Original article

Effects of different Familial Mediterranean Fever gene mutations and in vitro Colchicine treatment on Peripheral Blood Mononuclear sCells production of IL-6.

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Abstract:

Rationale: We wanted to study the effects of Familial Mediterranean Fever (FMF) genetic mutations in Northern Jordan population and in vitro Colchicine treatment on the peripheral blood mononuclear cells (PBMN) production of IL-6 as a marker of disease activity. Methods: - 17 FMF patients and 9 controls were studied. (4 patients had exon 10 mutations only (M680I and V726A), 5 patients had exon 2 mutations (R202Q and E148Q) only and 8 patients with both exon mutations (compound homozygous or heterozygous M694V and R202Q). PBMN cells were incubated with Lipopolysaccharide at 100ng/ml or colchicine 10 ng/ml alone or both. ***Results:*** The results showed higher IL-6 levels in the FMF group than control for all treatment modalities, (108.97 vs. 56.49 ng/ml for unstimulated cells) with the highest levels when both exons are involved. Exon 10 mutations were associated with a higher IL-6 level than exon 2 mutations only. Exon 2 mutations alone also were associated with a higher than control IL-6 levels suggesting that it is not a polymorphism phenomenon and is involved in the pathogenesis. In vitro Colchicine treatment caused an increase in the production of IL-6 - although not as high as with LPS - for all groups. ***Conclusions:*** Mutations occurring in exon 10 are more significant than mutations occurring in exon 2, although both are contributing to the disease. However colchicine was associated with a paradoxical increase in IL-6 levels. This observation needs confirmation with different colchicine levels in the culture medium and warrants thinking about its exact mechanism of action.

Keywords: MEFV genetic mutations, Colchicine, in vitro IL-6 Production

Introduction

Familial Mediterranean Fever (FMF, OMIM 249100) is the prototype and the most frequent hereditary periodic fever syndrome 1. The international medical community gave the disease its name (FMF) which reflects on the three classical aspects of the disease. First, familial because it is a hereditary inflammatory disorder. Second, Mediterranean as it affects predominantly populations from the Mediterranean area. Third, fever is the most common feature of the disease[2].

FMF is classically inherited as an autosomal recessive trait. It is characterized by recurrent short self-limiting attacks of fever, peritonitis, pleuritis, arthritis and skin lesions. Attacks usually last from 1-3 days. The disease can be complicated by AA amyloidosis that may lead to renal failure. Clinical symptoms of the attacks have intra- and inter-individual variances. FMF attacks generally start in childhood or adolescence in about 50% of the cases, but it can develop after the age of 30 years in some individuals with more benign disease profile [3]. Patients between attacks are free of symptoms although the biochemical markers for inflammation may persist [4]

MEFV (MEditerranean FeVer) is the gene involved in FMF disease, and was mapped to the short arm of chromosome 16p3 5. The French and International groups independently were able to clone this gene that encodes a protein called pyrin/marenostrin in 1997 6. Pyrin exact role is still unclear but in general it participates in the regulation of inflammation, apoptosis and cytokine processing [7, 8, 9, 10.]

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246 of mutations have been described in the MEFV gene until now. Most of these mutations are of missense type occurring in the C-terminal of pyrin (http://fmf.igh.cnrs.fr/infevers/ accessed on 2013-1-28). Five most common mutations - namely M694V, M694I, M680I, V726A and E148Q - were reported to account for three quarters of the MEFV gene mutations in all the affected populations [6].

Colchicine is the current effective treatment in FMF patients. Positive response to colchicine is used as one of the diagnosis criteria 11. Oral daily prophylactic colchicine intake of 1-2 mg was shown able to reduce the frequency and severity of the clinical manifestations in 90-95% of the patients. It was also reported to inhibit the development of amyloidosis and to reverse proteinuria even in colchicine non-responders 12. Recently, drugs as thalidomide, IFN-a and anti TNF-a had been administrated as colchicine adjutants in colchicine resistant patients in small-sized studies. However, none of these drugs is approved for clinical use [13].

IL-6 is a proinflammatory cytokine produced by lymphocytes, monocytes, fibroblasts and several tumor cells. It induces the final maturation of B cells into plasma cells and it is a major inducer of acute phase reactant. Different researchers reported elevated serum level of IL-6 in FMF patients compared to controls [14, 15, 16]. These finding suggest its involvement in the pathogenesis of the disease.

The general aim of this study was to determine the effects of different MEFV gene mutations in Jordanian subjects on the pathogenesis of the disease and the effects of in vitro colchicine. IL-6 production by the peripheral blood mononuclear cells in vitro was used as a marker for the disease activity in the study.

Materials and Methods:

Study Design and Patient's Selection:

The study design was first approved by Human Research Committee of Jordan University of Science and Technology and then by Human Research Committee of King Abdullah University Hospital. Our study depended on the previous work done by Princess Haya Biotechnology Center (PHBC) between 2003- 2009. PHBC provided us with their registry of MEFV gene mutations work in Northern Jordan. The registry included only patient's names and their mutational analyses. 324 subjects were tested over the years; 153 out of them were reported positive with at least one known mutation (heterozygous, homozygous or compound). Only 55 subjects were homozygous for at least one mutation. There were 55 patients with homozygous MEFV gene mutations (R202Q in 40, M694V in 20, and 14 individuals had both of these mutations. M681M, W148Q, M691I and V726A were also found in 4, 2, 2 and 1 patient). The electronic and hard copies of the medical records at King Abdullah University Hospital were reviewed to gather information about patient's contact. For our study, priority was given to patients in the homozygous state for any of the MEFV gene mutations. The initial study design intended to include five patients from each of the five most common MEFV gene mutations, but we were not able to do so because the number of patients in some mutations was less than five and some patients refused to participate in the study. Moreover, we could not contact many of the patient's due to missing information. Therefore, we extended our target to those carrying double heterozygous MEFV gene mutations. The inclusions criteria were: - being clinically confirmed FMF patients, having MEFV gene mutations, being in the attack free period, absence of other chronic diseases, current infections or use of antibiotics or any immunosuppressant drugs, and acceptance to participate in the study. The last colchicine dose was at least 12 hours before the sample collection.

We tried all our best to contact the 55 subjects with homozygous mutations by phone. If these subjects accepted to participate in the study, they were given appointments to the outpatient's clinic of King Abdullah University Hospital. Informed consent was obtained from each of them and /or their guardians. A treating physician interviewed the patients and confirmed the diagnosis clinically. The researcher filled a questionnaire regarding patient's clinical information either directly from them/their guardians or from their medical records.

Patients Groups: - Seventeen patients (7 males and 10 females) were classified into three groups according to the location of the exon of the MEFV gene mutation/s they have. The first group had mutations at exon 10 only (1 case of M680I and 3 cases of compound heterozygous V726A and M680I, total 4 cases). The second group had mutations at both exons 10 and 2 (7 cases of compound homozygous M694V and R202Q and 1 case of compound het-

erozygous M694V and R202Q, total 8 cases). The third group had only mutations at exon 2 (4 cases of homozygous R202Q and 1 case E148Q, total 5 cases).

Control Group: - Nine control subjects that are age and sex matched (5 females and 4 males) were enrolled in the study. None of the controls had the clinical symptoms or family history of FMF. Genetic studies were done and all the subjects were negative for any of these mutations (M694V, M694I, V726A, M680I, E148Q or R202Q).

Sample Collection: -20 ml of whole blood samples were collected from each of the patients and controls using vacutainer heparinized tubes under sterile condition for cell culture use.

Laboratory Analysis:

Cell Separation, Viability and Counting: - PBMNCs were isolated from whole blood using Histopaque-1077 (from Sigma-Aldrich) technique (Density 1.077g/ml) according to the manufacture's instructions. Histopaque-1077 solution was sterile-filtered, ready to use and cell culture tested. Then cells viability was checked by 0.4% trypan blue dye bought from Sigma-Aldrich, viability of the cells exceeded 95%. Cells count was determined using automated system (ABX 60 Micros counter) and adjusted to 2 x 106 cells/ml in each well.

Cell Culture: - Liquid RPMI-1640 media, sterile-filtered, cell culture tested and glutamine free supplemented with sodium bicarbonate was bought Sigma-Aldrich and was used for cell culture of the PBMNCs. L-glutamine-penicillin-streptomycin solution (cell culture tested, 200 mM L-glutamine, 10,000 U penicillin and 10 mg streptomycin/mL in 0.9% NaCl) was bought from Sigma-Aldrich and was added to the RPMI-1640 media at concentration of 10 ml/L. 10% of heat inactivated-fetal calf serum, sterile-filtered solution and cell culture tested was bought from Sigma-Aldrich and was added to the RPMI-1640 media as recommended by the manufacture.

Lyophilized Lipopolysacchride from Escherichia coli serotype O127:B8 purified by phenol extraction was bought from Sigma-Aldrich. LPS stock was prepared with sterile water in concentration of 104 ng/ml. 10 µl from the stock was added to each well to reach a final concentration of 100 ng/ml in the media [17]. LPS stock was stored in the refrigerator for use within one month while frozen aliquots were stored at -20 °C for longer periods.

Colchicine powder for laboratory use (with purity = 95% extracted by HPLC) was bought from Sigma-Aldrich. Primary stock was prepared by dissolving 45 mg of the powder in 1 ml of deionized distilled water and then sterilized by filtration units with 22 µm pore size. The primary stock was diluted to reach concentration of 450 ng/ml. 22 µl of the working stock (450 ng/ml) was added to each well with a final concentration of 10 ng/ml. This final concentration of colchicine was chosen according to previous studies. Serum level of oral colchicine after 120 mins of administration of 2 mg in healthy subjects ranged from 4.4-19.6 ng/ml [18]. Colchicine solution was stored in the refrigerator protected from light for use within one month.

Cells were cultured in flat bottom wells under four different conditions, each condition was carried out in duplicate. The four conditions were either only cells in media, or cells in LPS and media, or cells in colchicine and media, or cells in LPS, colchicine and media.

Specific cell suspension volume was transferred to cell culture wells to reach final concentration of 2 x 106 cells/ml after that, LPS and colchicine was distributed where appropriate and the volume of each well was adjusted to total 1ml by adding RPMI-1640 media. Finally, each plate was mixed gently, covered with aluminum foil and incubated at 37° C in 5% CO2 for 20 hours. Wells contents were transferred into eppendorf tubes and were centrifuged at 1500 RPM for 5 min. Supernatant was collected into new eppendorf tubes and stored at -65 °C until IL-6 ELISA was carried out.

IL-6 ELISA Assay: - Level of IL-6 from in vitro cell culture supernatant was detected using quantitative sandwich enzyme immunoassay kit (Quantikine, R&D Systems) ® according to the manufacturer's instructions. The sensitivity of the assay is 0.70 pg/ml.

Statistical Analysis: - Statistical analyses were performed using SPSS 17.0 software. IL-6 level in FMF patients and in controls was compared using "independent sample t-test". "Paired t test" was used to compare the level of IL-6 in different treatments models while "ANOVA test" was carried out to compare

the level of IL-6 from patients with different MEFV gene mutations; P < 0.05 was considered significant.

Results:

The results of the study are summarized the tables and figures attached. There were a total of 17 FMF and 9 controls. Table 1 describes the demographic characteristics like age and sex distribution. There was positive family history in all the FMF group but none in the control group. The presence of consanguinity was found much higher in the FMF group in 13 out of 17 but none in the control group which was un-intended. Table 2 describes the clinical characteristics of the patients and the rates of their symptoms by the order of their frequency and the severity of their attacks. The feature included fever, abdominal pain, joint pain and swelling, chest pain, vomiting, diarrhea, seizures and skin lesions.

Figure 1 shows the levels of IL-6 production comparing FMF patients and controls in all culture media types (unstimulated cells, or stimulated with LPS alone, or with Colchicine alone and with LPS and Colchicine together). The results are displayed showing the result of the statistical analysis P values of the comparisons. As expected the group of FMF patients had a significantly higher IL-6 levels produced at all treatments used mostly by unstimulated cells. It was also found that colchicine in vitro caused a rise in the IL-6 of 134.76 ng/ml vs. 108.97 for unstimulated cells in FMF patients vs. controls (P value 0.001). Figure 2 displays the results of the IL-6 production in relationship to the type of exons were mutation occurring. It compared LPS treatment vs. unstimulated cells for patients with exon 2, 10, both or controls.

Table 1: - Demographic characteristic of the FMF patients and healthy controls (mean ± SD).

Variables	FMF patients (N= 17)	Controls (N= 9)
Mean Age (range)	19.8±12 (5.0-44.0)	21.5±11 (7.0-42.0)
Sex (M/F)	7/10	4/5
Family history of FMF	17/17	0/9
On colchicine	14/17	None
Consanguinity in parents - First degree -Second degree	12/17 1/17	0/9 0/9

Table 2 - Distribution of the clinical features in FMF patients

Features	Frequency	Percent
Fever	15/17	(88.2%)
Abdominal pain	15/17	(88.2%)
Joint pain	13/17	(76.5%)
Chest pain	9/17	(52.9%)
Joint swelling	7/17	(41.2%)
Vomiting	7/17	(41.2%)
Appendectomy	5/17	(29.4%)
Diarrhea	4/17	(23.5%)
Seizures	3/17	(17.7%)
Skin lesions	2/17	(11.8%)
Age of onset -1-10 years -11-20 years ->20	 12/17 4/17 1/17	 (70.6%) (23.5%) (5.9%)
Attacks frequency in the past 2 years - 1-5/year - 6-10/year - 11-15/year - >15 /year	 6/17 3/17 4/17 4/17	 (35.3%) (17.6%) (23.5%) (23.5%)

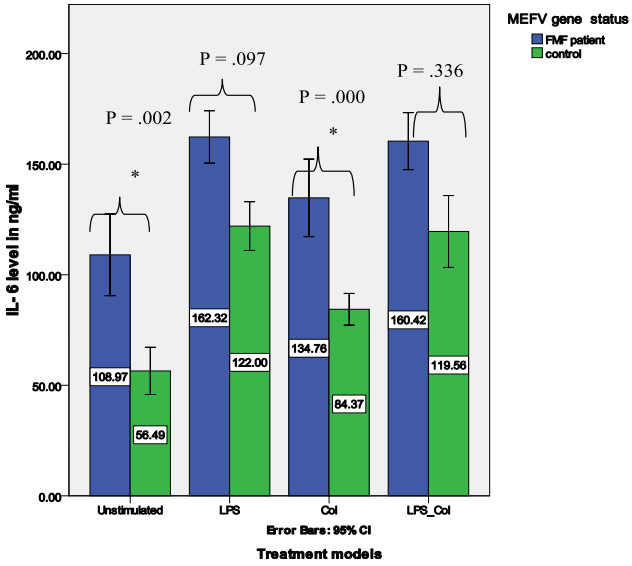

Figure 1 IL-6 levels in FMF patients and controls (from unstimulated, LPS stimulated, colchicine treated, and LPS + colchicine incubated cells together). * Represents P- value < 0.05

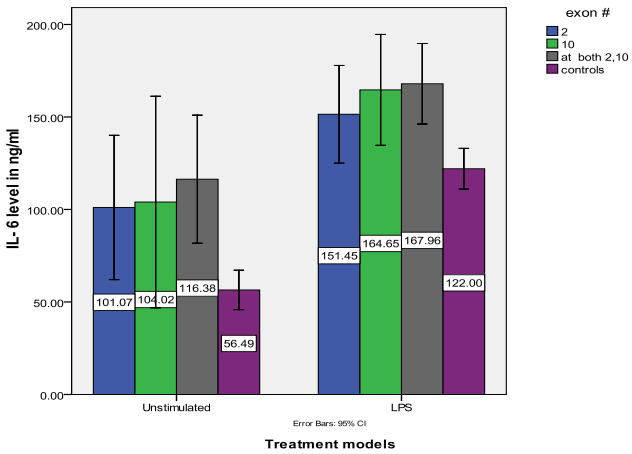

Figure 2 Effects of different MEFV gene mutations on the production of IL6. Mutations are classified according to their location on the MEFV gene.

Discussion:

Due to availability of subjects, our cohort consists of a small number of patients that are not representative of the population and not taken randomly. However, the small number of the study subjects is appropriate in reference to other similar in vitro studies. Data

from Princess Haya Biotechnology Center about male to female ratio in their 153 subjects who were positive for MEFV gene mutations was different (1.04:1) from that reported by Majeed and Barakat who found a slight predominance in females in the studied 88 Jordanian children [19]. However, Majeed's and our data do not support the suggestion that MEFV gene mutations may have incomplete penetrance in females. El-Shanti suggested patient's selection as the cause for such difference [6].

The high rate of consanguinity in our patient's families was similar to that reported by Al-Salem and Rawashdeh in their report from North Jordan [20]. The high incidence of consanguinity in Jordan resulted in a higher frequency of FMF than expected [21]. However, no consanguinity was reported in our control group and this was by chance and unintentional. 70.6% of our patients had an early childhood age onset before 10 years. However, Majeed reported that 86% of the 467 FMF children developed their first attack before age of 10 years in their study population [22]. His younger study population could explain this higher percentage.

The clinical picture of our patients is representative to that of the disease and close to what was described in reports from Jordan. Generalized abdominal pain and fever were the commonest seen in 88.2% of our study population. Similar to that reported by Majeed and Al Wahadneh [22, 23]. Joint pain was the second most common attack feature reported in 76.5% of our patients higher than mentioned by other studies from Jordan. The difference may be retuned to small size of our population. Pattern of joint pain involved the large joints mostly the ankle or knee similar to that reported by Al Wahadneh. Chest pain was reported in half of our study individuals less that mentioned by Majeed. when 42% of their study population was noticed with chest pain. Different percent may be explained by difficulties in describing this symptom in younger children. Skin lesions were presented in 11.8% of the patients consistent with that stated by Majeed and his co-workers [22].

Despite of the small size of our study population, one patient had renal failure as FMF complication. Majeed and his colleagues reported two cases of FMF renal failure in Jordan in their study. However, Al Wahadneh and Daharbeh documented no case in the 54 patients they diagnosed [23]. Furthermore, higher incidence of amyloidosis was reported in other ethnic groups than in Arabs. El-Shanti and colleagues in their review retuned the reason to underreporting as most of the data were collected following colchicine administration [6].

Studies that concentrated on the clinical picture of FMF in Arabs either were carried out before identification of the MEFV gene or did not incorporate molecular data in the analyses [6]. Our patient that suffered from renal complication was carrying compound homozygous mutations for M694V, R202Q. This finding is consistent with many reports from other ethnic groups in which a significant association had been noted between M694V mutation and the development of amyloidosis, especially in the homozygous state [24, 25, 26]. Moreover, seizures were noted only in 3 cases of our patients, 2 of them are homozygous for R202Q and the third had compound homozygous M694V and R202Q mutations. Similarly, skin lesions were only seen in two patients carrying homozygous R202Q mutation. However, due to low number of cases no statistical test was done. These are interesting observations that need further types of studies with larger sample size.

In our in vitro system, PBMNCs were incubated into optimal incubation period as the peak level of IL-6 from monocytes and lymphocytes needs 16 hours to develop and remains stable until 3 days. In all similar studies, the absolute concentration of IL-6 is not standardized. Additionally, the value of the level is useful in the comparisons made between groups or treatment models. IL-6 level from cell culture supernatant of FMF patients (both spontaneous release and LPS induced production by PBMNCs) was higher than controls. Our finding is compatible with that of Aminov et al. and Bagci et al. who reported similar results in serum IL-6 of FMF patients (both before and during the attacks) compared to the controls [27, 15]. Moreover, Notarnicola and his colleagues found that the mRNA expression of IL-6 in PBMNCs of FMF patients (in attack free period) was higher than control [28]. This high level in attack free period might reflect sustained activation of the immune cells of these patients even in the resting stage.

LPS was used in concentration of 100 ng/ml similar to many related previous studies in spite of its action in dose-dependent manner. Ideally, we should have performed an optimization step to choose the best concentration of LPS to overcome its purity and activity issues but because of IL-6 determination (by ELISA) expenses we could not do this step. LPS is a

well-known IL-6 inducer [29,30].

The anti-inflammatory activity of colchicine was reported to be mediated by direct interaction with the microtubules. However, its effect on IL-6 production is still unclear. 10 ng/ml of colchicine that was used in our system is similar to the expected steady state level of the drug in serum when administrated as prophylaxis (in dose of 1-2 mg/day) [17, 31]. Besides, we could not use various concentrations of colchicine due to budget limitations. Paradox to our hypothesis that colchicine may reduce IL-6 level in FMF patients. It showed a stimulatory effect on the in vitro production of IL-6 in both controls and patients when used in concentration of 10 ng/ml. This data doesn't support the findings of either Zesong et al. or Entzian et. al.. Entzian reported that colchicine failed to exert significant influence on production of IL-6 and TNF-a from in vitro cultured PBMNCs of healthy subjects stimulated by PHA (5 mg/ml) when it was used in concentrations of (0.1, 1.0, 10, 25 ng/ml) [32]. When Entzain used colchicine in concentration of 10 ng/ml, colchicine showed a slight increase in the level of IL-6. However, the difference did not reach a statistical significant level due to the smaller sample size. Zesong et al. noticed that the expression of IL-6 in Hepatic Setllate Cells (HSCs) was down regulated by 2.1 folds after 12 hours of treatment with 6.25 mg/L of colchicine [33]. These contradictory observations might suggest that colchicine affects IL-6 production in a dose-dependent manner. Moreover, the study of colchicine effect on the production of IL-6 in vivo showed different results. Kiraz et al. reported that two months of colchicine treatment (1 mg/day) reduced the level of serum IL-6 in FMF patients to statistically significant level (p-value < 0.05)[34]. By contrast, Notarnicola et al. showed that there was no difference in the level of IL-6 mRNA expression from PBMNCs between patients on colchicine and those off colchicine. This group suggested that the therapeutic effect of colchicine occurred by inhibiting the translation, processing or secretion of IL-6 leading to a reduction in serum soluble level [28]. These observations were different from ours and may be explained by differences in the methodology used, sample size and more complicated colchicine mechanism of action in vivo that is influenced by various factors. In conclusion, further investigations are required to clarify the nature of colchicine action on IL-6 production from PBMNCs using a bigger sample size.

Patients with mutations located at exon 10 had a higher level of IL-6 than those with mutations at exon 2. Additionally, those carrying mutations at both exons had the highest level. Although these differences did not reach significant level, they may suggest the importance of the location of these mutations in an active site essential for pyrin function. In agreement with our observation, Notarnicola found no significant difference in mRNA expression of IL-6 according to different genotypes when he compared those with compound homozygous for M694V to other genotypes as a group 28. Colak et al. also stated that there is no significant difference in serum level of IL-6 from patients with different MEFV genotypes 14. Patients with mutations located at exon 2 had a significant higher level of IL-6 compared to controls, these mutations should not be considered as just polymorphism phenomenon and further studies are needed in different ethnic groups. Patients with mutations at both exons 2 and 10 had most severe attack features characterized by earlier age onset. Patients with mutations at exon 10 had more severe form of the disease than those with mutations at exon 2. Patients homozygous for R202Q uniquely had seizures and skin lesions.

Conclusions

- In our study population from North of Jordan, FMF patients had higher level of IL-6 compared to the control group in both unstimulated and LPS stimulated cells suggesting involvement of this cytokine in the pathogenesis of FMF disease.
- Level of IL-6 in individuals with mutations occurred at both exons 10 and 2 of the MEFV gene was the highest followed by those with mutations located at exon 10 and 2, respectively. However, the difference didn't reach a statistical significant level.
- Patients with mutations located at exon 2 had a significant higher level of IL-6 than controls; this finding may suggest that this mutation can be disease causing mutation and not just a polymorphism phenomenon. Furthermore, the patients with R202Q mutation had seizures and skin lesions as part of their clinical picture unlike other mutations in our patients group.
- Paradox to our hypothesis, in vitro colchicine increased the production of IL-6 in both FMF patients and controls. This observation was also associated with the in vivo uptake of colchicine (IL-6 level from patients on colchicine was higher than those off colchicine) and it needs further investigations.

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