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ORIGINAL ARTICLE

Relationships between OPG, RANKL, bone metabolism, and bone mineral density in biliary atresia

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Abstract
Purpose Osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) have been implicated in osteoclastogenesis. However, the relationship between the OPG–RANKL system and bone status in biliary atresia (BA) has not, as yet, been clarified. Thus, the aim of this study has been to evaluate the relationship between the OPG–RANKL system and bone mineral metabolism in patients with BA.
Methods Fifty patients with BA and 13 healthy controls were investigated. The mean age of BA patients and controls was 7.3 ± 0.6 and 8.0 ± 1.1 years, respectively. Serum levels of OPG, RANKL, osteocalcin, and C-terminal telopeptide of type I collagen (CTX) were measured by sandwich enzyme-linked immunosorbent assay. Bone mineral density (BMD) of the lumbar spine was determined by dual energy X-ray absorptiometry.
Results Biliary atresia patients had significantly elevated serum OPG levels compared with controls (4.0 ± 0.3 vs. 3.0 ± 0.3 pmol/L, $P = 0.02$) and serum OPG levels in BA patients with jaundice were higher than in those without jaundice (4.6 ± 0.4 vs. 3.6 ± 0.4 pmol/L, $P = 0.04$). Likewise, serum RANKL levels were significantly higher in BA patients than in controls (2.9 ± 0.2 vs. 1.2 ± 0.7 pmol/L, $P = 0.001$). In addition, serum RANKL levels were increased in BA patients with jaundice compared to those without jaundice, but this difference was not statistically significant (3.2 ± 0.3 vs. 2.7 ± 0.2 pmol/L, $P = 0.2$). The serum osteocalcin levels in BA patients were not significantly different from those in the healthy controls, whereas the serum CTX levels were elevated in BA patients compared with the controls (0.4 ± 0.1 vs. 0.2 ± 0.1 ng/mL, $P = 0.02$). Furthermore, BMD of BA children with jaundice was significantly lower than that of BA children without jaundice ($P = 0.0005$). BMD of BA patients was inversely correlated with serum levels of OPG ($r = -0.452$, $P < 0.001$).
Conclusion Elevated serum OPG levels are associated with reduced BMD and the outcome of BA. The increase of serum OPG in BA patients with severe disease could reflect a compensatory response to bone loss.

Keywords Biliary atresia · Bone mineral density · Osteoporosis · Osteoprotegerin · Receptor activator of nuclear factor-κB ligand

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Introduction

Biliary atresia (BA) is one of the most common causes of chronic cholestasis in children and is a progressive,

inflammatory, fibrosclerotic cholangiopathy leading to complete obliteration of both intrahepatic and extrahepatic bile ducts [1]. The obstruction presents as a triad of jaundice, acholic stools, and hepatosplenomegaly. Without medical and surgical intervention, cholestasis results in hepatic fibrosis, portal hypertension, end-stage liver disease, and death within a few years [2]. Kasai portoenterostomy currently remains the treatment of choice for patients with BA. Although the etiology and pathogenesis of BA remain largely unknown, several mechanisms have been suggested including defects in biliary tract morphogenesis, defects in prenatal circulation, viral infection, exposure to environmental toxins, and immunological dysregulation [3]. A number of growth factors and cytokines have been recently documented in patients with BA including hepatocyte growth factor [4], connective tissue growth factor [5], stem-cell growth factor [6], and monocyte chemoattractant protein-1 [7].

Osteopenia and osteoporosis are common among patients with chronic liver diseases as well as BA. Both are characterized by low bone mass, microarchitectural deterioration of bone tissue, originating from an imbalance of skeletal turnover maintained by two opposite but normally balanced processes of bone formation and bone resorption that results in bone fragility and a consequent susceptibility to fracture [8]. Although the pathogenesis of bone disease in BA is not yet completely understood, it has been postulated that malnutrition, malabsorption of vitamin D and calcium deficiency may be responsible for the imbalanced action of bone-forming osteoblasts and bone-resorbing osteoclasts [9]. Recently, osteoporosis has been observed in a number of cholestatic liver diseases including alcoholic cirrhosis [10], hemochromatosis [11], Wilson disease [12], primary biliary cirrhosis [13], and BA [9, 14].

Receptor activator of nuclear factor-κB ligand (RANKL), a member of the tumor necrosis factor (TNF) superfamily, is a soluble or transmembrane ligand, which is produced by osteoblast lineage cells and activated T lympocytes. RANKL binds to its specific receptor RANK that is expressed on osteoclast precursor cells and mature osteoclasts. Interactions between RANKL and RANK are essential for both osteoclastogenesis and activation of bone-resorbing osteoclasts [15]. The effects of RANKL are counteracted by osteoprotegerin (OPG) which serves as a soluble neutralizing receptor. OPG is known to inhibit osteoclastogenesis by binding to RANKL, thereby preventing RANKL from interacting with RANK on osteoclasts [16]. Although the emerging knowledge of the biological effects of the OPG/RANKL system derived from in vitro and animal studies has generated a new comprehension of osteoclast biology and has provided novel insights into the molecular and cellular basis of various metabolic bone diseases, the contribution of the OPG/RANKL system to the pathogenesis of bone disease in BA as yet has not been completely elucidated. The purpose of this study, therefore, has been to investigate the OPG/RANKL system, biochemical markers of bone turnover, and bone mineral density (BMD) in patients with BA.

Materials and methods

The study was approved by the Ethics Committee on Human Research of the Faculty of Medicine, Chulalongkorn University. All parents of children with BA and of the healthy controls were informed of the study's objectives, and written informed consent was obtained from the parents prior to the children entering the study.

A total of 50 BA patients after Kasai operation were recruited into the study during their annual routine follow-up between July 2005 and March 2006. Bone density, biochemical parameters, and clinical characteristics of our subjects have been reported previously [14]. We have not previously reported serum levels of OPG and RANKL or the relationship of OPG and RANKL with BMD. The BA group included 30 girls and 20 boys with a mean age of 7.3 ± 0.6 years. The control group contained 13 healthy children, age and gender matched with a mean age of 8.0 ± 1.1 years (7 girls and 6 boys) among those who participated in an evaluation of hepatitis B vaccine during the same period. None of the BA patients in this study had undergone liver transplantation or exhibited signs and symptoms of fever or ascending cholangitis at the time of blood sampling. The serum specimens were collected and stored at −70°C until assayed.

In order to compare the outcome among BA patients, based on the status of jaundice, they were divided into two groups: patients with jaundice (TB $\geq$ 2.0 mg/dL, $n = 20$) and patients without jaundice (TB $<$ 2.0 mg/dL, $n = 30$).

Laboratory tests

Measurements of serum OPG and RANKL were performed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biomedica GmbH, Vienna, Austria) according to the manufacturer's instructions. The range of controls tested for OPG and RANKL were 0–30 and 0–50 pmol/L, respectively. Sensitivity levels for these markers were 0.14 pmol/L for OPG and 0.4 pmol/L for RANKL. Recombinant human OPG and RANKL were used to generate the standard curve. The intra-assay and interassay coefficients of variation (CV) were below 10% for both assays. Serum osteocalcin as a marker of bone formation was measured by the osteocalcin ELISA kit (Nordic Bioscience Diagnostics, Herlev, Denmark). Intra-assay and interassay CV values were 2.6

and 4.7%, respectively, with the detection limit at 0.5 ng/mL. Serum C-terminal telopeptide of type I collagen (CTX) as a marker of bone resorption was detected by the ELISA CrossLaps kit (Nordic Bioscience Diagnostics). Intra-assay and interassay CV values were 5.2 and 6.7%, respectively, and the detection limit was 0.01 ng/mL. The absorption was determined with an ELISA reader at 450 nm against 690/620 nm. In addition, liver function tests including serum albumin, total bilirubin (TB), direct bilirubin (DB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), and gamma glutamyl transpeptidase (GGT) were measured by the central laboratory using the automated Hitachi 912 test system.

Bone density measurements

All children underwent BMD measurements of the lumbar spine (anteroposterior lumbar vertebrae L1–L4) with dual energy X-ray absorptiometry (DEXA) using Hologic QDR 2000 (Hologic Inc., Waltham, MA, USA). BMD was expressed in absolute values (g/cm^2) and *Z*-scores. *Z*-scores of BMD were defined as numbers of standard deviations from the mean BMD of age-matched norms. Control data were obtained from Caucasian children provided in the software Hologic 2000. Children were classified into normal, osteopenia, and osteoporosis according to World Health Organization (WHO) guidelines. Osteoporosis was defined as a spinal BMD equal to or exceeding 2.5 standard deviations (SD) below the average values (Z score ≤ -2.5). Osteopenia was defined as a BMD below 2.5 SD but above 1 SD under the average values ($-2.5 < Z$ score < -1.0). Normal BMD was defined as a spinal BMD equal to or below 1 SD under the average values (Z score ≥ -1.0).

Statistical analysis

All the values are expressed as mean ± SEM. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software. Demographic and clinical data between groups were compared by Chi-square tests and unpaired Student's *t* tests, where appropriate. Comparisons of clinical data and biochemical markers among patients with normal, osteopenia, and osteoporosis were performed using one-way analysis of variance (ANOVA) with Tukey post hoc test if ANOVA showed significance. Correlation analysis was performed using the Spearman correlation test. P values < 0.05 were considered to indicate statistical significance. Multiple regression models were performed with the BMD as the dependent parameter, body mass index (BMI), OPG, RANKL, osteocalcin, and CTX as the independent parameters.

Results

The clinical characteristics and laboratory parameters between BA patients without jaundice and BA patients with persistent jaundice are demonstrated in Table 1. Fifty BA patients and 13 healthy controls participated in the present study. All BA patients had undergone Kasai operation. There were 30 BA patients without jaundice and 20 BA patients with persistent jaundice. There were no significant differences in age (7.3 ± 0.6 vs. 8.0 ± 1.1 years) and gender (F:M, 30:20 vs. 7:6) between the BA patients

Table 1 The clinical characteristics and laboratory parameters of healthy controls and BA patients

Characteristics	Controls	BA	Jaundice	Jaundice-free	*P*
N	13	50	20	30	
Female:male	7:6	30:20	10:10	20:10	0.3
Age (years)	8.0 ± 1.1	7.3 ± 0.6	5.8 ± 0.8	8.3 ± 0.7	0.01
Albumin (g/dL)	–	3.9 ± 0.1	3.4 ± 0.2	4.3 ± 0.1	0.0005
Total bilirubin (mg/dL)	–	4.5 ± 1.0	9.8 ± 1.9	0.9 ± 0.1	0.0005
Direct bilirubin (mg/dL)	–	3.0 ± 0.8	7.0 ± 1.5	0.3 ± 0.1	0.0005
AST (IU/L)	–	151.9 ± 17.5	243.4 ± 28.2	90.9 ± 14.0	0.0005
ALT (IU/L)	–	128.0 ± 16.4	176.2 ± 25.0	95.9 ± 19.8	0.008
ALP (IU/L)	–	508.1 ± 47.3	637.6 ± 58.7	421.8 ± 64.4	0.009
GGT (IU/L)	–	242.2 ± 33.3	311.0 ± 50.7	196.4 ± 42.6	0.05
Spine BMD *Z*-score	–	−1.3 ± 0.2	−2.3 ± 0.3	−0.7 ± 0.2	0.0005
OPG (pmol/L)	3.0 ± 0.3	4.0 ± 0.3	4.6 ± 0.4	3.6 ± 0.4	0.04
RANKL (pmol/L)	1.2 ± 0.7	2.9 ± 0.2	3.2 ± 0.3	2.7 ± 0.2	0.2
CTX (ng/mL)	0.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	0.2 ± 0.1	0.01
Osteocalcin (ng/mL)	16.3 ± 1.5	19.1 ± 2.8	20.9 ± 4.0	18.0 ± 3.7	0.6

Data are expressed as mean and SEM. *P* values for differences between the jaundice and jaundice-free patients

and the controls. The circulating concentrations of AST, ALT, AP, and GGT were significantly higher in the BA patients with persistent jaundice compared to those without jaundice, whereas the BA patients with persistent jaundice had lower levels of albumin than those without jaundice.

The mean serum levels of OPG in patients with BA were significantly higher than those in healthy controls (4.0 ± 0.3 vs. 3.0 ± 0.3 pmol/L, $P = 0.02$) (Fig. 1). Among the BA patients, serum OPG levels were significantly elevated in the jaundice group (4.6 ± 0.4 pmol/L) compared with the jaundice-free group (3.6 ± 0.4 pmol/L, $P = 0.04$) and the healthy controls (3.0 ± 0.3 pmol/L, $P = 0.004$) (Table 1). There was no significant difference in serum OPG levels between the jaundice-free group and the controls. Likewise, serum RANKL levels were significantly higher in BA patients (2.9 ± 0.2 pmol/L) than in controls (1.2 ± 0.7 pmol/L, $P = 0.001$) (Fig. 2). Although the BA patients with jaundice had higher serum RANKL levels than those without jaundice, the difference was not statistically significant (3.2 ± 0.3 vs. 2.7 ± 0.2 pmol/L, $P = 0.2$) (Table 1).

Serum osteocalcin levels did not differ statistically in BA patients (19.1 ± 2.8 ng/mL) and healthy controls (16.3 ± 1.5 ng/mL) (Fig. 3). However, serum CTX levels were significantly higher in patients with BA compared with the controls (0.4 ± 0.1 vs. 0.2 ± 0.1 ng/mL, $P = 0.02$) (Fig. 4). Furthermore, jaundice BA patients had greater serum CTX levels compared with non-jaundice patients ($P = 0.01$). Further studies showed that lumbar spine BMD Z-score of jaundice BA children was significantly lower than that of non-jaundice BA children ($P = 0.0005$) (Table 1).

The BA patients were categorized into tertiles according to the WHO criteria. The first tertile included 22 patients with BMD Z-scores from 0 to -1 (considered as normal), the second tertile included 14 patients with Z-scores from -1.0 to -2.5 (considered as osteopenic), and the third tertile included 14 patients with Z-score lower than -2.5

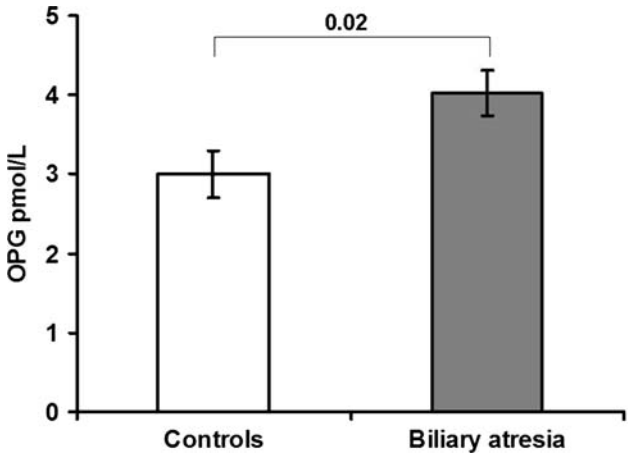

Fig. 1 Serum osteoprotegerin (OPG) levels in BA patients and in healthy controls. Data are expressed as mean and SEM

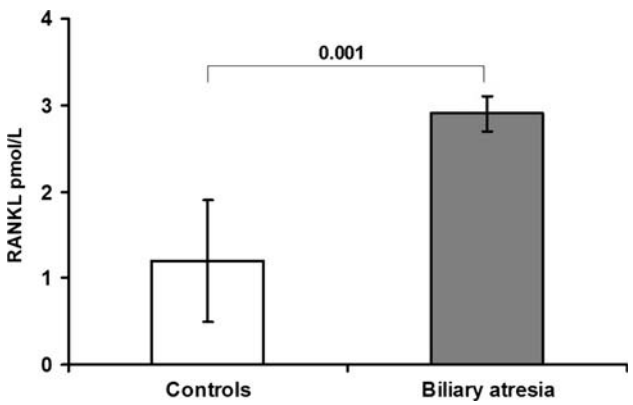

Fig. 2 Serum receptor activator of nuclear factor-κB ligand (RANKL) levels in BA patients and in healthy controls. Data are expressed as mean and SEM

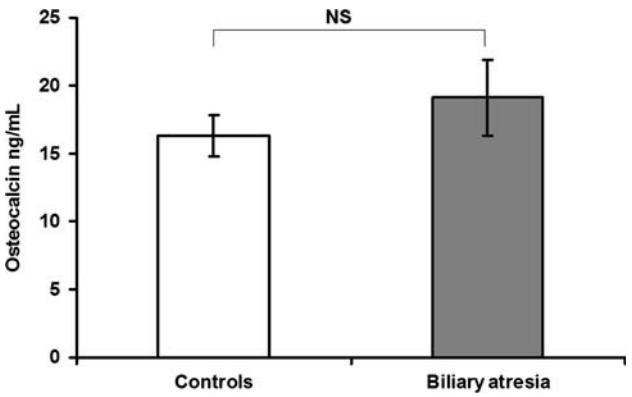

Fig. 3 Serum osteocalcin levels in BA patients and in healthy controls. Data are expressed as mean and SEM

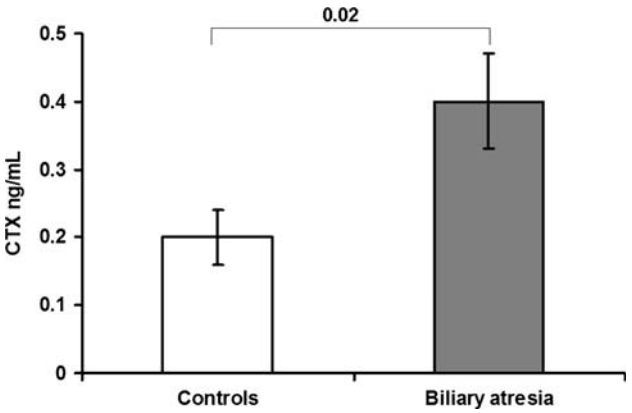

Fig. 4 Serum C-terminal telopeptide of type I collagen (CTX) levels in BA patients and in healthy controls. Data are expressed as mean and SEM

Table 2 Comparison of clinical characteristics and biochemical markers among BA patients with normal, osteopenic, and osteoporotic BMD *Z*-scores at the lumbar spine

Z-scores	Normal	Osteopenia	Osteoporosis	*P*
N	22	14	14	
Female:male	12:10	9:5	9:5	0.285
Age (years)	7.6 ± 0.7	7.5 ± 1.1	6.6 ± 1.1	0.732
BMI (kg/m^2)	16.5 ± 0.4	17.5 ± 0.7	16.3 ± 0.6	0.294
OPG (pmol/L)	3.3 ± 0.3	4.1 ± 0.6	5.1 ± 0.5	0.03
RANKL (pmol/L)	2.9 ± 0.2	2.8 ± 0.4	3.0 ± 0.4	0.915
Osteocalcin (ng/mL)	29.2 ± 9.2	27.1 ± 7.0	19.6 ± 4.3	0.687
CTX (ng/mL)	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.523

Table 3 Multiple regression analysis of the variables; lumbar spine bone mineral density as the dependent variable

Characteristics	Lumbar spine BMD	
	Beta	*P*
Body mass index	0.365	0.006
OPG	−0.366	0.011
RANKL	−0.020	0.885
Osteocalcin	0.092	0.478
CTX	0.044	0.738

(considered as osteoporotic). There was no significant difference in gender and age distribution and BMI between the three tertiles (Table 2). However, serum OPG levels differed significantly among these three patient groups. The highest OPG levels were observed in the third tertile (5.1 ± 0.5 pmol/L) and were significantly higher than in the second and first tertile (4.1 ± 0.6 and 3.3 ± 0.3 pmol/L, respectively; $P = 0.03$). No statistically significant difference was noted in the serum levels of RANKL, osteocalcin, and CTX among the three tertiles.

Multiple regression analyses were performed with lumbar spine BMD as dependent variable (Table 3), BMI and serum OPG levels were identified as a significant predictor for lumbar spine BMD. In contrast, serum RANKL, osteocalcin, and CTX levels were not correlated with lumbar spine BMD in multiple regression analysis. BMD of BA patients was inversely correlated with serum levels of OPG ($r = -0.452$, $P < 0.001$) (Fig. 5). There was no correlation between BMD and RANKL, osteocalcin, and CTX levels.

Discussion

Osteoprotegerin also known as osteoclastogenesis inhibitory factor, is a soluble member of the tumor necrosis

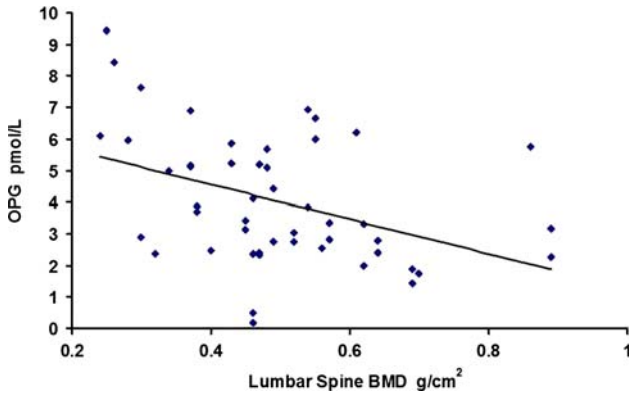

Fig. 5 Relationship between osteoprotegerin (*OPG*) and lumbar spine bone mineral density (*BMD*) in the BA patients. Lumbar spine BMDs were inversely correlated with serum OPG levels ($r = -0.452$, $P < 0.001$)

factor (TNF) receptor family and serves as a decoy receptor inhibiting the interaction between the receptor for activation of nuclear factor-κB (RANK) and its ligand (RANKL) [17]. The biological effects of OPG on bone cells include inhibition of the final stages of osteoclast differentiation, suppression of mature osteoclast activation, and induction of osteoclast apoptosis [18, 19]. Studies on transgenic mice overexpressing osteoprotegerin have shown that it decreased bone resorption and enhanced bone mass, whereas the OPG gene knockout mice developed severe osteoporosis due to an increase in osteoclastic bone resorption [20, 21]. It has recently been demonstrated that homozygous deletion of the OPG gene potentially leads to juvenile Paget's disease in some children [22]. In contrast, RANKL knockout mice displayed a reduced number of osteoclasts, increased BMD, even osteopetrosis [23]. A single administration of recombinant human OPG to young growing rats resulted in a significant increase in bone density and an inhibition of osteoclastic bone resorption [24]. These results indicate that OPG and RANKL are crucial physiological regulators of osteoclastogenesis.

In the present study, we investigated for the first time the circulating levels of OPG, RANKL, osteocalcin, and CTX in the serum of BA patients. Significantly higher serum levels of OPG were observed in BA patients, and in particular in BA patients with jaundice compared to BA patients without jaundice or healthy controls. The production of OPG and its ligand is regulated by a variety of inflammatory cytokines known to affect bone metabolism. Some of the inflammatory cytokines, such as IL-1, IL-6, and TNF-α, could enhance OPG production and might regulate osteoclast differentiation and activation through increased RANKL production [25, 26]. Although bone cells are the major source of OPG, other extraskeletal tissues may play a role in serum OPG elevation [18]. Possibly, other tissues or cells, such as inflammatory cells

and fibroblasts in the liver contribute to OPG production [27]. Another possible explanation may be that higher OPG levels are secondary to decreased clearance.

The present study has investigated alterations in the OPG–RANKL system in relation to lumbar spine BMD in BA patients. Subgroup analysis of BA patients based on jaundice status showed that the jaundice group displayed lower BMD than the jaundice-free group. These results suggest that decreased BMD in BA patients with jaundice could be the consequence of increased bone resorption. Intriguingly, high OPG levels tended to be associated with low BMD in the BA patients. Subsequent analysis showed that serum OPG levels were inversely correlated with BMD in that osteoporotic BA patients displayed substantial elevation in serum OPG levels. These findings support the hypothesis that OPG increases to compensate for bone loss, which is neither effective nor adequate, as it is reflected in the decreased bone density. Thus, the elevated OPG levels would subsequently reduce osteoclast activation and differentiation and would also enhance osteoclast apoptosis in BA patients.

Several studies have investigated serum OPG and RANKL levels in association with serum biochemical measurements in chronic liver diseases, but results have been inconsistent and conflicting. Szalay et al. [28] were the first to examine serum OPG and RANKL levels in patients with primary biliary cirrhosis (PBC). They have demonstrated that elevated serum OPG and low serum RANKL levels may, in part, represent a compensatory response to the negative balance of bone remodeling in PBC. Recently, Garcia-Valdecasas-Campelo et al. [29] have shown high serum levels of OPG with normal levels of RANKL in chronic alcoholic liver disease. They observed negative correlations between OPG and serum albumin and prothrombin activity, indicating a relationship between elevated OPG and deranged liver function. In our study serum OPG and RANKL levels were elevated in patients with BA, in accordance with Fabrega's finding. Fabrega and colleagues described raised OPG and RANKL serum levels in patients with advanced alcoholic liver cirrhosis [30]. The explanation for these conflicting results is obscure, but may be attributed to differences in disease advancement, populations or assays applied, or in incomplete control for confounding variables.

The reason why serum OPG and RANKL levels increase in BA patients is still not entirely clear, but it may be related to an imbalance between bone formation and resorption associated with disease severity in these patients. To further investigate whether bone loss in BA patients results from decreased bone synthesis or increased bone resorption, bone turnover markers, serum osteocalcin, and CTX were measured. The insignificant difference in the serum osteocalcin levels between the BA patients and controls reflects that there is normal osteoblastic function in patients with BA. However, serum CTX levels were significantly elevated in BA patients, which reflect increased bone resorption in BA.

The limitation of this study has been the absence of quantitative bone histomorphometry data which could have provided evidence as to whether bone was correlated with serum OPG and/or RANKL levels. Therefore, additional research will be necessary in order to clarify this point. In addition, serum OPG and RANKL levels may not consistently reflect the amount of bioavailable OPG and RANKL in the bone microenvironment. OPG and RANKL are synthesized locally in bone tissue by osteoblasts. Since the OPG–RANKL action is on the skeleton, skeletal OPG–RANKL production may have little influence on serum levels. Hence, alterations in the OPG–RANKL system within the bone microenvironment might be different from those in circulation. As this study was designed as a cross-sectional study, definite cause and effect relationship cannot be drawn and prospective studies are warranted to demonstrate the precise association of the serum levels of OPG, RANKL, and bone metabolism.

In conclusion, we found significant differences in the serum levels of OPG, RANKL, and CTX in children with BA when compared with healthy controls. Our statistical analysis has shown that the serum OPG levels were significantly associated with BMD and presence of jaundice in BA. These results suggest that OPG could be served as a screening tool for detection of BA patients with suspected low BMD who qualify for BMD evaluation. The increase of serum OPG in patients with severe disease could reflect a compensatory response to bone loss and osteoporosis. This study provides further evidence that the bone metabolism and bone density may be linked through the OPG/RANKL system. More research should address the relationships with serum and hepatic OPG/RANKL expression among BA patients and include the various groups of age-matched children with other severe liver diseases for comparison.

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