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Alterations in Gut Microbiota Composition in Familial Mediterranean Fever

(Submitted by academician K. G. Karageuzyan 16/IX 2006)

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Familial Mediterranean fever (FMF; MIM249100) is a recessively inherited disorder of the inflammatory pathway, manifested by acute self-limited recurrent episodes of fever and polyserositis [1]. The Mediterranean fever gene (MEFV), responsible for the disease, has been recently identified by positional cloning [2, 3]. Pyrin, the protein product of MEFV, consists of several conserved domains, including the N-terminal pyrin domain (PYD), which is found in a number of autoinflammatory proteins involved in the regulation of inflammation and apoptosis [4]. According to recent studies, autoinflammatory genes, such as MEFV, may represent an exaggerated innate immune response to various signals in vitro, including microbial products [5]. Indeed, the CARD15/NOD2 gene product belongs to the same superfamily of proteins [6], and its mutations have been found to underlie inflammatory bowel diseases (IBD), such as Crohn's disease, in which an inappropriate immune response to components of the commensal microbiota exists [7]. In this regard, it has been proposed to investigate the composition of gut microbiota in FMF to reveal a possible contribution of commensal bacteria to the onset and maintenance of the disease.

As a large majority of bacterial species is effectively unculturable, it is impossible for detailed examination of gut microorganisms to be achieved through traditional culture techniques. Molecular-genetic analyses of bacterial microbiota based on 16S ribosomal ribonucleic acid (rRNA) genes obviate the need for culture and have been shown to be powerful tools in determining microbial diversity in complex samples [8].

In the present study, the fecal bacterial composition has been for the first time examined in FMF by using microbial community analysis through sequencing of 16S rDNA libraries.

Fecal samples were collected from genetically ascertained FMF patients (12 patients in remission, 3 patients in attack periods) and 7 healthy individuals.

DNA was extracted from fecal samples of FMF patients and healthy subjects using QIAamp DNA Stool Mini Kit (Qiagen, UK), according to the manufacturer's instructions. DNA samples were transferred to the Rowett Research Institute (UK) where 16S rDNA clone libraries were generated, and phylogenetic analysis was performed. Bacterial 16S rDNA was PCR-amplified with universal primers covering most intestinal bacterial species (Table 1). The amplicons were cloned into *Escherichia coli* chemically competent cells using the pCR-4 TOPO TA Cloning Kit (Invitrogen, UK), according to the manufacturer's instructions. Recombinant colonies were randomly picked and sequenced on the automated DNA-sequencer (Beckman, USA) with 926R bacterial primer (Table 1). Alignment of sequences with reference 16S rDNA gene sequences from healthy gut microflora was performed using the multiple sequence alignment programme

CLUSTALX v. 1.83 [9]. Phylogenetic analyses were performed using the neighbor-joining algorithm [10]. Operational taxonomic units (OTUs) were identified by online Basic Local Alignment Search Tool (BLAST) program at the NCBI website [11], using search results of at least 99% sequence similarity.

Table 1

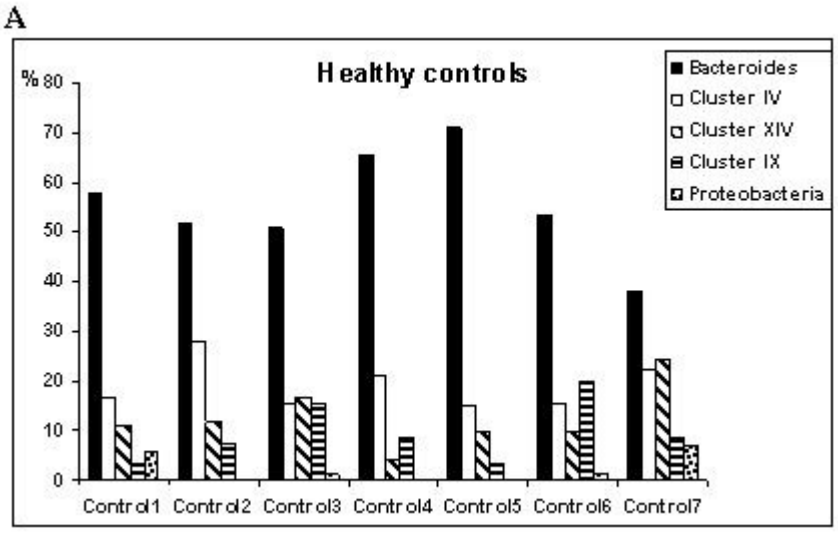
PCR and sequencing primers used in this study

Application	Primer	Position	Sequences (5'-3')
PCR	Fd1	8-27[1]	AGAGTTTGATCMTGGCTCAG
PCR	Rp2	1492-1510[1]	ACGGCTACCTTGTTACGACTT
Sequencing	926R	907-926[1]	CCGTCAATTCCTTTGAGTTT

[1]Position in E. *coli reference sequence*

Using a molecular approach, for the first time, the composition of fecal microbiota in FMF patients with both inactive and active stages of the disease, as well as in healthy subjects, was determined. It was demonstrated that fecal microbiota in FMF differed from that of the healthy state both in remission and attack periods of the disease.

Three 16S rDNA libraries from fecal samples of FMF remissions, acute FMF and healthy controls were generated. A total of 1328 clones (572 for healthy controls, 629 for FMF remission and 127 for FMF attack) were analyzed, and phylogenetic relationships of main bacterial phyla in each studied group were established (Fig. 1 A, B, C). Among the 1328 clones analyzed, there were 268 distinct OTUs, which fell into three major phyla: *Cytophaga-flavobacter-bacteroides* (CFB) group, *Firmicutes*, and *Proteobacteria*. The overall distribution of the three dominant bacterial phyla among the three subsets of subjects is shown in Table 2.



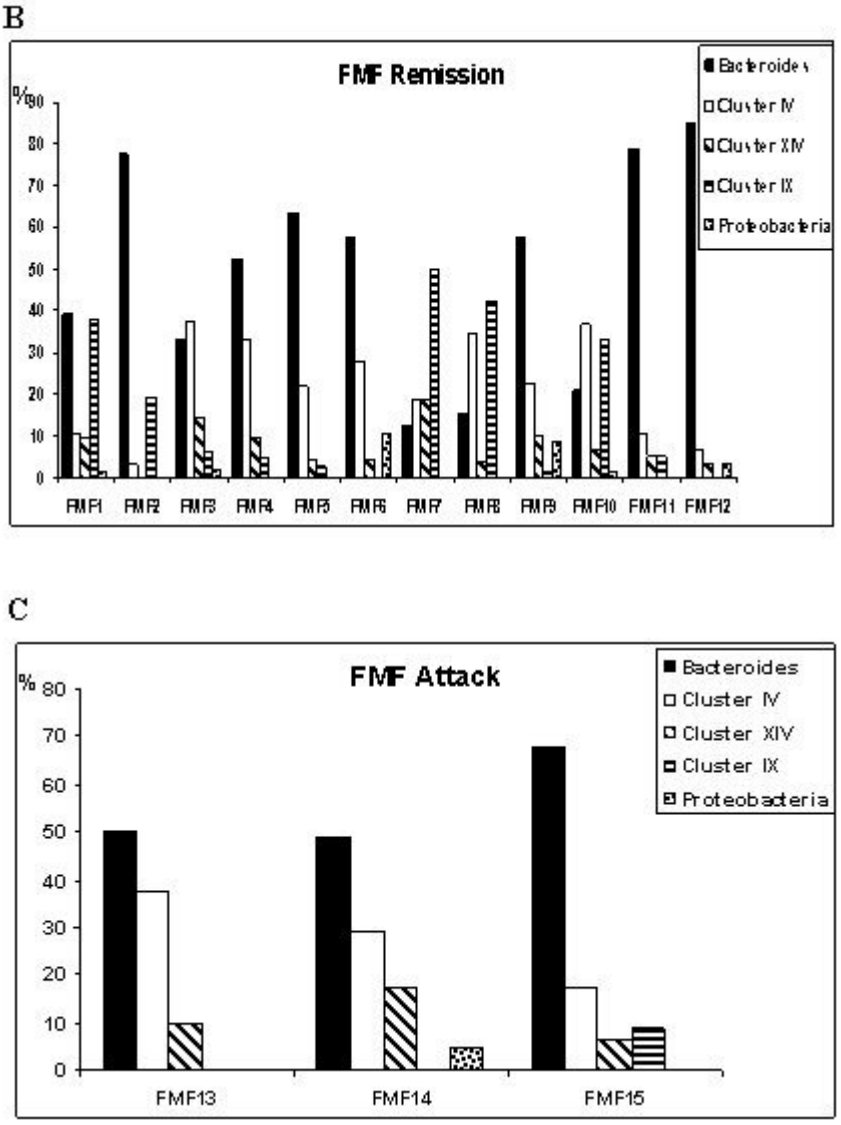


Fig. 1. Phylogenetic relationships among main bacterial phyla in fecal flora of healthy controls (A); FMF patients in remission (B); FMF patients in attack (C).

Table 2

Phylogenetic distribution of 16S rDNA libraries generated from healthy individuals and FMF patients in remission and attack periods

Phylum	Controls		Remission		Acute		Combined	
	OTUs	Clones	OTUs	Clones	OTUs	Clones	OTUs	Clones
CFB	54	324	57	319	21	71	99	714
% CFB	40.91	56.64	35.19	50.72	40.38	55.91	36.94	53.77
Firmicutes	73	237	98	289	29	54	158	580
% Firmicutes	55.30	41.43	60.49	45.95	55.77	42.52	58.96	43.67
Proteobacteria	5	11	7	21	2	2	11	34
% Proteobacteria	3.79	1.92	4.32	3.34	3.85	1.57	3.36	2.26
Total	132	572	162	629	52	127	268	1328

As shown in Table 2, *Bacteroides* was the most abundant group in all three cohorts, followed by the *Firmicutes*. The relative proportions of CFB and *Firmicutes* were not markedly different among the three groups; however, significant differences were detected in bacterial subgroups

within these main phyla (Fig. 1A, B, C). In Fig. 1B groups of FMF patients in remission were determined according to the biodiversity in the main phylogenetic groups, demonstrating high variability, in contrast to stable composition of gut bacteria in healthy state (Fig. 1A). Particularly, there is a group (FMF2, FMF4, FMF5, FMF6, FMF9, FMF11, and FMF12) overrepresented by OTUs belonging to the CFB phylum, which amounted up to 50-55% of gut bacteria in healthy subjects (Fig. 1A) and acute FMF (Fig. 1C). In the second group of FMF remissions (FMF1, FMF7, FMF8, and FMF10) there is a substantially higher proportion of cluster IX of Propionate-producing bacteria, as compared to healthy controls. Interestingly, these bacteria tended to disappear during the attack period (Fig. 1C). The pairwise comparisons of each 16S rDNA library to every other library also revealed significant alterations in gut microbiota composition in FMF compared to the norm (Table 3). In particular, the *Prevotellaceae* subgroup (within CFB) was significantly low in active stage of FMF as compared to FMF remission and healthy state (16.5%, 22% and 27.6%, respectively), in contrast to *Bacteroidaceae* (within CFB) subgroup (30.7%, 17.8% and 21.7%, acute, remission and healthy, respectively). The Butyrate-producing *Faecalibacterium* group was higher in active FMF compared to both FMF remissions and controls (14.2% in attack vs. 6.5%). *Gamma-proteobacteria* were 0.2% and 2.1%, in healthy controls and FMF remission, respectively, and there was a complete loss of these bacteria in the acute phase. The most striking difference was observed in the Propionate-producing *Acidaminococcaceae* subgroup (Clostridial cluster IX within *Firmicutes*). These bacteria were overrepresented in remission period compared to controls (16% vs. 10%), and tended to disappear in attack (3%), found only in FMF15 (Fig. 1C). Although in the latter group the bacterial sequences were the least diverse, which might be the consequence of a general inflammatory process, however representatives of the Butyrate-producing *Faecalibacterium* group in attack were significantly high compared to both FMF remission and healthy state (Table 3). Butyrate, which is produced by bacterial fermentation, has been shown to reduce inflammation in experimental colitis in animal models. It reduces inflammation through an inhibitory effect on proinflammatory cytokine expression, thus demonstrating anti-inflammatory properties [12]. Such increase of butyrate producers among acute patients implies that it could correspond to a compensative response.

Table 3

Comparison of the libraries derived from healthy controls and FMF patients in two stages of the disease

Bacterial subgroups	Healthy	Remission	Attack
Prevotellaceae (CFB)*	27.6%	22%	16.5%
Bacteroidaceae (CFB)**	21.7%	17.8%	30.7%
Faecalibacterium (Cluster IV)*,**,***	6.5%	6.5%	14.2%
Acidaminococcaceae (Cluster IX)*,**,***	10%	16%	3%
Gamma-proteobacteria***	0.2%	2.1%	0

*$p < 0.01$-in healthy/attack comparison

**$p < 0.01$- in remission/attack comparison

***$p < 0.01$- in healthy/remission comparison

We observed no specific microbial group pointing to the presence of bacteria, which could be specifically involved in disease activity. The 16S rDNA profile of the fecal microbiota was very stable under healthy conditions but unstable in FMF patients. It seems the alterations in gut microflora composition reflect a metabolic imbalance of the complex microbial ecosystem with severe consequences for the host immune system. How some bacteria may exert an inflammatory effect and others a protective role in FMF is yet uncertain. Is a breakdown in the balance between putative "protective" and "harmful" intestinal bacteria simply a secondary phenomenon in FMF, or is altered composition a primary modification, that is to say genetically determined, leading to an inflammatory process? Further studies may help to explain the complex relationships among bacteria, inflammation and genetics, which could provide new insights into the pathogenesis and treatment of FMF.

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Զ. Ա. Խաչատրյան

Աղիքային միկրոբիոտայի կազմի փոփոխությունները պարբերական հիվանդության ժամանակ

Հետազոտված է ՊՀ հիվանդների աղիքային միկրոֆլորայի կազմը հիվանդության ռեմիսիայի և սուր շրջաններում: Հայտնաբերվել են պրոպիոնատ արտադրող միկրոբների նշանակալի բարձր քանակություններ ռեմիսիայի շրջանում, և գրեթե դրանց բացակայությունը սուր շրջանում, ինչպես նաև բութիրատ արտադրող միկրոբների քանակության բարձրացում սուր շրջանում, համեմատած առողջ դոնորների հետ: Այս տվյալները վկայում են ՊՀ ժամանակ միկրոբային կազմի դիսբիոտիկ փոփոխությունների մասին, ինչպես նաև հնարավորություն են տալիս փնտրելու բուժման այլընտրանքային ուղիներ, որոնք ուղղված կլինեն աղիներում առկա միկրոբային դիսբալանսի կարգավորմանը:

З. А. Хачатрян

Изменения состава кишечной микробиоты при периодической болезни

Исследован состав кишечной микрофлоры у больных ПБ на стадиях ремиссии и атаки. Обнаружено значительное увеличение пропионат-продуцирующих бактерий в ремиссии и почти полное исчезновение этих бактерий у больных ПБ в острой стадии, а также увеличение бутират-продуцирующих бактерий в атаке, по сравнению с контролем. Эти данные свидетельствуют о дисбиотических изменениях в кишечной микробиоте при ПБ и дают возможность изыскания новых путей терапии, направленных на коррегирование дисбаланса в кишечнике.