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EVALUATION OF RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B LİGAND (RANKL) AND OSTEOPROTEGERIN (OPG) LEVELS IN PATIENTS DIAGNOSED WITH BRONCHIECTASIS AND OSTEOPOROSIS

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Abstract

Objective: Bronchiectasis is a condition characterized by irreversible abnormal dilation and anatomic breakdown of the bronchial tree. In our study we aimed to understand the causes of osteoporosis at molecular levels and to investigatethe usefulness of RANKL and OPG levels as markers in early diagnosis and follow-up of osteoporosis, RANKL inhibitors in the treatment of osteoporosis. Materials and Methods: 30 noncystic fibrosis bronchiectasis patients were diagnosed as osteoporosis with DEXA and applied to the Pediatrics Chest Diseases Department of Meram Medical Faculty of Necmettin Erbakan University between June 2015 and June 2016 were included in our study. BMD values were determined by DEXA and QUS in the patient group and only QUS in the control group. Results: In the patient group 56.6% (n: 17) were male, 43.4% (n: 13) were. There was no statistically significant difference between RANKL, OPG, RANKL / OPG ratios, BALP, OST, NTX values between the patient and control group ($p > 0.05$). The median serum Ca level was 9.51 (IQC: 0.74) in the patient group and 9.75 (IQC: 0.47) in the control group, there was a statistically significant difference between them ($p = 0.003$). There was a strong positive correlation between QUS z scores and DEXA z scores in the patient group ($p = 0.008$). Conclusion: In conclusion, we could not pinpoint the role of RANK / RANKL / OPG in the pathogenesis of osteoporosis in patients with noncystic fibrosis bronchiectasis. However, we think it is appropriate to conduct studies on a wider series in this topic.

EVALUATION OF RECEPTOR ACTIVATOR NUCLEAR KAPPA B LIGAND (RANKL) AND OSTEOPROTEGERIN (OPG) LEVELS IN PATIENTS WITH OSTEOPOROSIS AND BRONCHIECTASIS

INTRODUCTION

Bronchiectasis is a condition characterized by irreversible abnormal dilation and anatomic breakdown of the bronchial tree. Although the frequency pf bronchiectasis has been declining due to the improvement in nutrition and sanitation circumstances, increased rate of vaccination and early and frequent use of antibiotics, it still causes a serious health problem in developing countries (1,2). Pifferi et al. reported the frequency of bronchiectasis as 1/6000 in general pediatric population in their study in 2004 (3).

The most common cause of bronchiectasis in developed countries is Cystic Fibrosis (CF). Bronchiectatic patients with CF and noncystic fibrosis have many common features. Chronic and recurrent lung infections,

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chronic inflammation, inhaled corticosteroid use in inactivity and acute exacerbations, bronchial hyperreactivity and nutritional problems are the characteristic features of both groups of diseases (4).

Osteoporosis and osteopenia were reported to be more frequent in patients diagnosed with CF and noncystic fibrosis bronchiectasis compared to normal population and the frequency was reported to increase with increasing age (4-5).

Osteoporosis is a systemic bone disease characterized with decreased bone mineral density (BMD) and impairment of inner architecture. The amount of mineralization is normal in all forms of osteoporosis, while decreased bone volume in especially trabecular bone and decreased trabecular bone cycle is noticed. Osteoporosis is evaluated using BMD, dual-energy X-ray absorptiometry (DEXA), quantitative computed tomography (QCT) and quantitative ultrasonography (QUS). Osteoporosis is frequently encountered in chronic diseases, especially with long hospital stay, inadequate vitamin D intake and drug use. Vitamin K and D deficiency in cystic fibrosis is thought to be associated with osteoporosis (6).

Many mechanisms are responsible in the development of osteoporosis. Bone resorption secondary to the changes in RANK-RANKL-OPG system is in the forefront recently (7). OPG is a member of the receptor TNFR super family, and is also known as TNFRS11B. Another name of it is osteoclastogenesis inhibitory factor (OCIF). Original OPG molecule is a polypeptide composed of 401 amino acids, and the mature protein composed of 380 amino acids is formed by separation of the 21-amino acid propeptide part.

The association between osteoporosis and RANKL/OPG levels has been demonstrated in the literature in patients with CF in whom bronchiectasis is common. However, no study has been published evaluating the association between osteoporosis and RANKL/OPG levels in patients with noncystic fibrosis bronchiectasis. The aim of this study was to measure the RANKL and OPG levels in patients with noncystic fibrosis bronchiectasis and with osteoporosis. And to evaluate the applicability of using RANKL and OPG levels as markers in the early diagnosis of osteoporosis and its follow-up.

MATERIAL AND METHOD

Patients between 5-18 years of age and applied to the outpatient clinics of Pediatric Pulmonary Diseases with the diagnosis of non-cystic fibrosis bronchiectasis and osteoporosis were included in the study. Patients presented to the outpatient clinics of pediatric for routine follow-up with no chronic disease were included as the control group. DEXA measurement was performed in order to evaluate the bone mass of patients with bronchiectasis.

BMD value measured by DEXA is expressed by Z and T scores. Comparison of the measured bone mass with reference values according to age and gender and definition of it as standard deviation is the Z score. The standard deviation of comparison of the bone mass with the mean bone mass of the young adult reference population is the T score. BMD measured in children is evaluated as Z score. The length of the measured bone in addition to the height, pubertal phase, skeletal maturation, race and body composition is considered in order to correctly interpret the data obtained by DEXA method (8). Another method of evaluation in children is QUS (9).

Ethics board approval was obtained from the Ethics Board Committee dated January 8, 2016, and numbered 2016/399. An informed consent was obtained from all patients included in the study after explaining the aim and extent of the project in detail to each of them.

Collection and Storage of Samples and Study Method

Blood samples obtained were centrifuged at a cooling centrifugation device with the trademark Hettich Rotina 46R (*Hettich*Zentrifugen, *Tuttlingen* , *Germany)* at 4000 cycles/minute for 10 minutes and the sera were separated. Serum samples were stored in New Brunswick U570 (New Brunswick Scientific, New Jersey, ABD) refrigerator at -80°C. Enzyme Linked Immunosorbent Assay (ELISA) method was used in the serum samples using human RANKL (Biovendor Research and Diagnostic Products Karasek, The Czech Republic), OPG

Biovendor Research and Diagnostic Products Karasek, The Czech Republic, bALP (Quidel Corporation, San Diego, USA), OST (Biovendor Research and Diagnostic Products Karasek, The Czech Republic), NTX (Alere Scarborough, Scarborough, USA) kits.

The results were calculated according to the absorbance concentration calibration graphics using Biotek ELX 50 microplate washer (*BioTek* Instruments, Vermont, USA) and Bio-rad Microplate absorbance reader xMark (Bio-rad Laboratories, *California, USA*) system.

STATISTICAL ANALYSIS

All analyzes of the study were performed using SPSS 20.0 package program. Nominal scale (categorical) variables were presented using frequency and percentage; proportional scale (numerical) variables with normal distribution using mean±SS and with non normal distribution using median (interquartile range) in tables and graphics. All the proportional scale variables were checked for whether they were normally distributed or not using Kolmogorov-Smirnov and Shapiro-Wilk analyzes. Age, bALP, Bone US BMD and Mg values were found to be normally distributed in the groups (p>0,05), and the remaining values were found to be non-normally distributed with a high skewness value. Therefore, Student t-test was used in independent two-group comparison for parameters of Age, bALP, Bone US BMD and Mg since they were parametric variables and Mann-Whitney U test was preferred for nonparametric variables. Monte Carlo corrected chi-square analysis was used to determine the significance of the association between categorical variables. Pearson correlation coefficient was used when both parameters were normally distributed to establish the correlation between the numerical variables. Spearman's Rho correlation coefficient was used when any of the parameters was non normally distributed. Type-1 error level was accepted as 5 % in all the analyzes and p<0,05 was accepted as statistically significant.

RESULTS

Among the 30 patients included in the study, 56,6 % (n:17) were boys and 43,3% (n:13) were girls with a mean age of 13,3±3,8 (5-18) years. Among the 40 patients included as the control group, 35 % (n:14) were boys and 65% (n:26) were girls with a mean age of 11,8±3,5 (5-17) years. No significant differences were found in age and gender between the patient and control groups (p>0,05).

When the underlying cause was evaluated in the study group, 13 was found to be immune deficiency, 9 PSD, 4 infection, 3 idiopathic and 1 as Mac Leod Syndrome.

Laboratory values of the patient and control groups is given in Table 1-2-3.

Correlations are given in Table 4-5.

DISCUSSION

Bronchiectasis is increasingly being diagnosed with the introduction of high-resolution computed tomography. Primary cause of noncystic fibrosis bronchiectasis is postinfectious causes worldwide; however, this has changed in some regions with the efficient use of antibiotics and decreased prevalence of tuberculosis and childhood pneumonia. The etiology pf bronchiectasis could be detected in 40-63% of the cases in different studies performed (10-11).

Studies have been published in the literature suggesting the effect of RANK/RANKL/OPG system on the development of osteoporosis in patients with cystic fibrosis. No study has been published that demonstrates the association of osteoporosis and RANK/RANKL/OPG system in patients with noncystic fibrosis bronchiectasis.

OPG is a member of the receptor TNFR super family and is also known as TNFRS11B. It is released extracellularly as a soluble glycoprotein and functions as a trap receptor for RANKL (12). Osteoporosis was developed in rats with OPG failure (13). The osteoprotective role of OPG was confirmed with demonstration of a 100-kb homozygous deletion in the OPG gene (chromosome 8q24.2) in juvenile Paget's disease and deletion in the third exon of OPG in idiopathic hyperphosphatasia (14-15). RANKL gene is localized in the

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chromosome 13q14 and in the exon 6. It is a peptide formed of 317 amino acids composed of two soluble forms such as membrane bound cellular form in 40-45 Da and biologically active form in 32 kDa. RANKL is a key regulator in osteoclastogenesis and osteopetrosis is developed in rats without RANKL due to osteoclast deficiency. Presence of RANKL and M-CSF in the environment is required and sufficient for the conversion of osteoclast precursors to mature osteoclasts (12-16). RANKL receptor has been defined as RANK. RANK is a transmembrane protein composed of a total of 616 amino acids including a short transmembrane of 21 amino acids and large cytoplasmic parts (12,16,17).

Osteoclast precursors convert into a multinuclear cell with the effect of many factors such as cytokines, hormones and growth factors. These cells differentiate into active osteoclasts in the presence of MCSF and RANKL. Osteoclasts, once differentiated, start to destruct the bone surface to produce lacunae. OPG, the trap receptor of the RANKL inhibits the continuous binding od RANKL to RANK and causes apoptosis of the osteoclasts. Subsequently, preosteoblasts convert to osteoblasts for new bone formation (18). RANKL was defined by four independent groups and was named as TRANCE, ODF, osteoprotegerin ligand (OPGL) and TNFSF11.

RANKL receptor has been defined as RANK. Other names of it are TNF-related activation-induced cytokine receptor (TRANCE-R) or osteoclast differentiation and activation receptor (ODAR). RANK is a transmembrane protein with a total of 616 amino acids with the extracellular part composed of a 28-amino acid signal peptide, a short transmembrane, and large cytoplasmic parts. Osteoporosis is known to be frequently seen in patients with bronchiectasis. Among the mechanisms causing this condition in patients with CF have been held responsible are malnutrition, pancreatic failure, calcium malabsorption, Vitamin D and K deficiency, long term use of systemic or inhaled steroids, delayed puberty, chronic respiratory acidosis, cytokines increasing osteoclast activation and decreased sex steroids; however, molecular mechanisms playing role are yet unclear. In a study by Putman et al. mean BMD value in vertebra and distal radius of young adults was demonstrated to be always low in patients with CF and to be unchanged in a follow-up of 15 years in spite of the improvements in the respiratory functions of patients with CF due to new developments in the follow-up of and vitamin D support. Understanding the mechanisms in the development of osteoporosis might allow new therapeutic approaches (19-22).

Bronchiectatic patients with CF and noncystic fibrosis have many common features. Patients above 5 years of age and with bronchiectasis and who had osteoporosis diagnosed by DEXA were included in the patient group in this present study. The age of three of the patients included in the study were 8.5 years or below, and the cause for this was considered to be related with the required time for the development of osteoporosis. Low BMD values in patients with cystic fibrosis and with normal nutritional state, at earlier ages down to 6 years suggests that this situation might be associated with bone metabolism (23).

RANKL expression is controlled by many factors such as glucocorticoids, vitamin D and IL-1. Low RANKL levels in patients with immune deficiency might be associated with the significantly low levels of vitamin D in these patients compared to the control group.

OPG levels were lower in the patient group in this present study compared to the control group, though not significant. Median OPG in patients with PSD was found to be lower compared to the controls and patients with immune deficiency, though not statistically significant. No statistically significant differences were found in serum OPG levels when the patients with PSD and control group were analyzed separately and when patients with immune deficiency and PSD were analyzed separately. OPG is known to be expressed in many tissues such as the heart, kidneys and liver (12). We considered that osteoporosis seen in patients with PSD might be due to the decreased expression of OPG in lung tissue.

Similarly, no statistically significant difference was found in the RANKL/OPG ratio between the patient and control groups. In this present study, no significant correlation was found between serum RANKL and OPG levels and DEXA z scores. Shead et al. in their study in adult patients with CF, serum RANKL levels were analyzed in the period of clinical stability of the patients, on the day prior to the start of antibiotics during acute exacerbation and on day 14 of the completion of intravenous antibiotic treatment. No significant

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difference was found in the RANKL levels in the stable period of the patients compared to the controls; however, RANKL levels on day 14 of the acute exacerbation was found to be significantly high compared to the stable period in that study. Serum OPG levels were found to be significantly low in patients with CF compared to the controls in the stable period, and significantly high on day 14 of the acute exacerbation (24). Ambroszkiewicz et al. in their study in patients with CF, included patients with no lung findings, no steroid treatment, and clinically stable patients as controls. Serum RANKL values were found to be 2-folds in the patient group compared to the controls, and OPG and OPG/RANKL levels were found to be lower compared to controls in that study (25). The serum samples were collected at the stable period of the patients and no comparison was made with the acute exacerbation period and this might be the cause of no significant difference in the RANKL, OPG and RANKL/OPG levels in this present study.

Franchimont and Galluzzi et al. in their study serum OPG levels were found significantly high in patients with Crohn's disease and Type 1 Diabetes Mellitus (26-27). Association of the changes in the RANK/RANKL/OPG system with chronic inflammation might be the underlying mechanism in osteoporosis in chronic diseases. We suggest that chronic inflammation might be effective in the pathogenesis of the osteoporosis developing in bronchiectasis patients.

Effects of inhaled and systemic corticosteroid use on bone health is a subject of great interest. Corticosteroids cause decreased bone mineral density by also effecting the RANK-RANKL-OPG system. A total of 5 g of inhaled steroids is demonstrated to cause a loss of 1 SD in vertebral BMD. General opinion on the systemic steroid use is that is causes low BMD and morphometric fractures (28). In a study by Wasilewska et al. in patients with nephrotic syndrome and using steroids, corticosteroids were detected to dose-dependently elevate RANKL and RANKL/OPG ratio (29). Corticosteroids and mostly inhaler corticosteroids were frequently used in the patient group in this present study among patients with bronchiectasis. Corticosteroid use was ignored when designing the patient group. We consider that steroid use might have affected the results of the study.

In vitro studies have been performed in patients with CF recently in order to demonstrate the association between osteoporosis and RANKL/OPG and CFTR gene mutation. These studies support that CFTR gene mutation increases bone resorption due to chronic inflammation (25,30,31). If the association of CFTR gene mutation and RANKL/OPG system becomes apparent as a result of the future studies, it would be meaningful why no association was found between the control group and the patient group noncystic fibrosis in this present study.

Serum osteocalcin level which is a good predictor of bone production was found to be low in the patient group compared to the control group, however no statistical significance was found in this present study. No statistical significance was found between the subgroups of immune deficiency and PSD. Rossini et al. in their study in patients with CF and vertebral fractures, found low osteocalcin levels in 36% of the patients (32). Ambroszkiewicz et al. in their study in patients with CF, found low osteocalcin levels and high RANKL/OPG ratio in the patient group compared to the control group and this was suggested to reflect the increased bone destruction and decreased bone production (25). In this present study, similarity of osteocalcin levels in patient and control groups could be due to the clinical stability of the patients and an achieved balance between bone production and destruction.

A powerful and negative correlation was found between the serum osteocalcin and OPG levels in the patient group. An inverse correlation was found between serum OPG level and BMD and osteocalcin level in a study by Oh et al. in 80 Korean male patients between 42-70 years of age (33). A powerful and positive correlation was found between OPG and osteocalcin in patients with osteoporosis in a study by *Fahrleitner-Pammer* and found that low OPG levels were associated with vertebral fractures (34).

Vitamin D levels were found to be low in both patient and control groups, with no statistically significant difference between the groups in this present study. Considering the previous studies, low levels of vitamin D in the healthy control group might be attributed to the high incidence of Vitamin D deficiency and insufficiency in Turkey. Vitamin D level was found to be in the normal range (>30 ng/mL) in only 12.3%

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of the control group in a study was Dogru and Suleyman (35). Vitamin D deficiency and insufficiency was detected in 48 % of the healthy control group composed of healthy individuals ina study by Turkeli et al. (36). Vitamin D deficiency and insufficiency was found in 80.3 % and 11.7 %, respectively in healthy children included in the study at the end of winter season in a study by Erol et al. performed in Istanbul (37). Vitamin D levels were found to be significantly low in the subgroup of patients with immune deficiency compared to the patients with PSD and control group. When immune deficiency and control group was compared, Ca and P levels were found to be significantly high in the control group. We thought that this might be due to the prescription of vitamin D and Ca preparations for patients in case when osteoporosis was diagnosed subsequent to the required tests for osteoporosis due to the clinical high incidence of osteoporosis and osteopenia in patients with PSD.

Median serum Ca level was significantly different in the patient group and the control group in this present study. Serum Ca level was found to be significantly low in the subgroup with immune deficiency compared to the PSD subgroup and control group. No significant difference was found between the patient and control groups in terms of serum Ca levels in a study by Ambroszkiewicz et al. in patients with CF (25). We suggest that these results in this present study might be due to the low levels of vitamin D in the control group and the subgroup of immune deficiency.

Median spot urine Ca/Cr ratio was statistically significantly different between the patient and control groups in this present study. Spot urine Ca/Cr ratio was found to be significantly high compared to the control group. We found no positive results that might be helpful to associate calciuria and OPG/RANKL; however, we thought that this might be associated with other factors of osteoclastic activity and factors causing increased bone resorption.

A powerful and positive correlation was found in QUS z scores and DEXA, BMD z scores in the patient group. According to the literature data, QUS is generally considered not to be a substitution of DEXA, but it could be used as a screening method. Williams et al. found a powerful correlation between QUS and DEXA in their study in obese patients with CF; however, they concluded that QUS could not be used in place of DEXA, especially in obese patients (38). Schepper et al. compared QUS, DEXA and Perioheral Quantitative CT in adult patients with CF. QUS was deemed not to replace DEXA in that study; but it was stated that it could be used as a screening method in patients with a normal bone mass (39). Similarly, Flohr et al. proved that QUS had no high specificity and sensitivity to replace DEXA (40). Parallel to the literature findings, a correlation was found in the bone US and DEXA values in the patient group in this present study. This supports the applicability of QUS as a screening test in patients with bronchiectasis since it is easily applicable compared to DEXA in BMD measurements and includes no radiation exposure.

A negative correlation was found between serum RANKL levels and ALP in the patient group. A powerful and positive correlation was found between NTX and bALP when the patient group was evaluated in itself and with the control group. A positive and statistically significant correlation was found between NTX and spot urine Ca/Cr in the patient group. A positive and statistically significant correlation was found between the serum osteocalcin and bALP, ALP, NTX, and Ca when the patient and control groups were evaluated together. Positive and negative many correlations between the bone production and destruction markers were found in this present study in the patient group. This suggests a high bone turnover in patients with bronchiectasis.

Our limitations were determination of serum OPG and RANKL levels are challenging due to many reasons. The source of RANKL released in the circulation is many and might be in many forms. RANKL is present in the serum in free form and most of it as bound to OPG and the two molecules have a circadian rhythm. Therefore, the measured values in the circulation may not completely reflect the effects on the bone microframe. Serum samples were preferred to be obtained in morning hours in this present study; however, the study results might be affected from the circadian rhythm.

In conclusion, we could not find the definite role of RANK/RANKL/OPG system in the pathogenesis of osteoporosis developed in noncystic fibrosis bronchiectasis patients in this present study. However, future

studies in larger series would be appropriate since the number of cases is small in this study.

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