Toll-like receptor-4 gene variations in Egyptian children with familial Mediterranean fever

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Abstract

Background: Familial Mediterranean fever (FMF) is an autosomal recessive disorder affecting people in the region of the Mediterranean Sea. It is usually associated with mutation in Mediterranean fever (MEFV) gene that encodes the pyrin protein, which affects the innate inflammatory response. Toll-like receptors (TLR) are a family of pattern recognition receptors that recognize pathogenic microbes and activate antimicrobial defense mechanisms. Toll-like receptor 4 (TLR-4) is concerned with recognition of gram-negative organisms. There is growing clinical evidence suggesting a role for expression of TLRs in the immune pathogenesis of FMF. Thus, the aim of the current study was to evaluate the presence of TLR-4 (p.Asp299Gly) and TLR-4 (p.Thr399Ile) gene variants in association with Egyptian children having FMF, furthermore, its effect on disease course and severity.

Results: Seventy Egyptian children diagnosed as having FMF, together with 50 age and gender-matched controls were enrolled in the study. The TLR-4 (p.Asp299Gly) and (Thr399Ile) gene variants were determined by PCR-RFLP analysis for all studied patients and controls. TLR-4 p.Asp299Gly gene variant was detected in 1 (1.4%) of the patients and p.Thr399Ile gene variant was detected in 2 (2%). None of the controls had any of the two tested gene variants. All found variations were heterozygous. We could not find a statistically significant association with disease severity in cases with or without TLR-4 gene variants (P = 0.568). Patients with M694V gene mutation showed a higher disease severity (P = 0.035).

Conclusion: TLR-4 (p.Asp299Gly) and (p.Thr399Ile) gene variants were not found to have a link with the occurrence, the clinical picture of FMF, its severity, and response to colchicine treatment in Egyptian children. M694V gene mutation seems to be associated with higher disease severity. Further larger studies are needed to verify these results.

Keywords: Familial Mediterranean fever, Toll-like receptor 4, Gene variants

Background

Familial Mediterranean fever (FMF) is an auto-inflammatory autosomal recessive disease associated with mutation in MEFV (Mediterranean fever) gene, which is located on the short arm of chromosome 16. This gene encodes the expression of pyrin protein, which has a role in the innate immune response. It suppresses the production of inflammatory cytokines specially interleukin (IL–1 ß) [1, 2].

Familial Mediterranean fever (MEFV) gene mutations interrupt the expression of pyrin, leading to intermittent episodes of uncontrolled inflammation [3].

Toll-like receptors (TLRs) constitute a family of transmembrane proteins expressed by various cell types, including immune cells. They identify pathogens and initiate inflammatory signaling pathways [4]. Discovery of TLRs increased our understanding of how the innate immune response senses various structures of
involved in promoting apoptosis, thus allowing persist-
are unable to inhibit the production of IL-1ß, a cytokine
kappa B. Furthermore, mutant pyrin proteins in FMF
pathway. This activates nuclear transcription factor-
causes the activation of protein signal cascades, includ-
flammation [5].

TLR-4 (p.Thr399Ile) polymorphisms downregulate in-
negative bacterial cell wall. TLR-4 (p.Asp299Gly) and
TLR-4 (p.Thr399Ile) polymorphisms downregulate in-

Genetic variations of TLRs have been found to in-
crease the susceptibility to autoimmune and allergic dis-
eases [9]. Furthermore, expression of FMF disease might be affected by some unknown modifying genes, besides the well-established MEFV gene mutations [10].

Single nucleotide variations of the TLR-4 genes have been associated with receptor hypo-responsiveness and susceptibility to bacterial, fungal, and viral infections [1]. An important question had been raised; whether TLRs gene variations would affect the immune responses against infections, thus, affect the course of FMF [11].

There is some clinical evidence suggesting a role for expression of TLRs in FMF [12]. Furthermore, some studies worked on finding an association between the low prevalence of single nucleotide polymorphisms (SNPs) in TLR-4 genes and FMF disease [11, 13, 14].

Kawamoto et al. suggested that FMF patients, whose attacks are triggered by the menstrual cycle, might have fewer TLR4 promoter genotypes that allow gram-
negative bacteria to colonize the urinary tract without symptoms [1]. Thus, we hypothesized that TLR-4 (p.Asp299Gly) and TLR-4 (p.Thr399Ile) gene variations might affect FMF in Egyptian children.

The aim of this study was to evaluate the presence of TLR-4 (p.Asp299Gly) and (p.Thr399Ile) gene variants in association with Egyptian children having FMF, including its effect, if any, on disease course and severity.

Methods
This study was conducted on 120 children, including 70 diagnosed as having FMF. They were recruited from our Pediatric Rheumatology Clinic. The control group included 50 age and gender-matched children who came for follow-up at the outpatient surgery clinic of the same hospital for elective pre-operative assessment. The control group does not have any symptoms suggestive of autoimmune disease. The study was conducted during the period from January 2017 to December 2018. Laboratory investigations were done in Clinical and Chemical Pathology Department Laboratories, Kasr Al Ainy Hospital.

All patients were diagnosed according to FMF pediatric criteria [15]. Their age at time of study ranged from 2-16 years. Patients diagnosed as having an associated autoimmune disorder were excluded from the study.

Full history was taken from patients including history of parents’ consanguinity, family history of FMF and other autoimmune diseases, age at disease onset, age at disease diagnosis, and duration of illness. Number of at-
tacks per year, durations of attacks/hour, and clinical pattern of disease attacks (fever, abdominal pain, chest pain, arthritis, erysipelas-like rash, testicular affection, and history of vasculitis) were also included. History of colchicine dosing was taken in full details including dur-
ation of therapy/year, starting dose and current dose in mg/day, frequency of attacks/year, and duration of at-
tacks/hour before and after colchicine therapy and compliance. Finally, response to colchicine therapy was assessed by FMF-50 score [16].

Disease severity was assessed by FMF severity score [17]. Familial Mediterranean fever (MEFV) gene vari-
ation status of the patients was recruited from their files. Complete blood picture (CBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and urine analysis were done to all participants at the time of study.

The study was approved by the Local Ethical Commit-
tee of Faculty of Medicine, Cairo University. All partici-
ants in this study were informed and written consents were taken from their parents or legal guardian of the child.

Specimen collection and storage
Four milliliters of venous blood was withdrawn from all subjects and divided in to two parts: 2 ml were collected in a sterile vacutainer containing ethylene diamine tetra-
acetate “EDTA” which were used for DNA extraction. Samples were kept frozen at −20°C till time of DNA ex-
traction and analysis. Two milliliters were collected on plain tubes, left for 10 min to clot, and then centrifuged at 3000 rpm for 5 min, the separated serum was used for routine laboratory investigations including CBC, CRP, ESR, serum creatinine, and urine analysis to check for proteinuria.

Genomic DNA extraction
Extraction of genomic DNA was done from peripheral blood using a (TIAN amp Genomic DNA Kit Intro Bio-
technology) according to the manufacturer’s instructions.
Analysis of toll-like receptor 4 (TLR-4) and TLR-4 (p.Thr399Ile) gene variants was done using polymerase chain restriction-restriction fragment length polymorphism (PCR-RFLP) procedure.

Amplification of extracted genomic DNA (PCR) was done using the following primers: for TLR-4 (p.Asp299Gly), sense: 5′-GAT TAG CAT ACT TAG ACTACT ACC TCC ATG-3′, and anti-sense: 5′-GAT CAA CTT CTG AAA AAG CAT TCC CAC-3′. As for TLR-4 (p.Thr399Ile), sense: 5′-GGT TGC TGT TCT CCA AAG TGA TTT TGG GAG AA-3′, and anti-sense: 5′-ACC TGA AGA CTG GAG AGT GAG TTA AAT GCT-3′. The thermal cycler was programmed for initial denaturation at 95 °C for 10 min, 35 cycles of amplification consisting of denaturation at 94 °C for 30 s, annealing for 30 s at 61 °C for Asp299Gly (NOCI) enzyme, and 62 °C for Thr399Ile (HINF) enzyme and extension at 72 °C for 1 min. The amplified samples were then run in parallel on 2% agarose gel and visualized on Ultra Violet transilluminator to detect presence of amplified material.

Each PCR product was digested to completion with FastDigest (NOCI-HF) restriction endonuclease for TLR-4 (p.Asp299Gly) and FastDigest (HinfI) restriction endonuclease for TLR-4 (p.Thr399Ile).

The digested products were separated by electrophoresis on agarose 3% gel and visualized using ethidium bromide (Table 1), (Figs. 1 and 2).

### Statistical methods

Data were analyzed using SPSS® Statistics version 24 (SPSS® Corp., Armonk, NY, USA). Normally distributed numerical data were presented as mean ± SD and intergroup differences were compared using the unpaired t test. Skewed numerical data were presented as median and interquartile range and between-group differences were compared using the Mann-Whitney test. Categorical variables were presented as number and percentage. Fisher’s exact test was used to compare nominal data and the chi-squared test for trend to compare ordinal data. Association between FMF genes and disease severity were tested using rank biserial correlation (rrb). P values ≤ 0.05 were considered statistically significant.

### Results

Demographic, clinical, and laboratory characteristics of the studied groups are represented in Table 2. Mean of age at disease onset was 4.9 ± 2.9 (0.5-12) years, while the time between first presentation and disease diagnosis showed a mean of 2.0 ± 2.5 (0.0-12.0) years.

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**Table 1** Identified bands of Asp299Gly and Thr399Ile genotypes after trans-illumination by ultraviolet trans-illuminator

| Genotype                  | Size of fragment | Number of bands detected |
|---------------------------|------------------|--------------------------|
| Asp299Gly                 |                  |                          |
| Wild genotype             | 249 bp*          | One band                 |
| Homozygous mutant genotype| 223 and 26 bp    | Two bands                |
| Heterozygous genotype     | 249, 223, and 26 bp| Three bands              |
| Thr399Ile                 |                  |                          |
| Wild genotype             | 406 bp           | One band                 |
| Homozygous mutant genotype| 377 and 29 bp    | Two bands                |
| Heterozygous genotype     | 406, 377, and 29 bp| Three bands              |

*bp base pair

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**Fig. 1** Using RFLP restriction enzyme (NCOI-HF), the identified bands after transillumination by UV transilluminator. From left to right: Shows, M (DNA marker), DNA ladder (lane 1-10) in (50 bp ladder). Lanes (2, 4, 7) show heterozygous genotype (Asp/Gly). Lanes (1, 3, 5, 6, 8, 9, and 10) show a wild genotype (Asp/Asp).
Furthermore, disease duration had a mean of 4.4 ± 2.7 (1.0-12.0) years. Hemoglobin level had a mean value of 12.2 ± 1.0 (9.0-14.0) g/dl. Total leucocyte count had a mean of 7 ± 2 (4-14) × 1000/mm³. One patient (1.4%) had normocytic normochromic anemia and 4 patients (5.7%) had leukocytosis.

The mean duration of colchicine therapy was 2.7 ± 1.9 (0.5-9.0) years. The starting dose in cases showed a mean of 0.89 ± 0.44 (0.25-2.0) mg/day, while the current dose of colchicine at time of study had a mean of 1.13 ± 0.48 (0.25-2.0) mg/day. Median and IQ range seen in Table 3.

Our result showed that the frequency of attacks decreases after colchicine therapy from a mean of 6 ± 4 (1-36) attacks/year to a mean of 2 ± 2 (0.0-12) attacks/year. Similarly, duration of the attacks decreased from a mean of 35 ± 20 (6-72) hours to a mean of 10 ± 11 (0-48) hours. Response to colchicine per FMF50 scores [16] showed non-responsiveness in 10 patients (14.3%) compared to 60 (85.7%) being responsive. Three patients (4.3%) were non-compliant to therapy.

We found that 20 (28.6%) patients carried a heterozygous MEFV gene mutation, 35 (50.0%) were homozygous, and 15 (21.4%) had compound heterozygous mutation. The most prevalent FMF gene among patients was the E148Q, in 22 (25.2%) patients, M694I in 20 (22.9%), V726A in 18 (20.6%), M680I in 16 (18.3%), and M694V was present in 11 (12.6%) patients.

The MEFV gene M694V had a weak association with disease severity with a rank biserial correlation coefficient (rrb) of 0.253 (P value = 0.035). Otherwise, there was no statistically significant association between any of the other FMF genes and severity of disease (all P values > 0.05). Similarly, there was no statistically significant association between disease severity and presence of FMF gene mutation (rrb = 0.069, P value = 0.572), as seen in Table 4.

![Fig. 2 Using RFLP restriction enzyme (HinfI), the identified bands after transillumination by UV transilluminator. From left to right: M (DNA marker), DNA ladder (lane 1-10), and 50 bp ladder. Lanes (1, 3, 4, 5, 6, 7, 8, 9, and 10) show wild genotype (Thr/Thr). Lane (2) shows a heterozygous genotype (Thr/Ile)](image_url)

**Table 2** Demographic, clinical, and laboratory characteristics of the studied groups regarding age, gender, clinical manifestations, disease severity, and presence of TLR4 gene mutations

| Variables                        | Cases (n = 70) | Controls (n = 50) | P value |
|----------------------------------|---------------|------------------|---------|
| Age (years), mean ± SD           | 9.5 ± 3.5     | 9.4 ± 2.6        | 0.840   |
| Gender                           |               |                  |         |
| Male n (%)                       | 30 (42.9%)    | 28 (56.0%)       | 0.195   |
| Female n (%)                     | 40 (57.1%)    | 22 (44.0%)       |         |
| Abdominal pain                   | 62 (88.6%)    |                  |         |
| Recurrent fever                  | 55 (78.6%)    |                  |         |
| Arthritis                        | 55 (78.6%)    |                  |         |
| Chest pain                       | 30 (42.9%)    |                  |         |
| Myalgia                          | 29 (41.4%)    |                  |         |
| Skin rash                        | 4 (5.7%)      |                  |         |
| Degree of disease severity n (%) |               |                  |         |
| Mild                             | 14 (20%)      |                  |         |
| Moderate                         | 20 (28.6%)    |                  |         |
| Severe                           | 26 (51.4%)    |                  |         |
| Laboratory findings              |               |                  |         |
| Positive CRP                     | 6 (8.6%)      |                  |         |
| Proteinuria                      | 1 (1.4%)      |                  |         |
| Anemia                           | 1 (1.4%)      |                  |         |
| Leukocytosis                     | 4 (5.7%)      |                  |         |
| TLR-4 Asp299Gly genetic variation|               |                  |         |
| Normal                           | 69 (98.6%)    | 48 (96.0%)       | 0.570   |
| Heterozygous                     | 1 (1.4%)      | 2 (4.0%)         |         |
| TLR-4 Thr399Ile genetic variation|               |                  |         |
| Normal                           | 69 (98.6%)    | 50 (100.0%)      | 1.000   |
| Heterozygous                     | 1 (1.4%)      | 0 (0.0%)         |         |
The TLR-4 (p.Asp299Gly) gene variants were found normal in 69 (98.6%) patients compared to 48 (96.0%) of the controls. One (1.4%) of the cases had heterozygous variation compared to 2 (4.0%) of the controls, with $P = 0.570$.

TLR-4 (p.Thr399Ile) genetic variation was normal in 69 (98.6%) cases compared to 50 (100.0%) of the controls, while 1 (1.4%) of the cases carried a heterozygous variation compared to none of the controls ($P = 1.000$).

Odds ratio of exposure among cases versus among control showed a result of 0.71 with no statistical significance ($P = 0.732$).

We did not find a significant correlation between frequency and duration of attacks and colchicine dosage among cases with and without TLR gene variations (Table 5).

As for disease severity in cases with TLR4gene variations, one (7.1%) patient showed mild form of the disease severity, and another patient (2.8%) showed severe form ($P$ value 0.568). Regarding response to treatment, the two (3.3%) patients with TLR4gene variations showed good response to colchicine treatment.

**Discussion**

To the best of our knowledge, this is the first study about TLR-4 gene variants in Egyptian children with FMF. We could not find a significant correlation between TLR-4 (p.Asp299Gly) and (p.Thr399Ile) gene variants and clinical measures of FMF disease, its severity, and response to colchicine treatment. Furthermore, M694V gene mutation statistically correlated with higher disease severity ($P$ value = 0.035).

The low prevalence of TLR-4 gene variations that were found in this study was also reported in Egyptian patients with other autoimmune diseases. The Egyptian study by Taha et al. [4] did not find any of the TLR-4 (p.Asp299Gly) or TLR-4 (p.Thr399Ile) gene variations in patients with rheumatoid arthritis or systemic lupus erythematosus or in the control group. They stated that the TLR-4 gene variations display ethnic differences. Africans have the highest frequency (16%) [18], followed by Europeans (4-10%) [19], whereas Asians do not display this type of gene variations [20]. It seems that Egyptians have a low prevalence for these types of gene variations.

This study comes in agreement with the results reported by the study done in Greece [11] who found no association between TLR-4 gene variations and the frequency of attacks or disease severity. Speletas et al. [11] found the allele frequency of TLR-4 (p.Asp299Gly) to be lower in FMF patients compared with controls, while two other Turkish studies [13, 21] found a lower prevalence among healthy controls. The latter two studies

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**Table 3** Duration, starting dose, and current dose of colchicine therapy among cases

| Variable                        | Min | Max | Median | IQ range | 25th | 50th | 75th |
|---------------------------------|-----|-----|--------|----------|------|------|------|
| Duration of colchicine therapy (years) | 0.5 | 9.0 | 2.5 | (1.0-3.5) | 1.0 | 2.5 | 3.5 |
| Starting dose of colchicine (mg/day)     | 0.25 | 2.00 | 1.00 | (0.50-1.00) | 0.50 | 1.00 | 1.00 |
| Current dose of colchicine (mg/day)       | 0.25 | 2.00 | 1.00 | (1.00-1.50) | 1.00 | 1.00 | 1.50 |

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**Table 4** Association between FMF genes present in study subjects and FMF disease severity

| Variable       | Severity of FMF | Mild N | Mild Row % | Moderate N | Moderate Row % | Severe N | Severe Row % | Rank biserial correlation | Coefficient ($r_{bb}$) | $P$ value |
|----------------|----------------|--------|------------|------------|----------------|----------|--------------|--------------------------|-----------------------|-----------|
| FMF gene V627A| Absent         | 10     | 19.2%      | 14         | 26.9%          | 28       | 53.8%        | -0.069                   | 0.570                 |           |
|                | Present        | 4      | 22.2%      | 6          | 33.3%          | 8        | 44.4%        |                           |                       |           |
| FMF gene E148Q| Absent         | 11     | 22.9%      | 12         | 25.0%          | 25       | 52.1%        | 0.043                    | 0.726                 |           |
|                | Present        | 3      | 13.6%      | 8          | 36.4%          | 11       | 50.0%        | -0.099                   | 0.413                 |           |
| FMF gene M680I| Absent         | 9      | 17.0%      | 16         | 30.2%          | 28       | 52.8%        | -0.099                   | 0.413                 |           |
|                | Present        | 5      | 29.4%      | 4          | 23.5%          | 8        | 47.1%        |                           |                       |           |
| FMF gene M694V| Absent         | 13     | 21.7%      | 20         | 33.3%          | 27       | 45.0%        | 0.253*                   | 0.035                 |           |
|                | Present        | 1      | 10.0%      | 0          | 0.0%           | 9        | 90.0%        |                           |                       |           |
| FMF gene M694I| Absent         | 11     | 22.0%      | 13         | 26.0%          | 26       | 52.0%        | 0.029                    | 0.813                 |           |
|                | Present        | 3      | 15.0%      | 7          | 35.0%          | 10       | 50.0%        |                           |                       |           |
| FMF gene mutation| Absent   | 1      | 50.0%      | 0          | 0.0%           | 1        | 50.0%        | 0.069                    | 0.572                 |           |
|                | Present        | 13     | 19.1%      | 20         | 29.4%          | 35       | 51.5%        |                           |                       |           |

Data are counts (N) and row percentage (%).

*There is a weak association between FMF gene M694V and severity of FMF ($r_{bb}$, 0-253; $P$ value, 0.035)
found a higher prevalence of TLR-4 (p.Thr399Ile) gene variations among control. All genetic variations found were heterozygous, a finding similar to our study.

Soylu et al. agreed with our finding that the frequency of these gene variations did not differ between FMF patients and control [13].

Another study looking for the same TLR4 gene variants in Henoch-Schonlein purpura (HSP) observed a similar finding. They found a low prevalence of these variants in HSP with no effect on disease occurrence and presentation [22].

On the other hand, a meta-analysis study found a weak association between inflammatory bowel disease and the studied gene variants in Caucasians, but not Asians [23], highlighting the fact that the prevalence and effect of TLR gene variants are different among ethnicities.

Our study found an association between FMF disease severity and M694V gene mutation. This agreed with several Egyptian studies [23–25]. An Egyptian study by Alhaggar et al. suggested a link between this gene mutation and amyloidosis [26]. Researchers studying other ethnicities found a similar link [27–30].

In our study, the mean age of disease onset was 4.9 ± 2.9 years. This was similar to Talaat et al. [31]. However, Salah et al. [32] and El-Garf et al. [33] found slightly older age of onset. Mean age of onset was also higher in a Syrian study [34] and a Turkish study [35]. Meanwhile, the age of onset in three other Turkish studies was almost similar [36–38]. These differences may be related to different ethnicities and different genetic backgrounds.

The most common disease manifestation in this study was abdominal pain, followed by fever. Abdominal pain came to be the first symptomatic presentation in several studies [32, 34, 36, 39]. However, other studies [33, 40–42] reported more occurrence of fever than abdominal pain. This difference may be due to different sample sizes and different presentations in various regions.

Limitations of our study are the relatively small number of patients and control.

Table 5  Relation between TLR-4 gene variations and age at disease onset, frequency, and duration of attacks before and after colchicine therapy and colchicine dosage

| Variable                      | Negative TLR-4 gene variation (n = 68) | Positive TLR-4 gene variation (n = 2) | P value |
|-------------------------------|----------------------------------------|---------------------------------------|---------|
| Age at onset (years)          | 4.75 (0.5-12)                          | 5 (5-5)                               | 0.750   |
| Frequency of attacks per year before colchicine | 4 (1-36)                               | 6 (4-8)                               | 0.545   |
| Frequency of attacks per year after colchicine | 1 (0-12)                               | 1 (1-1)                               | 0.794   |
| Duration of attacks before colchicine (h) | 24 (6-72)                              | 42 (36-48)                            | 0.459   |
| Duration of attacks after colchicine (h) | 6 (0-48)                               | 15 (6-24)                             | 0.388   |
| Starting dose of colchicine (mg/day) | 1 (0.25-2)                             | 1.25 (1-1.5)                          | 0.152   |
| Dose of colchicine at time of study (mg/day) | 1 (0.25-2)                             | 1.75 (1.5-2)                          | 0.077   |

Conclusion

TLR-4 (p.Asp299Gly) and (p.Thr399Ile) gene variants were not found to have a link with the occurrence, the clinical picture of FMF, its severity, and response to colchicine treatment in Egyptian children. M694V gene mutation seems to be associated with higher disease severity. Further larger studies are needed to verify these results.

Abbreviations

FMF: Familial Mediterranean fever; MEFV: Mediterranean fever gene; CBC: Complete blood count; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; PCR-RFLP: Polymerase chain restriction-restriction fragment length polymorphism; IL-1: Interleukin-1; HSP: Henoch-Schonlein purpura; IBD: Inflammatory bowel disease

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Authors’ contributions

All authors have contributed significantly and all authors are in agreement with the content of the manuscript. YM: contributed in searching the literature, writing and editing the manuscript, and being the corresponding author. SS: contributed with the idea of the research, following the results, and writing the manuscript. HT: contributed in searching literature, collecting the data, and performing the statistical part of the research. MH: contributed in the laboratory part of the research and writing the methodology of the manuscript. HM: contributed in following the results and writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the ethical scientific committee of the Faculty of Medicine, Cairo University. Informed written consents were taken from the parents or the patient’s guardians according to guidelines of the ethical committee of the Faculty of Medicine, Cairo University.

Consent for publication

Written informed consents to publish the data contained within this study were taken from the parents or the patient’s legal guardians.

Competing interests

The authors declare that they have no competing interests.
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