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Metagenomic Analysis of Gingival Sulcus Microbiota and Pathogenesis of Periodontitis Associated with Type 2 Diabetes Mellitus

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Biofilm of the gingival sulcus from 22 patients with type 2 diabetes mellitus and periodontitis, 30 patients with periodontitis not complicated by diabetes mellitus (reference group), and 22 healthy volunteers without signs of gingival disease (control group) was studied by quantitative PCR. Quantitative analysis for the content of *P. gingivalis*, *T. forsythia*, *A. ctinomycetemcomitans*, *T. denticola*, *P. intermedia*, *F. nucleatum/periodonticum*, and *P. endodontalis* in the dental plaque was performed with a Dentoscreen kit. The presence of other bacterial groups was verified by metagenomic sequencing of the *16S rRNA* gene to evaluate some specific features of the etiological factor for periodontitis in type 2 diabetes mellitus. Specimens of the *Porphiromonadaceae* and *Fusobacteriaceae* families were characterized by an extremely high incidence in combined pathology. The amount of *Sphingobacteriaceae* bacteria in the biofilm was shown to decrease significantly during periodontitis. Metagenomic analysis confirmed the pathogenic role of microbiota in combined pathology, as well as the hypothesis on a possible influence of periodontitis on the course and development of type 2 diabetes mellitus.

Key Words: *16S sequencing; metagenomic analysis; microbiota; type 2 diabetes mellitus; periodontitis*

More than 2.5 billion people in Russian suffer from diabetes mellitus; 90% patients have type 2 diabetes mellitus (DM2). Approximately one year after the diagnosis of DM, 100% patients have the signs of chronic periodontitis with possible loss of teeth [1]. This association of periodontitis and DM2 results from fact that DM is accompanied by a variety of metabolic and immunological disturbances, including oxidative stress, change in the cytokine profile of the immune system, and modulation of cell receptors [5,7]. These abnormalities in combination with the increase in glucose content in the oral fluid contribute to changes of microbiota in the oral cavity, appearance of periodontopathogenic species and, therefore, development of periodontisis [12]. However, the primary factor for these relationships remains unclear. Does the pre-diabetic state induce periodontitis, or vice versa?

In this work, metagenomic technologies were used to study the pathogenetically important features of biofilm microbes in the gingival sulcus during periodontitis complicated by DM2.

MATERIALS AND METHODS

The study was performed in the A. S. Loginov Moscow Clinical Scientific Centre (Moscow Healthcare Department) and Central Research Institute of Den-

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tal and Maxillofacial Surgery (Moscow). The main group consisted of 22 patients (11 men and 11 women, 47-63 years) with DM2 in combination with periodontitis (confirmed by a detailed dental examination). The duration of DM2 was 3-23 years (less than 10 years in 44% patients, more than 20 years in 17% patients). The reference group included 30 patients (13 men and 17 women, 42-60 years) with periodontitis not complicated by DM2. The control group for genetic studies was composed of 22 clinically healthy volunteers (9 men and 13 women, 25-28 years) without signs of gingival disease. All participant of this study signed the informed consent form. Protocols of the study were approved by the Ethics Committees in these health facilities.

The samples were obtained from 4 regions of the gingival sulcus with sterile paper endodontic pins (No. 30) and placed in a tube with 0.2 ml physiological saline.

DNA was isolated with a DNA-Express kit (Litekh) in accordance with the manufacturer's recommendations. DNA was stored at -20°C when required. Quantitative analysis of *P. gingivalis*, *T. forsythia*, *A. actinomycetemcomitans*, *T. denticola*, *P. intermedia*, *F. nucleatum/periodonticum*, and *P. endodontalis* in the dental plaque was performed with a Dentoscreen kit (Litekh) in accordance with manufacturer's recommendations.

Total DNA was isolated from dental plaque samples with QIAamp DNA Investigator Kit (Qiagen) in accordance with manufacturer's instructions. The amplicon library for 16S metagenomic sequencing was prepared with primers of 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 801R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') in accordance with the protocol of 16S Metagenomic Sequencing Library Preparation (Part #15044223 Rev. B). Sequencing of the libraries and analysis of these data were performed with a MiSeq genetic analyzer (Illumina) and MiSeq Reagent Kit 2 in accordance with the manufacturer's instructions. The results were visualized with MEGAN5 software (http://ab.inf.uni-tuebingen.de/software/megan5/).

The results were analyzed by nonparametric statistical methods with SPSS 21 software; 95% confidence intervals of study parameters were calculated.

RESULTS

First, red complex bacteria were evaluated in the gingival sulcus microbiota. Patients of the main group and reference group were examined for the incidence of each bacterial species that exceeded the threshold values (10^4 for *A. actinomycetemcomitans*; 10^5 for *P. gingivalis*, *T. forsythia*, and *P. endodontalis*; and 10^6 for *T. denticola*, *P. intermedia*, and *F. nucleatum/periodonticum*; Fig. 1). Only in some volunteers of the control group, these parameters were above the threshold values. The absolute results of PCR (genome equivalent per 1 ml crevicular fluid) were compared between study groups (Table 1).

P. gingivalis (bacteria with the greatest pathogenicity) and *P. endodontalis* were the most abundant species of periodontopathogenic microorganisms. The

TABLE 1. Content of Periodontopathogenic Bacteria in the Crevicular Fluid of Patients from Study Groups (genome-equ/ml; Me (min; max))

Species of periodontopathogenic bacteria	Patients with DM2 and periodontitis	Patients with periodontitis	Healthy volunteers	Probability of differences χ^2 test		
				p_1	p_2	p_3
P. gingivalis	45,258 (0; 860,000)	27 (0; 71,900)	0 (0; 102)	0.001*	0.001*	0.238
T. forsythia	1215 (0; 750,000)	124 (0; 624,000)	0 (0; 293,000)	0.308	0.046*	0.313
A. actinomycetemcomitans	649 (0; 138,000)	177 (0; 2,990,000)	0 (0; 2,990,000)	0.355	0.865	0.539
T. denticola	54,200 (0; 1,160,000)	1053 (0; 2,890,000)	0 (0; 2,890,000)	0.005*	0.005*	0.769
P. intermedia	138 (0; 940,000)	76 (0; 471,000)	0 (0; 95,200)	0.300	0.261	0.920
F. nucleatum	72,800 (120; 732,000)	3000 (0; 2,320,000)	57,400 (0; 2,320,000)	0.030*	0.336	0.441
P. endodontalis	5840 (0; 880,000)	546 (0; 1,160,000)	0 (0; 1,160,000)	0.298	0.300	0.968

Note. p_1, probability of differences between DM2 patients and periodontitis patients; p_2, probability of differences between DM2 patients and healthy volunteers; p_3, probability of differences between periodontitis patients and healthy volunteers. *$p<0.05$, Mann—Whitney test.

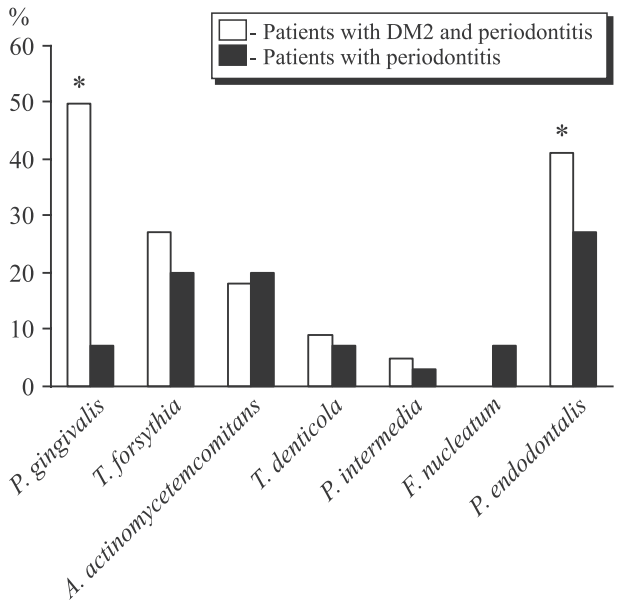

Fig. 1. Incidence of some periodontopathogenic bacteria in patients with DM2 and periodontitis and in patients with periodontitis. *$p<0.05$ in comparison with periodontitis patients.

incidence of these periodontal pathogens was highest during periodontitis complicated by DM2.

Detailed comparative quantitative analysis of periodontopathogenic microorganisms (as differentiated from the partial method in clinical dental practice) allowed us to evaluate a wide range of differences between patients and healthy volunteers.

For example, the development of periodontitis in DM2 patients was accompanied by a significant increase in the content of *P. gingivalis*, *T. forsythia*, and *T. denticola* in gingival sulcus fluid. These patients were also characterized by a greater amount of *F. nucleatum/periodonticum* as compared to the periodontitis group. No between-group differences were found in the content of *T. forsythia* and other identified species.

The involvement of bacteria from other groups (families and genera) in periodontal damage was studied by metagenomic sequencing of the *16S rRNA* gene.

Statistically significant differences were observed only in the content of specimens of the *Porphiromonadaceae* and *Fusobacteriaceae* families in the gingival sulcus microbiota. The incidence of these bacteria was 1.9-fold higher in patients with combined pathology (Fig. 2). These families include *P. gingivalis* and *F. nucleatum/periodonticum*, whose etiological role in DM2 and periodontitis was described previously. Independently on the concomitant disease, the amount of *Sphingobacteriaceae* specimens in biofilm samples was much lower during periodontitis (Fig. 2). These bacteria are probably involved in the formation of biofilm mantle components. These data provide a new explanation for differences between biofilms, which are formed under normal conditions and during periodontitis (as shown by scanning electron microscopy) [2].

Published data show that red complex bacteria and orange complex bacteria are often present in dental plaques during periodontal diseases [8]. Russian scientists believe that these bacteria are presented by periodontopathogenic species of the first order (*P. gin-*

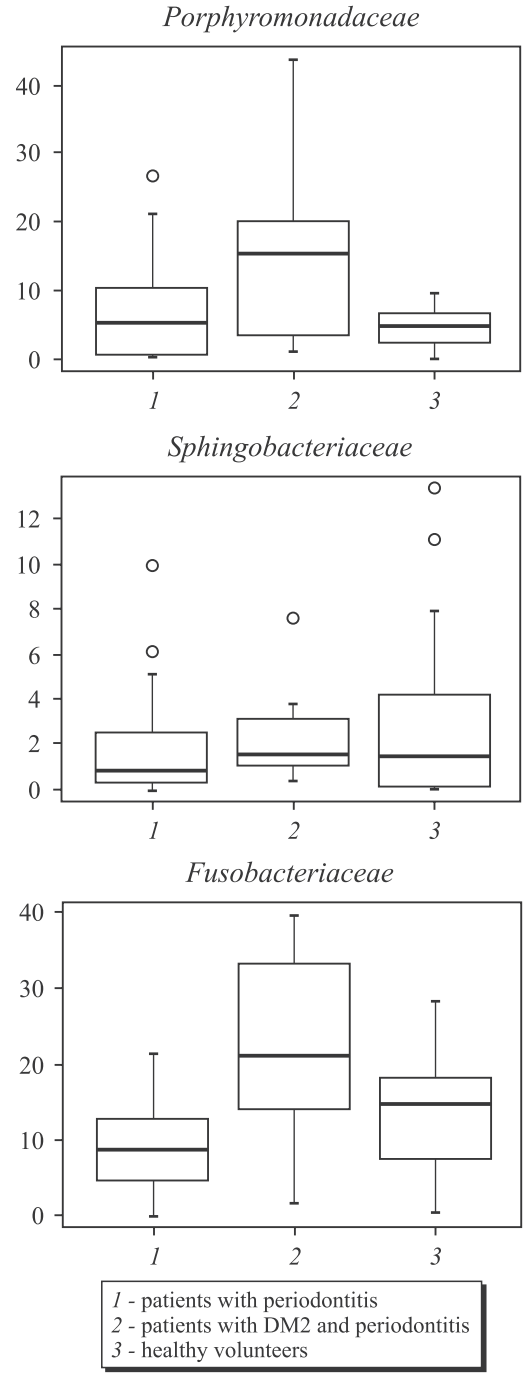

Fig. 2. Incidence of specimens of the *Porphiromonadaceae*, *Fusobacteriaceae*, and *Sphingobacteriaceae* families in the gingival sulcus microbiota. Circles illustrate the individual deviations of study parameters from the range of a 95% confidence interval.

givalis, *A. actinomycetemcomitans*, and *T. forsythia*) and second order (*T. denticola*, *P. intermedia*, *F. nucleatum/periodonticum*, *P. endodontalis*, *E. corrodens*, and *P. micra*) [2,3]. The introduction of new molecular biological technologies with PCR diagnostics and high-performance sequencing in laboratory practice allowed us to identify other microorganisms, which are not cultured and cannot be revealed in bacteriological studies [4].

Our results indicate that the most pathogenic species of *P. gingivalis*, which belongs to the first-order periodontal pathogens, plays an important role in the etiology of periodontitis in DM2 patients [2]. Second-order periodontal pathogens (*e.g.*, *F. nucleatum/periodonticum*) also have a certain etiological role in this disease. It should be emphasized that the role of *T. forsythia* and *T. denticola* in the etiology of periodontitis does not depend on the presence of concomitant disorders.

Many Russian and international epidemiological studies are performed to evaluate the relationship between periodontitis and obesity. The amount of red complex bacteria was shown to be associated with lipopexia in healthy individuals and development of DM2 [6]. However, the primary factor in these relationships remains unknown. The progression of diabetes is associated with the induction of proinflammatory cytokines that increase the resistance to insulin [10]. This concept was confirmed by other studies, which showed that a general inflammatory state during periodontitis contributes to the development of cardiovascular diseases and diabetes [11].

Our study showed that patients with DM2-associated periodontitis are characterized by quantitative features of the microbiota, but not by qualitative changes in the species composition of periodontopathogenic bacteria. These results do not contradict the concept that periodontitis can provoke pathological processes, which are typical of DM. Taking into account these data, it should be emphasized that the content of commensal bacteria of the *Sphingobacteriaceae* family in the biofilm is reduced during periodontitis. These bacteria are involved in the production and metabolism of sphingolipids and modulate the intensity of metabolic processes, including the sensitivity of cells to insulin [9]. This phenomenon confirms the concept that periodontitis has a primary role in the pathogenesis of DM2.

We conclude that modern technologies for studying the microbiota in oral cavity biofilm illustrate the absence of qualitative changes in the species composition during DM2-associated periodontitis (as differentiated from the biofilm under a normal somatic state). However, the content of periodontopathogenic *P. gingivalis* and *F. nucleatum/periodonticum* is high under these conditions. Moreover, periodontitis is characterized by a low amount of *Sphingobacteriaceae* bacteria in the biofilm. These bacteria do not exhibit pathogenicity, but modulate the cell sensitivity to insulin. Our results confirm the hypothesis that periodontitis plays an important role not only in the course, but also in the development of DM2.

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REFERENCES

1. Bogomolov MV. Periodontitis as a nonspecific complication of diabetes mellitus. Methods of prevention. Russ. Med. Zh. 2011;19(13):829-831. Russian.
2. Ippolitov EV, Didenko LV, Tzarev VN. The characteristics of morphology of biofilm of periodontium under inflammatory diseases of gums (chronic catarrhal gingivitis, chronic periodontitis, Candida-associated periodontitis) according results of electronic microscopy. Klin. Lab. Diagnost. 2015;60(12):59-64. Russian.
3. Nikolaeva EN, Tsarev VN, Ippolitov EV. Parodontopathogenic bacteria as indicators of the risk of periodontitis (part II). Stomatologiya dlya Vsekh. 2011;(4):4-7.
4. Rebrikov DV. Real-Time PCR. Moscow, 2014. Russian.
5. Lalla E, Papapanou PN. Diabetes mellitus and periodontitis: a tale of two common interrelated diseases. Nat. Rev. Endocrinol. 2011;7(12):738-748.
6. Matsushita K, Hamaguchi M, Hashimoto M, Yamazaki M, Yamazaki T, Asai K, Yamori M, Bessho K, Toda H, Hasegawa G, Nakamura N, Fukui M. The novel association between red complex of oral microbe and body mass index in healthy Japanese: a population based cross-sectional study. J. Clin. Biochem. Nutr. 2015;57(2):135-139.
7. Patil VS, Patil VP, Gokhale N, Acharya A, Kangokar P. Chronic periodontitis in type 2 diabetes mellitus: oxidative stress as a common factor in periodontal tissue injury. J. Clin. Diagn. Res. 2016;10(4):BC12-BC16.
8. Rôças IN, Siqueira JF Jr, Santos KR, Coelho AM. "Red complex" (Bacteroides forsythus, Porphyromonas gingivalis, and Treponema denticola) in endodontic infections: a molecular approach. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 2001;91(4):468-471.
9. Straczkowski M, Kowalska I. The role of skeletal muscle sphingolipids in the development of insulin resistance. Rev. Diabet. Stud. 2008;5(1):13-24.
10. Tilg H, Moschen AR. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends Endocrinol. Metab. 2008;19(10):371-379.
11. Wang GP. Defining functional signatures of dysbiosis in periodontitis progression. Genome Med. 2015;7(1):40.
12. Zhou M, Rong R, Munro D, Zhu C, Gao X, Zhang Q, Dong Q. Investigation of the effect of type 2 diabetes mellitus on subgingival plaque microbiota by high-throughput 16S rDNA pyrosequencing. PLoS One. 2013;8(4):e61516. doi: 10.1371/journal.pone.0061516.