CASE REPORT

# Perforin Gene Analaysis in an Iranian Family with Familial Hemophagocytic Lymphohistiocytosis

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## ABSTRACT

Perforin gene (*PRF1*) mutations have been reported in 20-30% of patients with familial hemophagocytic lymphohistiocytosis (FHL), an immune disorder of infancy and early childhood. Cytotoxic T and natural killer (NK) cell activities are remarkably reduced or absent in FHL patients. We report the first cases of familial hemophagocytic lymphohistiocytosis in an Iranian family with two siblings. Exons 2 and 3 of the *PRF1* gene were analyzed by polymerase chain reaction (PCR) amplification and direct sequencing. Perforin gene mutation(s) were detected in none of the cases. The result of our study indicates that not much evidence is present concerning a correlation between perforin gene defects and familial hemophagocytic lymphohistiocytosis etiology in these cases.

**Keywords: Familial Hemophagocytic Lymphohistiocytosis, Perforin gene, Cytotoxic T lymphocytes**

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Iran.J.Immunol. VOL. 4 NO. 2 June 2007

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## INTRODUCTION

Familial hemophagocytic lymphohistiocytosis (FHL), an inherited form of hemophagocytic lymphohistiocytosis (HLH) syndrome, is characterized by the overwhelming activation of T lymphocytes and macrophages invariably leading to death in the absence of treatment. It is difficult to differentiate primary from secondary cases of HLH on the basis of symptoms, which are very similar (1).

FHL is a heterogeneous disorder, with one known causative gene which codes for perforin, a cytotoxic effector protein responsible for the function of immune cells involved in control of viral infections. A second genetic mutation responsible for FHL involves the gene-encoding Munc13-4 protein, which is also involved in the function of NK and T cells (2). The third and most recent gene to be associated with HLH is *Syntaxin 11*. Defects in this gene have thus far only been reported in Turkish families. The function of the Syntaxin 11 protein has not yet been clearly linked to NK cell function (3). Approximately 50% of FHL in North America is caused by mutations in the gene-encoding perforin, *PRF1*. In other parts of the world, approximately 20-30% of FHL cases are caused by the *PRF1* mutations (4).

## SUBJECTS AND METHODS

**Clinical Assessment.** The project was approved by the medical university of Jondi Shapour's Ethics Board. After informed consent, all participants were questioned on their personal and medical history and a family tree was drawn.

**Patients.** A four member family, parents and their two twin female siblings (3.5 years), were admitted to Shafa hospital, Ahwaz, Iran. Unfortunately, we could not perform flow cytometry for analysis of natural killer cells, but all patients fulfilled the diagnostic criteria of HLH as described by the Histiocyte Society (5).The affected twins exhibited typical features of FHL, which included fever, liver and spleen enlargement, bicytopenia, hypertriglyceridemia, hypofibrinogenemia, and elevated ferritin level. We identified hemophagocytosis on examination of bone marrow, spleen, and lymph nodes. The parents had no clinically noticeable discomforts**.**

**Methods.** Blood specimens anticoagulated with 10% EDTA were processed immediately after collection and stored at -70 °C. Genomic DNA was isolated from 500 µL of peripheral blood leukocytes by standard procedures. Ten microliters of DNA eluate was used to amplify exons 2 and 3 of the *PRF1* gene (coding and non-coding regions) by polymerase chain reaction. The following primers as described by Ericson et al. (6) were used (Table 1).

The 25 µL reaction mixture contained 2.5 µL PCR buffer, 0.75 µL $MgCl_2$ (1.5 m*M*), 0.5 µL of all four deoxynucleoside triphosphates (each at 0.2 m*M*), 0.5 µL of each forward and reverse primers (each at 25 pmol/µL), and 2.5 U of *Taq* DNA polymerase. After an incubation (94°C for 3 minutes), 30 cycles (94°C for 30 seconds, 52°C (for $PRF_2$), 61°C (for $PRF_3$/1), 60°C (for $PRF_3$/2), and 56°C (for $PRF_3$/3), and 72 °C for 30 seconds) were performed and were followed by a final 5-minute extension at 72 °C in a thermal cycler (BIO-RAD thermocycler, Germany). The amplification products were visualized by staining with ethidium bromide after electrophoresis on 1.5 % agarose gel. The PCR products were cycle sequenced with the ABI PRISM Big Dye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems) (Table 2).

**Table 1. Perforin primer sequences for PCR amplification of exons 2 and 3**

| Region and Primer Sequence | Annealing Site |
|---|---|
| Exon 2: | |
| PRF/F$_2$: 5'-TGTGCCCTGATAATCTGTG-3' | 3171–3190 |
| PRF/R$_2$: 5'-GCAGCCTCCAAGTTTGA-3' | 3901–3917 |
| Exon 3: | |
| PRF/F$_3$/1: 5'-TCCTAGTTCTGCCCACTTAC-3' | 4886–4905 |
| PRF/R$_3$/1: 5'-GGGTTCCAGGGTGTAGTCCA-3' | 5471–5480 |
| PRF/F$_3$/2: 5'-ACTGCCCTGCGCACCTG-3' | 5164–5180 |
| PRF/R$_3$/2: 5'-GGTTGTTATTGTCCCACACG-3' | 5811–5828 |
| PRF/F$_3$/3: 5'-GTCACCACCCAGGACTGCTG-3' | 5659–5678 |
| PRF/R$_3$/3: 5'-GGCTCCCACTGTGAGA-3' | 6169–6184 |

**Table 2. Perforin primer sequences for cycle sequencing of exons 2 and 3**

| Region and Primer Sequence | Annealing Site |
|---|---|
| Exon 2: | |
| PRF/R$_2$: 5'-GCAGCCTCCAAGTTTGA-3' | 3901–3917 |
| Exon 3: | |
| PRF/F$_3$/1a: 5'-TCTCTTCTCGCAGTTTCCAT-3' | 4982–5001 |
| PRF/F$_3$/2a: 5'-GCCTCCTTCCACCAAACCTA-3' | 5323–5340 |
| PRF/F$_3$/3a: 5'-ATCCAAGCATGGGGCCTG-3' | 5719–5736 |

## RESULTS

Perforin gene silent mutations were found at codon 273 (GCC>GCT) coding for alanine and at codon 299 (CAC>CAT) coding for histidine in both cases. The gel electrophoresis of some PCR products is shown in figure 1. In none of the patients pathogenic mutations were detected at the perforin gene by analysis of sequencing. The partial sequencing result was illustrated in figure 2.
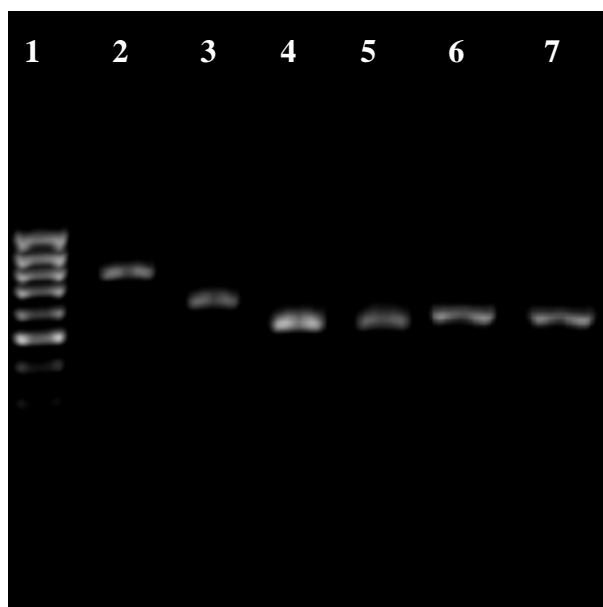


**Figure 1.** Agarose gel (1.5%) electrophoresis stained with ethidium bromide, showing the polymerase chain reaction products of the four *PRF* regions. *Lane 1*: DNA Ladder (100bp), *Lane 2*: PRF$_2$ product (746bp), and *Lane 3*: PRF$_3$/1 product (594bp) for one of the siblings, *Lane 4 - 7*: PRF$_3$/3 product (525bp) for the affected siblings, and their parents.

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CCTGACTTCAAGAGGGCCCTCGGGGACCTGCCCCACCACTTCAACGCCTCCACCCAGCCC  PCR PRODUCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCTGACTTCAAGAGGGCCCTCGGGGACCTGCCCCACCACTTCAACGCCTCCACCCAGCCC  PRF1

GCCTACCTCAGGCTTATCTCCAACTACGGCACCCACTTCATCCGGGCTGTGGAGCTGGGT  PCR PRODUCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GCCTACCTCAGGCTTATCTCCAACTACGGCACCCACTTCATCCGGGCTGTGGAGCTGGGT  PRF1

GGCCGCATATCGGCCCTCACTGCCCTGCGCACCTGCGAGCTGGCCCTGGAAGGGCTCACG  PCR PRODUCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGCCGCATATCGGCCCTCACTGCCCTGCGCACCTGCGAGCTGGCCCTGGAAGGGCTCACG  PRF1

GACAACGAGGTGGAGGACTGCCTGACTGTCGAGGCCCAGGTCAACATAGGCATCCACGGC  PCR PRODUCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GACAACGAGGTGGAGGACTGCCTGACTGTCGAGGCCCAGGTCAACATAGGCATCCACGGC  PRF1

AGCATCTCTGCTGAAGCCAAGGCCTGTGAGGAGAAGAAGAAGAAGCACAAGATGACGGCC  PCR PRODUCT
|||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||
AGCATCTCTGCCGAAGCCAAGGCCTGTGAGGAGAAGAAGAAGAAGCACAAGATGACGGCC  PRF1

TCCTTCCACCAAACCTACCGGGAGCGCCATTCGGAAGTGGTTGGCGGCCATCACACCTCC  PCR PRODUCT
|||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
TCCTTCCACCAAACCTACCGGGAGCGCCACTCGGAAGTGGTTGGCGGCCATCACACCTCC  PRF1

ATTAACGACCTGCTGTTCGGGATCCAGGCCGGGCCCGAGCAGTACTCAGCCTGGGTAAAC  PCR PRODUCT
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ATTAACGACCTGCTGTTCGGGATCCAGGCCGGGCCCGAGCAGTACTCAGCCTGGGTAAAC  PRF1
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**Figure 2.** BLAST result of the PCR product generated with PRF/$F_3$/1 and PRF/$R_3$ /1 revealed homology to the *PRF1* gene with NCBI reference number NM-005041.3. The gray highlighted positions indicate the C>T nucleotide changes in this PCR product.

## DISCUSSION

Identification of genetic defects responsible for FHL has been a major concern for a number of years. To date, more than forty pathogenic mutations have been reported for the *PRF1* gene reviewed by Ishii et al. (4). *PRF1* is a single gene organized in only three exons, of which exons 2 and 3 are translated (7). *PRF1* gene encodes a 60-kDa active protein (perforin) expressed by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, and has a crucial role in the CTL and NK-associated cytotoxic processes (8). Impaired cytotoxicity by NK cells and CTL is crucial in the pathophysiology of FHL (4). Perforin mutations account for approximately 20% of cases of FHL, with a some-what higher prevalence (30%) in children of Turkish descent. Chromosome arm 9q mutations account for approximately 10% of familial cases; the remainder of FHL cases are caused by mutations in as yet unidentified genes (6).

Some clinical symptoms helped in FHL diagnosis of the two young girls in our case report. However, the detected silent mutations (as mentioned before) can not be pathogenic and probably present a genomic variation. Therefore, both nucleotide changes observed in these 2 cases do not lead to changes in amino acids and therefore have no functional effects on the gene product.

To our knowledge, this is the first report on perforin gene analysis in an Iranian family diagnosed with FHL. Identification of a genetic defect in patients with HLH has diagnostic, prognostic, and therapeutic implications and should be pursued whenever possible. A genotype-phenotype relationship cannot be performed until a much larger number of

patients with and without *PRF1* mutations are identified. In this study, we are not able to correlate the defined polymorphisms to the etiology of FHL.

## ACKNOWLEDGEMENTS

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