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GENETIC MARKERS OF LOW BONE MINERAL DENSITY IN PATIENTS WITH CYSTIC FIBROSIS

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ABSTRACT

Introduction: failure to maintain bone mass density is a major problem in patients with cystic fibrosis (CF). CF is due to mutations in the CFTR gene and other genes may contribute to modifying the disease. Genetic and environmental factors may play a role in determining the variability of bone mass. **Aim of the study:** to analyse the association between polymorphic variants of genes considered to be risk factors of bone metabolism disturbances and decreased bone mineral density (BMD) in children and adults with CF in R. Macedonia. **Materials and methods:** the study included 80 clinically stable CF patients (age range 5-36y), who regularly attended the CF center at the Pediatric Clinic in Skopje, Macedonia. Three candidate genes likely associated with BMD variability were studied: the vitamin D receptor (VDR) gene, the estrogen receptor alpha (ESR1) and the type I alpha I collagen (COLIA1) gene. A complete bone and CF evaluation was obtained for all patients: 55 had normal BMD (group 1), 17 were osteopenic (group 2) and 8 were osteoporotic (group 3). **Results:** Low bone mineral density (Z score < -1SD) was found in 31.25% patients and in 10% of them BMD was below -2SD. Patients with low BMD had worse BMI, FEV1 and more severe symptoms of CF. No significant correlation was found between COLIA1 and VDR polymorphisms and BMD. **Conclusion:** There was no evidence that the genes under study may modulate bone phenotype in CF.

Key words: cystic fibrosis, osteoporosis, modifier genes,

INTRODUCTION

Since 1979 when low bone mineral density (BMD) in patients with cystic fibrosis (CF) was firstly described, a lot of studies are performed trying to understand the underlying reason for metabolic disturbance in bones in these patients [1, 2, 3, 4]. The term "bone disease" is used to differentiate the bone abnormalities seen in CF from postmenopausal osteoporosis [5, 6]. Adulthood is now common in CF and survival continues to increase, but they face major complications emerging with longer survival including diabetes, liver disease, osteoporosis and infertility [7, 8]. Osteoporosis is systemic skeletal disease characterized by low bone mass and micro architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture risk.

A lot of risk factors may influence over low bone density including malabsorption of calcium and vitamin D, malnutrition, delayed puberty, hypogonadism, diabetes, reduced physical activity, glucocorticoid therapy, frequent antibiotic therapy and chronic pulmonary infection [9, 10]. The origin of the low bone mass in patients with CF is incompletely understood.

The International Society for Clinical Densitometry defines low bone density in children and adolescents like Z score equal or lower than -2 SD, determined for age, sex and height [11].

Low serum 25 OHD concentrations were associated with lower BMD, suggesting that vitamin D deficiency may play a significant role in the pathogenesis of demineralization in cystic fibrosis [12, 13, 14, 15]. Influence of vitamin D receptor alleles on BMD suggests that these polymorphisms have a greater influence on BMD in childhood [16, 17, 18].

Many studies suggest that there is genetic component, independent from disease severity and nutritional deficits [19, 20]. They suggest that there is direct link between Δ508 mutation and CFTR protein in molecular process involved in bone formation and resorption [19]. In studies on mice and humans is found that CFTR is expressed on the surface of osteoblasts. Dysfunction of CFTR chloride canal in bone cells may have influence over disturbed regulation of expression of the genes involved in the process of bone formation [20]. Twin and family studies, as well as association studies in unrelated individuals, have evaluated several polymorphic markers associated with low BMD and increased risk of fractures [21].Several studies examined modifier genes candidate for modulating the severity of the pulmonary and gastrointestinal disease manifestations [22]. The others speculate that non-CFTR modifier genes may influence the degree of bone disease, finding correlations between BMD and some candidate genes [23]. Bone density is multifactorial foundation, where inherited factors affect the variability of the phenotype.80 % of age-specific variation in bone mass can be accounted for by genetic factors [24].

The aim of the study was to evaluate for the first time genotype and allele frequencies of five polymorphic markers within three candidate genes for bone density in patients with cystic fibrosis from Republic of Macedonia.

MATERIALS AND METHODS

Patients

The study included 80 clinically stable CF patients

(range 5-36y) who regularly attended the CF center at the Pediatric Clinic in Skopje. The diagnosis of CF was made by the presence of typical clinical characteristics of CF (chronic respiratory disease and/or pancreatic insufficiency) together with abnormal sweat chloride test (>60 moll/l) and/or the presence of two CFTR gene mutations.

Clinical assessment

The nutritional status of CF patients was expressed as body mass index (BMI) index for weight and height (kg/m^2). Values are compared with standard percentiles for age and sex.

Pulmonary functional tests were measured by Flow Screen-Jaeger Spiro meter. Forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were analyzed. The values were expressed as percent of predicted values for sex, age, weight and height;

Cystic fibrosis disease severity was assessed using the Shwachman-Kulczycki (S-K) system, which rates general activity level, pulmonary physical findings, growth and nutrition, and chest radiographic findings. Total S-K scores may range from 20 to 100; low scores representing greater illness severity.

Laboratory analyses

Calcium, phosphorus, and alkaline phosphatasis were measured in serum at the University Pediatric Clinic in Skopje. Serum osteocalcin (OC), β cross laps, 25OHD and PTH were determined electrohemiluminiscent method on the automatic immune analyzer elecsys 2010 roche at the University Clinic for Biochemistry in Skopje. Referral values for 25OHD are 15-44ng/ml. According Cystic Fibrosis Foundation levels for 25 OHD in CF patients below 30ng/ml are consider insufficient and levels beyond 20 mg/ml for deficiency.

For determining the polymorphisms of candidate genesa commercially available kit based on reverse hybridization technique was used. Briefly, after PCR amplification, the DNA is denatured and incubated with a nitrocellulose membrane containing attached hybridization probes. The following polymorphisms were determined: COL1A1 G2046T, ESR1 IVS-397, VDR-I352I (TaqI), VDR IVS-10 +354G>A (BsmI) and VDR M1T (FokI). The genetic analyses were performed at the Institute for immunology and human genetics, Medical Faculty, Skopje.

Bone density measurements

BMD was measured via dual energy-ray absorptiometry (DXA) scans with spinal scores recorded. They were expressed by Z or T scores depending of the age of patients.

Densitometry definition of osteoporosis is accepted by the European Foundation for Osteoporosis and World Health Organization (WHO) and is the golden standard for definition for osteoporosis. Osteoporosis is defined as a bone density <2SD of the mean BMD of a gender-matched, young healthy population. Osteopenia is an intermediate category of reduced bone density defined as a Z or T score within -1SD and -2SD.

Statistical analysis

Results are reported as mean value (M) and standard deviations (SD) for each group. Student's *t*-test was used for calculating significant differences between CF and control group. Pearson scores were used to determine correlation analysis between BMD and various clinical variables. Statistical significance was defined as $p<0.01$. For the comparison of the allelic and gene frequencies among the different BMD groups, the Chi square test with 1 degree of freedom and the Fisher exact test were used. Differences with $p<0.05$ were considered statistically significant.

RESULTS

The study included total 80 patients with cystic fibrosis who were divided in 3 groups depending Z or T score of bone mineral density (BMD) (Table 1).

Table 1. Distribution,mean age and frequency in CF groups depending on BMD

	Normal BMD	Osteopenia	Osteoporosis
Number	55(68.75%)	17(21.25%)	8(10%)
Mean age	13.4±6.54	16.29±7.6	15.62±7.44
BMD (Z/T) score	0.05±0.73	-1.21±0.23	-2.48±0.35

Most of CF patients (68.75%) have normal mineral density (BMD), 21.25% have osteopenia (Z or T score <-1SD) and 10%(<-2SD) were with osteoporosis (Table 2). The prevalence of total reduced BMD in our study was 31.25% (Figure 1).

Table 2. Distribution of ΔF508 mutation in CF patients

CF genotype	N	Average age	Frequency %
Homozygous for ΔF508 mutation	42	13.57±6.8	52.5
Heterozygous for ΔF508 mutation	28	15.67±7.6	35
No allele for ΔF508 mutation	10	13±4.7	12.5

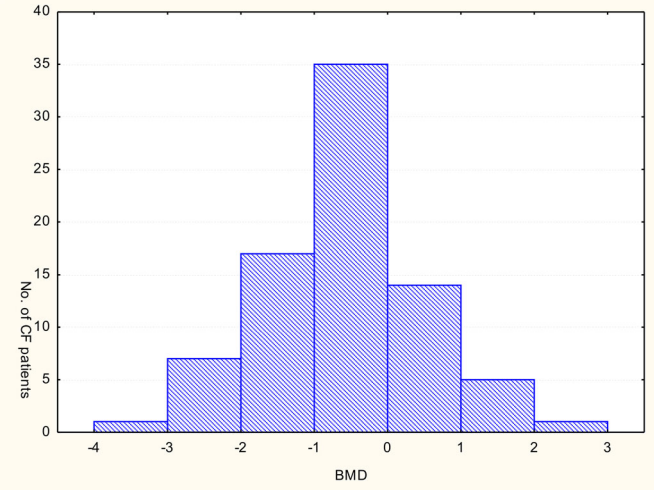

Fig. 1. Distribution of Z or T scores of DXA scans in CF patients

CFTR genotypes and their distribution in the three groups are shown on table 2. ΔF508 mutation is the most frequent with 87.5% (homozygous 52.5% and heterozygous 35%) in CF patients.

Table 3. Mean values for laboratory and clinical parameters in CF patientsdepending BMD values

	Normal BMD	**Osteopenia**	**Osteoporosis**
25OHD(ng/ml)	22.94±10.27	26.64±8.57	15.8±8.44
OC (ng/ml)	67.57±42.55	66.14±44.27	61.16±46.37
CTX(ng/ml)	1.24±0.74	1.24±0.84	1.05±0.48
PTH(pg/ml)	45.33±36.43	38.38±17.37	39.18±23.5
Ca(mmol/l)	2.35±0.14	2.37±0.12	2.25±0.2
P(mmol/l)	1.5±0.25	1.45±0.24	1.47±0.33
Alk.ph.(IE)	234.54±82.58	220.83±81.25	202.25±46.27
BMI(kg/m^2)	20.48±24.32	19.27±2.65	17.97±2.92
FEV_1	89.78±24.32	78.61±21.22	69.62±24.92
FVC	97.59±17.16	86.28±18.21	73.45±23.22
C-N score	8.76±2.1	9.52±2.78	11±3.6
S-K score	93.4±6.54	76.29±7.6	75.62±7.44

We found significant difference for 25OHD between the group with osteoporosis and osteopenia($p<0.01$).We didn't find significant difference between groups for OC, CTX and for the other biochemical analysis PTH, Ca, Pand AF.

Significant difference was found for BMI ($p<0.01$), FEV1 and FVC ($p<0.01$); C-N and S-K score($p<0.01$) between group with osteoporosis and the group with osteopenia and normal density.

Table 4. Genotype distribution for polymorphic markers in normal BMD group

Polymorphic marker	Genotype distribution(number of subjects) Normal			Allele frequencies (%)	
COL1A1	GG (12)	GT (24)	TT (1)	G (64.9)	T (35.1)
ESR1 (PvuII)	CC (11)	CT (29)	TT (6)	C (55.4)	T (44.6)
VDR (Taq1)	CC (0)	CT (45)	TT (3)	C (46.9)	T (53.1)
VDR (FokI)	CC (2)	CT (46)	TT (0)	C (52.1)	T (47.9)
VDR (BsmI)	AA (1)	AG (46)	GG (1)	A (50)	G (50)

Table 5. Genotype distribution for polymorphic markers in osteopenic group

Polymorphic marker	Genotype distribution (number of subjects) Osteopenic			Allele frequencies (%)	
COL1A1	GG (4)	GT (5)	TT (0)	G (72.2)	T (27.8)
ESR1 (PvuII)	CC (1)	CT (6)	TT (3)	C (40)	T (60)
VDR (Taq1)	CC (0)	CT (11)	TT (0)	C (50)	T (50)
VDR (FokI)	CC (0)	CT (11)	TT (0)	C (50)	T (50)
VDR (BsmI)	AA (0)	AG (11)	GG (0)	A (50)	G (50)

Table 6. Genotype distribution for polymorphic markers in osteoporotic group

Polymorphic marker	Genotype distribution (number of subjects) Osteoporotic			Allele frequencies (%)	
COL1A1	GG (4)	GT (2)	TT (0)	G (83.3)	T (16.7)
ESR1 (PvuII)	CC (0)	CT (4)	TT (2)	C (33.3)	T (66.7)
VDR (Taq1)	CC (0)	CT (5)	TT (1)	C (41.7)	T (58.3)
VDR (FokI)	CC (0)	CT (6)	TT (0)	C (50)	T (50)
VDR (BsmI)	AA (0)	AG (6)	GG (0)	A (50)	G (50)

On Table 4,5 and 6 are presented genotype distributions for different polymorphic markers in groups with normal BMD, osteopenic and osteoporotic CF patients.

DISCUSSION

Cystic fibrosis is the most frequent rare, autosomal recessive and lethal disease in Caucasian population. It is caused by mutation of the gene for cystic fibrosis transmembrans regulator (CFTR). The incidence is 1:2500 newborns[1, 2]. Mutation in CFTR gene results in defect chloride transport in epithelial cells in pancreas, gut, liver, lung, renal, bone and testicular canals. Clinical presentations in CF are chronic lung disease with recurrent infections who leads to respiratory insufficiency and eventually lethal end, malapsorption presented with frequent and oily stools, which are manifestation of pancreatic insufficiency and malnutrition which is an important determinant of growth and body development during childhood and adolescence [3, 4, 5, 6, 7]. Imbalance between bone formation and degradation in cystic fibrosis (CF) in childhood has become an important issue for developing osteopenia [8, 9, 10]. The development of CF bone disease leads to decrease in pulmonary function, caused by inability to cough and pain when they are doing physical therapy. Every vertebral or rib fracture makes difficult the clearance of secret from bronchi's what is necessary for prevention of exacerbations of pulmonary infections [11, 12, 13, 14]. Vitamin D, whose activity is determined by VDR gene, has influence over bone mass. The variants of alleles of VDR gene are ApaI (allele A/a), BsmI (allele B/b), FokI (allele F/f), and TaqI (allele T/t) [15, 16].

In the past decade, a lot of attention has been paid to the hypothesis that particular susceptibility genotypes determine the phenotype of the CF patients [16, 17]. One of the most important CF related conditions is the bone disease, which is nowadays acknowledged as a significant clinical complication of CF. Several risk factors connected to the pathophysiology of CF have been identified, and translated into a general rule, the more severe the disease, the worse the bone involvement. Several groups have investigated candidate gene polymorphisms proven to be valid for noted differences in the bone density [18, 19, 20]. In this study, we evaluate and present for the first time genotype and allele frequencies of five polymorphic markers within three candidate genes for bone density in patients with cystic fibrosis from Republic of Macedonia. These genes have previously been proven to show variation in the general Caucasian population [21, 22, 23, 24].

Since our group of patients is rather homogenous regarding the CFTR mutation status (the mutation is confirmed in more than 95% of the cases), we hypothesize that differences in the examined candidate genes might be associated with the bone density and responsible for the degree of the bone disease.

We confirm in our study that the degree of CF severity is correlated with an increased risk for low bone mineral density. Osteopenic and even more osteoporotic CF patients had worse pulmonary function and nutritional status compared to the normal T score group. However, we couldn't find significant difference in genotype or allelic distribution for the analyzed genes in normal, osteopenic and osteoporotic CF patients. We are aware of two limiting factors in our study. The first one is the small number of patients, especially in the osteopenic and osteoporotic groups. The second limitation is the lack of genotyped samples from general population that would serve as controls, having in mind that variations in the genotype frequencies have already been reported among different populations.

Due to these limitations, we cannot definitely exclude the role of these genes to the variability of bone phenotype in CF patients.

In **conclusion,** we looked for susceptibility alleles which may function as modifier genes for the osteoporotic phenotype in CF patients. No significant correlation was found between the VDR, CALCR, and COL1A1 gene polymorphisms and reduced BMD values in CF.

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