

## Quantitative evaluation of *DYT1* promoter methylation in dystonia patients

Sahereh Rahnavard<sup>1</sup>, Mohammad Hamid<sup>1,\*</sup>

<sup>1</sup>Department of Molecular Medicine, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

\*Corresponding author: Mohammad Hamid, Department of Molecular Medicine, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran. E-mail: hamidi@pasteur.ac.ir

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### ABSTRACT

This study aimed at assessing the methylation state of the *DYT1* gene in the pathogenesis of dystonia disease and evaluated the clinical feature. Thirty patients with no mutation in *DYT1* gene and 30 normal individuals were investigated. The methylation of promoter in *DYT1* gene was investigated using Real-time PCR technique. There was no significant difference in methylation status of *DYT1* gene promoter in the dystonia patients compared with normal individuals, showed that the methylation of promoter in *DYT1* gene had no important role in dystonia. Also, the methylation of promoter in *DYT1* gene was observed with higher frequency in the generalized type, suggesting that the methylation of this gene may play a role in pathogenesis of this type of dystonia.

**Keywords:** Dystonia, DNA methylation, *DYT1*, Real-time PCR

### INTRODUCTION

Dystonia is a movement disorder which is characterized by involuntary movements, unusual states and paralysis [1]. There are different disorders with various causes that may lead to dystonia. Generally, it involves

muscle contractions that cause repetitive twisting and unusual spasms [1,2]. Dystonia is divided into several groups according to the origin, onset age and body distribution [3].

On the basis of the origin, this disease is categorized into genetic and non-genetic

groups [4]. The genetic group of dystonia is also called dystonia type I or first dystonia [5]. They can show different patterns of inheritance (autosomal dominant, autosomal recessive, and X-linked) [6]. The non-genetic group of dystonia is also called dystonia type II or second dystonia. It has been known that dystonia type II may be caused by spinal and brain damages or insufficient oxygen at birth, stroke, tumor, infection, toxins and metabolic disorders at older ages [7].

Dystonia is classified according to onset age into early and late classes [8]. The symptoms of early onset dystonia begin from childhood that usually presented as generalized, while the late onset dystonia occurs during adolescence in which head and neck are usually involved [9,10].

Body distribution of dystonia can exist as focal, local or generalized [11]. The symptoms of focal type presented in the 4-5<sup>th</sup> decade of the life and some limited muscles were involved. The onset of local type was also 4-5<sup>th</sup> decade of the life and most of muscles were usually affected. The generalized type of dystonia started in the age less than 5 years and involved the most of muscles. It is the most inheritable type of dystonia [12]. The average onset age is 12.5 year and almost occurs before the age of 28 in every affected individual. It appears as a

focal dystonia and then distributes from hands or feet to all over the body [4].

The prevalence of dystonia in other countries has been previously studied. This disease showed a prevalence of about 77 %, 14 % and 8 % in China, Malaysia and India, respectively [13]. The most painful type of this disease is the early onset dystonia type I. *DYT1* and *DYT6* genes have been known to be involved in this type of dystonia [14]. The onset age is an important factor in the prognosis of the disease [15]. Primary dystonia is a dominant autosomal disease which is caused by deletion of GAG bases in the exon 5 of *TOR1A* gene (alias *DYT1*) in about 30 % of affected individuals [16]. Deletion one of two adjacent GAG trinucleotides in exon 5 had been found as the most common mutation in dystonia patients [17].

The prevalence of dystonia is 1 in 16000 individuals in Ashkenazi Jews and 1 in 20000 in non-Jews populations [18]. The mutations causing this disease have been investigated in numerous clinical and genetic studies [16]. Hamid *et al.* in 2010 had studied the correlation between genotype and phenotype of dystonia type I patients. This study was performed on 42 men and 30 women with Dystonia type I, The GAG mutation in *DYT1* gene was known in 10 % of the patients, but

no mutation in *DYT6* gene was found. They suggested that there could be other factors contributing to this disease which were not yet identified [19].

The methylation of CpG regions in *DYT1* gene could be considered as one of candidate causes of disease which has been unknown. With this aim in mind, the methylation pattern was investigated in the dystonia patients with no mutation in *DYT1* and *DYT6* genes.

**MATERIALS AND METHODS**

The peripheral blood was collected from 30 dystonia patients without any mutation in *DYT1* and *DYT6* genes and 30 normal individuals who had been referred to Hazrat Rasool Hospital in Tehran, Iran. Informed consent was signed and obtained from all participants following a detailed description of the purpose of the study. Then, DNA genomic was extracted by salting out method

[20] and DNA concentration was estimated by nanodrop spectrophotometer. The digestion was performed with *HpaII* (Fermentas, Hanover, NJ). Typically, 10 ng/ml of genomic DNA was incubated overnight with *HpaII* at 37 °C. This step was followed by Real- time PCR on digested and control DNA samples.

The PCR conditions consisted on 95 °C for 10 min, followed by 40 cycles of 30 s at each of steps 95 °C, 60 °C, and 72 °C for exon 1 of *DYT1* (target) and *PMP22* (Internal control for DNA digestion) genes. The samples were analyzed in triplicate. The primer sequences used in this study is shown in Table 1.

Fisher’s exact test was used for comparing the qualitative variables. *P* values lower than 0.05 were considered as statistically significant.

**Table 1.** Primer sequences used in the Real time PCR for *DYT1* and *PMP22* genes

Target	Forward Primer	Reverse Primer
<i>DYT1</i>	’3GAGCAGAACCGAGTTTCCG-5’	’3-CCATCTTCTTCAGGGCAGC-5’
<i>DYT1</i>	’3TCAGCGAGAGGAGGAACCG-5’	’3GACCCAGCTTCATGCCCG-5’
<i>PMP22</i>	3GGAGGAGAGAAGGCTTGAATGC-5’	’3-GTTCCACATGCACACAGAAACG-5’

## RESULTS

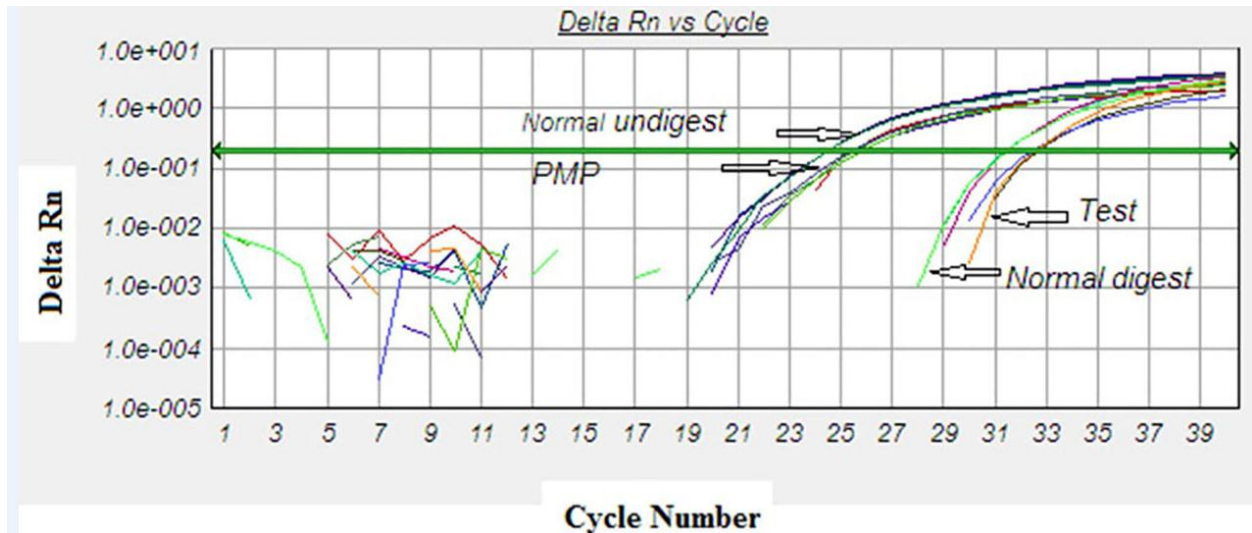
Promoter region of *DYT1* gene in 30 dystonia patients with no mutation and 30 normal individuals were studied through the quantitative analysis of DNA methylation using Real-time PCR (qAMP) technique. The *PMP22* gene as an internal control selected with no digestion site for *HpaII* enzyme. The average Ct value obtained from undigested and digested samples were estimated 25.25 and 25.28, respectively. The  $\Delta$ Ct calculated for *PMP22* gene was 0.13 that means the selected gene had no CCGG restriction site

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for *HpaII* enzyme. The average Ct value of *DYT1* gene and  $\Delta$ Ct were determined in undigested and digested samples of normal individuals (controls) and dystonia patients with no known mutation. As shown in Table 2, the  $\Delta$ Ct of *DYT1* gene was 7.13 and 7.15 for the studied controls and dystonia patients, respectively. The results obtained by 2- $\Delta$ Ct method showed that *DYT1* gene was methylated in dystonia patients as well as normal individuals (Table 2, Figure 1).

**Table 2.** The average Ct and  $\Delta$ Ct obtained in undigested and digested DNA of normal samples and dystonia patients

Gene	Groups	Ct		$\Delta$ Ct
		Digested	Undigested	
DYT1	Normal individuals	31.36	24.20	7.13
	Dystonia Patients	32.64	24.20	7.15
PMP22	Normal individuals	25.28	25.25	0.13
	Dystonia Patients	25.28	25.25	0.13



**Figure 1.** Sigmoidal-shaped plot obtained from Real-time PCR results in undigested and digested DNA of controls and dystonia patients.

## DISCUSSION

With respect to the previous studies which showed the frequency of dystonia patients with no known mutation, was observed six times more than dystonia patients with GAG mutation in exon 5 of *DYT1* gene. These observations suggested that it is likely that other genetic and environment factors may play a role in pathogenesis of this disease [1,8]. In the present study, the methylation status of promoter and exon1 *DYT1* gene was assayed in the dystonia patients with no known mutation. The results indicated that there was no

significant difference in the methylation status between the studied dystonia patients and normal individuals. However, the methylation pattern in these patients with different types of dystonia was also compared with each other.

The methylation rate of the studied region in generalized patients was 2 % more than the other types. The generalized type showed the most abundant followed by Multi-focal type in the studied dystonia patients. The methylation rate was reported 1 % in the patients with Multi-focal type of dystonia. The onset age can be seen as an important factor in multi-focal patients. However, the disease presented as well as

other types of dystonia. Furthermore, the ratio of men to women was 1:0.8 in Iranian patients with generalized and multi-focal type of dystonia while this ratio decreased in the other types of dystonia. Almost, the disease started from hand and right-side of the body in half of patients. Remarkably, as the onset age increased, the disease can also appear from the neck. The average age of onset was 13.4, twice more than *DYT1*<sup>+</sup> patients.

Dystonia disease is presented in 31.8 % of the studied patients aged below 12. Among them, the onset age <12 was observed in 38.4 % and 30.2 % of female and male patients with dystonia, respectively. Furthermore, 28.9 % of the patients with dystonia showed the disease from the age of 12 to 48. Based on our previous epidemiological investigation among people with no mutation in *DYT1* gene in Iran, as results showed, more than 28 % of the studied Iranian patients (with age range of birth 48) were family marriages children (cousins marrying cousins) [21]. Therefore, considering the fact that marrying to your cousin is legal and has high rate in some part of Iran [22,23], it is not so far from our speculation about patients (45 % with third degree kinship parents) [21].

In addition, that study cleared to us that patients also suffer from general distribution (45 %), multi focal (25 %), writhing cramp (5 %), focal (5 %) and segmental (20 %). Moreover, the outset of disease in patients with hemi-dystonia type with no family relationship, has begun from hand(45 %), leg(25 %), face(20 %) [21].

Most of these patients with no known mutation presented the generalized type of disease. The multi focal type of dystonia was observed in 14.7 % of the studied patients. Among them, the ratio of male to female was 2:1. Almost 10.14 % of the patients showed the focal type of dystonia, with ratio of male to female equal to 1:0.75. The frequency of segmental type was 10.14 % in the studied dystonia patients with no known mutation. The ratio of male and female was reported 1:1.35 in this type of dystonia. The hemi-dystonia type of dystonia was observed in 4 of the patients (3 female and one male). Only one man affected by dystonia showed the writing camp type and presented the disease at the age of 43.

In general, our results indicated that the alterations in DNA did not appear to be an important factor in the etiology of dystonia. However, the methylation of promoter in *DYT1* gene was observed with higher

frequency in the generalized type, suggesting that the methylation of this gene may play a role in pathogenesis of this type of dystonia. The study of other genetic and environmental factors may increase our understanding etiology of this disease.

### CONCLUSION

This study showed that methylation status of *DYT1* gene promoter in the patients suffering from Dystonia ( $\Delta C_t$ ; 7.15) compared with normal individuals ( $\Delta C_t$ ; 7.13) had no significant difference. Study results described that the methylation of promoter in *DYT1* gene had not an important role in Dystonia onset. Also, the methylation of promoter in *DYT1* gene was observed with higher frequency in the generalized type, suggesting that the methylation of this gene may play a role in pathogenesis of this type of dystonia. Finally, the examinations of other genetic and environmental factors were needed to increase our vision to the causes of this disease.

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