for gene sequencing by Biosune Biotechnology Co. (Shanghai, China), and the results were compared with the C5 genomic sequence of the C57BL/6J background that is available in the National Center for Biotechnology Information database (NC_000068.7, 34983331..35061449, chromosome 2, *Mus musculus* strain C57BL/6J).

To further confirm the genetic background of the harvested C5-deficient C57BL/6 mice, we also cut mouse tails from 2 females and 2 males C5-deficient mice (F9 in C57BL/6 background), 2 females and 2 males C5-deficient mice (F0 in C57BL/10Sn background) and 1 male wild type C57BL/6 mouse (positive control). The mouse tail DNA was isolated as describe above and sent for marker loci examination on each chromosome in a third-party company, Shanghai Biowing Applied Biotechnology Co., Ltd. (Wuxi, Jiangsu, China).

**ARMS-PCR and Semi-quantitative PCR**

Based on the mechanism of the ARMS-PCR, we designed forward primer 2 (F2) to amplify genomic fragments containing the mutated sequence when paired with the reverse primer (Fig. 1). Each PCR reaction was performed sequentially as follows: 95°C for 3 min followed by 35 cycles of 15 s at 95°C, 25 s at 62°C and 20 s at 72°C in the same reaction solution as mentioned above. And semi-qPCR was also performed to further validate the results of ARMS-PCR using a 360 bp β-actin genomic DNA fragment as internal control under the same condition but corresponding primer (Fig. 1). Ten microliters of each PCR product was electrophoresed through a 2% (wt/v) agarose gel that was then stained with ethidium bromide and photographed.

Considering that the quality and amount of DNA template may affect the amplification efficacy of ARMS-PCR and further assessment of the result, we performed DNA quality control by measuring OD260/OD280 ratio. Meanwhile, we optimized the amount of DNA template as indicated in Fig. 3E in ARMS-PCR with F2/R primers.

**Quantitative real time PCR (qRT-PCR)**

DNA extracts from Hc1/Hc1, Hc1/Hc0 and Hc0/Hc0 mice were used as templates, and forward primer 2 and the reverse primer served as the primer pair. qRT-PCR was performed in a total volume of 10 µl according to the manufacturer’s guidelines (Cat No.: 4367695, Invitrogen Co., Ltd.). The reaction program was set as follows: 10 min at 95°C followed by 45 cycles of 15 s at 95°C, 25 s at 62°C and 20 s at 72°C using an ABI 7900HT Fast Real-Time PCR System (Cat No.:7900HT, Applied Biosystems, Thermo Fisher Scientific Inc.). Each reaction included 3 replicates and a negative control with ddH2O in place of template.

**Complement-mediated hemolysis**

Human erythrocytes were collected from one healthy individual and stored in Alsever’s solution (Sigma-Al-

### Table 1.

The corresponding C5 gene sequences and primer design.

<table>
<thead>
<tr>
<th>Forward Primer 1</th>
<th>GAAGGATTTACACACACTGGAACGTGCA</th>
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<tbody>
<tr>
<td>Forward Primer 2</td>
<td>GAAGGATTTACACACACTGGAACGTGCTTA</td>
</tr>
<tr>
<td>C5 gene</td>
<td>18213:GAAGGATTTACACACACTGGAACGTGCA</td>
</tr>
<tr>
<td>18274:TAATATGTTATAATAACTAAGGGATT</td>
<td></td>
</tr>
<tr>
<td>18335:CAAGAAGAGGTGGGGGAGCACAGTGAC</td>
<td></td>
</tr>
<tr>
<td>GTAGTCATTGTGGATGACTAGTCGCTTG</td>
<td></td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>AAGCACTAGCTACTCAACAA</td>
</tr>
<tr>
<td>β-actin forward primer</td>
<td>ATGACCCAGGTGCAGTATCGGCTG</td>
</tr>
<tr>
<td>β-actin reverse primer</td>
<td>GAAACAGCTAAGTTCTGAGTCG</td>
</tr>
</tbody>
</table>

Fig. 1. The corresponding C5 gene sequences and primer design. The C5 gene sequences inside the rectangle from bp 18213 to 18391 contain the “TA” deletion (underlined and italic) in the inherited C5-deficient mice. F1 and the R were used in the PCR to amplify DNA for gene sequencing and ARMS-PCR, while forward primer 2 (F2) combined with R were used in the ARMS-PCR and qRT-PCR assays. β-actin forward primer and β-actin reverse primer was utilized in semi-qPCR as internal control corresponding to F2/R with Hc1/Hc1 and Hc1/Hc0 mice template.
drich, St. Louis, MO) at 4°C until use within three days. Mouse blood samples were obtained from the inferior vena cava of C57BL/10Sn and C57BL/6 C5-deficient, and C57BL/6 wild-type mice (3 mice in each group) after anesthetization with diethyl ether. After clotting for 45 min at room temperature, serum was harvested by centrifugation at 10,000 rpm for 10 min at 4°C, then pooled and used immediately. Then, hemolytic assays were performed to test complement function, as described previously [5].

Statistical analysis

The results of hemolysis were analyzed with two-way ANOVA analysis by statistical software GraphPad Prism 5.0.

Results

Identification of the C5-deficient mouse genotype using gene sequencing

Based on the complement C5 gene sequence, we designed forward primer 1 and a reverse primer, as shown in Fig. 1, to amplify the “TA”-containing DNA fragment by PCR. The expected PCR product sizes were 179 bp for Hc+/Hc+ mice and 177 bp for Hc0/Hc0 mice due to the “TA” nucleotide deletion, while heterozygous Hc+/Hc0 mice were expected to form both PCR products. It was difficult to discriminate between these two bands via agarose gel electrophoresis. Therefore, the fragments were purified and sequenced. The results clearly showed that the “TA” nucleotides were deleted in C5-deficient C57BL/10Sn mice (Fig. 2A) but were retained in C5-sufficient C57BL/6 mice (Fig. 2B). However, double-colored peaks appeared from the deleted “TA” position in the sequencing chromatograms of heterozygous mice resulting from crossing C57BL/10Sn with C57BL/6 mice (Fig. 2C) due to the presence of two different chromosomes either with or without this deletion. During the entire process of backcrossing heterozygous mice to the C57BL/6 background, we repeated the same procedure to verify the mouse genotype. The data demonstrated that all the offspring in each generation were Hc+/Hc+ or
Identification of the C5-deficient mouse genotype using ARMS-PCR

Although gene sequencing was able to identify the mouse genotype, this method was complicated and time-consuming. PCR is a widely used method for genotyping genetically engineered mice. Based on the deletion mutation in C5-deficient mice, we employed ARMS-PCR to identify this deletion using the specific forward primer 1, forward primer 2 and a reverse primer (Fig. 1). Forward primer 2 contains a 3-bp mutation at its 3’ terminus, i.e., one “A/T” mutation and two “AT” deletion mutations, which made it impossible to amplify the gene from C5-deficient mice at a given annealing temperature. The ARMS-PCR resulted in a clear band in the agarose gel when using DNA extracted from C5-sufficient C57BL/6 mice, but no band was evident when using template from C5-deficient C57BL/10Sn mice (Fig. 3A).

At each generation resulting from backcrossing the $Hc^1/Hc^0$ mice for nine generations (data not shown).
Hc\(^+\)/Hc\(^0\) mice to the Hc\(^+\)/Hc\(^+\) C57BL/6 background, the offspring demonstrated both the Hc\(^+\)/Hc\(^0\) and Hc\(^+\)/Hc\(^+\) genotypes. The ARMS-PCR products of Hc\(^+\)/Hc\(^0\) mice with F2/R primer pair may theoretically show a band of approximately half the intensity of that of the Hc\(^+\)/Hc\(^+\) mice with F1/R primer pair because only the single chromosome that lacks the mutation is an effective template in the PCR reaction. Indeed, the products amplified from DNA extracted from the Hc\(^+\)/Hc\(^0\) mice with F2/R primer pair were less bright than those from Hc\(^+\)/Hc\(^+\) mice with primer F1/R primer pair in an agarose gel (Fig. 3B). While using F1/R and F2/R primer pair respectively, the products of Hc\(^+\)/Hc\(^+\) mice were nearly identical resulting to the twins-like brightness. These results were further supported by semi-quantitative PCR and quantitative RT-PCR assays, in which products of β-actin control of Hc\(^+\)/Hc\(^+\) mice and Hc\(^+\)/Hc\(^0\) mice with its primer pair seemed to be the same, but there was a distinct consequence for F2/R primer pair with the same template (Fig. 3C). On the other hand, the delta normalized reporter (ΔRn) represents the magnitude of the signal generated by a given set of PCR conditions. As shown in Fig. 3D, the values of the ΔRn for Hc\(^+\)/Hc\(^+\) mice were 1.7- (4116/2433) and 4.5-fold (2623/583) higher than those for Hc\(^+\)/Hc\(^0\) mice at 45 and 35 cycles, respectively. However, the ΔRn value for Hc\(^0\)/Hc\(^0\) mice was approximately zero due to the lack of an effective template. Critically, all the results from the ARMS-PCR were consistent with those from gene sequencing at each generation of at least ten offspring, demonstrating the accuracy of ARMS-PCR in genotyping the inherited C5-deficiency. Therefore, this simple and rapid ARMS-PCR assay provides an alternative approach to the complicated gene sequencing method for genotyping inherited C5-deficient mice.

It should be noted that the principal of the current ARMS-PCR method for discriminating Hc mutant genotype depends on accuracy of band intensity. Only in heterozygous mice, the ARMS-PCR product bands with F2/R primers display half intensity of those with F1/R primers (Fig. 3B). Therefore, we recommend: 1) the ARMS-PCR with tetra-primer (F1/R and F2/R) appears better than with only one pair of primer (F/R), in which the F1/R primers can be at least regarded as the positive control; 2) the quality of DNA template is essential. The reliable commercial mouse tail DNA isolation kit and the subsequent OD\(_{260}/\text{OD}_{280}\) ratio measurement are required. In this study the OD\(_{260}/\text{OD}_{280}\) ratio of mouse tail DNA samples is 1.8 to 2.0 (data not shown); and 3) the template DNA amounts need to be optimized. The extremely-low or –high concentrated DNA templates may make a difficult even incorrect assessment. As shown in Fig. 3E, we chose 200 ng DNA templates for the experiments, the PCR band produced by which seems appropriate.

**Verification of the C5 deficiency in the C57BL/6 background**

After 9 generations of backcrossing C57BL/10Sn to C57BL/6 mice, we obtained C5-deficient mice in the C57BL/6 background. In each generation, we determined the mouse genotype via ARMS-PCR and further verified the genotype by gene sequencing (Fig. 2D). Moreover, the C5-deficient mice were functionally verified by complement-mediated hemolysis assays. Human erythrocytes were sensitized by rabbit anti-human erythrocyte polyclonal antibodies, and mouse serum from wild-type or C5-deficient mice of the C57BL/6 background was used as a source of complement. The data represent the mean ± sd, n=3 and were analyzed with GraphPad Prism software. **P<0.01 and * P<0.05 vs. WT.**
with C57BL/6 genetic background in comparison with database of wild type C57BL/6J, and found that all the marker loci are exactly same among them in all 20 chromosomes (data not shown). In addition, considering that tiny amounts of locus in C57BL/10Sn might be retained to C57BL/6J in Hc0/Hc0 mice, we randomly selected three SNPs (rs13477866, rs13476874 and rs6271003) between C57BL/6J and C57BL/10Sn to exclude this possibility. The results also revealed that three tested SNPs in four Hc0/Hc0 C57BL/6J mice are same as those in wildtype C57BL/6J mice, which are truly different from those in Hc0/Hc0 and wildtype C57BL/10Sn mice. Therefore, we successfully established a simple and rapid method for genotyping inherited C5-deficient mice and generated C5-deficient mice in the C57BL/6 background.

Discussion

C5 is a critical component in complement cascade, and the accumulating evidence implied that C5 plays a critical role in whole process of many inflammatory diseases and some kinds of tumors [7, 8, 10, 11, 13, 14, 18, 20]. Therefore, inhibition of C5a-C5aR axis has been demonstrated an effective therapeutic approach for some human diseases with uncontrolled and deleterious complement activation [1, 13, 27, 30]. The inherited C5-deficient human being families [4, 12, 22, 23, 25] and more importantly mouse strains [3, 16, 17, 19, 21, 24, 26, 27, 29] have already been reported, which triggers the field to produce a C5-deficient mouse strain for experiment study. Although Wetsel et al. revealed that a 2-base pair gene deletion resulted in the inherited C5 deficiency in some mouse strains including DBA/2J background [28], there is no available C5-deficient stains in the common genetic backgrounds such as C57BL/6J. Therefore, in this study we generated a simple ARMS-PCR-based method for rapidly genotyping the inherited C5-deficient mice in backcrossed C57BL/6 background. Meanwhile, using this method we successfully produced the inherited C5-deficient mice with pure C57BL/6 background by nine-generation backcross, which were further proved by gene sequencing, functional test and marker loci examination on each chromosome.

In our ARMS-PCR method, design of the specific second pair of primers F2/R is the most critical, in which F2 forward primer can induce three nucleotides mismatch in 3’ terminal only happening in the Hc0 mutant chromosome. Therefore, Hc1/Hc1 mouse DNA templates with two effective chromosomes could produce about 2-fold stronger bands than Hc1/Hc0 mouse with only one effective chromosome; while there was no visible band for Hc0/Hc0 mouse due to without any effective template. Given the limited number of amplification cycles for a certain amount of DNA template in ARMS-PCR method, the quality and amount of DNA templates should be the crucial determinant. Here, to successfully generate the ARMS-PCR method, we first controlled the quality of DNA templates by using a reliable mouse DNA isolation kit and further measuring the ratio of OD260/OD280. Next we still optimized the amount of templates as 200 ng with 35 amplification cycles in ARMS-PCR (Fig. 3E). Therefore, we strongly believe that the quality control and amount optimization of DNA templates are prerequisites for the successful of ARMS-PCR method. Together with the results of hemolytic assay, gene sequencing and marker loci examination on each chromosome, the ARMS-PCR results strongly demonstrated that the harvested mutant mice with nine-generation backcross are indeed inherited C5-deficient in C57BL/6 genetic background.

Therefore, ARMS-PCR is a simple, rapid and reliable method for mouse genotyping. It can also be used in the generation of inherited C5-deficient mice in another genetic background such as BALB/c. According to the sequence of the gene in the BALB/c strain, the same ARMS-PCR primers can be used without any changes (Fig. 2E). Furthermore, this method can be used to genotype other mouse strains with different inherited gene deletions.

Disclosures

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