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Contribution of the putative genetic factors and ANKH gene polymorphisms to variation of circulating calciotropic molecules, PTH and BGP

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It is well known that regulation of calcium homeostasis in bone remodeling is one of the most crucial factors for maintaining healthy bones. Parathyroid hormone (PTH) is probably the most important hormone that participates in the bone remodeling process. Another important biochemical factor governing bone metabolism is osteocalcin (BGP). Although the physiological functions of both of these factors are well known, there is still very little known regarding their specific genetic determination and in particular, the specific genes that may regulate the circulating concentrations of these substances. In the present study, we examined whether nine single nucleotide polymorphisms (SNPs) in the human homologue of the mouse progressive ankylosis gene (ANKH)—one of the key genetic factors involved in bone mineralization—can be associated with PTH and BGP levels in apparently healthy human populations. The study sample comprised 244 nuclear families (840 individuals). After adjustment of BGP and PTH for the significant covariates (sex, age and BMI), the contribution of the putative genetic effects was statistically significant ($P < 0.001$) for both biochemical factors: 45.27 $\pm$ 10.8% for PTH and 30.19 $\pm$ 12.6% for BGP. Application of transmission disequilibrium tests (TDTs) revealed a significant association ($P < 0.05$) between PTH and two SNPs: rs39968 and rs875525. However, the association became particularly significant for four TDTs (*P*-values ranging from 0.0025 to 0.0008) when the association with the haplotypes generated from the above SNP was tested. This association remained significant even after correction for multiple testing with a false discovery rate of 0.05.

INTRODUCTION

Age-related changes in bone and cartilage take place throughout human life. These changes cause bones to lose their mechanical integrity and eventually lead to an individual's diminished fitness and the increasing fragility of his bones. The molecular-genetic and metabolic mechanisms underlying this process are complex and are still not fully understood. It is, however, well-known that regulation of calcium homeostasis in bone remodeling is one of the crucial factors governing the health of bones. Parathyroid hormone (PTH) is probably the most important hormone participating in bone remodeling. This hormone is regulated by a negative feedback mechanism based on the blood's calcium levels. It produces both anabolic and catabolic effects in bone, depending on the mode of administration. Continuous treatment stimulates high bone turnover, resulting in net bone loss, whereas intermittent application increases bone mass by enhancing bone formation (1).

Another important biochemical factor associated with bone metabolism is osteocalcin (BGP–Bone Gla Protein). BGP is synthesized exclusively by osteoblasts, is well-known as a marker for differentiated mature osteoblasts and is an important determinant of the bone mineralization process (2,3). BGP concentrations increase according to the disease status and are characterized by high bone turnover such as renal osteodystrophy and osteoporosis (4). Several twin and familial studies have shown that both PTH and BGP circulating levels are strongly influenced by genetics, and this effect may modify genetic influences on bone mineral density (5).

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A wide variety of candidate genes have been investigated in relation to bone health status and osteoporosis outcomes (e.g. 6,7). The list of the candidate genes includes ERα, COL1A1, VDR, ANKH and many others. However, there are still very little data regarding the specific candidate genes that may regulate PTH and BGP circulating concentrations (5).

In the present study, we examined whether polymorphisms in the human homologue of the mouse progressive ankylosis gene (ANKH) are associated with PTH and BGP levels in apparently healthy human population. The ANKH gene is one of the key genetic factors involved in the regulation of hydroxipatite deposition and bone mineralization (8). It may therefore have a universal influence on the size and shape variation of different bones in the human skeleton. Indeed, some data suggest that the mutations in ANKH may cause ankylosing spondylitis and sclerosis of the craniofacial bones and abnormal modeling of the tubular bones (9). We have recently found that ANKH is strongly associated with bone size at various sites of the human skeleton (10,11). Previously, it was shown that ANKH is associated with familial calcium pyrophosphate dihydrate deposition disease, in which calcium-containing microcrystals are deposited in the joint cartilage and periarticular tissues (12). Thus, the ANKH gene appears to be a potentially promising gene that may affect PTH and/or BGP levels, deeply involved in calcium metabolism.

The major aim of the present investigation was therefore to ascertain the extent to which genetic factors contribute to variation of PTH and BGP circulating levels and to test whether the DNA polymorphisms of the ANKH gene can be associated with this contribution.

RESULTS

Descriptive statistics and familial effects

Descriptive statistics are presented in Table 1, showing the baseline characteristics for each studied trait according to gender. Biochemical indices data are presented in the original units before log-transformation. The plasma concentration in the total sample ranged from 10 to 144 pg/ml for PTH and 10–70.5 ng/ml for BGP. The average levels of PTH and BGP were higher in women than in men (Mann–Whitney U test, $P < 0.05$). The span of variation and the average values of all the variables were within the normal range for the respective gender. Correlations between each of the studied biochemical markers and potential confounders were examined, taking into account the familial structure of the studied sample (Table 2). Both PTH and BGP were significantly correlated with age. The distribution of both biochemical indices before and after adjustment for age and BMI were significantly skewed and therefore they were log-transformed before statistical–genetic analysis.

Familial correlations between the first-degree relatives were statistically significant ($P < 0.01$), suggesting a potential genetic influence on both variables. To examine more accurately the extent of the familial effects on the variation of each of the studied biochemical indices, we performed variance component analysis. The results of the analysis are given in Table 2, which shows maximum likelihood estimates of all potential sources of variation for PTH and BGP, respectively. In each case, the general model included the contribution of the potential covariates and familial effects. The corresponding most parsimonious models suggested a significant effect of age on the variation of both PTH and BGP, and in addition, a significant influence of BMI and stature on BGP variation. BMI effect on PTH variation was weak but statistically significant ($P < 0.05$). The additive genetic component explained 45.27 ± 10.8% of the variation of the PTH circulatory levels and 30.19 ± 12.6% of the BGP variation.

Table 1. Basic descriptive statistics (mean ± SD) for studied variables in the Chuvashian sample

Traits	Males, $n = 426$	Females, $n = 414$
Age (years)	45.5 ± 16.8	47.2 ± 16.4
Stature (m)	1.65 ± 0.06	1.54 ± 0.06
Weight (kg)	63.72 ± 10.4	59.71 ± 12.4
BMI (kg/m^2)	23.1 ± 3.2	25.1 ± 5.0
PTH (pg/ml)	45.3 ± 19.1	51.9 ± 17.5
BGP (ng/ml)	28.8 ± 11.1	30.9 ± 11.8

Adjusted for significant covariates, PTH and BGP showed a significant correlation only in females ($r = 0.2$, $P < 0.001$). The bivariate variance component analysis revealed that this phenotypic correlation is attributable to environmental factors shared by PTH and BGP ($r_E = 0.26 \pm 0.05$, $P < 0.001$), whereas the contribution of the pleiotropic genetic factors was negligible by likelihood ratio test ($P = 0.42$).

Association tests

The single nucleotide polymorphisms (SNPs) tested were the only ones that passed a Mendelian inheritance check and did not fail to be amplified in PCR reactions even after three times of repeated measurement. When testing the association of the nine SNPs, located inside and outside of the ANKH gene, with the studied biochemical indices, we found a significant association of PTH levels with the rs875525 marker, located between exons 6 and 7 and with the rs39968 marker, located some 150 kb downstream from ANKH (Table 3). Despite the 176.5-kb distance between these two markers, they were in a highly significant linkage disequilibrium ($D' = 0.48$, $P < 10^{-6}$). As shown in Table 3, the haplotype A–C of these two markers showed a consistently reliable association with PTH in all four implemented TDTs (with P-values ranging from 0.0025 to 0.0008).

At the second stage of association analysis, we included four additional DNA markers. Two added SNPs (rs3045, rs2288474) had very low MAF ($q = 0.04$ and 0.08, respectively) in our sample and were not used in the association analysis. Two other markers (rs2291943, M05HT1) were sufficiently polymorphic (Table 4) and used in the association tests. As seen in Table 3, all these markers and their haplotypes with rs39968 marker showed significant association with the PTH levels. Moreover, haplotypes generated from the different combinations between these new markers also showed significant association with PTH (Table 3). We used the false discovery rate (FDR) method to correct our results for multiple testing. For PTH, the total number of tests for

Table 2. Variance component analysis of PTH and BGP in Chuvashian pedigrees

Parameters	PTH		BGP	
	General	MPS	General	MPS
Regression coefficients				
α_m	−0.202 ± 0.070	−0.157 ± 0.054	−0.213 ± 0.068	−0.200 ± 0.067
α_f	0.253 ± 0.073	0.189 ± 0.053	0.126 ± 0.072	0.121 ± 0.051
$\beta_{1\text{-}m}$	0.208 ± 0.051	0.182 ± 0.043	−0.243 ± 0.052	−0.248 ± 0.050
$\beta_{1\text{-}f}$	0.126 ± 0.107	0.106 ± 0.045	0.176 ± 0.055	0.1818 ± 0.048
$\beta_{2\text{-}m}$	0.119 ± 0.059	0.118 ± 0.059	−0.182 ± 0.060	−0.158 ± 0.035
$\beta_{2\text{-}f}$	0.039 ± 0.043	[0]	−0.137 ± 0.042	−0.158!
$\beta_{3\text{-}m}$	0.068 ± 0.068	[0]	0.150 ± 0.068	0.145 ± 0.068
$\beta_{3\text{-}f}$	0.109 ± 0.075	[0]	0.012 ± 0.076	[0]
Variance components				
V_{AD}	0.299 ± 0.407	0.412 ± 0.099 (45.27 ± 10.8%)	0.222 ± 0.082	0.272 ± 0.114 (30.19 ± 12.6%)
V_{SP}	0.058 ± 0.096	0.079 ± 0.035 (8.68 ± 3.8%)	0.103 ± 0.035	0.106 ± 0.044 (11.76 ± 4.9%)
V_{HS}	0.039 ± 0.206	[0]	{0}	[0]
V_{SB}	0.036 ± 0.029	[0]	0.039 ± 0.026	[0]
V_{RS}	0.477 ± 0.302	0.419 ± 0.102 (46.04 ± 11.2%)	0.534 ± 0.079	0.523 ± 0.130 (58.05 ± 14.4%)
−LogLH	1089.34	1091.89	1123.27	1124.74
χ^2	5.1		2.94	
d.f.	5		4	
p	0.40		0.57	

Parameter estimates shown under general and most parsimonious (MPS) models.
$\alpha_{\text{-}m}$, $\alpha_{\text{-}f}$ are sex-specific intercepts (m-male, f-female); $\beta_{1\text{-}m,\text{-}f;}$ $\beta_{2\text{-}m,\text{-}f;}$ $\beta_{3\text{-}m,\text{-}f\ \text{-}}$ are sex-specific regression coefficients for age, body mass index and stature, respectively. Variance components: V_{AD}, additive genetic, V_{PR}, common parental environment, V_{HS}, common household, V_{SB}, common sibling, V_{RS}, residual. LogLH, log likelihood; df, degrees of freedom; parameter value (±) standard error estimate is shown; {0}, parameter reached the limit of possible range; [0], parameter was fixed at the indicated value; !, constrained to equal parameter in the above row.

Table 3. Association between PTH and markers of the ANKH gene (*P*-values of tests are given)

Marker	Stage 1			Stage 2				
TDT	rs39968	rs875525	[a]rs39968–rs875525	M05HT1 STR	rs2291943	[a]rs39968–M05HT1	[a]rs39968–rs2291943	[a]rs2291943–M05HT1
EOT	0.0098	0.0386	**0.0010**	**0.0010**	0.0110	**0.0032**	**0.0021**	**0.0009**
OTP	0.0256	0.1616	**0.0025**	0.0587	0.0653	**0.0023**	*0.0043*	0.0106
OT	0.0217	0.0604	**0.0020**	0.0645	0.0193	*0.0053*	**0.0028**	**0.0015**
FBAT	0.0165	0.0837	**0.0008**	0.0473	0.0172	**0.0032**	**0.0010**	**0.0003**

EOT, extreme offspring design *t*-test; OTP, the orthogonal test after adjustment of offspring phenotypes for parent phenotypes; OT, the orthogonal test without adjustment; FBAT, the family-based association test. Bold-face values are *P*-values significant on the FDR level 0.05; italic, FDR level 0.1.
[a]Haplotypes between the corresponding markers.

10 SNPs, 6 dichotomous schemes for STR alleles and 24 pairwise haplotypes multiplied by 4 employed TDTs gave 160 tests. In addition, the association was examined between the nine SNP and BGP but no significant association was found. So the total number of tests during both stages achieved 196. After FDR correction, 16 tests were significant at FDR = 0.1, with limiting $P = 0.0053$ (the *P*-value required to achieve significance level). Furthermore, 14 of these tests were significant with FDR = 0.05, limiting $P = 0.0032$ (Table 3).

To examine finally the consistency of the observed association of the PTH levels with the above chromosomal region, we divided the whole sample into two groups: (i) the genotypes carrying one or two copies of the haplotype A–C (rs39968 and rs875525 markers) and (ii) the genotypes lacking this haplotype. Figure 1 shows the consistent trend in the total sample and in both sexes, clearly suggesting that the A–C carriers tend to have lower PTH levels, regardless of sex. However, this trend is more prominent in females. Similar testing of other haplotypes confirmed these results, unequivocally suggesting significant association of the PTH variation with this chromosomal block (14.600–14.813 kb).

DISCUSSION

The major aim of our study was to establish the extent of genetic variation and covariation of the circulating levels of two main biochemical factors related to calcium metabolism and bone turnover: PTH and BGP. In addition, we examined the hypothesis whether the ANKH gene affecting variation of several skeletal traits (e.g. 9–11), also influences variation

Table 4. Characteristics of the chosen DNA markers relatively to ANKH gene location and their association with bone strength phenotypes

	Exons	SNP	SNP location (in Mb)	Rare allele frequency	Bone Phenotype	HB
		rs835141	14.941	q=0.43 (T)		B1
		rs835154	14.929	q=0.47 (G)	ST, WT, BB, VC, PC	
ANKH	1	hCV3191922	14.921	q=0.23 (A)		B2
		hCV11658675	14.829	q=0.35 (C)	WT, BB, VC, PC, AS	
	2				CC	
		rs258360	14.816	q=0.12 (T)		B3
		rs258215	14.816	q=0.45 (T)		
	4					
		rs2291943	14.804	q=0.42	**PTH,** BB, PC	
	5					
					WT, BB, PC	
	6	rs875525	14.799	q=0.18 (T)	**PTH**	
	8					
					AS	
	9					
	12				CC, BIS	
		rs2288474	14.795	q= 0.08		
		rs3045	14.764	q= 0.04		
		M05HT1	14.701	* q=0.48	**PTH**, WTS, BIS	
		(STR)		(6-alleles)		
		rs39968	14.623	q=0.37 (A)		
		rs152628	14.603	q=0.36 (C)		

The location of the studied SNPs within and in a vicinity of ANKH shown according to SNPbrowser™ (Software v.2.0.8). Relevant ANKH exons are indicated by numbers 1–12. Bone strength phenotypes significantly associated with the ANKH gene are denoted by their initial letters: ST and WT are body height and weight, BB, biacromial breadth; VC, vertebral column length, PC, first factor score of age-adjusted ST, WT, VC and BB; WTS and BIS, body weight and biiliac diameter adjusted for stature (10,11); AS, ankylosing spondulitis (19); CC, chondrocalcinosis (29); PTH, parathyroid hormone (present study). HB, are haploblocks ($B_{1,2,3}$) based on the International HapMap Project data for the population of the Western European origin.

[a]Frequency of the most frequent allele.

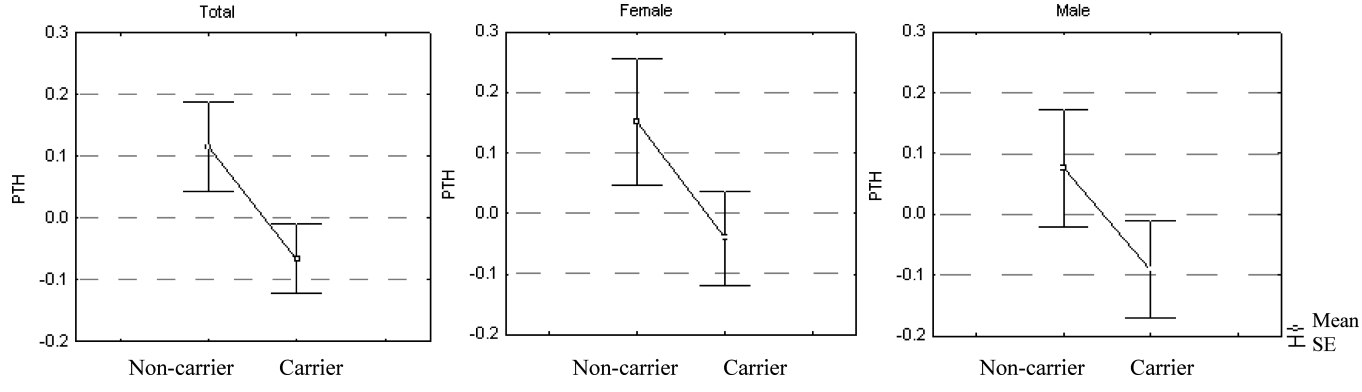

Figure 1. Comparison of the average PTH levels in carriers and non-carriers of the A–C haplotype of SNPs rs39968–rs875525 (the data were adjusted for age and standardized; group mean ± SE are shown).

of PTH and BGP. Our sample consisted of 244 nuclear families of apparently healthy volunteers, whose PTH and BGP variation was within the ranges of normal variation, as reported in other studies (13,14). The model fitting analysis of the present data (Table 2) showed modest, although significant, effects of age and BMI on the variation of both biochemical factors. The main source of the interindividual differences in the PTH level in our sample was putative genetic effects, which explained some 45.27% of the total variation. The contributions of genetic influences were also important for BGP variation (30.19%), and the statistical significance of the genetic effects was highly significant ($P < 0.001$) in both instances.

This substantial contribution of genetic factors to variation of PTH and BGP is in good agreement with the results provided in our former studies (15,16) as well as in other publications (17,18). The latter study (18), for example, reported somewhat higher but still comparable heritability estimates for these markers in the UK sample of twins. Similar to the present data, their estimates of the genetic effects for BGP were substantially lower than those observed for PTH (0.29 versus 0.61, respectively). Of particular interest was the fact that the above study also detected significant common sibs' environmental components for BGP variation (0.34), but as in our sample, it was not significant in PTH variation. It should be mentioned, however, that the phenotypic correlation between PTH and BGP was very low in the present sample ($r = 0.20$, $P < 0.05$) and was fully explained in shared environmental factors.

The lack of a genetic correlation was in agreement with our results of testing for an association with ANKH gene polymorphisms. A significant association was found only between some DNA markers and PTH. Of special interest here was the highly significant association with haplotype A–C generated from two SNPs: rs39968 and rs875525. The *P*-values by four different TDTs varied between 0.0025 and 0.0008. When we examined association of a few more polymorphic markers in this region with PTH, the obtained results were in full agreement with the above association (Table 3). Moreover, testing this association by graphical methods using contrasting carriers of haplotype A–C (rs39968–rs875525) versus non-carriers of this haplotype also well confirmed the observed association (Fig. 1). The reason for this association between the two SNPs separated by 176.5 kb is not obvious. It can probably be explained in a specific pattern of LD in this chromosomal region. According to the data available in International HapMap Project (http://www.hapmap.org), despite the apparent general tendency of disequilibrium decay with distance between markers, the disequilibrium pattern around ANKH is complex. The size of 'perfect' haploblocks in the region is <10 kb, but there are numerous SNPs showing high and significant LD levels, and they belong to different, often distant blocks. This may also be indicative of a few potential functional polymorphisms in the studied chromosomal area.

In attempting to clarify this issue, we combined all available published data on skeletal traits associated with ANKH, which are presented in Table 4. The figure shows that there are a number of bone and skeletal size traits associated with various polymorphisms in this gene. The situation with the association of these traits is also often complex. For example, ankylosing spondylitis is associated with different sex-specific markers separated by 60.8 kb (19). In fact, only in the male sample was an association found with the rs27356 and rs26307 markers located 16.8 kb apart. We have previously observed several bone mass traits associated with marker rs39968 and others in the vicinity (10,11). Interestingly, however, these associations were particularly significant after adjusting the mass traits for size (stature). For instance, body weight and biiliac diameter adjusted for stature showed a significant association with marker rs39968, with $P = 0.002$ and 0.003, respectively (11). This suggests that the above association with PTH is not likely to be spurious. However, examination of the available databases (OMIM, NCBI and dbSNP) showed no known functionally relevant genes in this chromosomal area. There are two genes with unknown function in this region [FLJ11127, TRIO (http://www.ncbi.nlm.nih.gov/mapview/)]. It is possible that they also involved in the regulation of calcification and affect PTH variation, in particular. Presently, there is a growing body of evidence suggesting that the gene regulatory elements may be distantly located from the structural gene itself (20). It is therefore clear that this region requires further narrowing down of the association peak with concomitant sequencing to establish potentially important functional polymorphism(s).

MATERIALS AND METHODS

Sample

The study cohort consisted of a community-dwelling sample of 840 healthy individuals: 426 men and 414 women, aged 19–75 who belong to 244 nuclear families. The subjects were derived from a large sample of pedigrees recruited within a framework of our general project in the field of human skeleton aging within the Chuvasha population.

The Chuvasha population resides mostly in a rural area with practically no exposure to outside influences such as genetic flow. These people are ethnically Caucasians (descendents of Bulgar tribes) living in numerous small villages on the Volga riverside in Russia. The inclusion criteria were as follows: apparently healthy individuals who had neither chronic or acute infection, nor hematological, metabolic or other bone-related diseases. In addition, they had not received prescription medications or steroidal anti-inflammatory drugs on a regular basis, nor had they consumed vitamin, mineral or other dietary supplements. Basic demographic information was collected including age, gender, occupation, smoking habits, alcohol use and medical history. In addition, blood samples and anthropometrical measurements were taken from each individual participating in the study. Further description of the sample is given in Livshits *et al*. (21). All participants were unaware of the specific hypotheses tested, and signed an informed consent document to participate in the study, which was conducted with the approval of the Ethics Committee of Tel-Aviv University.

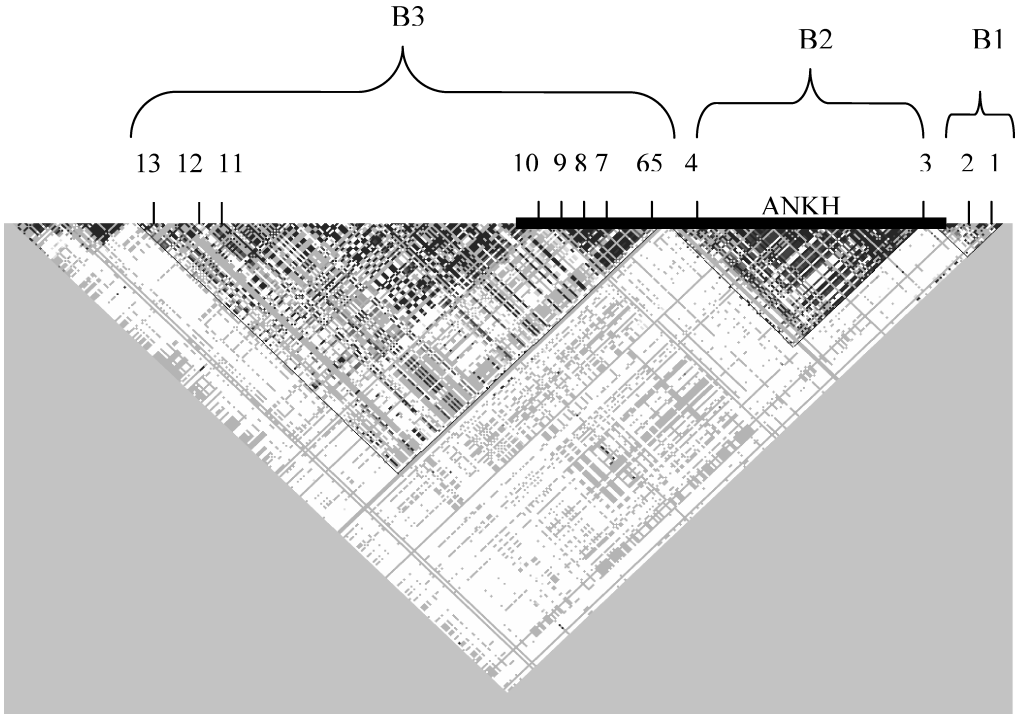

Figure 2. Diagram of the chromosomal location of the studied SNP at ANKH gene (5p15.2–p14.1). The upper part of the figure shows the location of 13 DNA markers used in the study: 1, rs835141; 2, rs835154; 3, hCV3191922; 4, hCV11658675; 5, rs258360; 6, rs258215; 7, rs2291943; 8, rs875525; 9, rs2288474; 10, rs3045; 11, rs39968; 12, M05HT1; 13, rs152628. The lower part illustrates the LD pattern showing the R^2 disequilibrium levels based on the International HapMap Project data for the population of the Western European origin. For further explanation see Table 4.

PTH and BGP measurements

Blood samples were collected by standard technique after a 12-h overnight fast. Plasma was separated from whole blood samples and stored at -80°C until assaying. Intact PTH was measured by immunoradiometric assay using the INTACT PTH DSL-8000 kit (Diagnostic Systems Laboratories, Webster, TX, USA) according to our previous protocol (22). Plasma-intact osteocalcin was measured by immunoradiometric assay using the ALSA-OSTEO kit (*CIS* Bio International, ORIS group, France) and following the manufacturer's instructions.

Genotyping

DNA was prepared from peripheral blood lymphocytes by standard techniques. Genotyping was performed by Pyrosequencing™ on the PSQ™HS96A system (Biotage AB, Uppsala, Sweden). All 840 individuals were genotyped for nine SNP markers in the ANKH gene from the NCBI dbSNP database and SNP browser™ Applied Biosystems. These markers were selected in vicinity of markers that were examined in our previous study of bone phenotypes and showed significant association with at least one bone phenotype (10). Additional criteria of the markers' selection included: (i) MAF $\geq$0.2, according to SNP browser, (ii) the tagging of the surrounding markers. The genotyping was carried out under the supervision of Dr M. Korner at the Center for Genomic Technologies, the Hebrew University of Jerusalem, Israel. Four additional markers—three SNP (rs2291943, rs3045, rs 228874) and one STR (MO5HT1) were previously tested by our team (10) and were used in the second stage of the present analysis. Table 4 and Figure 2 show the chromosomal location of the studied markers and frequency of the rare alleles. The genotype distribution in the total sample and offspring sample, whose phenotypic values were used in the association analysis were in H-W equilibrium. Further details on this gene were given by us recently elsewhere (11).

Statistical analysis

Basic preliminary statistical analysis and selection of the potential covariates (age, sex, stature and BMI) were conducted by implementing the MAN-7 statistical package (23) that takes into account familial composition of the sample. To estimate the extent of familial and possible genetic influences on PTH and BGP levels, we conducted two mutually complementary analyses. Analysis of familial correlations was performed after adjustment for significant covariates. In order to determine the putative genetic influences on the PTH and BGP levels, we performed a pedigree-based variance decomposition analysis (24). This method enables evaluation of the following components of variation: the additive genetic component (V_{AD}), three common family environment components, namely, the shared spouse environment component (V_{SP}), the common household environment component (V_{HS}) and the shared siblings environment (V_{SB}) that is specific for siblings raised together. The unexplained residual influences component was defined as V_{RS}. Next, we conducted

bivariate pedigree-based variance decomposition analysis for PTH and BGP. The application of the above method was described in numerous recent publications (24). MAN-7 package was used to conduct all family aggregation analyses.

Once the familial aggregation of PTH and BGP was established, we performed transmission disequilibrium tests (TDTs) of the selected DNA markers with PTH and BGP levels adjusted for significant covariates. We examined four types of this test. Each test takes as the unit of observation a nuclear family in which one or more parents is heterozygous at the marker polymorphism (this enables one to avoid the possible influence of population stratification). The first two types of the implemented TDT were two modifications of the orthogonal test (OT), described by Abecasis *et al*. (25). OT, based on the orthogonal decomposition of genotype scores, is a maximum likelihood test of the significance of the additive impact of a within-family genotype score on the phenotype. One of the test implementations is performed after adjustment of offspring phenotypes for parent phenotypes (OTP) and the other one is without it (OT). The third test is the family-based association test (FBAT), proposed by Horvath *et al*. (26).; it is implemented in the FBAT program. This test estimates a similar hypothesis: the independence of the phenotype from a specific genotype coding, but it is based on a different statistical algorithm. The fourth test is the extreme offspring design *t*-test (EOT), proposed by Malkin *et al*. (27).; it is implemented in the MAN-7 program. EOT evaluates the standardized difference in trait means between the 'extreme' offspring who have received a certain marker allele from their heterozygous parents and those to whom this allele has not been transmitted. For two adjacent markers that showed a significant association with PTH in some TDTs, we reconstructed pairwise haplotypes using MAN-7 (23) and analyzed the association of each of four possible haplotypes with PTH.

Finally, we used the FDR approach to correct for the total number of all tested markers. This approach was proposed by Benjamini and Yekutieli (28) for multiple testing under dependency. It was implemented to reject or accept a number of different null-hypotheses, some of which are probably true.

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Conflict of Interest statement. None declared.

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