

An Asymptomatic Case With MEN1 Slipping Through Genetic Screening by SNV-dependent Allelic Dropout

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Abstract

Context: Genetic testing is useful not only for the diagnosis of the MEN1 proband but also for determining the putative asymptomatic variant carriers to improve the prognosis or to avoid unnecessary medical intervention. However, we must be aware of the putative pitfalls of polymerase chain reaction (PCR)-based genetic testing in specific conditions that lead to medical mismanagement.

Objective: To warn of the putative pitfalls of PCR-based genetic testing, we report an overlooked case of MEN1 due to PCR allelic dropout.

Methods: A 69-year-old man was clinically diagnosed with MEN1, and genetic testing revealed that he had a pathogenic variant in the *MEN1* gene. His 36-year-old son was completely asymptomatic. As the son was 50% at risk of MEN1, he was willing to undergo genetic testing himself after genetic counseling.

Results: Genetic testing was carried out in 2 independent laboratories. Although laboratory A showed that he carried a pathogenic variant, laboratory B showed that he had the wild-type genotype of *MEN1*. The discrepancy in these results was due to PCR allelic dropout by single-nucleotide variations of the *MEN1* gene in the 5' region. The surveillance revealed that he had asymptomatic primary hyperparathyroidism and a neuroendocrine tumor of the pancreas.

Conclusion: PCR-dependent genetic analysis may be susceptible to PCR allelic dropout in an SNV-specific manner. We must be careful when genetically testing individuals of relatives with clinical MEN1 disease.

Key Words: MEN1, PCR, allelic dropout

Abbreviations: G4, G-quadruplex; PCR, polymerase chain reaction; SNV, single-nucleotide variation.

MEN1 is an autosomal dominant disorder that is due to pathogenic variants in the tumor-suppressor gene *MEN1* (OMIM No. 131100), which encodes menin [1]. The finding of MEN1 in a patient has important implications for family members, as first-degree relatives have a 50% risk of developing the disease and can often be identified by *MEN1* genetic analysis. Despite the advances in treatment of MEN1 tumors, the life expectancy of patients, especially with pancreatic neuroendocrine tumors larger than 2 cm, remains shorter than normal population [2]. The prognosis for MEN1 patients might be improved by early detection of the tumor, presymptomatic tumor detection, and the initiation of treatment specific for MEN1 tumors [3]. Thus, *MEN1* germline variant screening is recommended not only for the index patient with MEN1 but also for their first-degree symptomatic and asymptomatic relatives [4]. Single-site analysis using Sanger sequencing is the standard tool for at-risk family members. Even though polymerase chain reaction (PCR) is considered to be a robust technology that generally provides reliable results, erroneous

genotypes sometimes occur, which can result in overlooking the diagnosis of asymptomatic cases of genetic disease [5].

It has been reported that pathogenic variants in the coding region of the *MEN1* gene are detected in approximately 90% of familial cases [6]. In cases without the variant in the coding region of the *MEN1* gene, there are some cases with genetic conditions, such as variants in the promoter region of the *MEN1* gene and variants of the *CDKN1B/p27*, *p16*, *p18*, and *p21* genes [7, 8]. In addition, when using the PCR analysis, large deletion of the coding region of the *MEN1* gene can lead to a false-negative result. However, the frequency of these conditions is low [9].

If genetic abnormalities cannot be detected, technical problems regarding PCR analysis may be present. One problem associated with the PCR of diploid genomic DNA is the occasional amplification failure of 1 of the 2 alleles at a given locus, termed *allelic dropout* [10]. It seems that this phenomenon is not so familiar among endocrinologists. In this paper, we show a case with MEN1 slipping through genetic

screening with Sanger sequencing due to single-nucleotide variation (SNV, formerly single-nucleotide polymorphism [SNP]) (rs509606 NM_130799:c.-23-16C > G)-dependent allelic dropout.

Materials and Methods

Case Presentation

A 69-year-old male proband (II-2 in Fig. 1) had been clinically diagnosed with MEN1 with primary hyperparathyroidism, neuroendocrine tumor of the pancreas, and pituitary tumor. Genetic testing showed that he carried the germline pathogenic variant NM_130799: c.249_252del (NP_570711:p.Ile85Serfs*33) in the *MEN1* gene [11]. To the best of our knowledge, none of the family members had any clinical findings of MEN1 except his daughter (III-1 in Fig. 1), who had nephrolithiasis suspected to be related to primary hyperparathyroidism due to MEN1. After careful genetic counseling, the son of the proband (III-2 in Fig. 1) was willing to undergo genetic testing for *MEN1*.

Genetic Testing for *MEN1*

Genetic testing of III-2 was carried out in 2 independent laboratories (one in a commercial laboratory, laboratory A, and the other in an in-house laboratory, laboratory B). In both laboratories, PCR-direct sequencing was performed in exon 2 of the *MEN1* gene. As a side note, since laboratory A is a commercial laboratory, detailed conditions for PCR have not been disclosed.

Genetic Analysis in Laboratory B

Genomic DNA from the participants was extracted from the peripheral blood using a genomic DNA purification kit (Qiagen, or DNA) in accordance with the instruction manual. The proband's genomic DNA was screened for variants in *MEN1*. Protein-coding exon was amplified by PCR using LA Taq (Takara). The PCR primers used were: forward primer: 5'-agcggaccctgggaggagctccccggcg-3' (nucleotides [nt] 2197-2226 in accession No. U93237, GenBank) reverse primer: 5'-cctgccgaactcacaaggcttacctt-3' (nt2808-2838). The PCR conditions were 30 cycles of denaturation for 30

seconds at 95 °C, annealing for 30 sec at 65°C, and extension for 2 minutes at 72 °C. After purifying the PCR products using a QIAquick PCR purification kit (Qiagen), direct sequencing was performed using a 3500 Genetic Analyzer (Applied Biosystems).

Investigation of the Allelic Dropout

To examine the allelic dropout of the PCR, PCR was carried out by using Taq polymerase (Takara) with various magnesium concentrations (range, 1 mM-2.75 mM). PCRs were also carried out by using LA Taq at the same magnesium concentrations. The reaction profile and PCR primers were identical to those described previously.

Ethics

All procedures in this article were performed in accordance with the ethical standards of the Shizuoka General Hospital Ethics Committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from the patients for their anonymized information to be published.

Results

Genetic testing of III-2 was conducted in laboratories A and B. Laboratory A showed the wild-type sequence of *MEN1* in the region of the variant of the proband (Fig. 2, upper panel). However, the analysis of laboratory B showed the identical variant of the proband (see Fig. 2, lower panel). As PCR allelic dropout was suspected, we tried to reproduce the experiment of laboratory A. Fig. 3A and 3B show the structure of the *MEN1* gene and the schematic of intron 1 and exon 2, respectively. rs509606 is located 39 bp upstream of the start codon of exon 2. The proband showed the C/C genotype at rs509606 and c.249_252del in a heterozygous manner (Fig. 3C, upper panel). On the other hand, III-2 showed the C/G genotype at rs509606 and c.249_252del in a heterozygous manner (see Fig. 3C, lower panel). These results suggested that c.249_252del is in *cis* with the C allele of rs509606. To investigate the C/G allele effect of rs509606 on PCR efficiency, PCR was carried out at various Mg concentrations using Taq

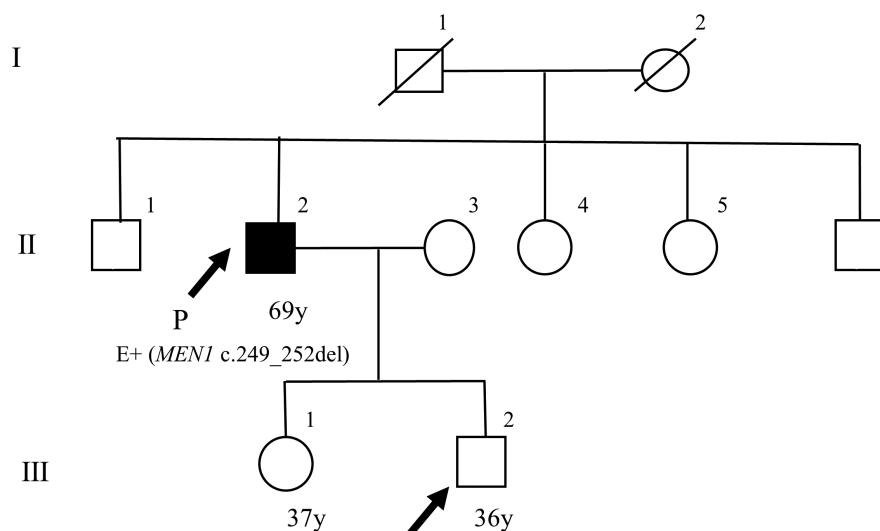


Figure 1. The pedigree. Black boxes indicate the presence of the *MEN1* pathogenic variant (*MEN1* c.249_252del).

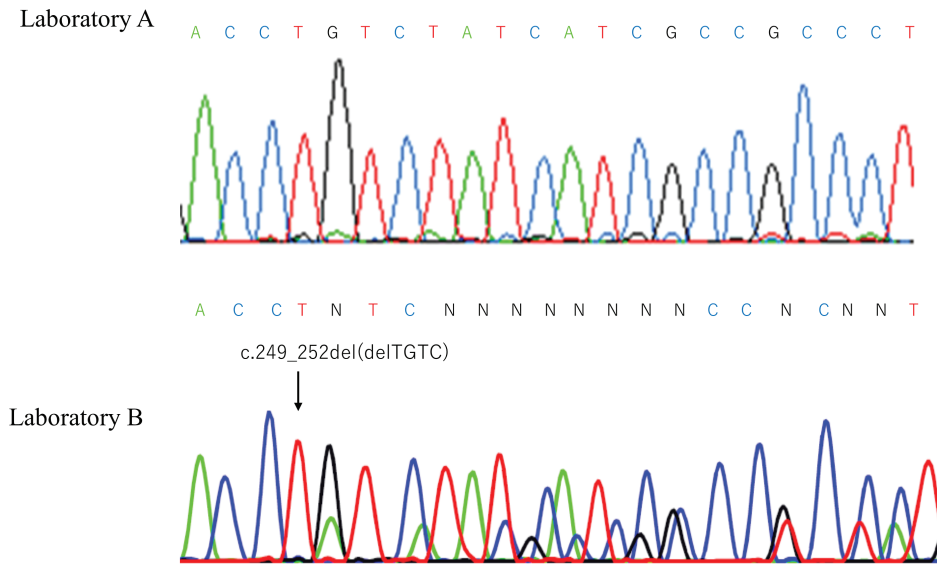


Figure 2. The result of genetic testing of III-2. The electropherograms of the *MEN1* gene around the region of the pathogenic variant of the proband. Laboratory A showed the wild-type sequence of *MEN1* (upper panel). Laboratory B showed the identical variant of the proband (lower panel).

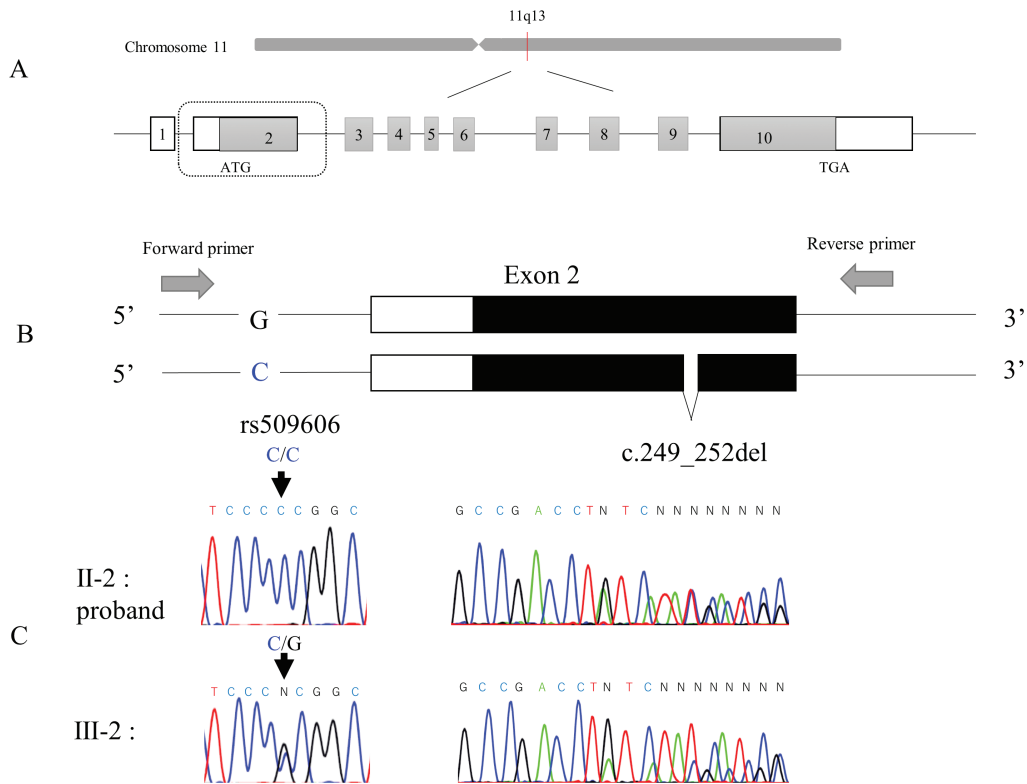


Figure 3. A, The structure of *MEN1* gene located on chromosome 11q13. B, The schematic of the intronic rs509606 and the c.249_252del in exon 2 of the *MEN1* gene. c.249_252del is in *cis* with C allele of rs509606. C, The electropherogram of the proband showed the C/C genotype at rs509606 and c.249_252del in a heterozygous manner (upper panel). The electropherogram of III-2 showed the C/G genotype at rs509606 and c.249_252del in a heterozygous manner (lower panel).

polymerase. Fig. 4A shows the results. No PCR product was detected at a 1-mM Mg concentration. The peak heights of the C allele of rs509606 were lowered in a Mg concentration-dependent manner. At 2.5-mM Mg, the C allele was completely lost. The sequencing result of exon 2 revealed the wild-type sequence at 2.75-mM Mg (Fig. 4B). These allelic dropouts were not observed when LA Taq polymerase was used (data not shown).

We tried these analyses on another *MEN1* sample that harbors c.164delC in exon 2 with the C/G genotype in rs509606 (Fig. 5A). The sequencing result of exon 2 showed a homozygous pattern of c.164delC at 2.75 mM Mg and a heterozygous pattern at a 1.25-mM Mg concentration (Fig. 5B). These results suggest that the c.164delC variant is in *cis* with the G allele of rs509606.

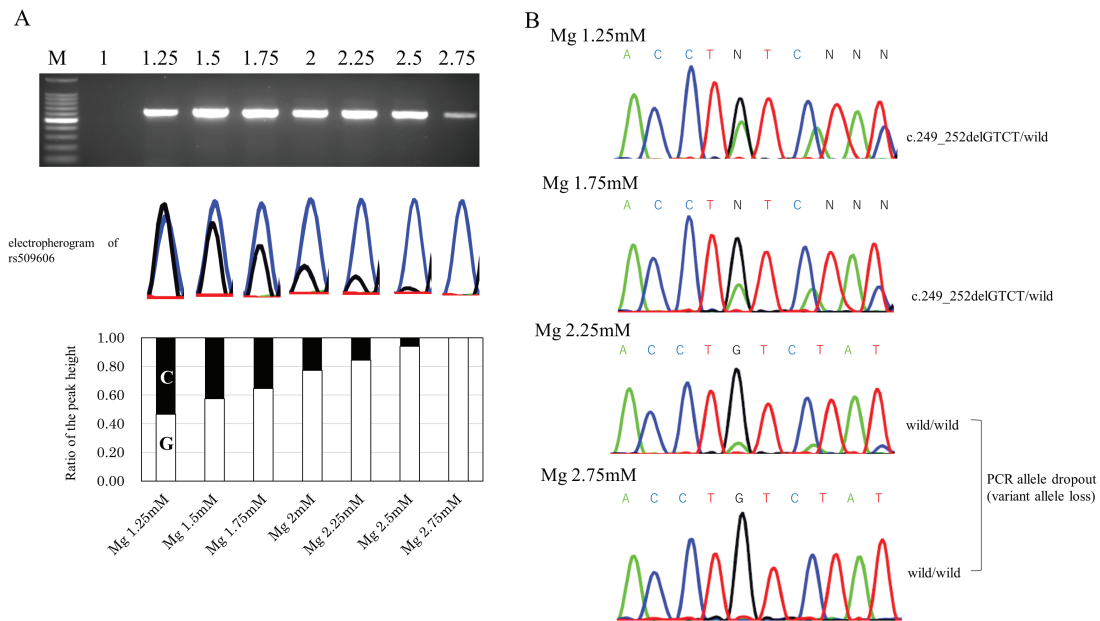


Figure 4. A, The polymerase chain reaction (PCR) results of the intronic rs509606 at various magnesium (Mg) concentrations using Taq polymerase. Agarose gel electrophoresis showed no PCR product at a 1-mM Mg concentration (upper panel). Electropherogram of rs509606. Blue line = G, black line = C (middle panel). The ratio of the peak height in rs509606. The peak height of the C allele was lowered in a Mg concentration-dependent manner (lower panel). B, The sequencing result of exon 2 in various Mg concentrations using Taq polymerase. The variant allele was completely lost at a Mg concentration of 2.75 mM.

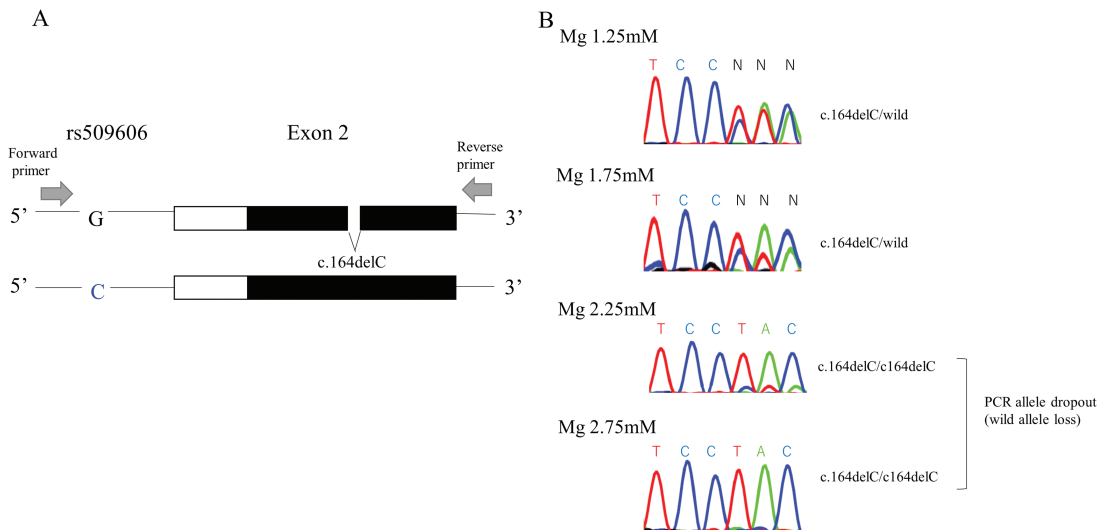


Figure 5. A, The schematic of the intronic rs509606 and the c.164del in exon 2 of the *MEN1* gene. c.164del is in *cis* with the G allele of rs509606. B, The sequencing result of exon 2 in various magnesium (Mg) concentrations using Taq polymerase. The wild-type allele was completely lost at a Mg concentration of 2.75 mM.

Clinical surveillance was initiated, and primary hyperparathyroidism and neuroendocrine tumor of the pancreas were suspected based on the high intact parathyroid hormone concentration despite a normal high calcium concentration (Table 1) and based on the detection of the small space-occupying lesion in the tail of the pancreas by the contrast-enhanced computed tomography, respectively (Fig. 6).

Discussion

MEN1 is an autosomal dominant, hereditary tumor syndrome with a high penetrance of endocrine tumors. Early presymptomatic genetic testing is a robust tool for improving

prognosis [3]. Single-site analysis using Sanger sequencing is the standard tool for at-risk family members of the genotyped proband.

Allelic dropout has been recognized as an important problem with PCR, which results in a false-negative or a false-positive outcome [10]. In the present case, the genotype of rs509606, located in intron 1 of the *MEN1* gene, can affect the PCR efficiency when using Taq polymerase with a high Mg concentration buffer and can result in allelic dropout. Although the allelic dropout problem with this *MEN1* variant has already been reported in a technical manner, our case is original because we illustrate this feature with the case of an asymptomatic patient who has undergone targeted genetic screening

Table 1. Clinical data of III-2

Endocrine profile			
	Value	Normal range	Units
Calcium	10.1	8.8-10.1	mg/dL
Phosphorus	2.5	2.7-4.6	mg/dL
iPTH	87.9	15-65	pg/mL
Plasma glucose	82	70-125	mg/dL
IRI	18.2	≤ 18.7	μU/mL
Gastrin	140	≤ 200	pg/mL
Glucagon	73.9	70-174	pg/mL
GH	0.05	≤ 2.47	ng/mL
IGF-1	123	99-275	ng/mL
Prolactin	14.0	4.29-13.69	ng/mL

Abbreviations: GH, growth hormone; IGF-1, insulin-like growth factor-1; iPTH, intact parathyroid hormone; IRI, immunoreactive insulin.

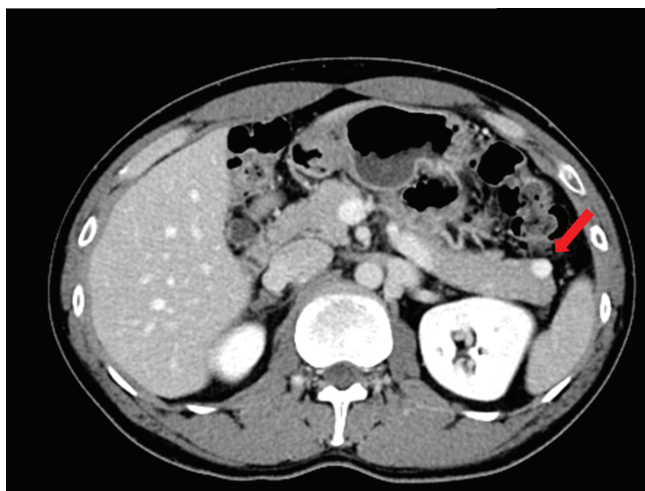


Figure 6. Contrast-enhanced abdominal computed tomography of III-2. The enhanced small tumor in the tail of the pancreas is a suspected neuroendocrine tumor (red arrow).

in a family context, and for whom a laboratory has given a false-negative result [12]. A false-negative result due to allelic dropout can occur in a patient who has the C/G genotype in rs509606 and whose pathogenic variant allele is in *cis* with the C allele of rs509606. The allele frequency of rs509606 (C > G) is 0.308 in the ExAC database. The C/G genotype in individuals is estimated to be approximately 42% in the general population by Hardy-Weinberg equilibrium. In such a population of MEN1 patients who were asymptomatic carriers, the variant allele on the C allele of rs509606 was lost, resulting in false negatives. The clinical effect of false negatives on asymptomatic or presymptomatic variant carriers is critical.

A few studies on allelic dropout have been reported. Blais et al [5] showed that genotyping errors such as allelic dropout in PCR assays occurred in 0.44% of genotypes. Most allelic dropouts, up to 90%, might be caused by unpredictable sequence-independent factors (eg, the quantity and quality of DNA, presence of PCR inhibitors, and thermocycler temperature). Allelic dropout can be resolved by simple re-analysis. On the other hand, sequence-specific allelic dropout occurs nonrandomly, and the mechanisms are usually related to the presence of an SNV situated inside the primer

sequence [13-15]. The primer binding site SNV causes failure of primer template annealing. Sometimes, an SNV outside the primer sequence can also cause amplification failure by forming a secondary structure, such as a hairpin formation and G-quadruplexes (G4s) [16, 17]. In the present cases, an intronic variant (rs509606) in the *MEN1* gene would result in a G-4- or i-motif-like sequence on the C-allele and form G4s that lead to preferential amplification of the G-allele. G4s are secondary nucleic structures formed in the region of G-rich DNA and RNA. Four guanines form a planar structure through Hoogsteen bonds, which are stacked to form the G4 structure [18]. Although G4s are widespread throughout the human genome and can affect chromatin architecture and gene regulation, the structures in the template DNA can inhibit amplification during PCR. It is known that the formation of G4s is influenced by the composition of the buffer, for example, the magnesium concentration, and whether the DNA containing G4s can be amplified depends on the PCR enzyme [19]. In this case, it was also shown that allelic dropout occurred in a high-magnesium concentration buffer when using Taq polymerase. LA Taq polymerase was able to amplify in any magnesium concentration buffer, suggesting that the allelic dropout occurred in a polymerase-dependent manner. As another solution to this problem, Sumner et al [20] have proposed a PCR method to use noncontinuous binding loop-out primers for avoiding problematic DNA sequence in PCR sequencing.

In our case, we considered that false-negative genetic testing of the pathogenic variant of *MEN1* can occur under the following conditions: 1) the pathogenic variant of *MEN1* is in exon 2; 2) the C/G genotype in rs509606; and 3) the variant allele is in *cis* with the C allele of rs509606. According to the Japanese multiple endocrine databases, 23% of 180 families with *MEN1* have pathogenic variants in exon 2 [6]. The genotype frequencies of C/C, G/C, and G/G of rs509606 were 49%, 42%, and 9%, respectively, according to the ExAC database. Therefore, when conducting PCR using Taq polymerase and high-magnesium concentration buffer, the estimated probability that the pathogenic variant cannot be detected in *MEN1* patients is theoretically up to 4.8% ($0.23 \times 0.42 \times 0.5 \times 100$). Furthermore, the same is true for combination with other exons and other SNVs. This frequency is not negligible. To avoid allelic dropout, we have to use low-magnesium concentration buffer when using Taq polymerase or change the DNA polymerase to, for example, LA Taq polymerase.

In this report, we showed a case with *MEN1* slipping through genetic screening by single-site analysis using Sanger sequencing due to SNV-dependent allelic dropout. Recently, next-generation sequencing has prevailed as a standard method for genetic analysis; however, the same error can occur with next-generation sequencing. Health care professionals should be aware of the putative pitfalls of PCR-based genetic testing in specific conditions, which may lead to medical mismanagement. Especially in the genetic testing of 50% of at-risk individuals with autosomal dominant diseases without symptoms, we should be careful when evaluating PCR results.

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Disclosures

The authors have nothing to disclose.

Data Availability

Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in “References.”

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