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GENETIC AND INFECTIOUS FACTORS PREDISPOSING TO OSTEOPENIA

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Osteopenia is a complex disease associated with decreased bone mineral density and characterized by a moderate risk of fracture, which in certain situations can lead to osteoporosis. Identification of predisposition factors for osteopenia may help to individualize prognosis, treatment, and prevention of fractures and their adverse outcomes. The purpose of this study was to examine the association of single-nucleotide polymorphisms of the TNFSF11, TNFRSF11B, SOST, ZNF385B, and LRP5 genes involved in bone metabolism and *Porphyromonas gingivalis* oral infection with risk for osteopenia. The study group included 18 patients with osteopenia. The control group included 18 people without osteopenia. The T-allele polymorphism of the TNFRSF11B gene increased the risk of osteopenia in allelic, dominant, and recessive inheritance models, while the A-allele polymorphism of the ZNF385B gene – in allelic and dominant inheritance models. The number of bacteria in the gingival sulcus fluid samples of patients with osteopenia significantly exceeded the same indicator in the control group

Key words: osteopenia, oral health, polymorphism, genotyping, oral bacteria.

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ГЕНЕТИЧНІ ТА ІНФЕКЦІЙНІ ФАКТОРИ СХИЛЬНОСТІ ДО ОСТЕОПЕНІЇ

Остеопенія являє собою комплексне захворювання, пов'язане зі зниженням мінеральної щільності кісткової тканини, що характеризується помірним ризиком переломів, яке в певній ситуації може призвести до остеопорозу. Ідентифікація факторів схильності до остеопенії може допомогти індивідуалізувати прогноз, лікування та профілактику переломів і їхніх несприятливих наслідків. Метою цього дослідження було вивчення асоціації однонуклеотидних поліморфізмів генів TNFSF11, TNFRSF11B, SOST, ZNF385B і LRP5, які беруть участь у метаболізмі кісткової тканини, а також інфікування ротової порожнини Porphyromonas gingivalis із ризиком розвитку остеопенії. Група, що досліджувалася, налічувала 18 пацієнтів з остеопенією. До контрольної групи було залучено 18 осіб без остеопенії. Т-алель поліморфізму гена TNFRSF11B підвищував ризик остеопенії в алельній, домінантній та рецесивній моделях успадкування, водночас А-алель поліморфізму гена ZNF385B – в алельній та домінантній моделях успадкування. Кількість бактерій у зразках рідини ясенної борозни пацієнтів з остеопенією достовірно перевищувала аналогічний показник у контрольній групі.

Ключові слова: остеопенія, здоров'я порожнини рота, поліморфізм, генотипування, бактерії порожнини рота.

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Osteopenia is a term that defines bone density that is neither normal nor as low as in osteoporosis [2]. According to World Health Organization (WHO) terminology, osteopenia is defined by bone densitometry as a T-criterion – the ratio of the obtained bone mineral density value to the statistical average (normal) value in young people – equal to –1 to –2.5. At the same time WHO characterizes osteoporosis as a systemic skeletal disease with low bone mass and microarchitectural deterioration of bone tissue followed by increased bone fragility and susceptibility to fracture, with a T-criterion of –2.5 and below [9]. Osteopenia and osteoporosis are definitely related. Osteopenia is an initial and often reversible decrease in bone mineral density due to an imbalance between resorption and bone formation in which resorption predominates, and which in some cases can lead to osteoporosis, just as gingivitis, an initial form of periodontal disease, can progress to periodontitis if untreated or in its acute form [6, 8]. At the same time, osteopenia is no less of a health and health care problem than osteoporosis because about 50 % of all fractures, about 50 % of all recurrent fractures and most of the comorbidities, mortality and associated financial costs occur in the larger population of patients with osteopenia, which is characterized by a moderate risk of fracture, rather than in the smaller population with osteoporosis, which is associated with a high risk of fracture [9].

There are many causes of osteopenia, including calcium and vitamin D deficiency, smoking, diabetes, hormonal levels, gender and age, and hypodynamia. In terms of genetic predisposition, decreased bone mineral density can be determined by numerous genetic factors with minor individual effects, the cumulative effect of which plays a more significant role in the variability of this trait compared to individual gene effects. Mutations and dysfunctions in pathways regulating bone tissue metabolism, such as TGF-

β/BMP and Wnt/β-catenin signaling pathways, affect the process of bone remodeling, which ultimately leads to a wide variety of metabolic bone diseases, including osteoporosis and osteopenia [9]. Identification of hereditary factors of these diseases is of great clinical importance for their early recognition and expansion of therapeutic opportunities.

Disturbances associated with decreased bone mineral density are referred to as one of the causes of periodontitis, a chronic inflammatory disease in which the connective tissue and bone supporting the teeth is destroyed [7]. The clinic of chronic periodontitis is characterized by such features as loss of gingival tissue attachment to the tooth, deepening of the gingival crevice or formation of the so-called periodontal pocket, degradation of the periodontal ligament, and loss of alveolar bone. It is assumed that the relationship between defects in bone metabolism and periodontitis is bidirectional. On the one hand, a general decrease in bone mineral density as a result of bone metabolic disorders may affect the alveolar bones of the maxilla and mandible as well, thus contributing to the pathogenesis of periodontitis [8]. On the other hand, periodontitis may be a cause of osteopenia and osteoporosis because the bacteria associated with the development of periodontitis, particularly Porphyromonas gingivalis, activate processes leading to alveolar bone resorption and affecting the overall bone mineral density in the body [4, 5].

The purpose of the study was to establish associations of single nucleotide polymorphisms rs2073617 950C>T of TNFRSF11B gene, rs865429 675C>T of SOST gene, rs2277438 –438 A>G of TNFSF11 gene, rs6757845 G>A of ZNF385B gene and rs41494349 266A>G of LRP5 gene with osteopenia. We also compared the number of P. gingivalis bacterial cells in the gingival sulcus of patients with osteopenia and controls.

Materials and methods. The study involved 36 patients aged 25–55 years. The study group included 18 patients with osteopenia. The control group included 18 people without osteopenia. Dental examination was conducted in the dental office at the Department of Epidemiology and Prevention of Major Dental Diseases, Pediatric Dentistry and Orthodontics of the SE “The Institute of stomatology and maxilla-facial surgery National academy of medical sciences of Ukraine” (SE “ISMFS NAMS”).

DNA isolation from buccal epithelial cells was performed according to a modified method using Chelex [11]. 200 µl of 5 % solution of Chelex 100 in distilled sterile water (Chelex in sodium form, 100–200 mesh, Bio-Rad) was added to a tube (Eppendorf) containing an applicator with a scraping of epithelial cells. Before adding the resin was mixed in a homogeneous state with a wide-bore pipette and an aliquot was taken directly during mixing. Incubated at 56° for 30 minutes with constant stirring on a thermoshaker. Then incubated at 96°C for 8 minutes, shaking occasionally. After incubation, centrifuge for 3 minutes at 12000 g (Eppendorf Centrifuge 5424). DNA concentration and purity were determined spectrophotometrically (Nanophotometr, Implen, Germany) by taking a 5 µl aliquot directly from the tube with the DNA solution. For polymerase chain reaction (PCR), 5 µl of the supernatant was taken. To study the rs2277438 TNFSF11 –438 A>G polymorphism, we used allele-specific three-primer PCR using two forward primers on the 5'-length DNA strand, one specific to the mutant allele and the other to the normal allele, and a common reverse primer on the 3'-length strand. Amplification of the studied gene sites was performed on an Analytik Jena thermal cycler (Flex Cycler, Germany) in parallel in two Eppendorf tubes for the normal and mutant alleles of each gene in 20 µl of buffer solution (Fermentas, Lithuania), 100 nm of each oligonucleotide primer (Metabion, Germany), and 100–150 ng of DNA. Allelic variants of the polymorphisms rs2073617 TNFRSF11B 950C>T, rs865429 SOST 675C>T, rs6757845 ZNF385B G>A and rs41494349 LRP5 266 A>G were evaluated by PCR-PDRF (restriction fragment length polymorphism) with the corresponding primers manufactured by Metabion (Germany). For detection of allelic variants of OPG gene polymorphisms (950T/C), the amplification products after PCR (thermal cycler “Analytik Jena”, Flex Cycler, Germany) were treated at 370 C for 4 hours with the appropriate restriction enzymes manufactured by Fermentas (Lithuania): HindII for rs2073617, Nco1 for rs865429, Eco321 for rs6757845, and PvuII for rs41494349. Fractionation of amplification and restriction products was performed in a horizontal 2 % agarose gel prepared on single tris-acetate buffer (1xTAE) at 100V for 45 minutes. DNA pUC19: Msp1 was used as a molecular weight marker. The agarose gel was stained with ethidium bromide and visualized in transmitted ultraviolet light. Samples of microbiological material were collected nathesessionally to the wound of the oral cavity using sterile papery endodontic pins (size №25), which were inserted into the gingival sulcus for 15 seconds with a tweezers. After collection, the material was placed neatly in sterile sealed Eppendorf tubes (1.5 ml) with 1 ml of physiological solution for transportation to the laboratory in a refrigerant thermocontainer. Bacterial DNA was isolated using the kit “DNA-EXPRESS” (NPF “Litech”) according to the instructions of the manufacturer. The presence and amount of P. gingivalis parodontopathogen was determined by the PLR method in real time using the kit “Dentoscreen for quantitative analysis of Fluoropol-RV format”, OneStep Strip package (NPF “Litech”) according to the method of the manufacturer on CFX96 Touch “REAL TIME” amplifier (Bio-Rad, USA).

Statistical processing of the obtained results, including the test for deviation from the Hardy-Weinberg equilibrium (HWE) and the assessment of the association of genotypes and alleles with the risk of periodontitis by the Pearson $\chi 2$ method, was carried out using the DeFinetti genetic statistics program on the website of the Institute of Genetics (Munich, Germany). Associations were characterized by odds ratio (OR) with 95 % confidence interval and Pearson's $\chi 2$ test. The difference was considered to be statistically significant at $p<0.05$ [1].

Results of the study and their discussion. Genotyping of rs2073617 TNFRSF11B 950C>T, rs865429 SOST 675C>T, rs2277438 TNFSF11 -438 A>G, rs6757845 ZNF385B G>A and rs41494349 LRP5 266 A>G polymorphisms was performed in the group of patients with osteopenia (experience, n=18) and the group of patients without osteopenia (control, n=18). The distribution of genotype frequencies and its consistency with the Hardy-Weinberg equilibrium (HWE) as well as differences between the groups in the distribution of genotype and allele frequencies were analyzed in the studied groups. Only the AA genotype of the rs41494349 LRP5 266A>G polymorphism was detected in both groups, so this polymorphism was excluded from further analysis. For the other polymorphisms, the frequencies of genotype distributions were consistent with those theoretically calculated for HWE in both groups ($p>0.05$, table 1, 2).

Table 1

Distribution and comparison of frequencies of genotypes and alleles of rs2277438 TNFSF11 –438 A>G and rs6757845 ZNF385B G>A polymorphisms in patient groups

Polymorphism	rs2073617 TNFRSF11B 950C>T					
Genotype, allele	CC	CT	TT	Alele C	Alele T	HWE p-value
Case, frequency	0.110	0.445	0.445	0.333	0.667	1.000
Control, frequency	0.445	0.445	0.110	0.667	0.333	1.000
Comparison of frequencies	T<>C	CT<>CC	CT+TT<>CC *DM*	TT<>CC+CT *RM*	–	–
OR (95 % CI)	**4.000 (1.501–10.658)**	4.000 (0.639–25.020)	**6.400 (1.124–36.437)**	**16.000 (1.788–143.150)**	–	–
$\chi 2$ p-value	**0.005**	0.126	**0.026**	**0.007**	–	–
Polymorphism	rs6757845 ZNF385B G>A					
Genotype, allele	GG	GA	AA	Alele G	Alele A	HWE p-value
Case, frequency	0.444	0.556	0.000	0.722	0.278	0.102
Control, frequency	0.889	0.111	0.000	0.944	0.056	0.802
Comparison of frequencies	A<>G	GA<>GG	GA+AA<>GG *DM*	AA<>GG+GA *RM*	–	–
OR (95 % CI)	**6.538 (1.318–32.442)**	**10.000 (1.756–56.933)**	**10.000 (1.756–56.933)**	1.941 (0.035–106.656)	–	–
$\chi 2$ p-value	**0.011**	**0.004**	**0.004**	1.000	–	–

Note. CI – confidence interval; DM – dominant model; RM – recessive model; HWE – Hardy-Weinberg equilibrium. Significant values of the odds ratio (95 % CI) and values of $p<0.05$ are highlighted in bold.

The distribution of genotypes and alleles of the single-nucleotide polymorphism of the TNFRSF11B gene rs2073617 950C>T as well as the ZNF385B gene rs6757845 G>A was different between the studied groups. The frequency of the T allele rs2073617 in the group of patients with osteopenia exceeded that in the control group: 0.667 and 0.333, respectively. There was also an excess frequency of the mutant A allele rs6757845 in the experiment compared with the control: 0.278 and 0.056, respectively. At the same time, AA homozygotes of the rs6757845 polymorphism were not detected in either the control or the experiment.

The T allele of the rs2073617 TNFRSF11B 950C>T polymorphism increased the risk of osteopenia in the T<>C allele model, OR=4.000 (95 % CI 1.501–10.658), $\chi 2$ value reliability p=0.005. The hetero- (CT) and homozygous (TT) state of the T allele was also associated with an increased risk of osteopenia, consistent with the dominant inheritance model of CT+TT<>CC, OR=6.400 (95 % CI 1.124–36.437), p=0.026. In addition, TT homozygote in isolation was associated with the risk of osteopenia, according to the recessive inheritance model of TT<>CC+CT, OR=16.000 (95 % CI 1.788–143.150), p=0.007.

The A allele of the rs6757845 ZNF385B G>A polymorphism was associated with the risk of osteopenia in the A<>G allelic model, OR=6.538 (95 % CI 1.318–32.442), p=0.011; GA<>GG heterozygous state, OR=10.000 (95 % CI 1.756–56.933), p=0.004; and in the GA+AA<>GG dominant model, OR=10.000 (95 % CI 1.756–56.933), p=0.004.

Table 2

Distribution and comparison of frequencies of genotypes and alleles of rs2073617 TNFRSF11B 950C>T and rs865429 SOST 675C>T polymorphisms in patient groups

Polymorphism	rs2277438 TNFSF11 –438 A>G					
Genotype, allele	AA	AG	GG	Alele A	Alele G	HWE p-value
Case, frequency	0.000	0.500	0.500	0.250	0.750	0.157
Control, frequency	0.000	0.556	0.444	0.278	0.722	0.102
Comparison of frequencies	G<>A	AG<>AA	AG+GG<>AA *DM*	GG<>AA+AG *RM*	–	–
OR (95 % CI)	1.154 (0.404–3.295)	0.905 (0.016–50.244)	1.000 (0.019–53.120)	1.118 (0.020–62.734)	–	–
χ^2 p-value	0.789	1.000	1.000	1.000	–	–
Polymorphism	rs865429 SOST 675C>T					
Genotype, allele	CC	CT	TT	Alele C	Alele T	HWE p-value
Case, frequency	0.056	0.333	0.611	0.222	0.778	0.879
Control, frequency	0.111	0.611	0.278	0.417	0.583	0.275
Comparison of frequencies	T<>C	CT<>CC	CT+TT<>CC *DM*	TT<>CC+CT *RM*	–	–
OR (95 % CI)	2.500 (0.894–6.987)	1.091 (0.081–14.664)	2.125 (0.175–25.775)	4.400 (0.319–60.614)	–	–
χ^2 p-value	0.076	0.947	0.546	0.243	–	–

Note. CI – confidence interval; DM – dominant model; RM – recessive model; HWE – Hardy-Weinberg equilibrium. Significant values of the odds ratio (95 % CI) and values of p<0.05 are highlighted in bold.

The differences between the studied groups in the distribution of genotype and allele frequencies of the rs865429 SOST 675C>T and rs2277438 TNFSF11 -438 A>G polymorphisms were not statistically significant.

Figures 1 and 2 show the quantitative distribution of Porphyromonas gingivalis in gingival sulcus fluid samples from 8 (fig. 1) and 10 (fig. 2) patients with osteopenia and 8 (fig. 1, fig. 2) individuals without osteopenia.

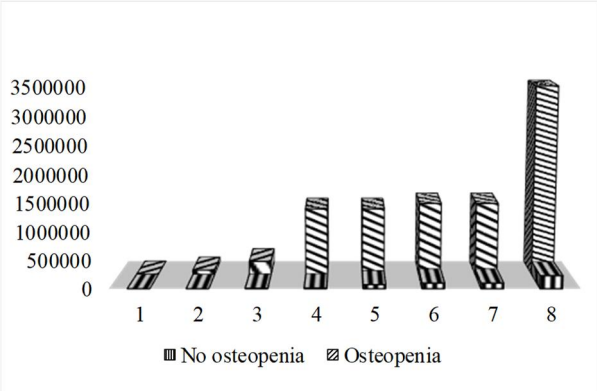

Fig.1. Quantitative distribution of *Porphyromonas gingivalis* in gingival sulcus fluid samples from 8 patients with osteopenia and 8 individuals without osteopenia.

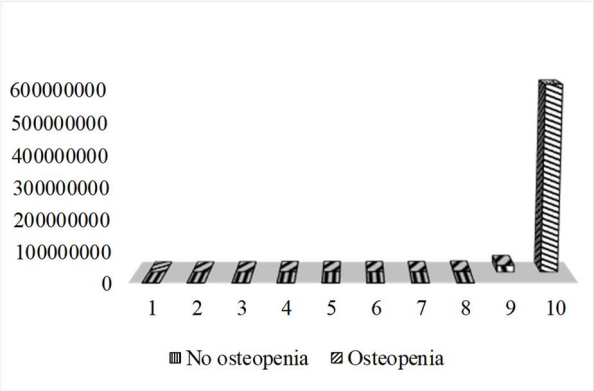

Fig.2. Quantitative distribution of *Porphyromonas gingivalis* in gingival sulcus fluid samples from 10 patients with osteopenia and 8 individuals without osteopenia

Quantification of P.gingivalis in gingival sulcus fluid samples from patients without osteopenia (control, n=8) and with osteopenia (experiment, n=10) showed a median bacterial cell count of 4.05×104 and 1.25×106 in the studied groups, respectively. The number of bacteria in the experimental samples was significantly higher than that in the control group (Mann-Whitney U-test = 15.5, p<0.05; Fig. 1, 2). In the control group, the maximum number of bacteria in the sample was 2.3×105; in 2 of 8 samples, the number of bacteria was below the high sensitivity threshold (<103 copies of bacterial DNA in the sample). In the experimental group, only 1 of 10 samples had a bacterial cell count below the high-sensitivity threshold; in 2 samples in this group, the bacterial count was several orders of magnitude higher than in the remaining samples: 2.2×107 and 6×108 (Figure 2).

Osteopenia is a complex human disease associated with decreased bone mineral density and characterized by a moderate risk of fracture, which in certain situations can lead to osteoporosis. Identification of gene variants that contribute to osteopenia or determine responses to therapy may ultimately help to individualize prognosis, treatment, and prevention of fractures and their adverse outcomes. Our study found a significant association of TNFRSF11B rs2073617 950C>T and ZNF385B

rs6757845 G>A polymorphisms with osteopenia. According to the results of our study, the single-nucleotide polymorphism of one of these genes TNFRSF11B (OPG) is a genetic predisposition factor for osteopenia. Another polymorphism of this gene rs2073618 1181G>C was previously recognized as a risk factor for osteoporosis in postmenopausal women [2]. In the same study, the GG-genotype of the rs2277438 TNFSF11 (RANKL) -438 A>G polymorphism was associated with osteopenia, but we were unable to confirm this association. It is possible that a similar association may be found in a larger sample, since the G allele of the rs2277438 polymorphism has been associated with an increased risk of rheumatoid arthritis, radiographic progression of rheumatoid arthritis, and risk of ankylosing spondylitis in the literature, while the AG genotype was associated with decreased serum 25-hydroxyvitamin D levels in osteoporotic patients [2, 10]. The ZNF385B gene belongs to a large family of genes encoding numerous proteins with a “zinc finger” domain, which are involved in the regulation of transcription, ubiquitin-mediated protein degradation, signal transduction, DNA repair, cell migration and many other processes. The ZNF385B rs6757845 G>A polymorphism is poorly studied; this polymorphism is known to be associated with the formation of nonsyndromic orofacial clefts in the Chinese population [3]. The association of rs6757845 with osteopenia that we identified needs further experimental confirmation and study using a more representative sample of patients. Our study also revealed an increased content of P. gingivalis in the oral cavity of patients with osteopenia compared to the control group. P. gingivalis is a gram-negative anaerobe with black pigmentation parasitizing in the gingival epithelium and periodontal tissues, which is part of the so-called “red complex” of the main periodontal pathogens along with Tannerella forsythia and Treponema denticola [4]. It has been shown that P. gingivalis can invade osteoblasts and inhibit their maturation and mineralization in vitro [7]. P. gingivalis cells have the ability to bind red blood cells and, when entering the bloodstream, move through the vessels under their cover, which helps avoid phagocytosis, and colonize the surrounding tissues, taking part in the development of systemic diseases, such as rheumatoid arthritis, Alzheimer's disease, atherosclerosis and oncological diseases. Oral infection with P. gingivalis not only causes local alveolar bone loss, but also increases the reduction of articular bone mineral density in mice with arthritis [5]. Based on the above data as well as the results of our study, we suggest that the presence of P. gingivalis in the oral cavity may cause the development of osteopenia.

Conclusions

1. The single-nucleotide polymorphisms rs2073617 950C>T in the TNFRSF11B gene and rs6757845 G>A in the ZNF385B gene as well as oral colonization by the periodontopathogen P. gingivalis are risk markers for osteopenia, but this needs further experimental confirmation.

2. According to our research, the polymorphisms rs865429 675C>T of the SOST gene, rs2277438 –438 A>G of the TNFSF11 gene and rs41494349 266A>G of the LRP5 gene are not associated with the risk of osteopenia. It is possible that the possible minor effects of these polymorphisms were not sufficiently pronounced in the study group of patients.

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