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Polimorfizm genu VDR - efektywny marker molekularny ryzyka osteoporotycznych złamań kości w grupie kobiet po menopauzie pochodzących z rejonu Wielkopolski

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Streszczenie

Osteoporoza jest ważnym problemem zdrowotnym dzisiejszych czasów. W większości przypadków dotyczy kobiet w okresie pomenopauzalnym. Rozwija się zwykle powoli i początkowo ma bezobjawowy przebieg. Często pierwszym jej objawem i groźnym powikłaniem jest złamanie kości. Skutki tego zdarzenia mogą wpływać negatywnie na jakość życia oraz mogą być przyczyną zwiększonej umieralności tej populacji. Efektywna prewencja i leczenie osteoporozy polega na identyfikacji i ocenie indywidualnego ryzyka złamania kości. Do tego celu można wykorzystać wiele metod diagnostycznych, w tym również metody genetyczne. Celem badania była ocena, które z różnych wariantów genotypu związane są z występowaniem choroby oraz mają wpływ na gęstość mineralną kości. Obserwacji poddano 261 pacjentek z osteoporozą pomenopauzalną. W badaniu ocenie poddano polimorfizmy następujących genów: OPG, VDR, ESR1, TGFB1, COL1A1 oraz BMP2.

Znamienność statystyczną pomiędzy wartościami gęstości mineralnej kości a polimorfizmem genu wykazano tylko dla allelu T TaqI genu VDR. W populacji kobiet z osteoporozą pomenopauzalną, zamieszkujących teren Wielkopolski, stwierdzono częstsze występowanie genotypu aa dla ApaI, bb dla Bsm i TT dla Taq genu VDR u pacjentek z większym ryzykiem złamania kości.

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Słowa kluczowe: *osteoporoza, złamanie kości, polimorfizm*

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Polymorphism of VDR gene - the most effective molecular marker of osteoporotic bone fractures risk within postmenopausal women from Wielkopolska region of Poland

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Abstract

The major public health problem which will arise is a frequency of osteoporosis. The first manifestations of this disease are often bone fractures. Identification and evaluation of individual bone fracture risk will be the most effective way of solving the problem. Genetic determination of osteoporosis is unquestionable. The aim of this study is to detect which variants of genotypes lead to illness. We investigated 187 patients with osteoporosis (161 women, 26 men) and 19 healthy subjects. Polymorphisms of the following genes were investigated: *OPG, VDR, ESR1, TGFB1 COL1A1,* and *BMP2*. The statistically significant relationship between BMD value and T allele of Taq I VDR gene were found. Genotypes: aa, bb, TT of VDR gene occur more frequently in polish osteoporotic population in Wielkopolska region within patients with higher risk of bone fractures.

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Key words: *osteoporosis, bone fractures, gene polymorphisms*

Abbreviations:

OPG – Osteoprotegerin gene
VDR – Vitamin D receptor gene
ESR1- Estrogen receptor gene 1
TGFB1 - Transforming growth factor beta 1 gene
COL1A1 – Collagen type 1 alpha 1 gene
BMP2 – Bone morphogenetic protein 2 gene
BMD – bone mass density

Introduction

The frequency of osteoporosis is in proportion to increasing number of elderly people. Changes in bone structure cause skeleton distortions, bone aches, difficulties in everyday activity, and what is the most dangerous, bone fractures. The aim of osteoporosis treatment is to prevent such events. Demographic data indicate that the problem of osteoporosis will increase. Even now due to convincing data osteoporosis is underdiagnosed and underrated [1]. Diagnosis of osteoporosis due to definition of IOF (International Osteoporotic Foundation) is based on Bone Mass Density (BMD) index. The estimation of risk of bone fractures due to low BMD index remains still not enough accurate. This occurs due to many different factors involved in origin of osteoporosis. These factors include: micro architecture, the quality of matrix, bone flexibility and the pace of metabolic processes [2].

Achievements of molecular biology provide new prospectives in research on origins of many diseases. Genetic determinations of osteoporosis are unquestionable. Many studies on variability of candidate genes were performed. The subjects of these studies include genes which code proteins of bone metabolism. They are i.e.: vitamin D receptor (*VDR*), estrogen receptor (*ESR1*), calcitonin receptor (*CALCR*), parathyroid hormone receptor 1 (*PTHR1*), collagen type I alpha 1 (COL1A1), tumor necrosis factor (*TNF*), interleukin 6 (*IL6*), interleukin 1 receptor antagonist (*IL1RN*), transforming growth factor beta 1 (*TGFB1*), low density lipoprotein receptor related protein 5 (LRP5) and osteoprotegerin (*OPG*) [3, 4, 5, 6, 7, 8, 9, 10, 11].

The question is simple: what variants of genotype lead to illness? Unfortunately the answer is complicated. There are differences in the results of studies. Interpretation of the results are difficult, because of few possible explanations: fenotypical manifestation of damaged genes depends not on one single gene, but on the group of them; influences of environment during life time can change genetic predisposition so much, that visible effects can be different. It is important to find in population who are susceptible to osteoporosis. But even more important issue is to isolate in population of osteoporotic patients those with the highest risk of bone fractures. Such proceeding can change the form of treatment and allows setting individual therapy for single patient. This would be a great opportunity because every therapy is potentially harmful. Treatment of osteoporosis is long-lasting, provides side effects and is expensive.

The aim of this study was to estimate genetic predisposition to osteoporosis and/or fragility within postmenopausal women from Wielkopolska region of west Poland.

Material

187 patients were included in the study (161 women and 26 men). Mean age of women 67.6, age from 45 to 85, weight from 39 to 82, mean weight 60.2. Mean age of men 67.4, age from 47 to 84, weight from 57 to 84, mean weight 72.4.

In group with presence of fractures there were: 56 patients with at least one osteoporotic bone fracture in history (51 women, age from 50 to 85 mean age 69.7; weight from 49.5 to 82 kg mean weight 63.4 kg and 5 men, age from 48 to 76, mean age 58.2; weight from 63 to 84 kg, mean weight 74 kg) and 131 patients with osteoporosis but without bone fractures in history (110 women, age from 45 to 84, mean age 66.3; weight from 39 to 81 kg, mean weight 58.8 kg and 21 men, age from 53 to 79, mean age 63.7; weight from 57 to 81 kg, mean weight 72 kg).

187 patients were also divided in groups of 141 patients with osteoporosis and 46 patients with osteopenia by BMD measurements as a basis of this division.

The study was also performed in group of 19 women (age from 52 to 77 years, mean age 63.2; mean weight 59.7) who come for their periodical examination with correct bone mass (mean T-score: -0.63 SD, measured in femoral neck). None of them had osteoporotic fractures or been pharmacologically treated.

In all cases the polymorphisms of the following genes were examined: *OPG, VDR, ESR1, TGFB1 COL1A1,* and *BMP2*. (Table 1)

Methods

***DNA analysis*.** DNA was isolated from peripheral blood lymphocytes. PCR primers were designed using genomic GeneBank sequences of *OPG, TGFB1* and *BMP* genes (OPGF-AAg gTg CAA AgT TTg gTC CAg g; OPGR-gTC TTC CAT AAA gTC AgC Agg; TGFB1F-TCC ggg CTg Cgg CTg CAg C; TGFB1R-gTT gTg ggT TTC CAC CAT TAg; BMP2F-CCC CAC ggA ggA gTT TAT CAC; BMP2R-CCg ggg gAg CCA CAA TC). For *VDR, ESR1, COL1A1* genes the primers were designed according to literature data [11, 12].

The 20 µl reaction mixture contained 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH

8.3), 1.5 mM $MgCl_2$, 0.25 mM dNTP, 30 ng of each primer and 0.6 unit of Taq DNA polymerase. The reaction was performed using cycling protocol of 94°C for 40 s; annealing for 40 s (OPG-55°C; TGFB1-57°C first 5 cycles and 54°C next 30 cycles; BMP2-59°C); and 72°C for 100 s, for 35 cycles. In table 1 analyzed polymorphisms and methods used for their genotyping are listed. Genotyping by SSCP was performed for 9G/C polymorphism of *OPG* gene. PCR products with 60% formamide were denatured and then resolved on native, 20×20 cm 8% polyacrylamide gels (49:1 acrylamide:bisacrylamide) in 0.5×TBE buffer throughout 16h, 65V. Other polymorphisms were genotyped by RFLP. For polymorphism of Sp1 site in *COL1A1* gene the primers were Cy5 labeled and fractionation was carried out on ALFexpress (Amersham-Pharmacia). Digestions with restriction enzymes were preformed following the manufacturer's instructions. Polymorphisms and the method of genotyping are presented in Table 1.

Table 1. *Polymorphisms in analysis and the method of genotyping. (SSCP – single strand conformation polymorphism, RFLP – restriction fragment length polymorphism)*

Gene	Polymorphism	The method of genotyping
OPG	9G/C	SSCP
VDR	TaqI	RFLP with restrictase TaqI
	ApaI	RFLP with restrictase Bsp120I
	BsmI	RFLP with restrictase Mva1269I
	FokI	RFLP with restrictase BseGI
ESR1	PvuII	RFLP with restrictase PvuII
	XbaI	RFLP with restrictase XbaI
TGFB1	29T/C	RFLP with restrictase PvuII - restriction site was introduced by modified primer
COL1A1	Polymorphism of Sp1 site	RFLP with restrictase MlsI- restriction site was introduced by modified primer
BMP2	570T/A	RFLP with restrictase BseNI

Results

The bone mineral densities (BMD) in femoral neck and in lumbar L_2-L_4 spine in patients with bone fractures and without them are presented on the Fig.1.

The differences between T–score measurements in groups of patients with and without bone fractures are not statistically significant. These data is presented in Table 2.

Table 2. *The differences between mean T-scores in patients with bone fractures and without them measured in femoral neck and in lumbar spine L_2-L_4.*

mean T-score	Fractures	no fractures	difference
neck	-2.51	-2.44	-0.07
spine	-3.62	-2.93	-0.69

The frequency of osteoporotic fractures in compare with sex of patients is presented on Fig. 2.

The relation of bone fractures to whole group of osteoporotic patients is presented on Fig. 3.

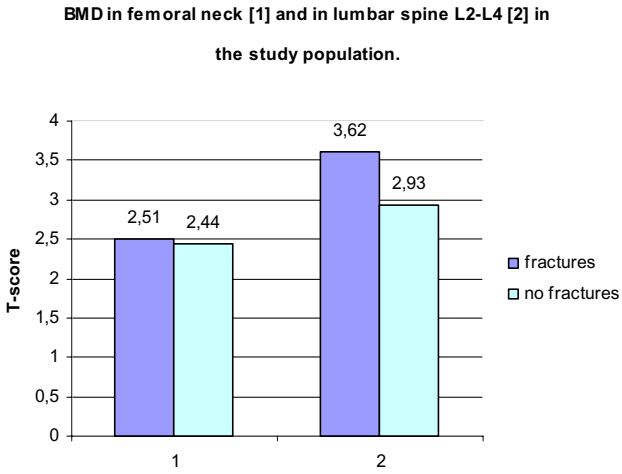

Fig. 1. *Bone mineral density measured in femoral neck [1] and in lumbar spine L_2-L_4 [2] in population of patients with bone fractures and without them.*

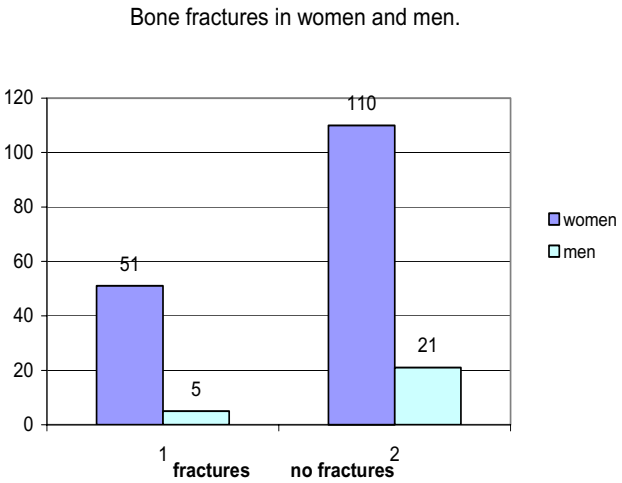

Fig. 2. *The frequency of bone fractures in group women and men.*

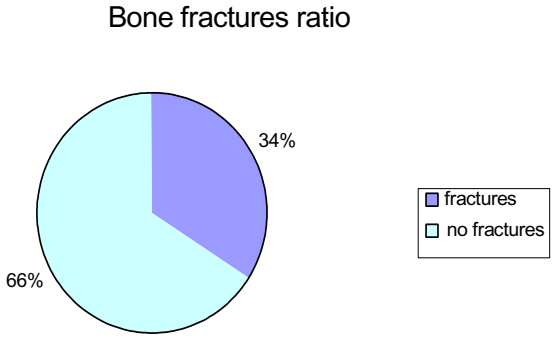

Fig. 3. *Bone fractures in patients with osteoporosis (Neck T-score < -2.5 SD).*

There were investigated polymorphisms of genes: *OPG, ESR1, VDR, TGFB1 COL1A1,* and *BMP2* in patients with osteoporosis, osteopeny and in healthy subjects. Statistically significance results in T- allele Taq 1 VDR gene were found. These results are presented in the Table.3.

Table 3. *Dispositions of polymorphisms of investigated genes in patients with osteoporosis, osteopeny and in healthy subjects. (p<0.05)*

VDR Taq I	Osteoporosis	Osteopenia	Healthy	
TT	64	19	2	
Tt	56	20	11	
tt	21	7	6	**P 0.036**

The groups of osteoporotic patients with bone fractures and without them were investigated to molecular variants of *OPG, ESR1, VDR, TGFB1 COL1A1,* and *BMP2* genes.

Statistical methods.
The Fisher-Freemann-Halton test for nonparametric data was used to evaluate the distribution of genotypes. The significance level was set at p<0.05. We have found statistically significant relationship between BMD and T-allele Taq I variant of VDR gene. The statistically significant differences were also found in polymorphism variants: a-allele Apa I, b-allele Bsm I and T-allele Taq I of vitamin D receptor gene in osteoporotic patients with bone fracture to compare to osteoporotic one without fracture. The results are shown on Fig. 4, 5 and 6.

Discussion

The VDR gene is one of the most important candidate gene involved in osteoporosis development, but not the only one. Finding genetic susceptibility to osteoporosis is one of ways to identify individual predisposition to illness and can be a way to early protection. Environment factors can influence bone turnover mass in a few different ways. It is necessary to check populations in order to find any connections between genotype and an "osteoporotic fracture phenotype". There are papers in which these kind correlations are detected [13, 14].

The important role of vitamin D3 in bone metabolism regulations is proven by results of several studies investigating its influence on osteoporosis risk. There are reports confirming relation between genotype of VDR and bone mineral density [15, 16]. In study on polish population from Wielkopolska, we have found the predominance of T allele of the Taq I polymorphism of VDR gene in osteoporotic patients. This result is in accordance to ours earlier observations in polish population in pilot study [17]. Other data concerning European and Mexican

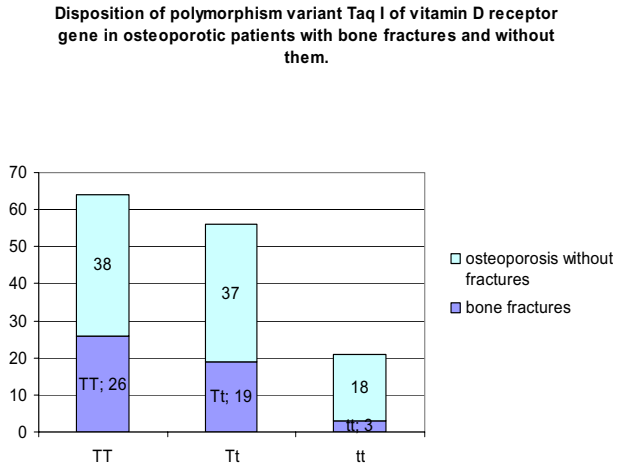

Fig. 4. *Genotypes T-allele, Tt, t-allele of Taq I polymorphism in VDR gene in osteoporotic patients with bone fractures (p=0.08)*

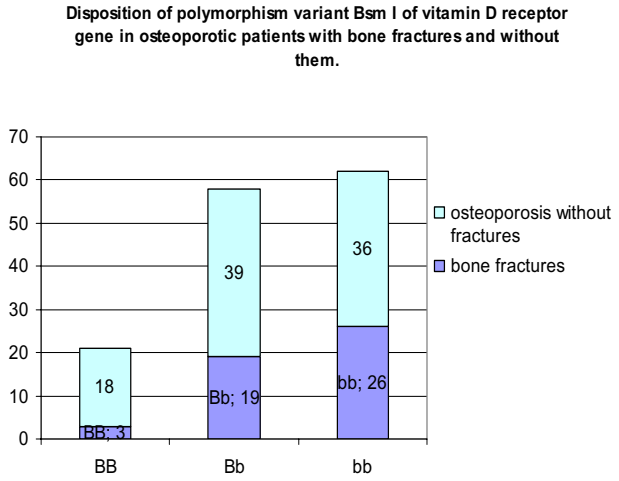

Fig. 5. *Genotypes B-allele, Bb, b-allele of Bsm I polymorphism in VDR gene in osteoporotic patients with bone fractures (p=0.06).*

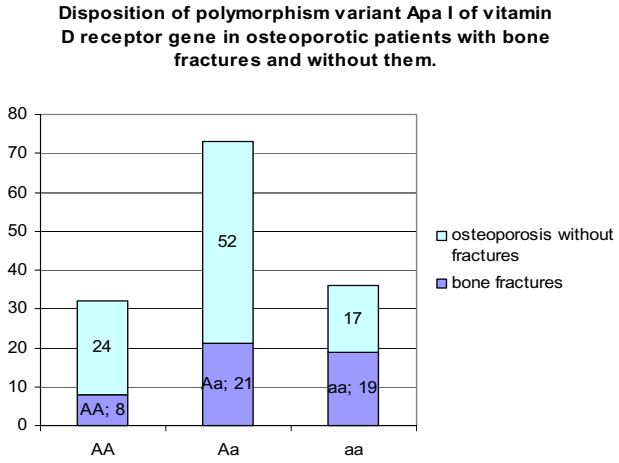

Fig. 6. *Genotypes A-allele, Aa, a-allele of polymorphism Apa I in VDR gene in osteoporotic patients with bone fractures (p=0.02).*

American population conclude similar results confirming relationship between VDR polymorphism and the risk of osteoporosis [18, 19]. There are also a reports in which relationship between polymorphism Taq1 VDR gene and bone density were not found [20] North American study which have not found any dependence between BMD and polymorphism of VDR gene. Possible explanation of American results is that in their study Taq1 variant of VDR gene was not investigated [21]. The study in China also no confirm any significant relationships between VDR gene and BMD index even they reported Taq1 polymorphism. The variance of results must be explained by presence of differences between populations or another possibilities that environment factors can change so much genetic predisposition that the final phenotypes are so different [22, 23]. The consequence of gene mutations can be enhanced or inhibited because of activation of other genes and their products which play role in similar metabolic tracts [24]. That kind of activity can explain results of Morrison study in which relationship between VDR allel B and the risk of osteoporosis was found. In other studies the determination of low BMD is assigned to allel b VDR [19]. In our results although presence of b – allele of Bsm I of VDR gene was not exactly statistically significant, but it was not far from that (p-0.41), and it was frequent in patients with osteoporosis 44% vs. 35% healthy and osteopenic subjects. It is possible that this result depends on changes in enzymatic activity of vitamin D3. Japanese authors also noticed correlation between dietary intake of vitamins and calcium and phenotype [28].Supplementation of vitamin D3 in population living in area of high sun activity can be performed with less active vitamin D. But in population with low calcium diet the same metabolic activity of vitamin D3 is insufficient for proper calcium concentration [38]. In polish population level of vitamin D3 is very low in compare to other populations (French, Irish) [13].

In our study relationship between BMD and variants of other candidate genes such as: estrogen receptor (*ESR1*), osteoprotegerin (*OPG*), collagen type I ά 1, TGFβ1 and BMP1 have not been found. There are results of other studies confirming such correlation [26, 21, 27, 28]. There are also reports in agreement to our conclusion, in which that correlation was not found [29, 28]. Again we suppose that, this is the result interpopulation differences in environment and genetic factors and their influence on determining bone metabolism.

The analysis of differences between variants of VDR gene in polish osteoporotic patients from Wielkopolska in Poland, with bone fractures resulted in finding the significant predominance of genotype a – allele of Apa 40% vs. 18%, b – allele of Bsm I 54% vs.39% and T-allele of TaqI 54% vs.41% VDR gene with p=0.02; 0.06; 0.08 respectively. The results of similar studies on population of United States confirmed the dependence between *VDR* genotype and risk of osteoporotic bone fractures [30, 31, 29]. Polymorphisms in exon 2, intron 8 and exon 9 of VDR gene were studied in Dutch population. They found only that B – allele of the Bsm I in 3 untranslated regions of the VDR was associated with low BMD in the hip and tended to be associated with fracture. On the other hand, any relationship between bone fractures and VDR polymorphism gene were not found in British, Spanish and Italy populations [32, 33 34, 35]. In Dutch population it was found again only that B – allele of Bsm I VDR is connected with predisposition to bone fracture. They also check other polymorphisms as: Fok I, Apa I and Taq I with negative results [31]. Results confirming correlations a-allele, b-allele and T-allele with BMD and higher risk of bone fracture come from Uitterlinden study performed in Nederland's population [29].

Investigating populations' predisposition to illness we detected many discrepancies between neighbor's populations. It may be important to discover the reason why they exists. However polish population is located in central-eastern Europe we detected differences.

Osteoporosis is a multifactor disease with undoubted genetic predisposition. It is possible like in other genetic illnesses which are visible in older age that the influences of many environment factors are even stronger that genetic predisposition to final phenotype.

It is important to find who and why will be defected with osteoporosis. Strategy of investigation of gene polymorphism is based on knowledge of bone metabolic consequences of gene activity. The studies are often not successful because many past and present factors can change the final result. For example we have not found any correlation between bone fractures and BMD index in investigated population in *OPG* gene with so strong influence on bone metabolism in theoretical point of view and studies confirming this correlation [13, 29].

In polish population from Wielkopolska we also have not found statistically significant correlation between genotypes of genes *ESR1, OPG, TGFB1, COL1A1*, *BMP2* and the risk of bone fractures. There are studies in which relationship between polymorphisms of these genes and bone fracture were found [38, 39]. Even there are also numerous studies reporting lack of such association [40, 41, 42, 43]. It is possible that gene-gene relations (epistasis) can have so strong influence to final phenotype.

Nevertheless estimation of individual risk of bone fractures of patients with osteoporosis can be a proper way of setting a group of patients to whom a specific treatment should be applied. In whole

group of osteoporotic and osteopeny patients there were 56 cases (34%) with bone fractures. Using a sex of patient as an indicator we can predict a bone fracture in 5% of women and 10% of men. Although we found a difference between mean T-scores (measured in spine L_2-L_4) in patients with bone fractures (mean T-score -3.62) and without them (mean T-score -2.93). This difference (-0.69) is not significant and not can be useful in estimation of bone fracture risk. The difference (-0.07) in mean T-scores (measured in femoral neck) in patients with bone fractures and without them was even lower and only slightly perceptible (Table.2.). Generally BMD value examination, as was published in many reports, does not allow prediction of bone fracture risk. Divide patients with similar value of BMD can not be done with satisfactory precision.

On this basis, we can assume, that molecular markers allow estimating bone fracture risk more precisely. The estimation of bone fracture risk probability using a method of molecular markers will be more precision comparing to the other. It is confirmed that among all genes polymorphisms the strongest relation to osteoporosis was detected in IL-6 gene and *COL1A1* promotor gene region. But it is also unquestionably that interactions with dietary (very low intake of vitamin D3 in polish old women population) and lifestyle factors (exercise, smoking) can modulate bone metabolism and can be a reason of different results. Besides DNA mutations discovered in promotor or exon regions influence protein synthesis. We can also expect that probably there will be difficulty to recognize by mutations in introns or especially in 3′ UTR untranslated region gene.

Conclusion

1. In polish populations from Wielkopolska region relation between bone mass value and molecular variant Taq 1 o VDR gene was confirmed.
2. Patients with osteoporotic bone fractures in Wielkopolska region o Poland were more frequently a-allele of Apa 1, b-allele of Bsm 1 and T-allele of Taq 1 VDR gene.

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