VARIATION IN THE COX-2 GENE MAY MODIFY THE EFFECT OF ALENDRONATE ON VERTEBRAL FRACTURE PREVENTION

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Bisphosphonates such as alendronate, which are potent specific inhibitors of osteoclast-mediated bone resorption, are widely used for treatment of postmenopausal osteoporosis as well as other diseases related to bone remodeling. We evaluated whether the reportedly functional PTGS2 (prostaglandin-endoperoxide synthase 2/cyclooxygenase [COX] 2) genotypes influence the efficacy of alendronate on vertebral fracture prevention. Sixty postmenopausal osteoporotic women participated in this interventional study. The extent of vertebral fracture was evaluated in all participants before and after intervention using X-ray imaging. Alendronate (10mg/day), calcium (1gr/day) and vitamin D (400mg/day) were given to participants for 2 years. Laboratory measurements included circulating crosslaps, osteocalcin, PTH, osteoporotegrin, RANKL, vitamin D, TNF-α, IL-6, IL-1 levels. Hip and spine BMD (bone mass density) were measured using DEXA. Genotyping for cox-2 gene SNP (-765G/C) was performed using PCR- RFLP method. Genotype frequency of homozygous major allele (GG), heterozygous (GC) and homozygous minor allele (CC) were 61.7%, 33.3% and 5% respectively. Evaluation of vertebral fracture before alendronate therapy in participants demonstrated no significant difference between carriers of G and C alleles, although the difference appeared near to significant after alendronate therapy at the end of 2 years. Serum PTH level and L2-L4 BMD were significantly different between subjects with different alleles. Moreover, IL-1 had prominently higher concentration in C allele carries. Furthermore, there was a significant difference in terms of the extent of vertebral fracture between two allelic groups after two years of treatment. Since bone remodeling process has been proved to be affected by inflammatory factors; it appears that variation in COX-2 genotypes may influence alendronate efficacy in fracture prevention among postmenopausal osteoporotic women.

Postmenopausal osteoporosis is a chronic, progressive disorder in which bone resorption exceeds its formation. This results in decreased bone mass and weakened bone structure and is followed by its increased susceptibility to fractures (1). There are similar pathways between fetal bone development and fracture healing except for inflammation which is an early phase of fracture healing and does not occur during development (2). Prostaglandins are known to have a role in fracture healing events. Alternating several pathways, prostaglandins can stimulate bone formation and resorption, thus contributing

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to bone metabolism (3). Different cell stimuli can modify the amount of prostaglandins produced by osteoblasts (4). Cyclo-oxygenase 2 (COX-2) is produced at inflammation sites and is categorized as a pro-inflammatory prostaglandin (2). Besides, it is known as a key enzyme for prostaglandin E2 (PGE2) production, which in turn induces the nuclear factor kappa B (NF-B) activation, thereby facilitating fracture healing (5). Moreover, prostaglandins affect bone metabolism and formation. It is noteworthy that inflammation is an early physiological response to bone fracture (2).

Cox2 is known to be of paramount importance as a key enzyme in fracture healing (2, 6) as it catalyzes the synthesis of prostaglandins from arachidonic acid in the initial inflammatory response to the skeletal trauma (6-7). Prostaglandins stimulate both bone formation and resorption by affecting replication and differentiation of osteoclast and osteoblast cells (2, 6). Findings from animal studies have demonstrated increased prostaglandin concentrations around the healing callus of bone (8), therefore, different COX-2 genotypes might have different effects on prostaglandin synthesis which is necessary for normal bone fracture healing.

Alendronate, a bisphosphonate and anti-resorptive agent, is widely used to treat osteoporosis. It inhibits bone resorption and reduces biochemical markers of bone remodeling (9). Bisphosphonate therapy has been demonstrated to increase skeletal micro-damage by reducing remodeling which appears to be the main responsible mechanism for the damage (10). Moreover, results of recent research have revealed that daily alendronate therapy increases micro-damage in vertebral bone after 3 years (11).

The extent of micro-damage is increased with age (12). Not only various animal studies have demonstrated significant increases in microdamage after bisphosphonate treatment (10). This could indicate that within the therapeutic-dosage range there exists a risk, although low, of a continuous and progressive decrease in bone turnover that, in the long run, might contribute to bone weakness.

It has been demonstrated that bisphosphonates release proinflammatory cytokines and are involved in selective receptor-mediated activation of ,-T cells, thus leading to their proliferation (13).

For two reasons, 1) the effects of prostaglandin on fracture healing, and 2) the role of cytokines in alendronate healing pathway, we designed the present study to evaluate the effect of cox-2 gene in preventing vertebral fractures that may be a side effect of alendronate treatment in women who are being treated for postmenopausal osteoporosis, and looked into the treatment's potential role in explicating this process by measuring bone turnover marker and inflammatory factors.

MATERIALS AND METHODS

Study subjects

Subjects were female patients recruited from the osteoporosis clinic of Shariati Hospital from January 2006 to June 2008. Inclusion criteria were age≥45 years and diagnosis of osteoporosis defined according to WHO criteria that was described in a previous study (14). Women with diabetes mellitus, ischemic heart disease, cerebrovascular disease, thyroid dysfunction, chronic liver, and renal and inflammatory diseases as well as other skeletal diseases (Paget's disease, osteogenesis imperfecta and rheumatoid arthritis) were excluded from the study. In addition, those on medication such as corticosteroids were excluded as they increase bone loss. Menopause was defined as the absence of menstruation for at least 12 months. Written informed consent was obtained from all participants. The study protocol was approved by the research ethics committee of EMRC, the Medical Ethics Research Center.

Demographic data, life style information, and medical and drug history were obtained using a questionnaire. Body weights were measured using a balanced scale while the subjects were lightly dressed and were barefoot. Height was measured using a stadiometer. Body mass index was calculated as weight divided by the square of height expressed in kg/m2.

Alendronate therapy and prescription of calcium and vitamin D supplements

Initially, we evaluated the density of vertebral bones in all participants. Then alendronate, (10mg/day), calcium (1gr/day) and vitamin D (400mg/day) were given to participants for 2 years. We followed them every 2 months in the osteoporosis clinic of Shariati hospital. Participants who showed any evidence of exclusion criteria were excluded from the study during each visit before the end of intervention time.

Laboratory measurements

Following an overnight fasting, 6 ml of peripheral

blood was taken and centrifuged in order to separate serum for measurement of serum parathyroid hormone, 25 hydroxyvitamin D, osteocalcin and cross laps.

Serum concentration of 25-hydroxy vitamin D3 was measured using a Biosource kit (Biosource Europe S.A, Belgium); intra- and inter-assay coefficients of variation (CV) were 5.2% and 7.5%, respectively (normal range: 2.5-75 ng/ml). Serum PTH concentration was also measured using a Biosource kit (Biosource Europe S.A, normal range: 13-66 pg/ml), with an intra- and inter-assay CV of 6.3% and 5.7%, respectively.

Osteocalcin, a bone formation marker, was measured by immunoassay (ELISA) using a Bioscience kit (Nortic Bioscience Diagnostic A/S, Denmark). The intra- and inter-assay CV were 2.6% and 4.7%, respectively. Another marker of bone resorption, namely the serum C-terminal telopeptides of type I collagen: serum crosslaps, was measured by immunoassay technique (ELISA) using a Bioscience kit (Nortic Bioscience Diagnostic A/S, Denmark), with intra- and inter-assay CV of 5.1% and 6.6%, respectively. Osteoprotegerin was measured by immunoassay (ELISA) using an Immunodiagnostic kit. The intra- and inter-assay CVs were 6.6% and 5.7% respectively. Serum sRANKL was measured by immunoassay (ELISA) using a Biomedical kit, with intra- and inter-assay CV of 4.1% and 5.1%, respectively. Serum concentration of interleukin-1beta (IL-1β) was measured by immunoassay (ELISA) using a R&D system kit (R&D system, USA); intra and inter-assay coefficients of variation (CV) were 4.8% and 4.1%, respectively. Serum Interleukin-6 (IL-6) concentration was also measured using a R&D system kit (R&D system, USA), with intra- and inter-assay CV of 2.4% and 4.7%, respectively. Serum TNF-α was measured by immunoassay (ELISA) using a R&D system kit (R&D system, USA); intra- and inter-assay CV were 4.8% and 6.1%, respectively.

Measurement of BMD

BMD was measured by DXA using Lunar DPX-MD device (Lunar Corporation, Madison, Wisconsin, 53713, USA) at lumbar spine (vertebrae L2-L4) and hip of all subjects. The DXA device was calibrated daily using appropriate phantom methods. Bone density was calculated based on gr/cm^2. The coefficient of variation for longitudinal BMD measurements in the DEXA machine averaged at 1.04%. Normal bone mass was defined as BMD measurements at or above -1 standard deviation (SD) from the optimal peak bone density (T-score) of healthy young adult of the same sex. BMD measurements at or below -2.5 SD from the optimal peak bone density of healthy young adults of the same sex was considered osteoporotic, as per World Health Organization standard (15).

Evaluation of vertebral fractures

Data on standardized radiographs for detected vertebral fractures was collected before intervention. The results of vertebral-fracture were also presented among women who continued in the study after 2 years. All X-rays were reported by the same radiologist. Fractures were identified by quantitative morphometry according to the guidelines of the US National Osteoporotic Foundation Working Group on Vertebral Fractures as previously described (16).

Extraction of genomic DNA

DNA extraction was carried out using FlexiGen Kit (QIAGEN Inc. Valencia, CA) from whole blood according to the manufacturer's instructions. The extracted DNA was stored at 4°C until it was used for PCR and RFLP analysis.

Genotyping

Genomic DNA from all subjects were analyzed for the presence of the G or C nucleotide at -765G/C of the cox-2 gene by a polymerase chain reaction (PCR) based Restriction Fragment Length Polymorphism (RFLP) assay. PCR amplification of a 309 bp fragment of the upstream region of the cox-2 gene containing the polymorphism was analyzed using previous study design (17) and used before PCR. Five µl of each PCR product, including the controls, were verified on a 2% agarose gel to ensure that the expected 309 bp product was generated. Restriction digest for the DNA fragment was carried out using *SsiI* restriction enzyme. The product of the restriction digest was verified on a 3% agarose gel. Difference between genotypes was determined by twith piece. The presence of a G at this piece generated a 309 bp fragment, while the 309 bp fragment was divided into three types of 309, 209 bp and 100 fragments. 100 bp and 209 bp fragments appeared when twith piece contained C. heterozygous, GC genotype. We used positive and negative samples as controls in all reactions and validity of this PCR-RFLP for COX-2 as well as genotyping was confirmed by sequencing of 15% of total PCR products.

Statistical analysis

Results are reported as the mean ± SD All statistical analyses were performed using the SPSS version 16 software. Student *t*-test was used to compare the means of quantitative variables but for comparing qualitative variables Chi-Square was used. ANOVA was used to compare the quantitative variable in different genotypes. P values less than 0.05 were considered to be statistically significant.

RESULTS

Sixty menopausal women with osteoporotic

bone disease participated in this study. The mean ± SD of age, BMI and menopause age of participants were 57.96± 8.71 years, 26.16±3.77 kg/m2 and 45.75±9.02 years respectively. Genotype frequencies of homozygous major allele (GG), heterozygous (GC) and homozygous minor allele (CC) were 61.7%, 33.3% and 5%, respectively.

Evaluation of vertebral fracture prior to alendronate therapy in participants demonstrated no significant differences between G and C alleles (21.2% in G allele *vs* 20% in C allele, p value=0.9). However, the difference appeared near to significant after alendronate therapy at the end of 24 months (67.7% in G allele *vs* 88.9% in C allele, pvalue=0.07). Furthermore, 78.3% patients had G allele and 21.7 % had C allele.

Table I demonstrates demographic and biochemical characteristic of participants according to allele. We detected significant differences in serum PTH levels and L2-L4 BMD between those with different alleles. As shown in Table II, our results demonstrated higher concentrations of inflammatory factors in C allele, compared to G allele. Besides, IL-1 proved to have higher concentrations in those with C allele. As shown in Fig. 1, a significant difference was noted between individuals with different alleles in assessment of the extent of fracture amongst participants (p=0.003).

DISCUSSION

Previous publications confirm findings of the present study, suggesting that variations in single nucleotide polymorphism in COX-2 gene might be responsible for the extent of vertebral fracture in menopausal women with osteoporotic disease (18).

We found that the 78.3% patients had G allele and 21.7 % had C allele. The results of a recent study by Sitarz et al. (19) reported the C allele was present in 41% in the control group among subjects from the Netherlands.

Our results show significant differences between

Table I. *Demographic and biochemical characteristics of participated according to allele.*

Characteristic	Allele		P value
	G	C	
Age (years)	57.53±8.45	59.95±9.83	0.2
BMI (Kg/m^2)	25.65±3.71	24.56±3.64	0.1
Serum 25-hydroxyvitamin D3 (nmol/dl)	31.55±19.36	28.38±19.85	0.6
Serum PTH (pmol/L)	16.10±5.04	30.92±14.46	0.001*
Hip BMD(gr/cm2)	0.88±0.09	0.84±0.1	0.1
BMD L2-L4 (gr/cm2)	0.98±0.16	0.89±0.13	0.01*
Serum Crosslaps (ng/mL)	0.67±0.42	0.74±0.42	0.9
Serum Osteocalcin (ng/mL)	18.02±9.06	15.87±8.29	0.3

Values are expressed as mean±SD
**Pvalue less than 0.05 is significant*
PTH: Parathyroid hormone; BMD: Bone Mass Density

Table II. *Serum concentrations of cytokines according to allele.*

Serum concentrations	Alleles		P value
	G	C	
Serum IL-1 (ng/mL)	0.5±0.04	0.96±0.02	0.03*
Serum IL-6 (ng/mL)	2.13±1.35	2.19±1.47	0.9
Serum TNF-α (ng/mL)	0.68±1.05	1.06±1.08	0.1
Serum OPG (pmol/L)	6.12±1.65	5.84±2.03	0.5
Serum RANKL (pmol/L)	0.54±0.63	0.64±0.63	0.2

Values are expressed as mean±SD
** P value less than 0.05 is significant*
IL_1: interleukin-1; IL_6: interleukin-6;TNF-α: tumor necrosis factor-α; OPG: Osteoprotegerin; RANKL: Receptor Activator for Nuclear Factor κ B Ligand

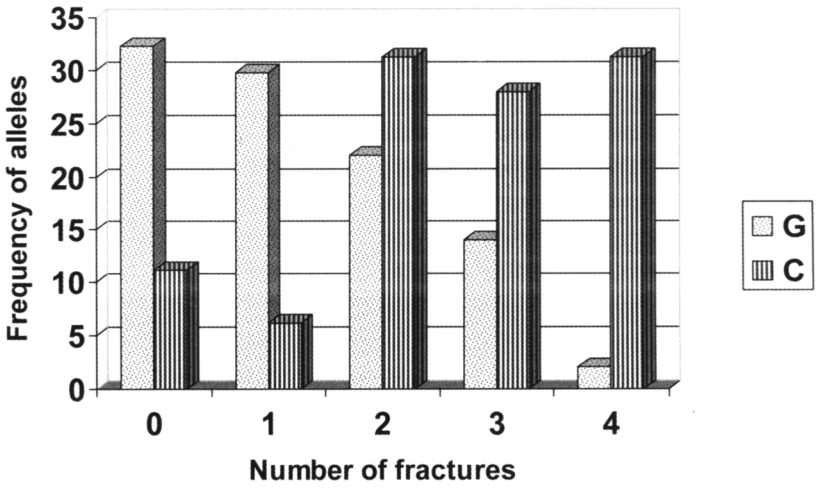

Fig. 1. *The frequency of fractures after 2 years intervention with alendronate with respect to the alleles.*

PTH concentrations in regard to different alleles. There is increasing evidence indicating a role for parathyroid hormone, in stimulating bone resorption and formation. Moreover, there is evidence indicating that PTH secretion is modified by PGE2 (20). This, in turn, takes place as a consequence of COX-2 function via the cyclic AMP-protein kinase, a finding that might explain a significant difference of PTH concentration between different alleles.

Moreover, our results demonstrate that variations in Cox-2 gene may be of some value in preventing vertebral fracture in osteoporotic patients who take alendronate. This might be explained partly by its effect on BMD. Thus, it appears that cox-

2 gene polymorphisms regulate the response to anti-resorptive treatments also in terms of severity of vertebral fracture. This finding highlights the importance of the patient s genetic background when selecting an anti-osteoporotic treatment.

Differentiation of pre-osteoclast cells into matured osteoclasts depends on stimulation of the receptor activator of nuclear κB (RANK), which is presented on pre-osteoclast cells, by RANK ligand (RANKL), expressed by osteoblasts (21). OPG (osteoprotegerin) binds to RANKL, thereby inhibiting its interaction with RANK, resulting in decreased osteoclast formation (22-23). Our findings demonstrate these significant correlations in G and C allele carriers. Carriers of G alleles had higher OPG and lower crosslap concentrations, a finding consistent with previous results.

The interdependent roles of IL-1, TNF-α and RANKL in osteoclast formation are recently demonstrated by Wei et al (24). It has been shown that TNF-α can stimulate osteoclast formation in the absence of osteoblasts without sufficient RANKL levels (25). This indicates the importance of inflammatory factors in bone turnover. Our results are similar; a higher TNF-α concentration was correlated with increased crosslap levels in C allele carriers and decreased levels in the G ones after 2 years of treatment with alendronate.

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