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A Novel Mismatched PCR-Restriction Fragment Length Polymorphism Assay for Rapid Detection of *gyrA* and *parC* Mutations Associated With Fluoroquinolone Resistance in *Acinetobacter baumannii*

Naoki Kakuta, M.D.[1,*], Ryuichi Nakano, Ph.D.[1,*], Akiyo Nakano, M.S.[1], Yuki Suzuki, M.S.[1], Ayako Tanouchi, M.S.[1], Takashi Masui, M.D.[1,2], Saori Horiuchi, M.S.[1], Shiro Endo, M.D.[3], Risako Kakuta, M.D.[4], Yasuo Ono, M.D.[5], and Hisakazu Yano, M.D.[1]

[1]Departments of Microbiology and Infectious Diseases and [2]Otolaryngology-Head and Neck Surgery, Nara Medical University, Nara, Japan; [3]International University of Health and Welfare, Shioya Hospital, Tochigi, Japan; [4]Department of Otolaryngology-Head and Neck Surgery, Tohoku University Graduate School of Medicine, Miyagi, Japan; [5]Department of Microbiology and Immunology, Teikyo University School of Medicine, Tokyo, Japan

Background: Mutations in the quinolone resistance-determining regions (QRDRs) of *Acinetobacter baumannii* DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) are linked to fluoroquinolone (FQ) resistance. We developed a mismatched PCR-restriction fragment length polymorphism (RFLP) assay to detect mutations in the *gyrA* and *parC* QRDRs associated with FQ resistance in *A. baumannii*.

Methods: Based on the conserved sequences of *A. baumannii gyrA* and *parC*, two primer sets were designed for mismatched PCR-RFLP to detect mutations in *gyrA* (codons 83 and 87) and *parC* (codons 80 and 84) by introducing an artificial restriction enzyme cleavage site into the PCR products. This assay was evaluated using 58 *A. baumannii* strains and 37 other *Acinetobacter* strains that have been identified by RNA polymerase β-subunit gene sequence analysis.

Results: PCR amplification of *gyrA* and *parC* was successful for all *A. baumannii* strains. In 11 FQ -susceptible strains, the *gyrA* and *parC* PCR products were digested by the selected restriction enzymes at the site containing *gyrA* (codons 83 and 87) and *parC* (codons 80 and 84). PCR products from 47 FQ-resistant strains containing mutations in *gyrA* and *parC* were not digested by the restriction enzymes at the site containing the mutation. As for the non-*baumannii Acinetobacter* strains, although amplification products for *gyrA* were obtained for 28 strains, no *parC* amplification product was obtained for any strain.

Conclusions: This assay specifically amplified *gyrA* and *parC* from *A. baumannii* and detected *A. baumannii gyrA* and *parC* mutations with FQ resistance.

Key Words: Quinolone resistance-determining regions, PCR-restriction fragment length polymorphism, *Acinetobacter baumannii*, Fluoroquinolone resistance

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Corresponding author:
Ryuichi Nakano, Ph.D.
Department of Microbiology and Infectious Diseases, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan
Tel: +81-744-29-8839
Fax: +81-744-29-8839
E-mail: rnakano@naramed-u.ac.jp

*These authors contributed equally to this work.

INTRODUCTION

Fluoroquinolones (FQs) are widely used to treat various bacterial infections [1, 2]. FQ resistance has increased globally in *Acinetobacter* species, which are clinically important pathogens that frequently cause infections among intensive care unit patients [2-5].

In *A. baumannii*, FQ resistance occurs mainly through muta-

tions in the quinolone resistance-determining regions (QRDRs) of DNA gyrase (*gyrA*) and topoisomerase IV (*parC*), although overexpression of efflux pumps can contribute to FQ resistance [6-10]. The most frequently described mutations in *A. baumannii* are within the QRDRs at Ser-83 in GyrA and at Ser-80 and Glu-84 in ParC [6-8, 11]. In particular, a double mutation, affecting the Ser-83 of GyrA and Ser-80 or Glu-84 of ParC, renders *A. baumannii* highly FQ resistant [7, 11]. The single mutation affecting Glu-87 of GyrA, an important mutation associated with FQ resistance in other gram-negative microorganisms [12], has rarely been found in *A. baumannii* [8]. Detection of these mutations is therefore important for assessing FQ resistance in *A. baumannii* and epidemiological studies of resistant strains.

Although DNA sequencing is a reliable technique for detecting mutations, it is costly, time-consuming, and laborious when analyzing numerous clinical strains. As an alternative, PCR-restriction fragment length polymorphism (RFLP) has been used to detect mutations associated with FQ resistance in *A. baumannii* [6, 7, 13]. However, this approach was insufficient for identifying significant mutations linked to high-level FQ resistance because it determined only the presence or absence of *gyrA* mutations at codon 83 or *parC* mutations at codon 80.

We have previously reported a mismatched PCR-RFLP assay for detecting *gyrA* and *parC* mutations associated with FQ resistance in *Enterobacteriaceae* [14]. We aimed to develop a mismatched PCR-RFLP assay to detect mutations in *gyrA* (codons 83 and 87) and in *parC* (codons 80 and 84) associated with FQ resistance in *A. baumannii*.

METHODS

Bacterial strains

We used 58 *A. baumannii* strains and 37 non-*baumannii Acinetobacter* strains, including some of the strains used in previous studies [15, 16]. These strains were collected between 2009 to 2018 and stocked in our laboratory. The 37 non-*baumannii Acinetobacter* strains included 10 *A. nosocomialis*, eight *A. pittii*, two *A. calcoaceticus*, two *A. soli*, two *A. ursingii*, two *A. colistiniresistens*, two *A. johnsonii*, two *A. gerneri*, one *A. radioresistens*, one *A. bereziniae*, one *A. towneri*, one *A. grimontii*, one *A. junii*, one *A. tandoii*, and one *A. haemolyticus* strain. These strains were identified by RNA polymerase β-subunit gene sequence analysis [17]. All strains were used to confirm the practicality of the mismatched PCR-RFLP assay. *A. baumannii* strain ATCC 19606 was used for comparison with the *A. baumannii* strains. According to the ethical guidelines for epidemiological studies released by the Ministry of Health, Labour, and Welfare in Japan [18], ethical approval and written or verbal informed consent are not required for this type of study.

Antimicrobial susceptibility testing

Susceptibility was tested using an agar dilution assay according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. The minimum inhibitory concentrations (MICs) of levofloxacin and ciprofloxacin were determined. To examine the effect of efflux pumps in FQ resistance, MICs were also measured in the presence of the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which was incorporated into the Mueller–Hinton agar at a concentration of 12.5 μM [20].

Amplification and DNA sequencing of the *gyrA* and *parC* QRDRs

The sequence of *A. baumannii* strain ATCC 19606 was used as the reference susceptible strain. The *gyrA* and *parC* QRDRs from 58 *A. baumannii* strains were amplified using PCR and sequenced using Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA), as previously described [21]. The results were compared with those of the mismatched PCR-RFLP.

Development of the mismatched PCR-RFLP assay

Based on the conserved sequences of *A. baumannii gyrA* and *parC*, we designed two sets of mismatched PCR-RFLP primers to detect mutations in *gyrA* (codons 83 and 87) and *parC* (codons 80 and 84) by introducing an artificial restriction enzyme cleavage site into the PCR products, as described previously [22]. The reverse primers for *gyrA* and *parC* are located immediately downstream of the nucleotide sequences, corresponding to GyrA87 and ParC84, with mismatched nucleotides to create recognition site (*Xmn*I), respectively (Fig. 1). The primer sequences and PCR conditions were expected to yield 143 and 120 bp DNA fragments for *gyrA* and *parC*, respectively. We performed PCR amplification of *gyrA* or *parC* from each strain using the AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions and as described in Table 1. The *gyrA* and *parC* PCR products were digested with *Hinf*I or *Xmn*I at 37°C for one hour, and the digested products were analyzed by electrophoresis using 3.0% MetaPhor Agarose gels (Takara, Shiga, Japan).

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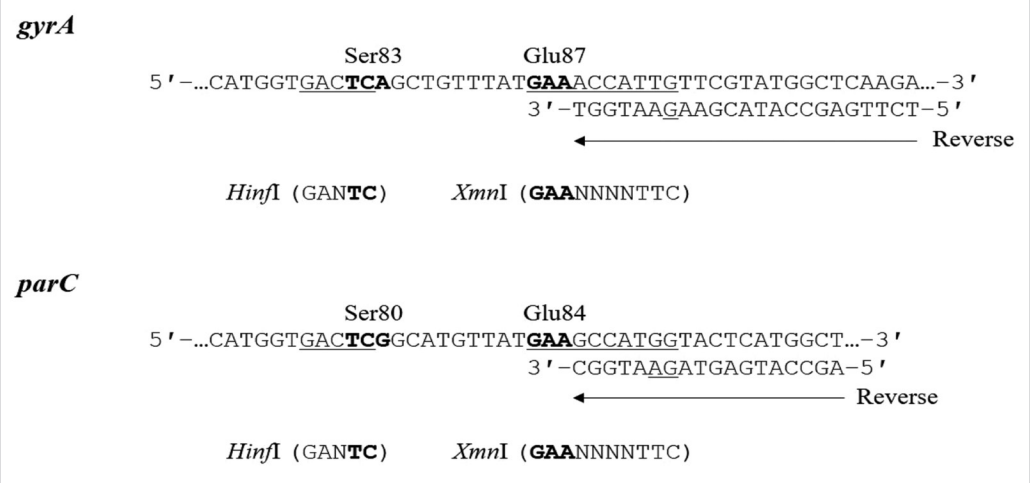

Fig. 1. Strategy used for mismatched PCR-RFLP of *gyrA* and *parC* QRDRs in *A. baumannii*. The reverse primers for *gyrA* and *parC* are located immediately downstream of the nucleotide sequences corresponding to GyrA87 and ParC84, respectively. The reverse primer for *gyrA* was designed with one mismatched nucleotide to create an *Xmn*I recognition site (GAANNNNTTC) in the *gyrA* region containing the codon for Glu-87 (GAA). The reverse primer for *parC* was designed with two mismatched nucleotides to create an *Xmn*I recognition site in the *parC* region containing the codon for Glu-84 (GAA). Boldface represents codons 83 and 87 of *gyrA* and codons 80 and 84 of *parC*. Underlined DNA sequences indicate restriction sites present in the QRDRs of FQ-susceptible strains.
Abbreviations: FQ, fluoroquinolone; QRDR, quinolone resistance-determining region; RFLP, restriction fragment length polymorphism.

Table 1. Primer sequences and restriction enzymes for mismatched PCR-RFLP

Target	Primer	Oligonucleotide sequence (5′ to 3′)*	PCR conditions	Product size (bp)	Restriction enzyme (Recognition site)†	QRDR amino acid (codon)‡
gyrA	Forward	GTGCTTTATGCCATGCACGAAT	95°C for five minutes and 35 cycles of 95°C for one minute, 48°C for one minute, and 72°C for 30 secconds	143	*Hinf*I (GANTC)	Ser83 in GyrA (TCA)
	Reverse	TCTTGAGCCATACGAA**G**AATGGT			*Xmn*I (GAANNNNTTC)	Glu87 in GyrA (GAA)
parC	Forward	GAGCTAGGCTTAAAAAGCAGTGG	95°C for five minutes and 35 cycles of 95°C for one minute, 48°C for one minute, and 72°C for 30 seconds	120	*Hinf*I (GANTC)	Ser80 in ParC (TCG)
	Reverse	AGCCATGAGTA**GA**ATGGC			*Xmn*I (GAANNNNTTC)	Glu84 in ParC (GAA)

*Boldface represents mismatched nucleotides that introduce artificial restriction sites. Underlined nucleotides indicate restriction sites; †Underlined nucleotides correspond to QRDRs in the *gyrA* or *parC* genes; ‡Underlined nucleotides indicate restriction sites.
Abbreviations: QRDR, quinolone resistance-determining region; RFLP, restriction fragment length polymorphism.

RESULTS

Susceptibility testing and DNA sequencing of the *gyrA* and *parC* QRDRs

We analyzed 58 *A. baumannii* strains (47 FQ-resistant and 11 FQ-susceptible). Point mutations were present only in *gyrA* (codon 83) and *parC* (codons 80 and 84). The strains with no mutations (N=11) resulting in amino acid changes at Ser-83 and Glu-87 in GyrA and at Ser-80 and Glu-84 in ParC were susceptible to levofloxacin and ciprofloxacin. The FQ-resistant strains (N=47) carried two mutations, in *gyrA* (codon 83) and *parC* (codon 80 or 84), and all but one strain had ciprofloxacin MICs ≥32 μg/mL (Table 2). Of these 47 FQ-resistant strains, 45 possessed *gyrA* codon 83 TCA (Ser)→TTA (Leu) and *parC* codon 80 TCG (Ser)→TTG (Leu) mutations, and the remaining two possessed *gyrA* codon 83 TCA (Ser)→TTA (Leu) and *parC* codon 84 GAA (Glu)→AAA (Lys) mutations. CCCP did not appreciably affect the levofloxacin and ciprofloxacin MICs for the *A. baumannii* strains. The FQ-resistant strains were still classified as resistant based on the CLSI breakpoints for levofloxacin and ciprofloxacin, even in the presence of this efflux pump inhibitor. The mutations identified in *gyrA* and *parC* and the MICs of levofloxacin and ciprofloxacin are shown in Table 2.

Mismatched PCR-RFLP

We performed PCR-RFLP on 58 *A. baumannii* strains and *A.*

Table 2. Levofloxacin and ciprofloxacin MIC ranges, amino acid (codon) changes in GyrA and ParC QRDRs, and mismatched PCR-RFLP results for *A. baumannii* strains

Strain (N)	MIC range (μg/mL)		Amino acid (codon) change at position*				Mismatched PCR-RFLP†			
			GyrA		ParC		*gyrA*		*parC*	
	Levofloxacin	Ciprofloxacin	83	87	80	84	*Hin*fI	*Xmn*I	*Hin*fI	*Xmn*I
ATCC 19606	0.5	0.5	TCA (Ser)	GAA (Glu)	TCG (Ser)	GAA (Glu)	+	+	+	+
(10)	0.125–0.25	0.125	–	–	–	–	+	+	+	+
(1)	1	0.5	–	–	TC**T** (Ser)	–	+	+	+	+
(2)	8	32	T**T**A (Leu)	–	–	AAA (Lys)	–	+	+	–
(45)	8–128	16–256	T**T**A (Leu)	–	T**T**G (Leu)	–	–	+	–	+

*Compared with ATCC 19606. Underlining indicates point mutations; –, no change.
†+ indicates PCR products that were digested by the restriction enzyme; – indicates PCR products that were not digested by the restriction enzyme.
Abbreviations: QRDR, quinolone resistance-determining region; RFLP, restriction fragment length polymorphism; MIC, minimum inhibitory concentration.

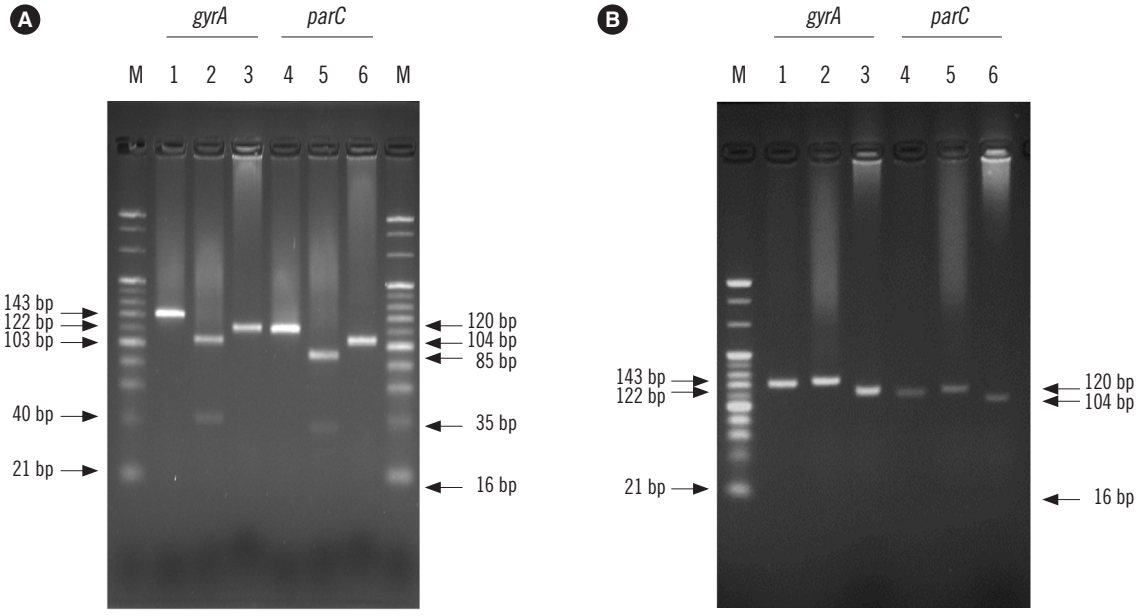

Fig. 2. PCR-RFLP patterns obtained following digestion with *Hin*fI or *Xmn*I for *gyrA* and *parC*. Lanes 1 to 3 and 4 to 6 show PCR-RFLP results for *gyrA* and *parC*, respectively. Lane: M, 20 bp DNA ladder marker. (A) PCR-RFLP results for *A. baumannii* ATCC19606. Lanes: 1, undigested (143 bp); 2, *Hin*fI-digestion (103 bp and 40 bp); 3, *Xmn*I-digestion (122 bp and 21 bp); 4, undigested (120 bp); 5, *Hin*fI-digestion (85 bp and 35 bp); and 6, *Xmn*I-digestion (104 bp and 16 bp). (B) PCR-RFLP results for the representative FQ-resistant *A. baumannii* strain possessing mutations in *gyrA* (83) and *parC* (80). Lanes: 1, undigested (143 bp); 2, *Hin*fI-digestion (143 bp); 3, *Xmn*I-digestion (122 bp and 21 bp); 4, undigested (120 bp); 5, *Hin*fI-digestion (120 bp); and 6, *Xmn*I-digestion (104 bp and 16 bp).
Abbreviations: FQ, fluoroquinolone; RFLP, restriction fragment length polymorphism.

baumannii strain ATCC 19606. Amplification products with the expected sizes of 143 bp for *gyrA* and 120 bp for *parC* were successfully obtained for all strains. The PCR products of *gyrA* from FQ-susceptible strains contained both one natural *Hin*fI recognition site and one artificially created *Xmn*I recognition site. Similarly, the PCR products of *parC* from FQ-susceptible strains contained both one natural *Hin*fI recognition site and one artificially created *Xmn*I recognition site (Fig. 1). Consequently, *Hin*fI and *Xmn*I digested the amplified 143 bp fragment to generate two fragments of 103 and 40 bp and of 122 and 21 bp, respectively. The 21 bp fragment, which was produced by *Xmn*I digestion, was not visible in Fig. 2; however, it was easy to recognize that the amplified 143 bp fragment was digested by *Xmn*I. Similarly, *Hin*fI and *Xmn*I digested the amplified 120 bp fragment to generate two fragments of 85 and 35 bp and of 104 and 16 bp, respectively. The 16 bp fragment, produced by *Xmn*I digestion,

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was not visible in Fig. 2; however, it was easy to recognize that the amplified 120 bp fragment was digested by *Xmn*I. *Hinf*I and *Xmn*I failed to digest the PCR products at the site containing the mutations that resulted in amino acid changes at Ser-83 in GyrA or at Ser-80 or Glu-84 in ParC.

Furthermore, we applied this assay to 37 non-*A. baumannii* strains. While *gyrA* amplicons were obtained for 28 strains, namely 10 *A. nosocomialis*, eight *A. pittii*, two *A. calcoaceticus,* two *A. ursingii*, two *A. gerneri*, two *A. johnsonii* strains, one *A. grimontii*, and one *A. tandoii*, no *parC* amplicons were obtained in any of the 37 strains tested.

DISCUSSION

We developed a mismatched PCR-RFLP assay to detect mutations in *gyrA* (codons 83 and 87) and *parC* (codons 80 and 84), which are associated with FQ resistance in *A. baumannii*. This assay specifically detected significant mutations associated with reduced susceptibility to FQs in *A. baumannii* and accurately classified all the FQ-resistant and FQ-susceptible strains according to the MIC results.

The regions containing the mutation site resulting in amino acid change within the Ser-83 codon in GyrA or Ser 80 codon in ParC have a naturally occurring *Hinf*I restriction site; thus, these regions are amplified by PCR, and the mutations at these positions are detected when the PCR products are not digested with *Hinf*I, as analyzed by electrophoresis on agarose gels [6, 7]. However, the mutation sites within the Glu-87 codon in GyrA and the Glu-84 codon in ParC are not involved at any restriction cleavage site. To detect mutations in *gyrA* (codon 87) and *parC* (codon 84), we introduced base substitutions near the mutation sites to create *Xmn*I cleavage sites using the primer-specified restriction site modification method [22]. Our results demonstrated that while *Hinf*I and *Xmn*I digested the *gyrA* and *parC* amplicons from FQ-susceptible strains, they did not digest those from FQ-resistant strains with mutations in *gyrA* and *parC*. The mutations detected by this assay were concordant with the DNA sequencing results shown in Table 2. The high specificity of the restriction enzyme *Xmn*I to each set of the three nucleotides for codon 87 (Glu) of GyrA and codon 84 (Glu) of ParC in FQ-susceptible strains allows our assay to accurately detect mutations at codon 87 in *gyrA* and at codon 84 in *parC*. Therefore, our assay can identify significant mutations in *gyrA* and *parC* QRDRs linked to high-level FQ resistance in *A. baumannii* without the need for DNA sequencing; this assay may thus serve as an alternative to other PCR-RFLP assays that are limited by their ability to detect mutations at only codon 83 of *gyrA* or codon 80 of *parC* [6, 7].

Our assay accurately identified FQ-susceptible strains and FQ-resistant strains. When our assay did not detect mutations in *gyrA* (codons 83 and 87) and *parC* (codons 80 and 84), the MICs showed susceptibility to FQs. On the other hand, when our assay detected mutations in both *gyrA* and *parC*, the MICs showed FQ resistance. In addition, our assay enables simultaneous analysis of many strains and provides results within four hrs. Thus, it could aid in the rapid identification of FQ-resistant *A. baumannii* strains in the clinical setting.

Previous studies have reported several rapid assays for detecting *gyrA* and *parC* mutations associated with FQ resistance in *A. baumannii* and determining FQ resistance, including PCR followed by electrospray ionization mass spectrometry and pyrosequencing assay [21, 23]. These assays successfully detected FQ resistance mutations in *gyrA* (codon 83) and *parC* (codons 80 and 84) and identified FQ-resistant strains. However, these assays require expensive equipment; our assay does not.

Another important and advantageous finding was that the mismatched PCR-RFLP primers for *parC* amplified *parC* from *A. baumannii* strains, whereas these primers did not amplify *parC* from any non-*baumannii Acinetobacter* strains. This assay may therefore differentiate *A. baumannii* from other *Acinetobacter* species in bacterial colonies, and determine FQ resistance for *A. baumannii* without the need for precise species identification within the genus *Acinetobacter*. This could also be an advantage of our assay. However, further studies are needed to confirm this finding.

The limitation of our assay is that it is unable to detect mutations at other locations of *gyrA* and *parC* or in other genes. Nevertheless, our data suggest this assay specifically amplifies *gyrA* and *parC* from *A. baumannii* and allows for simple, specific, rapid, and inexpensive detection of significant FQ resistance mutations. Thus, this assay may be useful for rapid assessment of FQ resistance in *A. baumannii* and for epidemiological studies of resistant strains in the clinical setting; moreover, it might be used to differentiate *A. baumannii* from other *Acinetobacter* species.

Author Contributions

The contributions of the authors are as follows: Research conception and design: Nakano R, Yano H. Data acquisition: Kakuta N, Nakano R, Nakano A, Suzuki Y, Tanouchi A, Masui T, Horiuchi S, Endo S, Kakuta R, Ono Y, Yano H. Data analysis and interpretation: Kakuta N, Nakano R, Nakano A, Suzuki Y, Tanou-

chi A, Masui T, Horiuchi S, Endo S, Kakuta R, Ono Y, Yano H. Manuscript writing (original draft): Kakuta N, Nakano R. Manuscript writing (review and editing): Nakano R, Yano H. All authors have accepted their responsibility for the entire content of this manuscript and approved submission.

Conflict of Interest

None declared.

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ORCID

Naoki Kakuta	https://orcid.org/0000-0001-9508-577X
Ryuichi Nakano	https://orcid.org/0000-0003-2086-2591
Akiyo Nakano	https://orcid.org/0000-0003-1379-384X
Yuki Suzuki	https://orcid.org/0000-0002-2509-8513
Ayako Tanouchi	https://orcid.org/0000-0002-6096-7630
Takashi Masui	https://orcid.org/0000-0002-9567-2947
Saori Horiuchi	https://orcid.org/0000-0002-4143-5807
Shiro Endo	https://orcid.org/0000-0003-0103-415X
Risako Kakuta	https://orcid.org/0000-0002-4594-908X
Yasuo Ono	https://orcid.org/0000-0002-5211-8102
Hisakazu Yano	https://orcid.org/0000-0003-2085-7194

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