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Journal of Shahrekord University of Medical Sciences
doi:10.34172/jsums.2019.44 2019;21(6):254-257 http://j.skums.ac.ir

Original Article

Investigation on the deletion and duplication of PMP22 gene in patients with Charcot-Marie-Tooth using Real-time PCR in Chaharmahal and Bakhtiari and Isfahan Provinces

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Abstract
Background and aims: Charcot-Marie-Tooth (CMT) is a common sensory-motor polyneuropathy with a prevalence of 1/2500. It is divided into different subgroups and has various hereditary patterns. Among the different subgroups of CMT, type 1A is the most prevalent form of the disease, which is created due to the duplication of the PMP22 gene. In patients has a deletion in the PMP22 gene, the hereditary neuropathic disease is known to be liable to pressure. The aim of this study was to identify the patients affected by the disease with the new, simple, and fast qPCR method and to investigate the appropriateness of this method in evaluating these types of mutations.
Methods: In this analytical-descriptive study (code:IR.SKUMS.REC.1394.152), gene duplication and deletion in the patients were studied using the Excel software. The blood samples of 15 families afflicted with CMT and 49 healthy individuals were collected in EDTA anticoagulant tubes and analyzed. DNA extraction and quantitative real-time PCR method were applied for the PMP22 gene as the target gene and the albumin gene as the internal control gene.
Results: Two genes were compared in each patient, and it was found that 46% of the subjects had duplication in the PMP22 gene.
Conclusion: The qPCR method is an easy and fast way to detect gene duplication and deletion in CMT patients. It does not require any statistical software and can be performed without needing for parental DNA. In addition, the results of this study are consistent with the results of various studies in some countries of the world where the highest levels of deletion and duplication in PMP22 gene are seen.
Keywords: CMT1A, PMP22 gene, Quantitative real-time PCR

Received: 25 July 2018, Accepted: 4 August 2019, ePublished: 31 December 2019

Introduction

The Charcot-Marie-Tooth (CMT), HNPP (hereditary neuropathy with liability to pressure palsy), and Dejerine-Sottas are among the sensory-motor polyneuropathies that are genetically and clinically heterogeneous. CMT, with a prevalence of 1 in 2500, is the most common type of neuropathy with a genetic cause (1, 2). Some forms of CMT are inherited in an autosomal dominant manner; however, some other forms are inherited in an autosomal recessive manner. Based on the progression of the disease, it can be categorized into 2 types: CMT1 (the demyelinating form) and CMT2 (the axonal form). According to electrophysiological criteria, it can be stated that CMT1 has a nerve conduction velocity of less than 38 m/s, and demyelination and remyelination symptoms are seen in the biopsy of the sural nerve. In CMT2, the nerve conduction velocity is normal or slightly reduced (3). Many genes and loci have been identified for each subgroup. In demyelinating CMT with the dominant autosomal hereditary, the cause of the disease in many cases is the PMP22 gene duplication at locus 17p11.2, which leads to CMT1A disease, but mutations in other genes can also be the cause of this subtype, including the MPZ gene at locus 1 q22, the EGR2 gene at locus 10 q21, and the LITAF gene at locus 16 p13 (4). CMT1A is the most common form of CMT disease with a prevalence of 1/3300. About 70% of CMT1A cases are associated with 1.5-Mb duplication at locus 17p11.2-12, where the peripheral myelin protein (PMP22) gene is located (5, 6). Molecular methods have been widely used to study the structure and function of genes (7-15). To investigate deletions and duplications of the PMP22 gene, various methods such as Southern blotting, FISH, PFGF, STR-PCR, and the quantitative PCR can be used

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through SYBR Green I or TaqMan methods. Since STR-PCR analysis is fast and cost-effective, it is widely used, but low sensitivity is considered a disadvantage thereof. Quantitative PCR methods are fast in case TaqMan is used, and also are proprietary because of the use of the probes. The SYBR Green PCR method can be used to investigate various deletions and duplications in different organisms (16). The aim of this study was to determine the frequency of PMP22 gene deletion and duplication mutations using real-time PCR method in patients with CMT1A and to examine the appropriateness of this method for studying these types of mutations.

Materials and Methods

Patients

Peripheral blood samples were collected in EDTA anticoagulant tubes from 15 families (49 individuals) including 5 children, 13 women, and 31 men, out of whom one was affected by the disease. Moreover, 49 healthy volunteers, out of whom no one was affected by neuropathy, were sampled as normal controls. The criteria for entering the study were evaluating the speed of conduction of the nerve and muscle by the specialist and giving the demyelinating CMT report.

DNA extraction

DNA extraction of the collected samples was performed. DNA was extracted from EDTA anticoagulant blood samples using the recommended method by DNA extraction kit (Cinnagen, Iran). The quality and quantity of the extracted DNA were examined using the agarose gel and spectrophotometer (Nanodrop 2000 Thermo Scientific).

Real-time PCR

Exon 4 was considered as the target sequence for the PMP22 gene, and Exon 12 was selected as the reference sequence for the albumin gene. The sequence of the primers designed in this study was obtained from a similar article (17), and after being reviewed in the Vector NTI software for the formation of a hair pin and primer dimer, as well as the correctness of primers for the desired genes, the sequence was ordered to be synthesized by the Korean Macrogene Company (Table 1) (17).

At the beginning of the experiment, different concentrations of DNA were prepared with a factor of 1 to 5. The real-time PCR was performed on the albumin gene at concentrations of 120, 24, 8.4, 0.96, and 0.19 ng for a healthy control sample and repeated three times. For each reaction, 5 µL of cyber green, 3 µL of water (Fermentas), and forward and reverse primers (made by Macrogene Company) were added to the final concentration of 1 pM. Then, 9 µL of Master Mix and different DNA concentrations were added to each microtube. Thereafter, the Rotor-Gene Q device (Qiagen) was used to amplify the intended parts to be assured of the specificity of the primers, the optimization of DNA concentration, as well as the efficiency of the primers.

The real-time PCR program was performed according to the SYBR Green protocol as follows: Initial activation at 95°C for 10 minutes, with 40 cycles including unwrapping at 95°C for 15 seconds, connecting the primers at 60°C for 20 seconds, and expanding at 72°C for 25 seconds. The temperature was kept in the range of 72 to 95°C to form the melting curve. For final confirmation, the PCR products were placed on the 8% polyacrylamide gel to determine the accuracy of the amplified parts (Figure 1).

Analysis of real-time PCR data using ΔΔCT

To use this method, 4 CTs (cycle of threshold) are needed. The CTs of the desired gene in the control (healthy) and under study (patient) samples and CTs of the internal control gene in the control and under study samples were

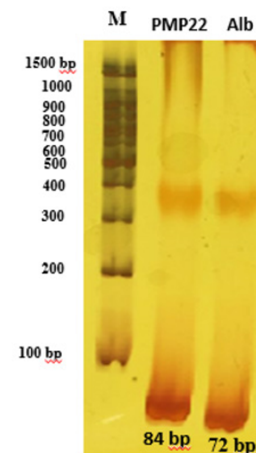

Figure 1. The Specificity of the Primers in Healthy Samples.
M: Marker of 100 bp DNA ladder, Alb: The albumin gene amplified by the real-time PCR method in a healthy person, PMP22: Peripheral myelin protein gene amplified in a healthy person.

Table 1. The designed primers specifications for PMP22 and albumin genes

Gene	Primer sequence	Primer	Length of the amplified part	TM	Primer length
Albumin (Exon12)	TGTTGCATGAGAAAACGCCA	Forward	72 bp	55	20
	GTCGCCTGTTCACCAAGGAT	Reverse		53.8	20
PMP22 (Exon4)	TCTGTCCAGGCCACCATGA	Forward	84 bp	54.3	19
	GAAGAGTTGGCAGAAGAACAGGA	Reverse		54.3	23

Table 2. The results of real-time PCR for both internal control and target genes.

CT PMP22	CT Alb	CT Alb-PMP22(Con)	CT Alb-PMP22(Sam)	ΔΔCT	$2^{-ΔΔCT}$
21.82	22.41	-0.06	0.59	-0.65	1.56
22.31	22.88	-0.06	0.57	-0.63	1.54
23.59	23.69	-0.06	0.1	-0.16	1.11
22	22.64	-0.06	0.64	-0.70	1.63
21.44	21.42	-0.06	-0.02	-0.04	1.02
21.07	21.7	-0.06	0.63	-0.69	1.61
21.28	21.39	-0.06	0.11	-0.17	1.12
21.44	21.38	-0.06	-0.06	0	1

examined. Then, according to the Livak formula, data analysis was performed, and finally the obtained results were examined compared with the control population and the internal control gene (17). For each patient, two target and internal control genes were compared and Ct difference was calculated. Afterwards, the difference in the healthy control population was also calculated. The difference between these two CTs was placed in the Livak Formula ($2^{-ΔΔCt}$), and the resulting values being about 1.5 were identified as individuals with duplication.

ΔΔCt=[Ct albumin-Ct PMP22 (control sample)]-[Ct albumin-Ct PMP22 (unknown sample)]

Results

The real-time PCR was performed and the products were placed on the 8% polyacrylamide gel to examine the specificity of the primers, which is shown in Figure 1.
The graphs of amplification of this gene in healthy and patient samples are presented in Figure 2, as follows:
The graphs of both genes in the healthy person are almost in agreement, and Cts are almost identical in both genes, while the Cts obtained from a patient with PMP22 gene duplication are smaller.

The real-time PCR was conducted, and the results were transferred to Excel software. Then, the calculations were performed using the Livak formula. In control subjects, the mean values of CT for the albumin and PMP22 genes were 22.91 and 22.97, respectively. In the following, these values were calculated separately for all patients using the formula $2^{-ΔΔCt}$, and the results were interpreted. It was revealed that 46% of the studied population had a duplication in the PMP22 gene.

The results of real-time PCR are presented in Table 2, showing that individuals number 1, 2, 4, and 6 have duplication in the PMP22 gene.

Discussion

CMT is a common neurological disorder that occurs due to different mutations in different genes. PMP22 and MPZ are the two genes with higher mutation rates found in various gene communities. PMP22 and MPZ genes have been studied as two very important genes involved in diseases in many countries around the world, such as the United States, Finland, France, Korea, Russia, Spain, Sweden, and Japan, to identify the patients with CMT using various methods. The number of families varies from 11 to 227 and the frequency of duplication in the PMP22 gene has been the highest in all studies. As noted, extensive studies have been conducted around the world on the CMT disease, including the study of chromosomal rearrangement in the 17p11.2 region. In Ukrainian patients, several cases of duplication in this region without a family history of disease were found, with the greatest cause reported to be duplication in the PMP22 gene, which accounted for 95% of the cases (18). In another study, 119 families from Slovakia with CMT were examined, out of whom 40 had gene duplication and 7 had gene deletion (19). In a large study in the United States of America, 95% of the positive results belonged to four genes: PMP22, MPZ, MFN2, and GJB1. Therefore, it was concluded that it would be better to examine these four genes first in the patients (20). In general, in most countries where this disease has been studied, the frequencies of various mutations in the PMP22 gene, including the duplication and deletion, have ranked the highest number, except for Japan where the mutation in the PMP22 gene did not have the highest frequency due to the founder effect. In this study, the PMP22 gene duplication and deletion on the short arm of chromosome 17 were investigated using the qPCR method and it was found that 46% of patients had duplication in the PMP22 gene. Thanks to the ease, fastness, and also lack of need for parental DNA, qPCR is a cost-effective method used in this study, while other methods such as Southern Blotting, STR-PCR, and MLPA have been applied in other earlier studies. It has been shown in this study that the PMP22 gene duplication is the most common cause of CMT1A.

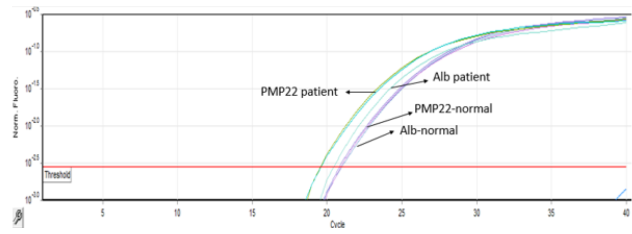

Figure 2. The Amplification Plot of Alb and PMP22 genes obtained from a healthy person and a person with the PMP22 gene duplication.

Conclusion

Considering the prevalence (1/2500) of CMT disease, qPCR was used as a quick, easy, and cost-effective method in this study, which can replace old diagnostic methods. The prevalence of duplication of this gene among the other genes involved in the development of CMT is of the highest rate and considered the main cause of this disease. In the

study conducted on 15 affected families in Chaharmahal and Bakhtiari and Isfahan provinces, 46% of patients were diagnosed with CMT1A.It seems that duplication of the PMP22 gene has the highest rate of prevalence among the causes of the disease in these two provinces like most countries in the world.

Conflict of interests

None.

Ethical considerations

This manuscript was approved by the Ethics Committee of Shahrekord University of Medical Sciences (code: IR.SKUMS.REC.1394.152).

Acknowledgments

The authors would like to thank all patients, the Welfare Organization of Chaharmahal and Bakhtiari Province, Dr. Basiri, Mr. Mohammad Kardi, the respectable staff of the Cellular Molecular Research Center, and all those who contributed to the plan. The paper was derived from the dissertation with the code of 1953.

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