Biotechnol Lett (2013) 35:515–522
DOI 10.1007/s10529-012-1115-0

ORIGINAL RESEARCH PAPER

Detection of single nucleotide polymorphisms by PCR conformation-difference gel electrophoresis

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Received: 13 September 2012 / Accepted: 28 November 2012 / Published online: 19 December 2012

Abstract The most common genetic variations in the human genome, single nucleotide polymorphisms (SNPs), are ideal biomarkers and are used extensively in disease research. Here we introduce a novel method of PCR-conformation-difference gel electrophoresis (PCR-CDGE) used for detecting SNPs. The principle of PCR-CDGE relies PCR products from different homozygous DNA samples showing dissimilar migration patterns upon PAGE due to their conformational differences. PCR products from heterozygous DNA samples may exhibit two or more bands in PAGE because of the existence of DNA homoduplexes and heteroduplexes. In this study, analysis of two SNPs showed that PCR-CDGE is an accurate, simple, rapid, low-cost, and high-throughput genotyping method that could be used in most laboratories.

Keywords Conformation-sensitive gel electrophoresis · High throughput · PCR-conformation-difference gel electrophoresis · Single nucleotide polymorphisms

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Introduction

Single nucleotide polymorphisms (SNPs) are the most common genetic variations in the human genome, with approximately 15 million detected to date (Altshuler et al. 2010b). The occurrence, development, and prognosis of multiple diseases have been associated with certain SNPs (Zhang et al. 2011; Palmer et al. 2011; Ho et al. 2011; Lubbe et al. 2012; Stanulla et al. 2007; Smits et al. 2011; Li et al. 2012). Moreover, some SNPs may be associated with the efficacy and toxicity of drugs used to treat certain illnesses (Shon

et al. 2011; Park et al. 2011; Schroth et al. 2009). Therefore, accurate detection of SNPs is important in diagnosing and treating disease.

SNP genotyping can be accomplished using several different methods, including restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), allele-specific oligonucleotide analysis (ASO), allele-specific amplification (ASA), denaturing gradient gel electrophoresis (DGGE), TaqMan probes, high-resolution melt (HRM) analysis, denaturing high-performance liquid chromatography (DHPLC), SNP chips, and DNA sequencing (Voisey and Morris 2008; Kinoshita-Kikuta et al. 2002). However, these genotyping methods involve complicated processes, specific apparatus, expensive reagents, and/or a high skill level (Kinoshita-Kikuta et al. 2002). Therefore, an accurate, simple, rapid, low-cost, and high-throughput SNP genotyping method that can be performed in standard laboratories is desirable.

Conformation-sensitive gel electrophoresis (CSGE) is mainly used to detect DNA mutations (Ganguly 2002). According to the principle of this method, mild denaturing solvents can amplify the tendency of single-base mismatches to produce conformational changes. This increases the differences in migration of DNA homo- and heteroduplexes during polyacrylamide gel electrophoresis (PAGE) (Ganguly et al. 1993). CSGE is also used to detect SNPs (Blesa et al. 2004; Leung et al. 2001). However, in most cases, a single CSGE protocol cannot distinguish between two types of DNA homoduplex associated with the same SNP, because the mobility of DNA homoduplexes is similar. Therefore, to differentiate these DNA homoduplexes, a second CSGE (CSGE-2) protocol using 1:1 mixtures of PCR product corresponding to one band, and PCR product of a control homozygous sample to allow heteroduplex formation, should be undertaken (Blesa et al. 2004; Leung et al. 2001). Leung et al. (2001) found that CSGE could not only distinguish SNP DNA homo- and heteroduplexes, but that it could also distinguish DNA homoduplexes of the c. −1081A>G polymorphism in *MYOC*. Compared with standard PAGE, the crosslinker for CSGE, 1,4-bis (acryloyl)piperazine, is expensive. Furthermore, the running time for CSGE gels is longer than for standard PAGE analysis (Ganguly 2002). Therefore, if SNPs could be directly genotyped using standard PAGE analysis, the cost of the experiment would be reduced. Additionally, because of the shorter running time, the detection efficiency would be improved with the use of standard PAGE techniques. In this study, we used standard PAGE to analyze the *NQO1* rs1800566 polymorphism and the *CHRNA3* rs12910984 polymorphism. Based on the results of these analyses, we conclude that standard PAGE, which we refer to as PCR-conformation-difference gel electrophoresis (PCR-CDGE), can be used for SNP genotyping, and provides an accurate, simple, rapid, low-cost, high-throughput SNP genotyping method that can be performed in most laboratories.

Materials and methods

Participants

Oral swab samples were provided by 130 undergraduate students (58 males and 72 females, age range: 18–23 years) who attended Nanchang University, China. Participants rinsed their mouths with clean water then rotated a sterile cotton swab against the buccal mucosa ten times. All participants signed a written informed consent. This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Nanchang University (approval ID: 2011022).

DNA extraction

Genomic DNA was extracted from oral swab samples using the salting out method (Lahiri and Nurnberger 1991), with slight modifications.

PCR amplification

PCR primers were designed using Primer3 software (http://fokker.wi.mit.edu/primer3/). The sequences of the primers used to detect the polymorphisms were as follows: the *NQO1* rs1800566 polymorphism forward primer 5′-GAAGCCCAGACCAACTTCTG-3′ and reverse primer 5′-AGGCTGCTTGGAGCAAAATA-3′; the *CHRNA3* rs12910984 polymorphism forward primer 5′-TGCACCTGGCCTGATTTTTA-3′ and reverse primer 5′-GGGCTAGTTCACCACTTTGC-3′. The size of the amplification product was 247 bp for the *NQO1* rs1800566 polymorphism, and 237 bp for the *CHRNA3* rs12910984 polymorphism. PCR was carried out in 10 μl containing 5 ng genomic DNA template, *Pfu* master mix (Tiangen Biotechnology Co., Beijing, China), and 0.2 μM of each primer (Invitrogen, Shanghai, China). PCR reactions were performed

using a MG96G/Y thermal cycler (LongGene Scientific Instruments, Hangzhou, China). Thermal cycler parameters were as follows: an initial denaturing step of 94 °C for 3 min, 35 cycles of 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 15 s, followed by a final extension of 72 °C for 5 min.

PAGE

A DYCZ-20C DNA sequence analysis electrophoresis tank (Liuyi Instrument Factory, Beijing, China) was used. The gel was 30 × 34 cm in size and 0.4 mm in thickness. A 100-well sharkstooth comb was used. The gel contained the following chemical ingredients: TBE (Tris/borate EDTA), 13.3 ml acrylamide-bisacrylamide (49:1), 26.5 ml deionized water, 0.04 % ammonium persulfate, 20 µl *N*,*N*,*N*′*N*′-tetramethylethylenediamine. The gel was polymerized at room temperature for about 1 h. A pre-run was conducted in TBE for 10 min at 300 V, and then 0.6 µl PCR product was loaded into each well. Gels were then run at 800 V for 2.5 h. Following electrophoresis, gels were fixed in 10 % (v/v) ethanol for 5 min, and then briefly rinsed with distilled water two times, for 20 s each time. Gels were then pretreated in 1 % (v/v) nitric acid for 2 min before being quickly rinsed twice with distilled water for 20 s each time. Gels were stained in 0.2 % $AgNO_3$ for 5 min and then briefly rinsed twice with distilled water for 30 s each time. Gels were developed in a 100 ml containing 3 % (w/v) Na_2CO_3 and 0.1 % formaldehyde. When the desired band intensity was achieved, development was stopped by addition of 100 ml 10 % (v/v) acetic acid. Stained gels were rinsed with distilled water and then dried on filter paper at room temperature. Dried gels were scanned using a LiDE200 scanner (Canon, Tokyo, Japan).

For high-throughput detection, gels were loaded with five rows of PCR products with ten samples per row, allowing 10 min between rows. The entire electrophoresis process was completed in 2.5 h.

Restriction enzyme digestion-based genotyping

The *NQO1* rs1800566 polymorphism was genotyped by restriction digestion in a reaction of 15 µl, containing 1 µl 10× FastDigest Green Buffer, 4 µl PCR product, and 1 µl FastDigest *Hin*fI (Fermentas, Vilnius, Lithuania), at 37 °C for 5 min. The rs12910 984 polymorphism was digested in 15.5 µl, which included 1 µl 10× Buffer Tango, 4 µl PCR product, and 0.5 µl *Alu*I (Fermentas). Ten of the 130 samples were digested with *Alu*I overnight in a water bath at 37 °C. The digestion products were separated on a 2 % (w/v) agarose gel containing 0.5 µg ethidium bromide ml^{-1}. Finally, the gel was photographed using a JS-680B gel imaging system (Shanghai Peiqing Science and Technology Co., Shanghai, China).

DNA sequencing

Samples that showed different banding patterns on PAGE were amplified and then sequenced by the Beijing SinoGenoMax Genomic Center (Beijing, China).

Results

CDGE

Electrophoresis of the *NQO1* rs1800566 polymorphism gave three band patterns (Fig. 1a): a single slow-moving band, a single fast-moving band, and double bands, which were a combination of the two single bands. Among the 130 samples, 38, 30, and 62 samples showed the slow-moving, fast-moving, and double band patterns, respectively.

Electrophoresis of the *CHRNA3* rs129109843 polymorphism produced three band patterns (Fig. 1b): a single slow-moving band, a single fast-moving band, and triple bands, which were a combination of the two single bands with a third, slower-moving band. Among the 130 samples, 42, 29, and 59 samples showed the fast-moving, slow-moving, and triple band patterns, respectively.

Figure 2 shows a representative example of the DNA band patterns from CDGE analysis of the *NQO1* rs1800566 polymorphism. Based on the results of electrophoresis, the three band patterns in five rows of loading could be clearly separated (Fig. 2).

Restriction enzyme digestion

Using *Hin*fI digestion to genotype the *NQO1* rs1800566 polymorphism, we observed that 62, 38, and 30 samples had the CT, CC, and TT genotypes, respectively. Figure 3a shows the results of restriction enzyme digestion of ten samples, corresponding to the samples

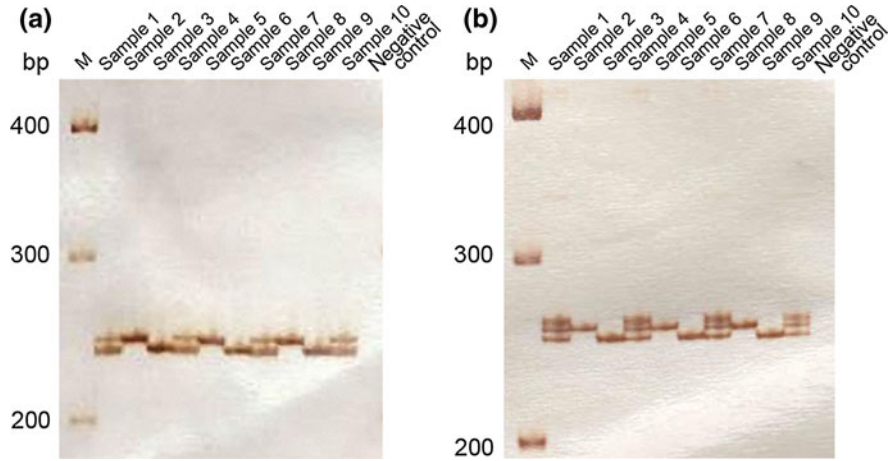

Fig. 1 Gel image of **a** the *NQO1* rs1800566 polymorphism and **b** the *CHRNA3* rs12910984 polymorphism following 2.5 h of electrophoresis. *M* is DNA Marker I, and *lanes 1*, *4*, *7*, and *10* show double bands. *Lanes 2*, *5*, and *8* show single slow-moving bands, while *lanes 3*, *6*, and *9* show single fast-moving bands. *Lane 11* is the negative control

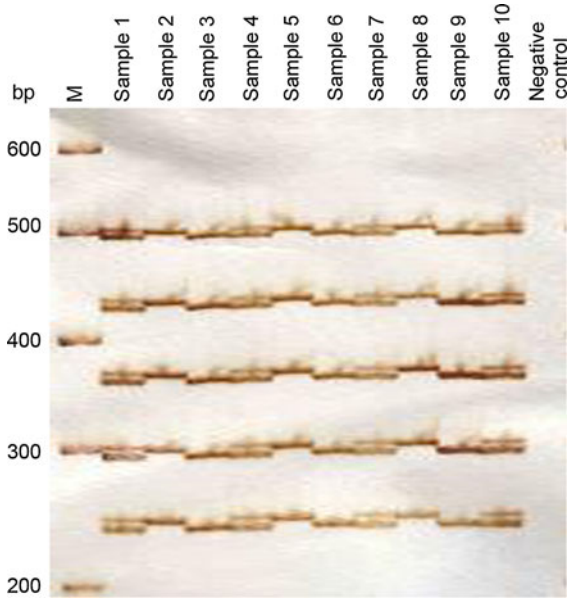

Fig. 2 High-throughput CDGE analysis. CDGE gel showing ten amplicons from the *NQO1* rs1800566 polymorphism that were analyzed in five rows. The image shows sufficiently high resolution to allow correct interpretation. *M* is DNA Marker I, and *lanes 1*, *4*, *7*, and *10* show double bands. *Lanes 2*, *5*, and *8* show single slow-moving bands, and *lanes 3*, *6*, and *9* show single fast-moving bands. *Lane 11* is the negative control

described in Fig. 1a. Figure 3b shows the results of restriction enzyme digestion of the ten samples in Fig. 1b, for the *CHRNA3* rs12910984 polymorphism.

Comparison of CDGE and restriction enzyme digestion results

We determined that the restriction enzyme cleaved the single slow-moving band seen in CDGE for the *NQO1* rs1800566 polymorphism but not the fast-moving band. Double bands in CDGE analysis produced two bands upon enzyme digestion.

While investigating the *CHRNA3* rs12910984 polymorphism, we found that the restriction enzyme digested only the samples which showed a single slow-moving band pattern in CDGE, but not those that produced a fast-moving band. Three bands were produced by restriction enzyme digestion of samples showing triple bands in CDGE.

DNA sequencing results

While investigating the *NQO1* rs1800566 polymorphism, we found that homozygous TT and CC samples corresponded with the single slow-moving and fast-moving bands in CDGE, respectively, whereas samples showing double bands were heterozygous CT. These results consistently agreed with the restriction enzyme digestion results. Figure 4a–c show the DNA sequencing results for the different band patterns for the *NQO1*rs1800566 polymorphism.

For the *CHRNA3* rs12910984 polymorphism, we determined that homozygous GG and AA samples corresponded with the single slow-moving and single fast-moving band patterns in CDGE, respectively, whereas triple bands corresponded with heterozygous AG samples. Again, these results consistently agreed with the restriction enzyme digestion results. Figure 4d–f show the DNA sequencing results for the different band patterns for the rs12910984 polymorphism.

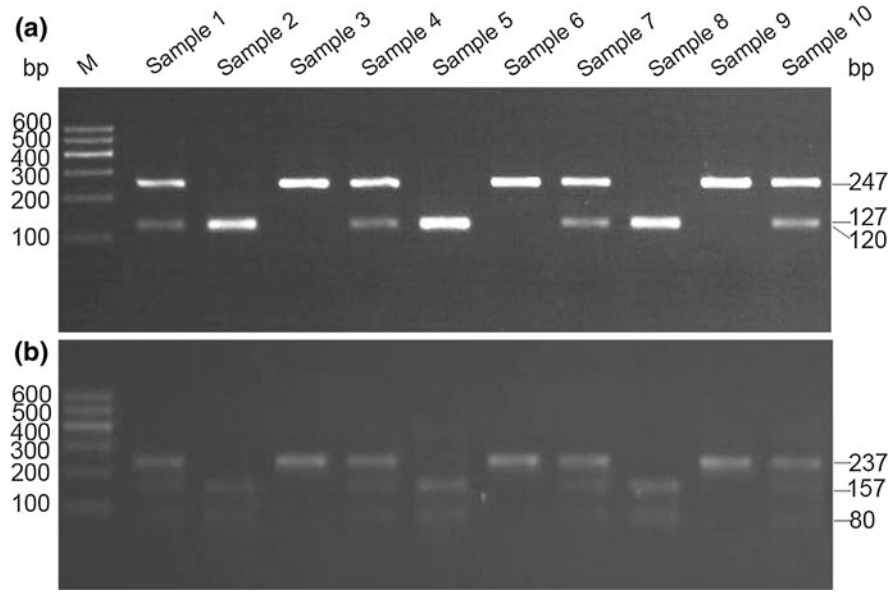

Fig. 3 Gel images of **a** the *NQO1* rs1800566 polymorphism and **b** the *CHRNA3* rs12910984 polymorphism genotyping by PCR–RFLP. *M* is DNA Marker I, and *lanes 1*, *4*, *7*, and *10* show CT genotype. *Lanes 2*, *5*, and *8* show TT genotype, *lanes 3*, *6*, and *9* show CC genotype

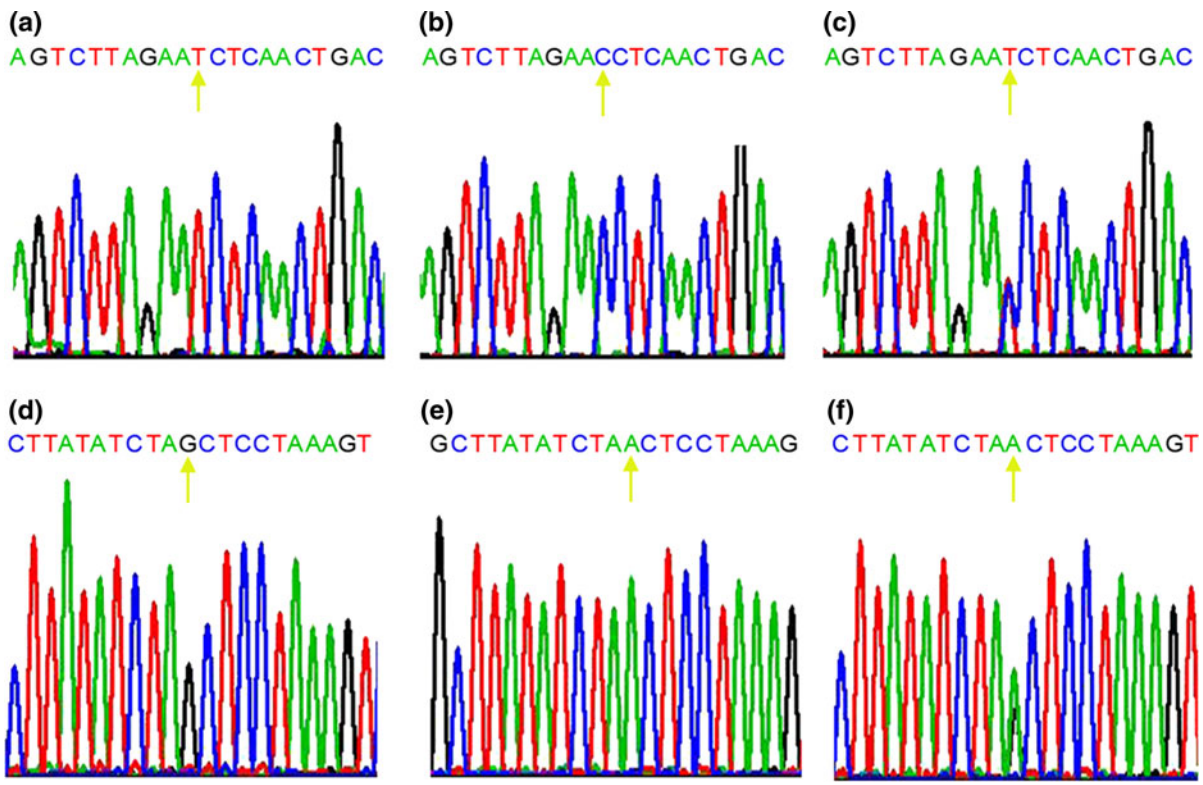

Fig. 4 DNA sequencing of **a**–**c** the *NQO1* rs1800566 polymorphism and **d**–**f** the *CHRNA3* rs12910984 polymorphism. **a** TT genotype, **b** CC genotype, **c** CT genotype, **d** GG genotype, **e** AA genotype, and **f** AG genotype

Discussion

The PCR products of homozygous samples contained only a single type of homoduplex DNA molecule. Therefore, these molecules appeared as single bands in CDGE. However, different homozygous samples showed different mobilities due to variations in their conformation. Therefore, the two types of homozygous samples could be differentiated by CDGE. In addition to these two homoduplexes, PCR products from heterozygous samples included two types of heteroduplex. Thus, depending on the mobility of these four DNA molecules, two to four bands could be produced by electrophoresis of heterozygous samples. We therefore designated samples showing two or more bands in electrophoresis as heterozygotes. As genotyping of SNP was performed by PAGE based on differential conformation of PCR products, we named this technique polymerase chain reaction conformation-difference gel electrophoresis (PCR-CDGE).

In this method for SNP genotyping, methylene diacrylamide was used as the crosslinker, no denaturant was added to the gel, and standard TBE buffer was used for electrophoresis. Therefore, the cost of CDGE is much lower than that of CSGE. Moreover, CDGE can be performed in a significantly shorter time. Although CDGE does not use a denaturant, results for the *CHRNA3* rs1290984 polymorphism showed that it can differentiate DNA homo- and heteroduplexes. Because CDGE is a SNP detection technology based on differential conformation of genotypes, it cannot directly distinguish the homozygous genotype resulting from two types of single-band samples. However, subsequent direct DNA sequencing can differentiate between the two homozygous samples.

With the completion of the human genome project (Lander et al. 2001), HapMap 3 (Altshuler et al. 2010a), and the 1,000 genomes project (Altshuler et al. 2010b), a great number of SNPs have been associated with the occurrence and treatment of disease. For example, the *NQO1* rs1800566 polymorphism is associated with a variety of malignant tumors (Fagerholm et al. 2008; Zhang et al. 2003; Lanciotti et al. 2005; Malik et al. 2011; Sameer et al. 2010), susceptibility to benzene poisoning (Chen et al. 2007), multiple sclerosis (Stavropoulou et al. 2011), and response to breast cancer treatment (Jamieson et al. 2011). Therefore, it is necessary to establish accurate, rapid, low-cost, and high-throughput SNP detection methods that are easy to perform. PCR–RFLP is a method that is commonly used for SNP genotyping. The results of CDGE of the two SNPs studied here consistently agreed with the findings of PCR–RFLP. This confirmed the reliability of CDGE in SNP genotyping.

Unlike PCR–RFLP, CDGE does not require endonuclease, which significantly reduces the cost of the technique. Even if the electrophoresis buffer and silver staining reagents used in this process are freshly prepared (the electrophoresis buffer, the ethanol solution, the nitric acid solution, and the $AgNO_3$ solution can be used more than ten times), the cost of the entire CDGE process is approximately 36 US cents. In comparison, the cost to genotype one sample for the *NQO1* rs1800566 polymorphism by PCR–RFLP is approximately 54 US cents. As an example of high-throughput detection of DNA variations using CSGE, 380 amplicons from exon 1 of human *TIMM8A* were analyzed in 12 rows, resolving 32 samples per row (Blesa et al. 2004). Based on the original CSGE protocols, Leung et al. (2001) developed an improved high-throughput CSGE (HTCSGE) method for safe and reliable screening of both heterozygous and homozygous SNPs or mutations in a large number of DNA samples.

This study also showed a satisfactory result for high-throughput CDGE analysis of the *NQO1* rs1800566 polymorphism. With a 100-well sharks-tooth comb and five rows of samples, CDGE could separate nearly 500 samples at one time. This further decreases the cost and increases efficiency. Compared with SSCP, CDGE does not require denaturation of PCR products, so the manipulation is simpler and the cost is lower. Furthermore, the electrophoresis time required by CDGE is much shorter than that required by SSCP. Compared with DGGE, HRM, DHPLC, and DNA sequencing, CDGE requires no special instruments or expensive reagents, other than pouring a standard polyacrylamide gel in TBE buffer. In summary, PCR-CDGE is an accurate, simple, fast, low-cost, and high-throughput SNP genotyping method, which can be used in standard molecular biology laboratories.

Acknowledgments The authors are very grateful to the students who provided DNA samples in this study. This work was partially supported by the National Natural Science Foundation of China (Grant No. 30860319), the Jiangxi Science and Technology Support Program (Grant No. 2009BSB09502),

and the Jiangxi Provincial Health Department of Science and Technology Plan (Grant No. 20072023).

Conflict of interest The authors declare that they have no conflict of interest.

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