

# Poly thymidine polymorphism and cystic fibrosis in a non-Caucasian population

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**Abstract.** *Background:* Cystic fibrosis is a monogenic recessive disorder found predominantly in Caucasian population. This disease arises from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. In this study we consider poly T polymorphism c.1210-12T[5], c.1210-12T[7], c.1210-12T[9] (T<sub>5</sub>, T<sub>7</sub>, T<sub>9</sub>) in the intron 8 of CFTR gene in normal individuals and cystic fibrosis patients in the north of Iran.

*Material and methods:* 40 CF patients and 40 normal individuals were screened for poly T polymorphism in intron 8 of CFTR gene using Reverse Dot Blot method which was also used to detect p.Phe508del among CF patients.

*Results:* T<sub>7</sub> allele is the most prevalent in both normal and CF patients. Its abundance is approximately 75%. T<sub>9</sub> and T<sub>5</sub> represent approximately 20% and 5% of alleles respectively. T<sub>7</sub>/T<sub>7</sub> genotype is the most present in both normal and CF patients with 72.5% and 60% prevalence respectively. p.Phe508del was present in 13 CFTR alleles belonging to 7 patients with either homozygote T<sub>9</sub>/T<sub>9</sub>, T<sub>7</sub>/T<sub>7</sub> or compound heterozygote T<sub>7</sub>/T<sub>9</sub> genotypes.

*Conclusion:* Contrary to the Caucasians, T<sub>7</sub> allele is more frequent in Northern Iranian CF patients. The presence of p.Phe508del and T<sub>7</sub> allele in the same framework is reported for the first time in this part of the world. Further investigations of other populations will help to understand whether p.Phe508del arose by selection pressure in this part of the world or was imported from European countries. The abundance of T<sub>5</sub>, T<sub>7</sub>, T<sub>9</sub> alleles indicates that this polymorphism can be used as one of the informative markers for detection of normal and mutant alleles in prenatal diagnosis or carrier assessment in families with previous history of the disease in regions with high degree of CFTR mutation heterogeneity.

Keywords: Cystic fibrosis, IVS8 polyT, polymorphism, p.Phe508del, Iran

## 1. Introduction

Cystic fibrosis is a monogenic recessive disorder present predominantly in Caucasians. This disease arises from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, that encodes chloride channel in the apical membrane of exocrine epithelial cells [1–3]. The gene comprises 27 coding exons, spanning over 230 kb on chromosome 7q31.2

that encode a protein with 1480 amino acids, with a molecular weight of ~ 170 KD [4,5]. CFTR protein is found in various cell types including lung epithelium, submucosal gland, pancreas, liver, sweat ducts and reproductive tract. Although CFTR functions mainly as a chloride channel, it has many other regulating roles, including inhibition of sodium transport through the epithelial sodium channel, regulation of ATP channels [6, 7], regulation of intracellular vesicle transport and inhibition of endogenous calcium activated chloride channels [8–10]. CF disease diagnosis is performed on the basis of clinical symptoms and high concentration of chloride in sweat [11,12]. Epithelial cells disorder is a dominant event in CF disease [13], causing a de-

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fect in transportation of electrolytes, water and other solutions from the cell membrane, leading to many different clinical symptoms such as lung infections, pancreas insufficiency, infertility in men and increased chloride concentration in sweat. Although cystic fibrosis is the most common autosomal recessive disease in many Caucasian populations, including those of Europe and the United States with the carrier frequency of about 1:25, it seems that cystic fibrosis prevalence in Iran isn't rare, and in one investigation it has been shown that the carrier frequency is about 1:40 [14]. More than 1800 CFTR mutations and polymorphisms have been identified (CF genetic analysis consortium <http://www.genet.sickkids.on.ca/CFTR/>). These mutations have different frequencies in different populations. The most common mutation in CFTR gene is p.Phe508del. Its frequency in Caucasian populations is about 70% but its frequency in Arab [15,16], Indian [17], Iranian [18] and Turkish populations [19] varies between 44% and 13%. Previous molecular studies demonstrated that the Iranian population is very heterogeneous regarding CFTR alleles; as about 50 different mutations were reported in less than a total of 200 CF patients. Many of these alleles were present at a frequency lower than 2% and at least 20% of mutations remained undiagnosed [18,14,20]. As molecular diagnosis is the unique approach to perform prenatal diagnosis in at risk couples, it is though necessary to apply segregation analysis of CFTR polymorphic markers for those families presenting a previous history of the disease with one or two unknown mutations. Also, even if both mutations are detected, it is still recommended to perform fetus genotyping during prenatal diagnosis both directly by mutation analysis and indirectly by studying multiple inter or intragenic microsatellites or RFLPs to reduce errors due to methods of mutation detection, maternal contamination or human manipulations [21]. As most microsatellites analysis necessitate either the use of capillary electrophoresis or sequencing [22,23] which may not be available in many laboratories, using polymorphic markers easily detectable with routine laboratory methods such as RFLP or Reverse Dot Blot could be advantageous for those laboratories.

PolyT polymorphism was previously reported in CFTR gene. Genetic studies have shown that the polymorphic poly pyrimidine (Tn) locus located within the 3' splice site of intron 8 in the CFTR gene is associated with a variable efficiency of exon 9 splicing [24]. There are three common alleles at this locus, with 5, 7 and 9 thymidines (c.1210-12T[5], c.1210-12T[7], c.1210-

12T[9]) which we shortly annotated as T<sub>5</sub>, T<sub>7</sub>, T<sub>9</sub>. Several studies performed on patients genotypes confirmed an association between bilateral absence of vas deferens (CBAVD) and T<sub>5</sub> allele. Indeed in nasal epithelium and vas deferens the T<sub>5</sub> allele is associated with a high proportion of alternatively spliced CFTR mRNA, lacking exon 9 [24]. This polymorphism is commonly investigated on infertile CF men [25–27].

However, the fact that the T<sub>5</sub> allele is not found exclusively in patients suffering from CBAVD but is also present in healthy subjects indicates that this mutation has partial penetrance probably due to simultaneous presence of other mutations or polymorphisms [24]. The T<sub>7</sub> allele is the most frequent in normal individuals with an approximate frequency of 80% in Caucasians [28,29] and 90% in East Asians [30]. However the frequency of poly T polymorphism in CF patients is controversial in different population studied. In Caucasians the T<sub>9</sub> is predominant, while in Indians the T<sub>7</sub> allele is predominant [17,29]. This difference could be explained by different spectrum and frequency of mutations among those populations but the haplotype studies of p.Phe508del and polyT polymorphism appeared again to be controversial among different populations [17,29,31]. Thus further population studies seems necessary to elucidate the multi-origin of p.Phe508del.

In this study we consider polyT polymorphism (T<sub>5</sub>, T<sub>7</sub>, T<sub>9</sub>) in the intron 8 of CFTR gene in normal individuals and cystic fibrosis patients in the north of Iran and its association with p.Phe508del, the most common Iranian mutation.

## 2. Materials and methods

Subjects: 40 normal adult fertile males or females and 40 patients that were diagnosed as cystic fibrosis based on clinical evaluation (pulmonary complications and pancreatic insufficiency) and elevated sweat chloride values (> 60 mEq/L) were screened for poly T polymorphism in the intron 8 of CFTR gene. The patients were aged between one month to 14 years recruited from pediatric hospital of Babol medical university and all subjects were from the north of Iran.

Molecular analysis of CFTR gene: Genomic DNA was extracted from peripheral blood leukocytes using Alkaline lysis method.

DNA was amplified by PCR using 5' biotinylated Forward 5'-CATAAAACAAGCATCTATTGAAAAT-3' and 5' biotinylated Reverse 5'-CACTACACCCATAC

Table 1  
Prevalence of T<sub>5</sub>, T<sub>7</sub> and T<sub>9</sub> alleles in intron 8 of CFTR gene of normal individuals and CF patients

	Alleles of normal subjects(%)	Alleles of CF patients(%)
T <sub>5</sub>	6.25	5
T <sub>7</sub>	83.75	75
T <sub>9</sub>	10	20
Total	100	100

ATTCTCCT-3' primers. PCR amplifications were carried out in 50  $\mu$ l reaction volumes containing approximately 250  $\mu$ M dNTPs, 2 mM MgCl<sub>2</sub>, 200 nM each forward and reverse biotinylated primers, 1.5 unit Taq DNA Polymerase. The amplification conditions included initial denaturation at 94°C for 3 minutes, followed by 30 cycles, each consisting of a 1 minute denaturation at 94°C, annealing at 58°C for 1 minute and extension at 72°C 45 second, followed by final extension at 72°C for 5 minutes. PCR products of 413 bp long were visualized after electrophoresis on 1.5% Agarose LE gel under 302 nm UV lamp.

Reverse Dot Blot reaction was performed according to Lappin et al. [32]. In this assay, oligonucleotide probes were synthesized using a C6-amino-linker on the 5' end of the product and were attached onto Biodyne C nylon membrane (from Pall Corporation) activated by 1-Ethyl 3-Dimethyl Aminopropyl Carbodiimide (EDAC). The target DNA is amplified with 5' biotinylated primers and hybridized to immobilized oligonucleotides on the membrane. Hybridization is detected by adding streptavidin-horseradish peroxidase to the membrane where it will bind to any biotinylated DNA that has hybridized to the oligonucleotides. A positive signal can then be obtained by a simple non-radioactive colorimetric reaction in the presence of tetramethylbenzidine dihydrochloride (TMB) substrate and dilute solution of hydrogen peroxide.

In this method biotinylated PCR products of intron 8 were used for hybridization with specific T<sub>5</sub>, T<sub>7</sub> and T<sub>9</sub> probes fixed on Biodyne C membrane. The sequence of probes are: IVS 5T: 5'-GTGTGTGTTTTTAAACAGG-3', IVS 7T: 5'-TGTGTGTTTTTAAACAGG-3' and IVS 9T: 5'-TGTGTGTTTTTAAACAG-3' [33]. Primers and probes were from MWG Biotech, Germany. The hybridization pattern of each PCR product allowed the genotype determination of different subjects (in the intron 8 of CFTR gene). p.Phe508del mutation was also screened for all CF patients using a Reverse dot blot reaction [32].

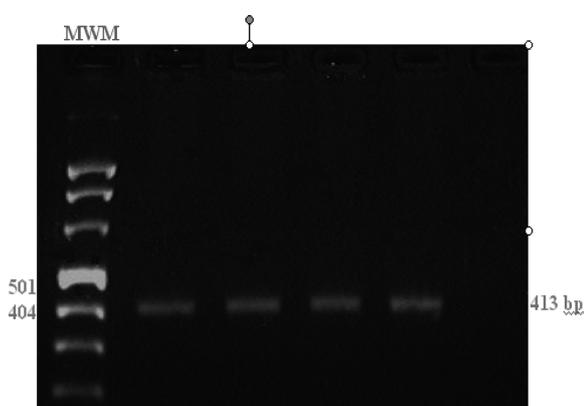


Fig. 1. Result of PCR reactions. The fragment of DNA with 413 bp length containing poly thymine in the intron 8 of CFTR gene was amplified with specific primers. The size of fragment was confirmed by comparing to molecular weight marker (MWM).

### 3. Results

40 normal individuals (80 alleles) and 40 patients (80 alleles) were screened for polyT polymorphism in the intron 8 of CFTR gene and the genotypes of normal individuals and patients were determined after PCR and Reverse Dot Blot reactions. The results of PCR and Reverse Dot Blot reactions are shown in Figs 1 and 2. The prevalence of each allele in normal individuals and CF patients is indicated in Table 1. T<sub>7</sub> allele is the most prevalent in normal individuals and CF patients with 83.75% and 75% abundance respectively. T<sub>5</sub> allele shows the lowest prevalence and its abundance in normal individuals and patients is 6.25% and 5% respectively. Table 2 shows the abundance of different genotypes. T<sub>7</sub>/T<sub>7</sub> genotype in normal individuals and CF patients is the most prevalent with 72.5% and 60% abundance respectively. T<sub>5</sub>/T<sub>9</sub> and T<sub>5</sub>/T<sub>5</sub> genotypes were not found in this study. 22.5% of normal individuals and 30% of CF patients had heterozygote genotypes (T<sub>7</sub>/T<sub>5</sub> or T<sub>7</sub>/T<sub>9</sub>).

We also compared the relationship between p.Phe508del mutation and polyT polymorphisms. Seven patients were homozygote for p.Phe508del and one was compound heterozygote for p.Phe508del. Five homozygote patients had T<sub>9</sub>/T<sub>9</sub> and one had T<sub>7</sub>/T<sub>7</sub> genotype. One compound heterozygote patient had T<sub>7</sub>/T<sub>9</sub> genotype.

### 4. Discussion

40 normal individuals and 40 cystic fibrosis patients from the north of Iran were investigated for polyT poly-

Table 2  
Prevalence of various genotypes in CF and normal individuals

	T <sub>7</sub> /T <sub>7</sub> (%)	T <sub>7</sub> /T <sub>9</sub> (%)	T <sub>7</sub> /T <sub>5</sub> (%)	T <sub>9</sub> /T <sub>9</sub> (%)	Total (%)
<b>Normal individuals</b>	<b>72.5</b>	<b>10</b>	<b>12.5</b>	<b>5</b>	100
<b>Patients</b>	<b>60</b>	<b>20</b>	<b>10</b>	<b>10</b>	100

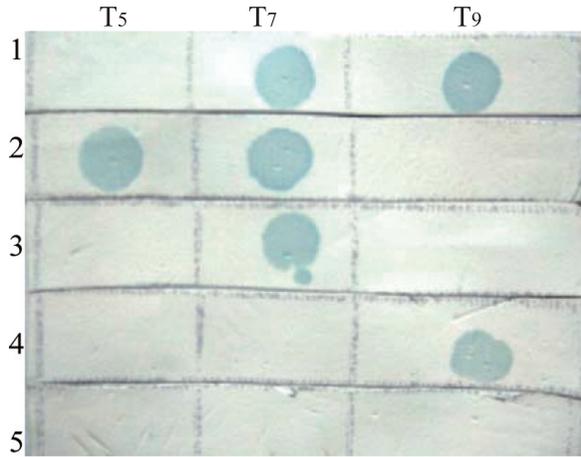


Fig. 2. Results of Reverse Dot Blot reaction. Rows 1, 2, 3, 4 indicate the Reverse Dot Blot reaction respectively for individuals with T<sub>7</sub>/T<sub>9</sub>, T<sub>7</sub>/T<sub>5</sub>, T<sub>7</sub>/T<sub>7</sub>, T<sub>9</sub>/T<sub>9</sub> genotype. Row 5: negative control (without PCR product).

morphism in the intron 8 of CFTR gene by Reverse Dot Blot method. This method is a simple, rapid and reliable method which allows simultaneous detection of up to 20 different mutations or polymorphisms in a single hybridization assay [32]. There are three common alleles at this locus with 5, 7 and 9 thymidines (T<sub>5</sub>, T<sub>7</sub>, T<sub>9</sub>) that could influence the processing of exon 9 in the mRNA of CFTR gene. In this study we found that T<sub>7</sub> allele is the most prevalent in both normal individuals and CF patients (83.75% and 75% respectively) while T<sub>5</sub> allele showed the lowest prevalence: 6.25% and 5% in normal individuals and CF patients respectively. These frequencies are very similar to those reported in Caucasians and American populations [34,35]. Four genotypes including T<sub>7</sub>/T<sub>5</sub>, T<sub>7</sub>/T<sub>7</sub>, T<sub>7</sub>/T<sub>9</sub> and T<sub>9</sub>/T<sub>9</sub> were found in both studied groups. Among these genotypes the T<sub>7</sub>/T<sub>7</sub> genotype is the most prevalent: 72.5% and 60% in normal individuals and CF patients respectively. The prevalence of T<sub>9</sub>/T<sub>9</sub> in normal individuals and CF patients was 5% and 10% respectively. Nearly 20% to 30% of genotypes were compound heterozygote (T<sub>7</sub>/T<sub>5</sub> and T<sub>7</sub>/T<sub>9</sub>). T<sub>5</sub>/T<sub>5</sub> and T<sub>5</sub>/T<sub>9</sub> genotypes were not found in this study. As the diagnosis of CF was certain according to Rosenstein and Farrell [11, 12], for those 4 CF patients presenting T<sub>5</sub> allele, there should be a CFTR mutation in cis with T<sub>5</sub> as CFTR re-

lated diseases did not apply to these patients. All studies on polyT polymorphism showed a predominance of T<sub>7</sub> allele in normal individuals [17,24–31] but Caucasians and Indians CF patients seemed to have different frequency of T<sub>7</sub> allele. This could be due to different spectrum of mutations in those populations and a high prevalence of p.Phe508del which is tightly linked to T<sub>9</sub> allele in Caucasians [17,29]. However in one study performed in the Maronite group in Lebanon, 66.7% of the p.Phe508del chromosomes were found to be associated with T<sub>7</sub> allele [31] suggesting a different origin of p.Phe508del in this later population. In the few studies performed in Iranian CF patients, various mutations were reported in CFTR gene. p.Phe508del is the most frequent mutation reported in Iran representing nearly 16% to 20% of mutations [14,18,20,36]. In this study the linkage between p.Phe508del mutation and polyT polymorphism was investigated. From seven cases with p.Phe508del mutation, six were homozygote for p.Phe508del with either T<sub>9</sub>/T<sub>9</sub> or T<sub>7</sub>/T<sub>7</sub> genotype and one patient was compound heterozygote for p.Phe508del with T<sub>7</sub>/T<sub>9</sub> genotype. A similar study performed in Indian population showed complete linkage between p.Phe508del mutation and T<sub>9</sub> allele [17]. Our results show a linkage between p.Phe508del and T<sub>7</sub> allele in few cases indicating that Iranian populations are more heterogenous than other populations so far studied in this part of the world. This heterogeneity may be due either to migrations of non-European CF individuals or recombination events in this population. More extensive studies in populations of East Asia or middle east is needed to elucidate whether p.Phe508del has different origins. Furthermore our results indicated that about 20% to 30% of individuals studied had compound heterozygote genotypes at IVS 8 poly T locus. Due to considerable heterogeneity in CFTR mutations among Iranian patients, prevention of the disease by prenatal diagnosis is very time consuming and using even sequencing or Multiplex Ligation-dependent Probe Amplification (MLPA) methods may not be always conclusive as many non disease causing polymorphisms or large insertions, deletions or rearrangements or mutations in introns which are not routinely sequenced in CF patients may exist. The use of segregation analysis of intragenic polymorphic markers such as IVS8 polyT to track the defective allele regardless

of the type of CFTR mutation, is technically straight forward, yet the method has certain limitations in its application in families lacking a prior history of cystic fibrosis and / or informative polymorphic makers. Thus, prior to performing linkage analysis, it is recommended to obtain confirmation of the clinical diagnosis of CF in the family. Linkage analysis may also be used in addition to direct mutation search for prenatal diagnosis, as suggested by European CF guidelines [21]. Moreover, regarding individual families with previous history of CF disease, the feasibility of the analysis highlights its potential use to provide relevant information regarding carrier status for unaffected relatives of individuals with unknown genotypes, without obligation to perform direct mutation testing. IVS8 polyT polymorphism had 30% and 22.5% heterozygosities in CF patients and in healthy control individuals respectively. Thus one would expect the analysis of the IVS8 polyT polymorphism to be informative in 22.5% to 30% of cases of linkage diagnosis, in order to discriminate the non-affected chromosome from the CF chromosome in the parents of the CF patients. Thus in about 20% to 30% of cases polyT polymorphism could be used as a useful complementary genetic marker for carrier detection or prenatal diagnosis in families with previous history of the disease. Although the IVS8 polyT polymorphism has a limited heterozygosity, the T9 allele is present in about 90% of chromosomes with the p.Phe508del mutation while it is present in 10% of wild-type chromosomes. Thus, T9 allele can be strongly informative and may be used in segregation analysis as a complementary test for mutation tracking in prenatal diagnosis or carrier testing in those families with p.Phe508del mutation.

As polyT detection is also included in many commercial kits such as ELUCIGENE CF-Poly-T, Tepnel diagnostics, UK; INNO-LiPA CFTR, Innogenetics, Belgium; Cystic fibrosis v3 5/7/9T OLA ASR, Abbott diagnostics, USA, it could also be investigated in segregation analysis in those laboratories using these commercial kits. However as in rare cases, other polyT alleles such as T2, T3 or T6 were reported [37–39] one should note that in rare cases, homozygosity for common alleles detected by these methods may be artifactual and due to the fact that all these polyT detection methods assume that only three common length variants exist in the general population. Preliminary molecular investigation of previously reported mutations with a frequency higher than 2% in Iranian population in these CF patients showed that the population is highly heterogeneous and except p.Phe508del, does not present

the most frequent mutations reported worldwide or in other parts of Iran (data not shown). Further investigations of other mutations and polymorphisms within or near CFTR gene on a larger number of CF patients will facilitate disease prevention in CF families with rare mutations and the identification of haplotypes associated with CF-causing mutations in highly heterogeneous populations.

### Acknowledgments

We thank all of the patients and their families for consenting to participate in this study. We also acknowledge Mr. Ali Banihashemi, Mrs. Mandana Azizi and Mrs. Beheshteh Asghari for their technical assistance. This study was authorized by the Ethic committee of Babol University of Medical Sciences. This work received supports from the Endocrine research center of Tehran University of Medical Sciences and the Research Council of Babol University of Medical Sciences.

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