SELÇUK TIP DERGİSİ
SELCUK MEDICAL JOURNAL

Araştırma Makalesi / Research Article

Selcuk Med J 2023;39(1): 24-28
DOI: 10.30733/std.2023.01605

NEÜ YAYINEVİ

Identification of the Mutation in *DCLRE1C* Gene by PCR-RFLP

DCLRE1C Genindeki Mutasyonun PCR-RFLP ile Tanımlanması

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Geliş Tarihi/Received: *5 August 2022*
Kabul Tarihi/Accepted: *15 January 2023*

Öz

Amaç: *DCLRE1C* genindeki mutasyonlar Artemis proteininin fonksiyonel olarak bozulmasına neden olur ve T/B hücre gelişimi olumsuz etkilenir. Bu mutasyonun bir sonucu olarak, genellikle ağır kombine ve kombine immün yetmezlik (CID) kliniği ortaya çıkar. Akraba evliliğinin yaygın olduğu bölgemizde bu mutasyona bağlı CID vakalarına sıklıkla rastlanmaktadır. Bu nedenle şüpheli hastalar ilgili gen mutasyonu açısından vakit kaybetmeden değerlendirilmelidir. Mutasyonların tespitinde daha karmaşık ve maliyetli yöntemlerin kullanılmakla birlikte daha ucuz ve hızlı yöntemlere ihtiyaç olduğu açıktır. Bundan dolayı çalışmada *DCLRE1C* geni ekzon 3 (c.194C>T; p.T65I) ve ekzon 14 (c.1669_1670insA; p.T577Nfs*21) mutasyonlarının Polimeraz Zincir Reaksiyonu-Restriksiyon Parça Uzunluk Polimorfizmi (PZR-RFLP) yöntemi kullanılarak belirlenmesi amaçlandı.
Hastalar ve Yöntem: Çalışma 2017-2020 yılları arasında kliniğimizde DCLRE1C mutasyonu ile takip edilen 14 hasta, 2 ebeveyn ve 10 sağlıklı kontrol dahil edildi. Mutasyon bölgeleri ve uygun restriksiyon enzimleri içeren primerler ile PZR-RFLP analizi gerçekleştirildi.
Bulgular: Analiz sonucunda 12 hasta *DCLRE1C* geni ekzon 3 açısından homozigot mutant, 2 ebeveyn ekzon 3 açısından heterozigot, 2 hasta ekzon 3 ve ekzon 14 açısından compound heterozigote genotipde olduğu bulundu. Mutasyonlar, Sanger DNA dizilimi ile doğrulandı. PZR-RFLP yöntemi ile ilgili bölgedeki mutasyonlar hızlı ve güvenilir bir şekilde belirlendi.
Sonuç: Çalışma, PZR-RFLP yönteminin primer immün yetmezliklerde özellikle bilinen mutasyonların tespiti ve aile taraması gibi durumlarda kullanılabilecek ucuz, güvenli ve hızlı bir yöntem olduğunu göstermiştir.

Anahtar Kelimeler: Artemis, *DCLRE1C*, PZR-RFLP

Abstract

Aim: Mutations in the *DCLRE1C* gene result in functional impairment of the Artemis protein and T/B cell development is adversely affected. As a result of this mutation, a clinic of severe combined and combined immunodeficiency (CID) generally occurs. In our region where consanguineous marriage is common, CID cases due to this mutation are frequently encountered. Therefore, suspected patients should be evaluated promptly for the relevant gene mutation. It is clear that more complicated and costly methods are used in the detection of mutations and there is a need for cheaper and faster methods. Therefore, in this study, it was aimed to determine the mutations of *DCLRE1C* gene exon 3 (c.194C>T; p.T65I) and exon 14 (c.1669_1670insA; p.T577Nfs*21) by using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method.
Patients and Methods: The study was carried out between 2017 and 2020 and study included 14 patients followed up with *DCLRE1C* mutation in our clinic, 2 parents and 10 healthy controls. PCR-RFLP analysis was performed with primers containing mutation sites and appropriate restriction enzymes.
Results: As a result of the analysis, 12 patients were homozygous mutant for *DCLRE1C* gene exon 3, 2 parents were heterozygous for exon 3, and 2 patients were heterozygous for exon 3 and exon 14 and were found to be compound heterozygous genotype. Mutations were confirmed by Sanger DNA sequencing. Mutations in the relevant region were determined quickly and reliably by the PCR-RFLP method.
Conclusion: The study showed that the PCR-RFLP method is a cheap, safe and fast method that can be used in cases such as family screening, especially for the detection of known mutations in primary immunodeficiencies.

Key words: Artemis, *DCLRE1C*, PCR-RFLP

Cite this article as: Karaselek MA, Kuccukturk S, Kapakli H, Hazar E, Guner SN, Keles S, Kurar E, Reisli I. Identification of the Mutation in *DCLRE1C* Gene by PCR-RFLP. Selcuk Med J 2023;39(1): 24-28

Disclosure: *None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this article. The research was not sponsored by an outside organization. All authors have agreed to allow full access to the primary data and to allow the journal to review the data if requested.*

INTRODUCTION

The *DCLRE1C* (DNA Cross-Link Repair 1C) gene is a gene located on the short arm of the tenth chromosome, 47.2 kb in length and consists of 14 exons. This gene encodes a protein called Artemis, which is a nuclease with 5'-3' exonuclease activity on single-stranded DNA (1,2). Artemis also plays an important role in repairing double strand breaks through non-homologous end joining and in V(D) J recombination (3,4). Mutation(s) in the DCLRE1C gene result in functional impairment of Artemis and T/B cell development is adversely affected, cause severe combined immunodeficiency (SCID) resulting in increased sensitivity to ionizing radiation (5,6). Although mutations in this gene result in the SCID phenotype, some hypomorphic mutations in Artemis have been shown to cause combined immunodeficiency (CID) with autoimmunity, granulomatous inflammation, lymphoproliferative disease and malignancy (6,7).

Consanguineous marriages are common in our region. The incidence of primary immunodeficiency (PID), many of which are autosomal recessive, is increasing accordingly. Therefore, rapid screening of mutations in individuals with a family history of consanguineous marriage is of great importance. DNA sequencing methods are widely used in the identification of relevant mutations in these patients. However, considering the cost and duration of these methods, it is not considered appropriate to use them for screening purposes. For this purpose, based on our previous experience, it was thought that the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method might be suitable for detecting *DCLRE1C* mutation (8). From this point of view, we aimed to use a fast, reliable and low-cost method of mutation in exon 3 (c.194C>T; p.T65I) and exon 14 (c.1669_1670insA; p.T577Nfs*21) previously detected in our patients. It was aimed to determine by the PCR-RFLP method. In addition, the obtained results were confirmed by Sanger DNA sequence analysis.

PATIENS AND METHODS

Patients

The prospective study was carried out between 2017 and 2020. A total of 14 patients with mutations in exon 3 and exon 14 of the DCLRE1C gene, 2 parents and 10 healthy controls were included in the study. Patient recruitment and the studies reported herein were approved by Institutional Review Board (2017/803). Written informed consent was obtained from participating patients' guardians and healthy controls. The methods applied to patients and controls are shown in Figure 1.

PCR and PCR-RFLP

PCR primers were designed to cover mutation sites in exon 3 (forward primer, 5'-GTTAGTCACCAAGATGGCTCATT-3' and reverse primer, 5'-GGCTCGTTAACAACAACTCCT-3') and exon 14 (forward primer, 5'-GGCTGGGACAGCCAATCAGATA-3' and reverse primer, 5'- AGAGTAAGTATCCTTTGGG-3).

A PCR protocol for exon 3 was used and cycling conditions were initial denaturation of 94°C for 6 minutes followed by 30 cycles of 94°C for 30 seconds, annealing beginning at 66°C for 30 seconds and 72°C 30 seconds. A final extension of 72°C for 10 minutes was applied. Resulting PCR products were visualized by 2% agarose gel. PCR-RFLP method was applied to PCR products. The PCR products were incubated for 1 hour at 37°C with DdeI (New England Biolabs®Inc.).

A PCR protocol for exon 14 was used and cycling conditions were initial denaturation of 94°C for 6 minutes followed by 30 cycles of 94°C for 10 seconds, annealing beginning at 59,6°C for 100 seconds and 72°C 10 seconds. A final extension of 72°C for 10 minutes was applied. Resulting PCR products were visualized by 2% agarose gel. PCR-RFLP method was applied to PCR products. The PCR products were incubated for 1 hour at 37°C with PstNI (New England Biolabs®Inc.).

Sanger DNA Sequence Analysis

The 3 genotypes determined by the RFLP method were confirmed by DNA sequencing. The mutation

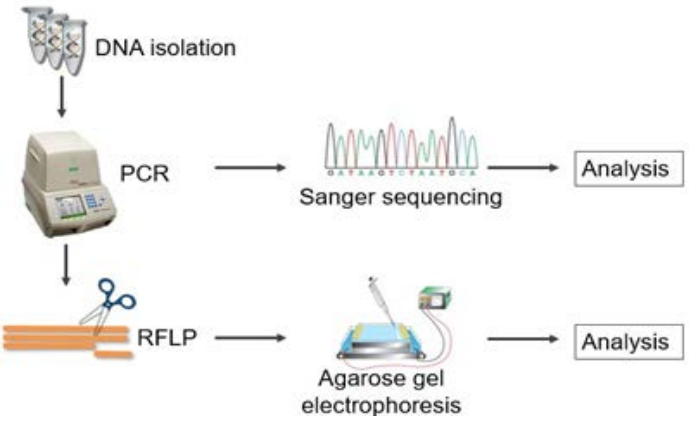

Figure 1. The methods applied to patients and controls

Table 1. Laboratory findings of patients with mutations (*: Patients with exon 14 mutations)

Patients	Age of onset	ALC (cell/µl)	B cell (cell/µl)	T cell (cell/µl)	CD4 T cell (%)	CD8 T cell (%)	Ig (mg/dl)
P1	3 years	1000	40	680	34	26	IgG:892; IgM:944; IgA:23
P2	4 years	6000	480	2940	6,2	1,2	IgG:1710; IgM:159; IgA:6.6
P3	2 years	2000	16	540	4,9	6,7	IgG:135; IgM:15; IgA:25
P4	4 years	200	0	13	6	52	IgG:240; IgM:127;IgA:6
P5	5 years	1240	13	603	6,3	6,2	IgG:1040;IgM:20.9;IgA:24
P6	5 years	900	36	558	23	27	IgG:560;IgM:54;IgA:19
P7	4 years	1500	85	820	19	18	IgG:450;IgM:52;IgA:20
P8	9 years	2440	24	2025	22	36	IgG:1440;IgM:86.5;IgA:6.6
P10	2 years	800	22	547	1,4	1,9	IgG:240;IgM:35;IgA:6.6
P16*	6 years	791	31	490	4,3	5,6	IgG:489;IgM:132;IgA:64.3
P17*	3 years	2170	238	1388	12,4	11,9	IgG:1340;IgM:157;IgA:6.6
P28	2 years	1370	123	643	20	38	IgG:65;IgM:113;IgA:6
P45	6 mounts	2000	18	1200	52	19	IgG:202;IgM:46;IgA:25
P46	6 years	400	76	304	57	21	IgG:456;IgM:179;IgA:6

region of the DCLRE1C gene in exon 3 and exon 14 was amplified with the primers used in the RFLP method, and sequence analysis was performed with the Sanger DNA sequence analysis method.

RESULTS

The M/F ratio of the patients included in the study was 5/9. The mean age of 14 patients was 4±2.17 years. Ten individuals were included in the study as healthy controls, and the M/F ratio was 5/5 and the mean age was 5±2.01 years. The laboratory and clinical findings of the patients are shown in Table 1 and Table 2.

PCR-RFLP

As a result of PCR-RFLP analysis, 12 patients were homozygous mutant for exon 3, 2 parents were heterozygous for exon 3, and 2 patients were heterozygous for exon 3 and exon 14 and were found to be compound heterozygous genotype.

DNA amplicons of 117 bp were obtained after PCR amplification of exon 3 mutation region. DdeI enzyme recognition region 5'….C^TNAG….3'; 3'….GANT^C….5' is selected according to the wild type allele. In the absence of mutation, enzyme digestion was performed in both alleles 94 and 23 bp DNA fragment was obtained after PCR-RFLP and these results were interpreted as normal homozygous genotype (C/C). If the patient is homozygous mutant genotype (T/T) 117 bp DNA fragment was obtained. In the case of heterozygote (C/T), three DNA fragments will be obtained: 117 bp, 94 bp and 23 bp. Accordingly, 12 patients were found to be homozygous mutant, 2 parents heterozygous, and 10 healthy controls homozygous wild genotype (Figure 2).

Table 2. Clinical features at admission of patients with mutations (*: Patients with exon 14 mutations)

Patients	Age of onset	Clinical features at admission
P1	3 years	Recurrent infections, anal wart
P2	4 years	Recurrent infections
P3	2 years	Recurrent infections, unidentified skin lesions, aphthous stomatitis
P4	4 years	Recurrent infections, unidentified skin lesions
P5	5 years	Recurrent infections
P6	5 years	Verruca vulgaris infection
P7	4 years	Recurrent infections
P8	9 years	Recurrent infections, severe varicella infection, Verruca vulgaris infection
P10	2 years	Verruca vulgaris, mycobacterial skin infection
P16*	6 years	Recurrent infections, granulomatous skin lesions, vitiligo
P17*	3 years	Recurrent infections, disseminated varicella infection, vitiligo
P28	2 years	Recurrent infections
P45	5 years	Recurrent infections
P46	6 years	Recurrent infections

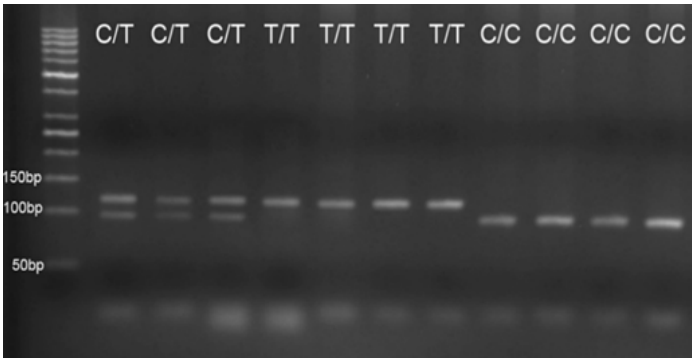

Figure 2. RFLP result of 3 different genotypes for DCLRE1C gene exon 3. (C/T): Heterozygous, (T/T): Homozygous mutant, (C/C): Homozygous normal.

DNA amplicons of 157 bp were obtained after PCR amplification of exon 14 mutation region. PstNI enzyme recognition region 5'….CAGNNN^CTG….3'; 3'….CTG^NNNGAC….5' is selected according to the mutant type allele. In the absence of mutation, enzyme digestion was not performed in both 157 bp DNA fragment was obtained after PCR-RFLP and these results were interpreted as homozygous wild genotype. If the patient is homozygous mutant genotype 136 bp and 21 bp DNA fragment was obtained. In the case of heterozygote, three DNA fragments will be obtained: 157 bp, 136 bp and 21 bp. According to these results, 2 patients were heterozygous (-/A) for exon 14 and the other patients were found to be homozygous wild genotype (-/-) (Figure 3).

DNA Sequence Analysis

Mutations identified by PCR-RFLP were confirmed by DNA sequence analysis and the presence of mutations was confirmed.

DISCUSSION

This study showed that RFLP was a fast and safe method for detecting the homozygous and heterozygous mutations in exon 3 (c.194C>T; p.T65I) and exon 14 (c.1669_1670insA; p.T577Nfs*21) previously detected in our patients with DCLRE1C gene defect and was also low-cost method.

Artemis protein, encoded by the DCLRE1C gene, is an endonuclease that plays a critical role in the unfolding of hairpins during V(D)J recombination in T and B cell development (9). In addition to this critical role, Artemis also plays a key role in repairing double-stranded DNA breaks, resulting in increased radiosensitivity as a result of Artemis gene mutation (1,6,10). Although hypomorphic mutations arising

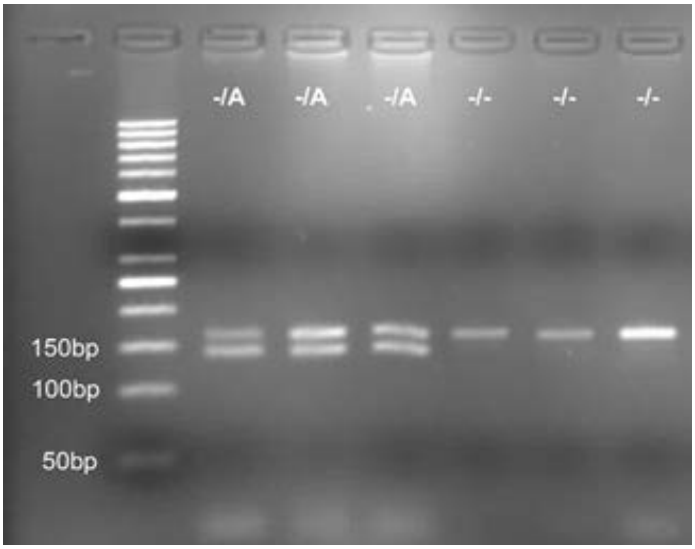

Figure 3. RFLP result of 2 different genotypes for DCLRE1C gene exon 14. (-/A): Heterozygous, (-/-), Homozygous normal.

from the DCLRE1C gene result in decreased V(D)J recombination, mutations that completely abolish Artemis expression or function also seriously affect T and B cell development. Many mutations have been detected in the DCLRE1C gene so far, and SCID is generally seen in patients with this mutation. On the other hand, in some mutations in this gene, patients appear in the CID table (6).

Since 2010, 17 patients with CID due to DCLRE1C mutation have been identified in our center (3,7,8,11). It is noteworthy that this number is higher than other regions in our country. This is due to the prevalence of consanguineous marriages, the geographical characteristics and the ethnic origin of the population in our region. Therefore, it is very important to identify these mutations as soon as possible. Although there are many methods such as DNA sequence analysis, exome analysis, array analysis, RT-RCR and RFLP for the identification of mutations at the DNA level, DNA sequence analysis method is among the most preferred methods. However, the RFLP method, which is less costly, requires less laboratory equipment, and is easier to apply, can be used to identify known mutations. CD19 deficiency, which is a PIY deficiency due to CD19 gene mutation, was defined by us in 2010 (12). Thereupon, a family scan was made with the PCR-RFLP method for this mutation, and the relevant mutation in the CD19 gene was detected (13). Therefore, in our study, the RFLP method based

on the RE cutting logic was preferred. Although the PCR-RFLP method is a reliable method, it is currently used for the identification of some bacterial species (14-16). By using PCR-RFLP method, homozygous mutant in 12 patients, heterozygous in 2 parents and compound heterozygous genotype in 2 patients were detected in DCLRE1C gene exon 3.

As a result, molecular identification of mutations in patients who are thought to be diagnosed with primary immunodeficiency in line with clinical and laboratory findings is of great importance for patients' lives. Because hematopoietic stem cell transplantation is among the life-saving treatments for these patients. Therefore, we believe that the RFLP method can be used as a rapid screening method in such patients, especially in patients with a family history in known mutations.

Conflict of interest: *Authors declare that there is no conflict of interest between the authors of the article.*

Financial conflict of interest: *Authors declare that they did not receive any financial support in this study.*

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