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Analysis of the Relationship of Allele Frequencies in the Risk of Aggressive Periodontitis

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ABSTRACT

A hereditary trait of aggressive periodontitis was revealed in long-term monitoring. The improvement of modern laboratory research methods facilitated active studies of the relationship of the individual's genetic makeup with their disease risk.

Key words: aggressive periodontitis, genetic polymorphism, SNP, collagen, MMP

INTRODUCTION

In recent decades there has been a tendency of increase in the number of juvenile age patients with the diagnosis of aggressive periodontitis. Nevertheless the progress achieved by the manufacturers of oral hygiene products does not have any substantial effect on the decrease of disease spread dynamics [7].The exact sequence of events leading to aggressive periodontitis remains undiscovered, but it is certain that fiber destruction, advanced destruction of the alveolar bone and consequential tooth loss among young patients is a result of their body response to the invasion of periodontopathogens[1]. The aggressive periodontitis diagnosis is based upon the patient's complaints, age, clinical tests – the definition of gingival pockets depth and subgingival zone condition as well as the results of X-ray examination of alveolar bone destruction degree [2].

Long-term monitoring showed a pattern of aggressive periodontitis among the members of a single family. Thus it is possible that there may be a genetic predisposition to the disease [7]. A great amount of information concerning the interconnection of certain allelic variants of genes of the individuals and the condition of his health was accumulated due to the recent research in the field of human genetics and genomics. The difference between people at the level of certain genetic traits determines predisposition to hereditary and infectious diseases, development of physical and mental abilities, possibility of long-term work in hazardous work conditions, reaction to pharmaceuticals etc. [3].

That's why searching for the new interconnections of the human genotype and his genes are very important. Now it's beyond any doubt that if we know the individual features of the DNA, we can predict the development of serious diseases and, what is also important, take early measures to prevent them. Modern medicine is achieving a new level – the preventive medicine.Doctors' task is

no more just to treat a disease, but to prevent it by the detailed examination of the patient's body and adequate preventive measures. Fast accumulation of the information about individual polymorphism of human genome and well-structured data bases create the prerequisites for intensive study of the associations of the genetic and phenotypic component at the individual level. Now we already know a few genes which have the allelic condition positively influencing the possibility of human developing periodontitis and the speed of progression and seriousness of the disease. However the research of the genetic factors of periodontitis is now at its initial stage. Though the disease is socially very important and a lot of attention is paid to the prevention of dental problems, genetic predisposition (resistance) to aggressive forms of periodontitis is still an openquestion. So it is very important now to look for prognostically significant molecular-genetic markers of development of aggressive forms of periodontitis as well as to develop diagnostic test-systems for determination of the necessary genetic markers.

MATERIALS AND METHODS

The clinical researches were performed in the periodontology department of the FSBI Central Research Institute of Dental and Maxillofacial Surgery of the Ministry of Healthcare and Social Development of the Russian Federation and LLC NGO DNA-Technology, Russia. 171 patients aged 18 to 45 without any severe general pathologies took part in the research. Patients were divided into two groups. The first group included 48 patients diagnosed with aggressive periodontitis, the second 123 healthy patients without severe physical conditions.

Criteria for inclusion:

• Individuals of both sexes belonging to the Europoid race, aged 18 to 45 yearsof age, residing in the territory of Moscow and Moscow region.

Exclusion criteria were as follows:

• Systemic diseases of connective tissue;
• Malignant disease, chemotherapy and radiation therapy in history;
• Acute infectious and viral diseases;
• Polyvalent allergy;
• Pregnancy and lactation;
• People who do not understand the purpose of the study and signed informed consent.

To determine the periodontal status the patients were examined with the help of clinical and X-ray methods. The patients were diagnosed with "aggressive periodontitis" basing on their complaints and time when they started, clinical examination and additional methods, such as orthopantomogram.One of the basic symptoms was an early age when a patient developed such complaints as pyorrhea, "temporal swellings" and developing of "bags" on the gums. Patients often complained about fever that they had together with these symptoms. All this was clinically accompanied by severe affection of the gingival junction. The medical history showed that all patients had a hereditary load on this disease, and noticed the first symptoms of it such as pyorrhea, "swelling" - abscess formation at the age of 14 – 17. One of the clinical indicators of AP was a gingival pocket which means loss of the gingival junction and volume of the bone tissue of the alveolus. Other anatomic-topographic features were, for example, hypermineralization of enamel, lack of abradabilityand the divergence of the roots showed at the X-ray picture, that was earlier mentioned in I.V. Bezrukova's work (2001) as typical signs of AP and was taken into account when diagnosis was made.Saliva was used as biomaterial during molecular-genetic research. Conditions of having biomaterial tested were explained to all the patients in advance. Patients were not allowed to brush their teeth, eat, or drink 30 minutes before the procedure. The biomaterial was collected in the dental office by the patients themselves who put 1 ml into special test tubes. Each test tube got a reference number, which was also put on the individual form of the patient. So the biomaterial

(saliva) then went to the lab in a depersonalized way. At the end of the procedure the test tube was put into a freezing chamber where the temperature was -10 ºC. The biomaterial was transported to the lab in special thermal containers where the temperature was 4 ºC.

Genetic Typing of the Samples of genomic DNA

When test-systems for mapping SNP were constructed, a principle of kissing probes was used. It was based on the analysis of the intensity of fluorescence versus fusion temperature curves with the use of allele-specific probes, marked respectively with fluorophores and dampers. For marking with fluorophores two probes were used with the active end nucleotide complying with one of the two possible allele variants of the genomic human DNA. The probe marked with the damper complied with the invariant sequence which adjoined the polymorphous position. To determine the allele condition of polymorphous positions within the genes selected for the analysis 23 sets of oligonucleotides were used. 18 of them were used in the dental practice for the first time, and 5 were taken in a modified way from the doctoral thesis of O.A. Zorina.

The Method of PCR "in Real Time"

The PCR reaction and result evaluation were done with the help of amplifier DT-96 (OOO NPO DNK Technologia, Russia), equipped with an optical system. The following parameters of the program for PCR were used: 94˚C – 10 s, 64˚C – 20 s, 72˚C – 10 s, there were 40 cycles of the reaction. The signal of fluorescence depending on the temperature was measured in the interval of 25˚C up to 80˚C. The reaction was repeated twice to make the results more accurate.

Statistical analysis

Statistical analysis of the results is done with the help of WINPEPI v. 10.7 (is spread without limits for non-commercial use). The statistical validity of the differences was done according to the Pirson criterion χ^2 with Yates correction for continuity. The correlation value between genotype and phenotype (risk of aggressive periodontitis) was calculated according to the value of the relative risk. OR parameter (odds ratio) is calculated with the confidence interval which is 95%. Candidate genes for molecular-genetic research were chosen based on the knowledge of periodontal fibers morphology [3,4]. These are the genes that encode basic types of protein: fibrillarand shapeless proteins of the matrix, metalloproteinases and their inhibitors, local signalling factors of periodontal and general signalling factors in blood circulation.

Table 1 represents polymorophous positions in genes selected for population genetic study of the association of certain allele variants of the genes with the risk of developing periodontitis. It's necessary to point out that when selecting the polymorphous positions we tried to use those, that have allele pollymorhysm connected with the biggest part of the population. Positions, where frequency of change was expected to be less than 5%, were not used.

Table 1.Nucleotide marker selected for the research

Gene	Function	Polymorphic positions that are studied
1. Metalloproteinases		
MMP2	Gelatinises	Rs243865 Rs2285052

MMP3	Stromelysins	Rs3025058
MMP9	Gelatinises	Rs17576 Rs3918242
MMP12	Metalloelastase, trapping factor of macrophages	Rs2276109
ADAM33	Membrane-bound MMP	Rs2280090
2. Chemokine and other signal factors of local action		
CCL2	Inflammatory chemokine	Rs1024611
CCR2	Inflammatory chemokine	Rs1799864
CCR5	Inflammatory chemokine	Rs333
IL8	Inflammatory chemokine	Rs4073
EPAS1	Anti-inflammatory chemokine (synonyms bHLHe73; HIF-2alpha; HIF2A; HLF; HRF; MOP2)	Rs1867785
LEPR	Leptin receptor (from the surface of macrophages)	Rs1137100 Rs1137101 Rs8179183
3. Lymphokines and other signal factors of generalized action		
LAMC1	γ-laminin, matrix receptor on the surface of fibroblasts	Rs10911193
LSP1	Lymphocyte-specific receptor of endotheliocytes	Rs3817198
CNTF	Vibratile factor of neutrophils, prevents the cytokine-dependant apoptosis	Rs1800169
LEP	Leptin – lymphokine of the type TH1, agonist TNFα	Rs7799039
IL18	Lymphokine of the mixed TH1/TH2 type	Rs187238

IL12B	Lymphokineof the TH1-type	Rs3212227
IL1R1	Lymphocyticreceptorofinterleukine 1	Rs2234650
TNP1	Factor of remodeling with nucleosis	Rs13387042

During the research the genotype was defined by means of genetic polymorphism with the help of real-time PCR using adjoining kissing probes to find out associations of aptitude markers with aggressive periodontitis. Previously in the work of O.A. Zorina (2011) it has been established that the withdrawal method of melting profiles with the fluorescence-labeled allele-specific oligonucleotide probes after the reaction allows for more reliable data [5]. Thus, based upon previous research, we developed test systems for identification of genetic polymorphisms as described above. Using statistical analysis of the results, we began with defining whether the ratio of hetero- and homozygotes occurrence frequency in the researched alleles complied with the Hardy-Weinber principle, which allows the use of the methods of parametritic statistics. Having established that the parametritic methods of statistical analysis were applicable, we researched the correlation between the chosen alleles and the genotypes with the risk of aggressive periodontitis.

It has to be taken into consideration that to define the true purpose of genetic factors in a pathogenic pathway, one has to use 2 models for each case:

1. The dominant model, based on the analysis of the occurrence frequency of the alleles of each locus;
2. The recessive (co-dominant) model, based on the analysis of the occurrence frequency of each locus genotype. Following the results of these calculations, the discovery of the most significant statistical differences between the samples allows us to choosebetween the models (the model with the better definition is chosen).

Below is a part of the data resulting fromthe analysis of the allele frequency.

The Analysis of the Interrelation between the Allele Frequency and the Phenotype

Table 2 represents the results of the statistical analysis of the data.

Table 2. The distribution of the gene alleles in the selection of the case and control group (dominant model)

	Gene	Polymorphism	Alleles[1]	Allele Frequency				χ^2 (p)
				n	Case	n	Control	
0	1	2	3	4		5		**6**
1	MMP2	rs243865	C	48	0,771	123	0,797	0,28 (0,6)
			T		0,229		0,203	
2	MMP2	rs2285052	A	48	0,396	123	0,370	0,20 (0,66)
			G		0,604		0,630	
3	MMP3	rs3025058	d	48	0,521	123	0,467	0,79(0,38)
			T		0,479		0,533	
4	MMP9	rs17576	A	48	0,552	123	0,695	6,25 (0,01)
			G		0,448		0,305	

5	MMP9	rs3918242	C	48	0,677	123	0,817	7,83 (0,005)
			T		0,323		0,183	
6	MMP12	rs2276109	G	48	0,854	123	0,841	0,09 (0,77)
			A		0,146		0,159	
7	ADAM33	rs2280090	G	48	0,906	123	0,841	2,40 (0,12)
			A		0,094		0,159	
8	CCL2	rs1024611	A	48	0,719	123	0,671	0, 74 (0,39)
			G		0,281		0,329	
9	CCR2	rs1799864	A	48	0,073	123	0,114	**1,26 (0,26)**
			G		0,927		0,886	
10	CCR5	rs333	I	48	0,865	123	0,882	0,20 (0,66)
			D		0,135		0,118	
11	IL8	rs4073	A	48	0,500	123	0,488	0,04 (0,84)
			T		0,500		0,512	
12	EPAS	rs1867785	G	48	0,615	123	0,659	0,58 (0,45)
			A		0,385		0,341	
13	LEPR	rs1137100	G	48	0,677	123	0,736	1,18 (0,28)
			A		0,323		0,264	
14	LEPR	rs1137101	A	48	0,771	123	0,829	1,55 (0,21)
			G		0,229		0,171	
15	LEPR	rs8179183	G	48	0,563	123	0,508	0,82 (0,37)
			C		0,438		0,492	
16	LAMC1	rs10911193	C	48	0,896	123	0,882	0,13(0,72)
			T		0,104		0,118	
17	LSP1	rs3817198	T	48	0,594	123	0,603	0,02 (0,89)
			C		0,406		0,398	
18	CNTF	rs1800169	D	48	0,875	123	0,841	0,61 (0,43)
			T		0,125		0,159	
19	LEP	rs7799039	G	48	0,552	123	0,533	0,11 (0,74)
			A		0,448		0,467	
20	IL18	rs187238	G	48	0,729	123	0,715	0,06 (0,8)
			C		0,271		0,285	
21	IL12B	rs3212227	A	48	0,854	123	0,821	0,53 (0,46)
			C		0,146		0,179	
22	IL1R1	rs2234650	C	48	0,708	121	0,665	0,58(0,45)
			T		0,292		0,335	
23	TNP1	rs13387042	A	48	0,542	123	0,626	2,05 (0,15)
			G		0,458		0,374	

[1] Variant alleles (mutations) are represented in the lower cells of the relevant DNA-markers,
[2]levels of significance of pfrequencies of the alleles between the groups (*p<0,05)

In the process of calculations based on the patients' samples we received reliable figures for the MMP9 gene in the rs17576position for allele A and in the rs3918242 position for allele C. Among the patients with progressiveperiodontitis the occurrence frequency of the allele A of MMP9 genein the rs17576 position was 55.2%, as compared to 69.5% in the control group. Thusthe allele A risk factor is 0.54 (95% CI 0.33-0.88). The fact that it is lower than 1 confirms that allele A has a

protective action. In the rs3918242 position of the MMP9 genethe occurrence frequency of allele C was 67.7% in the case group and 81.7% in the control group. The allele C risk factor is OR=0.47 (95% CI 0.27-0.80), which also shows that the allele has protective action. In the process of data calculation for the rest of the researched genes we did not observe any significant differences. That confirms that it is not associated with the aggressive periodontitis pathway. For example the occurrence frequency of the main Allele A of the CCL2 (rs1024611), CCR2 (rs1799864), MMP2(rs2285052), IL12B (rs3212227), IL8 (rs4073) genes was higher in the samples of the patients with the aggressive periodontitis than in the samples of the healthy control group. But the difference was insignificant – only about 2 to4%. In the process of calculating the data on all of the genes we found out that the occurrence frequency of allele Awas higher in the samples of the patients with the aggressive periodontitis. The occurrence frequency of alleleG of the LEPR (rs8179183), MMP12 (rs2276109), EPAS (rs1867785), LEP (rs7799039), LEPR (rs1137100), ADAM33 (rs2280090) and IL18 (rs187238) genes was also no more than6% in both samples. In the samples with the aggressive periodontitis the allele G of the LEPR (rs8179183) gene showed in 56.3% cases, while in the control group it only showed in 50.8% cases. On the contrary, the same allele of the EPAS (rs1867785) gene in the diseased group showed a smaller percentage of occurrence – 61.5% against 65.9% in the control group. The calculation of the occurrence frequency of the allele G of the LEPR (rs1137100) gene showed a similar picture: 67.7% for the diseased and 73.6% for the healthy. The difference in the occurrence frequency of all the other genes in both groups was minimal. The calculation of the occurrence frequency data for the allele Cof the genes like MMP2 (rs243865), LAMC1 (rs10911193) and IL1R1 (rs2234650) revealed insignificant deviations in the group withaggressive periodontitis and the control group as well. The occurrence frequency of the allele I of the CCR5 (rs333) gene in the samples of the patients with aggressive periodontitis amounted to 86.5%, as opposed to 88.2% in the samples of the healthy patients. Analysis of the occurrence frequency of the allele Tof the LSP1(rs3817198) gene among the patients with aggressive periodontitis and the control group showed similar results: 59.4% and 60.3%. The allele d (deletion) of the CNTF (rs1800169) gene occurred in 87.5% cases among the diseased and in 84.1% cases among the healthy, although the difference was again insignificant. The occurrence frequency of the allele d (mononucletoid deletion) of the MMP3 (rs3025058) gene in the group of patients with aggressive periodontitis amounted to 52.1% while the healthy had a slightly lower percentage – 46,7%. It should be noted that the work of Menezes-Silva et al, 2012 shows a significant impact of mutations in the positions of rs639752 (P = 0.03) and rs679620 of the MMP3 gene on the risk of caries and the depth of caries lesion [6].

RESULTS AND CONCLUSION

The results of the research showed the reliable association of the patients with rare alleles MMP-3 rs639752 (P = 0,03) and rs679620 (P=0,004) being more prone to caries and its complications, which are expressed through the damage of periapical tissues. Rare allele ofMMP-2 gene (P = 0.000004) was also found, which causes high risk of developing complications of the carious process. The important conclusion is the significance of genes MMP-2 and MMP-3 from the point of view of the development of oral cavity diseases. Besides, authors proved the possibility of getting statistically valid results using PCR technology “in real time”, which allows to analyze just some selections (a little bit more than 100 patients) compensating for it by the possibility of parallel analysis of quite a big number of marker SNP (16 pieces). That means that in case of caries unlike periodontitis MMP-3 gene is a more informative marker than MMP-9. These data show also a high level of multiformity of genes stromelysins significant to influence the risk of development of socially important diseases. Thus we have indirect data confirming the role of MMP3 in the control of the remodeling of the collagen and mineral matrix of oral cavity.

Molecular genetic research methods have become much more available for scientists and practitioners in the last decade. This allowed us to gain knowledge of some genes with the allele status which supposedly influences the probability of periodontitis development. However that mostly concerns the typical periodontitis because its aggressive form is less frequent, which makes it more difficult to find the necessary number of patients for research. Thus scientists and practitioners are still searching for genetic markers of the riskof aggressive periodontitis development.

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