Association of Estrogen Receptor-α Gene PvuII Polymorphisms with the Effect of Calcium Supplementation on Skeletal Development in Chinese Pubertal Girls

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Objective To investigate the association of estrogen receptor alpha (ER-α) PvuII polymorphisms with the effect of calcium supplementation on bone development in Chinese pubertal girls, and to study the importance of calcium supplementation by maximizing the peak bone mass at their pubertal stage for bone development and osteoporosis prevention and the role of estrogen in regulating bone mass. **Methods** Ninety-four pubertal girls were recruited in the study and divided into two groups and three sub-groups according to the ER-α PvuII polymorphisms. One year before and after calcium supplementation, bone mineral density (BMD) was measured by DEXA, while BGP, BAP, TRACP5b, and 25-OH-VitD_3, as well as estrogen were detected by ELISA. Analysis of covariance was used to examine the effect of ER-α polymorphisms on bone development. **Results** The absolute increase and percentage change of BGP were significantly higher in the supplemented group than in the control group (P<0.05). In the intervened group, The increase and percentage change of the total body and radio distal 1/3 BMD were higher in PP than in PP genotype (P<0.05), and the increase of BAP in Pp was also higher than PP in the same group (P<0.05). **Conclusion** PP genotype shows a better response to calcium supplementation than the other PvuII polymorphisms.

Key words: Pubertal girls; PvuII polymorphisms; Calcium supplementation ; Skeletal development

INTRODUCTION

Osteoporosis is one of the major global public health problems, particularly in women[1]. Low bone mineral is an important risk factor for osteoporosis-related fracture[2]. Calcium is the major mineral in bone, and increasing dietary calcium intake has been proposed as an effective way to increase the peak bone mass. However, age and developmental stage at which calcium supplementation produces the greatest bone effect remain controversial. Randomized controlled trials of calcium supplementation found that increasing calcium intake can elevate the bone calcium accretion during childhood and adolescence, reduce fragility fractures, particularly in the elderly and probably in adolescents as well[3].

Recent studies also suggest that accretion of peak bone mass during adolescence and young adulthood is essential because at least 90% of bone masses are formed at this period of time[4], and may be difficult for a woman to accrue an additional bone mass later in life. Therefore, optimizing bone health and maximizing the peak bone mass during adolescence can reduce future risk of osteoporosis in later maturity years

It was reported that both attainment of peak bone mass in childhood and adolescence and bone loss after the completion of growth are under genetic control[5]. Among many other candidate genes, estrogen receptors (ER) have been proved to regulate bone mass[6]. Although a number of studies have dealt with the relationship between XbaI and PvuII polymorphisms of ER genes and bone mass and bone loss in pre-and postmenopausal women[7-9], few have focused on such relationships in adolescent girls, especially on the association of ER polymorphisms with the response to calcium supplementation.

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This study was to investigate the effect of calcium supplementation on bone mineral density (BMD) and bone turnover markers in Chinese pubertal girls. Special attention was paid to the association of ER-α PvuII polymorphisms with the response to calcium supplementation.

MATERIALS AND METHODS

Subjects

Ninety-four female students at the age of 13-15 years were recruited from middle schools in Huairou District of Beijing. Those with any family history of osteoarthrosis or on drugs for bone mass, or on blood infusion were excluded.

This study was approved by the Ethical Committee of the National Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention. All the individuals volunteered to participate in the study and informed consent was obtained from the participants and their parents or guardian.

Study Design

The subjects were randomly divided into control group and calcium supplementation group. The subjects in each group were stratified into three subgroups according to the PvuII polymorphisms. Recall of a 24 h diet for 3 consecutive days (two school days and one weekend day) was used to obtain the dietary intake. Calcium intake per day was calculated based on the China Food Composition Table[10]. The subjects in calcium supplementation group were supplied with fruity calcium chewable tablets (each containing 250 mg calcium carbonate and 60 IU vitamin D) postprandial post cibum for 12 months. The subjects in control group received the same taste placebo in a similar way. During the school days, tutors checked the compliance per day while the parents helped to check the calcium supplementation in weekend days.

At the baseline and endpoint, height and weight of the subjects in indoor clothing without shoes were measured at a standing position following a standardized procedure by trained interviewers. Body mass index (BMI) was computed as weight in kilograms divided by height in square meters (kg/m^2). Pubertal development was self-evaluated using the Tanner scale for sexual maturation.

Genotyping

Genomic DNA was extracted from leukocytes in 5 mL of peripheral blood with the standard phenol-chloroform procedure (DNA extraction kits, Huashun Biotech Co., Shanghai). The PvuII sites in estrogen receptor genes were detected using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Primers of amplified fragments were designed as previously described[11]. The sequences of upstream and downstream primers are 5'-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3' and 5'-TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA-3', respectively. PCR was started with an initial denaturation at 94 ℃ for 5 min, followed by 30 cycles with each at 94 ℃ for 30 s, at 61 ℃ for 40 s, at 72 ℃ for 90 s and a final extension at 72 ℃ for 10 min. After the PCR products were digested with restriction enzyme PvuII, genotypes were identified by electrophoresis of the DNA samples on 1.5% agarose gels. The PP genotypes produced a 1.3 Kb fragment and pp genotypes produced two fragments (850 bp and 450 bp). The Pp genotypes contained the above three fragments.

Bone Turnover Markers

Bone turnover rate was assessed by measuring serum osteocalcin (BGP, IDS Biotech Co., British), bone specific alkaline phosphatase (BAP, DSI Biotech Co., USA) as well as tartrate-resistant acid phosphatase 5b (TRACP5b, IDS Biotech Co., British) by ELISA. 25-OH-VitD_3 (DiaSorin Co., USA) and estrogen (Beifang Co., China) in serum were also measured by radio-immunity at the baseline and 12 months after the intervention.

BMD Measurements

The BMD of the total body and non-dominant forearm sites (distal 1/10, distal 1/3 and distal 1/3 radius) was measured by dual-energy X-ray absorptiometry (DEXA, A NORLAND XR-36, USA) at baseline and at the end of 12 month study period. Quality control was performed every day during the study period according to the manufacturer's instructions. The coefficient of variation (CV) % was 0.56-0.65.

Statistical Analysis

All statistical analyses were performed with SPSS 11.5. The variables were expressed as mean± SD. The effect of ER polymorphisms on BMD and bone turnover markers was evaluated by ANCOVA after adjusting for weight, vitamin D, BMI, and sex characteristics. Mean absolute and percentage changes from the baseline to the endpoint in the two groups were compared using independent *t* test and paired-samples *t* test was used to compare the mean

absolute and percentage changes in subjects of the same group. $P<0.05$ was considered statistically significant.

RESULTS

Characteristics of the Subjects

Among the 94 adolescent girls, 84 could be genotyped and analyzed for changes in BMD and bone turnover markers after 12 months, the other 10 were not counted because seven dropped out for being unable to continue to take calcium tablets as required, one was missed for transferring to another school, two were expelled for absence from blood sampling and BMD measurement on the final day of our study. The baseline characteristics of all subjects are summarized in Table 1. The average age of the subjects in control and calcium supplementation groups was similar (14.1 ± 0.8 and 13.8 ± 0.7 respectively). There was no significant difference in baseline height, vitamin D, and estrogen level between the two groups. However, the weight and BMI were higher in control group than in calcium supplementation group.

The subjects with pp genotype in control group had a higher weight than those with Pp genotype ($P<0.05$), and the subjects with PP genotype in calcium supplementation group had a higher Vitamin D level than those with pp genotype ($P<0.05$).

One year after intervention, no significant difference was found in the absolute change of all indexes ($P>0.05$, data not shown). On the other hand, the daily calcium intake was 326 mg according to the 24-h dietary records for successive 3 days and the final total calcium intake was about 1 000 mg per day ($326+250\times3=1076$ mg).

Bone Development Status after 1 Year Intervention

As shown in Table 2, bone mineral density (BMD), bone mineral content (BMC), and scan area (data not shown) in the total body, distal 1/10 forearm, distal 1/3 forearm and distal 1/3 radius all increased at the endpoint. However, the absolute change and annual percentage of BMD at all sites were similar in the two groups ($P>0.05$).

Serum BAP and BGP, markers of bone formation, all increased at the endpoint, while serum TRACP5b, a marker of bone resorption, reduced in both groups. However, the absolute change and annual percentage BGP were significantly higher in calcium supplementation group than in placebo group ($P<0.05$).

Association of PvuII Polymorphisms with the Change of BMD

As shown in Table 3, the BMD, BMC, and bone area (data not shown) at all sites increased significantly at the endpoint ($P<0.05$). After adjustment for weight and BMI, the absolute change and annual percentage of BMD at all sites were similar in those of control group with different PvuII genotypes.

After adjustment for weight, BMI, and vitamin D, the absolute change and annual percentage of the total body BMD and the annual percentage at distal 1/3 radius BMD were higher in PP than in Pp of calcium supplementation group.

Association of PvuII Polymorphisms with the Change of Bone Turnover Markers

After adjustment for weight, second sex characteristic development status and vitamin D, no significant difference was found in the absolute change and annual percentage serum BAP, BGP, and TRACP in the control group with the three PvuII genotypes (Table 4).

After adjustment for the same covariances, the absolute change and annual percentage of BAP of the PP genotype were significantly higher in calcium supllementation group than those of the Pp genotype ($P<0.05$).

DISCUSSION

Intervention with calcium supplementation at the pubertal stage for the peak bone mass plays an important role in the development of bone and prevention of osteoporosis.

Calcium functions as a threshold nutrient. In other words , calcium retention improves as calcium intake rises up to some threshold intake value, above which no further increased amount of its intake will alter its retention and below which skeletal accumulation varies with the amount of its intake[12], indicating that the effect of calcium supplementation is associated with calcium threshold, which may differ in different races. LA Jackman[13] reported that intake of 1 300 mg of Ca/d is the smallest amount that allows some adolescent females to achieve 100% of maximal calcium retention. Furthermore, Matkovic and Heaney[14] suggested that calcium retention in white adolescents reaches a plateau (threshold intake) at 1 500 mg of Ca/d. The threshold for black adolescents only ranges 400-600 mg[15]. In addition, a trial of 16-month calcium supplementation in a group of Chinese girls at the age of 13.6 years (n=154) indicated that subjects receiving 1 000 mg of Ca/d

TABLE 1

Baseline Characteristics of the Subjects ($\bar{x} \pm s$)

	Control Group				Supplemented Group			
	PP	pp	Pp	Total	PP	pp	Pp	Total
N (Baseline)	10	18	18	46	10	21	17	48
N' (1 Year Later)	10	14	16	40	10	18	16	44
Height (cm)	157.1±4.6	159.5±7.5	155.2±7.1	157.2±6.8	155.0.±7.6	158.3±5.9	157.4±4.4	156.3±5.9
Weight (kg)	49.4±6.1	55.2±14.1*	46.3±9.7*	50.2±11.3	44.2±7.1	49.1±10.7	48.4±7.8	47.0±8.9Δ
BMI (kg/m^2)	20.0±2.0	21.6±4.7	19.1±2.9	20.2±3.6	18.3±1.9	19.5±3.6	19.5±2.8	19.3±3.0Δ
Estrogen (pg/mL)	85.85±40.88	86.76±35.04	79.76±25.53	82.59±35.67	77.84±43.64	84.24±40.92	82.32±30.15	80.43±34.39
Vitamin D (ng/mL)	16.42±8.76	18.02±9.77	20.05±15.64	18.19±12.04	24.82±7.63*	13.73±5*	16.9±8.21	16.47±7.62

Note. $^{\Delta}P<0.05$ *vs* control group. $^{*}P<0.05$ *vs* other PvuII gene polymorphisms in the same group.

TABLE 2

Change in Biochemical Markers of BMD and Bone Turnover in the Two Groups 1 Year after Calcium Interventionn

Item	Control Group	Supplemented Group	*P* Value
BMD (g/cm^2)			
Total Body BMD			
Baseline	0.8234±0.0774	0.8034±0.0769	/
1 Year Later	0.8770±0.0706	0.8451±0.0641	/
Δ*	0.0535±0.0301	0.0493±0.0304	0.519
Δ%*	6.69±3.93	6.34±4.17	0.681
Distal 1/10 Forearm			
Baseline	0.2941±0.0482	0.2825±0.0437	/
1 Year Later	0.3297±0.0483	0.3182±0.0483	/
Δ*	0.0364±0.0195	0.0436±0.0202	0.09
Δ%*	7.33±1.15	7.73±1.11	0.053
Distal 1/3 Forearm			
Baseline	0.6083±0.0556	0.6101±0.0529	/
1 Year Later	0.6555±0.0519	0.6496±0.0514	/
Δ*	0.0475±0.0228	0.0448±0.0223	0.574
Δ%*	8.02±4.23	7.46±3.87	0.513
Distal 1/3 Radius			
Baseline	0.6125±0.0523	0.6120±0.0512	/
1 Year Later	0.6585±0.0508	0.6510±0.0505	/
Δ*	0.0465±0.0250	0.0435±0.0233	0.552
Δ%*	7.77±4.50	7.2±4.0	0.527
Bone Turnover Biochemical Markers			
BAP			
Baseline	27.93±13.69	27.24±18.65	/
1 Year Later	48.85±30.93	41.97±21.14	/
Δ*	23.27±20.98	17.77±12.94	0.144
Δ%*	42.6±20.3	41.2±24.1	0.777
BGP			
Baseline	36.08±16.63	32.99±13.47	/
1 Year Later	47.57±18.66	63.28±36.32	/
Δ*	17.12±11.36	32.10±33.57[a]	0.01
Δ%*	35.29±19.99	43.61±16.85[a]	0.041
TRACP			
Baseline	9.35±4.28	9.89±4.23	/
1 Year Later	2.73±1.37	2.96±1.38	/
Δ*	-6.62±3.78	-6.94±4.18	0.74
Δ%*	67.51±16.22	65.41±18.59	0.62

Note. *: Δ= (1 year later)-baseline; Δ% =Δ/baseline ×100%; [a]: $P< 0.05$ *vs* control group.

TABLE 3

Bone Mineral Density Change in Different PvuII Polymorphisms of the Two Groups ($\bar{x} \pm s$)

BMD	Control			Supplemented		
	PP	pp	Pp	PP	pp	Pp
Total Body						
0 Month	0.822±0.047	0.813±0.097	0.834±0.074	0.78±0.09	0.82±0.08	0.8±0.07
12 Months	0.872±0.043	0.867±0.087	0.889±0.066	0.83±0.07	0.86±0.08	0.84±0.05
Δ[a]	0.048±0.014	0.057±0.09	0.054±0.01	0.091±0.014*	0.043±0.024*	0.054±0.031
Δ%[b]	0.058±0.018	0.075±0.012	0.062±0.013	0.125±0.027*	0.054±0.033*	0.069±0.043
Distal 1/10 Forearm						
0 Month	0.301±0.043	0.280±0.039	0.304±0.058	0.29±0.05	0.29±0.04	0.28±0.04
12 Months	0.341±0.034	0.311±0.034	0.342±0.062	0.29±0.06	0.33±0.05	0.32±0.04
Δ[a]	0.041±0.009	0.036±0.006	0.044±0.006	0.056±0.008	0.047±0.025	0.043±0.018
Δ%[b]	0.146±0.033	0.135±0.022	0.139±0.023	0.220±0.057	0.173±0.089	0.163±0.072
Distal 1/3 Forearm						
0 Month	0.623±0.028	0.607±0.068	0.6±0.056	0.61±0.07	0.62±0.06	0.61±0.05
12 Months	0.664±0.026	0.65±0.06	0.656±0.056	0.64±0.06	0.66±0.06	0.65±0.04
Δ[a]	0.036±0.011	0.041±0.007	0.053±0.007	0.062±0.007	0.041±0.02	0.051±0.025
Δ%[b]	0.058±0.019	0.072±0.013	0.086±0.013	0.111±0.021	0.067±0.034	0.085±0.044
Distal 1/3 Radius						
0 Month	0.623±0.027	0.609±0.065	0.610±0.053	0.61±0.05	0.62±0.06	0.61±0.04
12 Months	0.662±0.025	0.654±0.062	0.661±0.052	0.65±0.05	0.66±0.06	0.65±0.04
Δ[a]	0.034±0.010	0.042±0.007	0.047±0.007	0.069±0.019	0.037±0.019	0.05±0.025
Δ%[b]	0.055±0.018	0.072±0.012	0.076±0.013	0.123±0.041*	0.061±0.032*	0.083±0.042

Note. Δ=(1 year later)-baseline; Δ%=Δ/baseline ×100%; [a,b]: adjusted by weight, VitD, and BMI; *: $P<0.05$ *vs* other PvuII polymorphisms in the same group.

TABLE 4

Change of Bone Turnover Markers in Different PvuII Polymorphisms of the Two Groups ($\bar{x} \pm s$)

		Control			Supplemented		
		PP	pp	Pp	PP	pp	Pp
BAP (μg/L)	0 Month	27.37±11.92	32.61±14.84	24.19±13.27	29.10±16.22	29.03±24.34	24.94±13.91
	12 Months	42.86±20.00	55.59±34.40	46.99±34.44	52.29±22.28	42.97±23.59	37.15±17.78
	Δ[a]	17.33±9.71	25.03±5.45	13.83±5.73	23.59±4.83*	14.33±3.29	9.99±2.47*
	Δ%[b]	48.1±9.3	38.8±5.2	34.3±5.5	47.4±10.3*	38.8±7.0	30.5±5.3*
BGP (μg/L)	0 Month	29.28±7.24	42.58±20.05	34.63±16.33	32.98±16.96	35.42±14.27	30.79±11.22
	12 Months	46.53±11.96	53.16±18.23	43.42±22.25	59.39±20.81	68.62±47.32	60.44±31.54
	Δ[a]	23.91±6.99	16.18±3.92	18.90±4.13	22.37±6.16	14.01±4.20	24.25±3.15
	Δ%[b]	42.1±11.4	31.1±6.4	46.8±6.7	38.7±7.4	32.6±5.0	44.1±3.8
TRACP (U/L)	0 Month	9.62±3.69	10.66±4.18	10.19±4.1	10.85±4.86	9.25±3.75	10.26±4.49
	12 Months	2.36±0.44	3.12±1.26	3.09±1.48	2.8±1.09	3.08±1.44	2.90±1.43
	Δ[a]	-8.13±1.89	-7.39±1.06	-5.18±1.11	-8.48±2.23	-7.95±1.52	-6.36±1.14
	Δ%[b]	77.3±7.4	66.6±4.2	67.3±4.4	73.3±8.4	72.6±5.7	62.9±4.3

Note. Δ=(1 year later)-baseline; Δ% =Δ/baseline×100%; [a,b]: adjusted by weight, VitD, and second sex characteristic development status. *: $P<0.05$ *vs* other PvuII polymorphisms in the same group.

have a higher bone mass annual percentage than those receiving less than 1 000 mg of Ca/d[16]. In our study, BMD was not significantly increased in those who received 1 000 mg of Ca/d for one year. Whether this level can reach a plateau in Chinese adolescent girls needs to be further studied. It may be appropriate to explore the possible alternative nutritional interventions, such as increasing vitamin D concentrations[17].

On the other hand, supplementation time is an important factor for the effect of calcium intervention. Bone rebuilding is a long process. Heaney[18] pointed out that bone metabolism may take 6-8 months to achieve a new balance under a normal intervention, and BMD change may occur after at least 2 years of calcium intervention[19-20]. However, in a very recent meta-analysis[21] of 19 studies involving 2 859 children at the age of 3-18 years, calcium supplementation (lasting at least three months and having bone outcomes measured after at least six months of follow-up) had no effect on bone density at the femoral neck or lumbar spine, but a slight effect on the total body bone mineral content and upper limb bone density, suggesting that the percentage of BMD at all measured sites is similar between the two groups 1 year after calcium supplementation, which is consistent with the reported data[21].

Biochemical markers of bone turnover can be used as indirect measures for bone formation and reabsorption[22]. It was reported that biochemical markers are more sensitive than BMD, and can respond to calcium supplementation in a shorter span of time[23]. It has been shown that increased bone reabsorption evaluated by specific biochemical markers is associated with increased risk of hip, spine and non-vertebral fractures independently of BMD[24]. Thus, serum BAP, BGP, and TRACP-5b were used to assess the association between PvuII polymorphisms, peak BMD, and effect of calcium supplementation. In our study, the absolute change and change percentage of BGP were higher in calcium supplementation group than in control group, which is consistent with the reported data[23-24]. The fact that BAP and TRACP were similar in those with different PvuII genotypes may be attributed to the high SD. Further study is needed to confirm our results by prolonging calcium supplementation time.

Although ER-α polymorphism is related with BMD, the results are controversial. Few reports have dealt with the association of ER polymorphisms with response to calcium supplementation. Zhang[25] reported that the XX genotypes have a poorer therapeutic response to calcium supplementation than the other two XbaI genotypes of ER in postmenopausal Chinese women. As founding in this study, the percentage changes of BAP, total body BMD and distal 1/3 radius BMD for PP were higher than for Pp and pp in the supplementation group, indicating that PP genotype has a good response to calcium supplementation and can be considered in later calcium supplementation intervention.

Since the adolescence is at a fast development stage, the effect of calcium intervention will be affected by a number of factors. Besides intervention time, threshold level and factors such as exercise, smoking, and other lifestyles might also contribute to their development[26]. Since this study was limited by the lack of assessment of physical activity, the role of physical activity could not be excluded.

In conclusion, assessment of the association between calcium supplementation and bone development needs to focus on more genetic factors. Further study is needed to confirm our findings.

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