

# Bone Mineral Density-Affecting Genes in Africans

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**Background:** We have recently reported the role of environmental exposure in the ethnic diversity of bone mineral density (BMD). Potential genetic difference has not been adequately assessed.

**Purpose:** To determine allele frequencies of BMD-affecting genes and their association with BMD in Africans.

**Methods:** Allele frequencies at 18 polymorphic sites in 13 genes that affect BMD in Asians and/or Caucasians were determined in 143 recent immigrants (55 men and 88 women, 18–51 years of age) from sub-Saharan Sudan to the United States. Genetic association studies were performed.

**Results:** Among the 14 single-nucleotide polymorphisms (SNPs), 10 were significantly different in allele frequency between Sudanese and Asians, and 10 between Sudanese and Caucasians. Only the osteocalcin gene was not significantly different in allele frequency among Sudanese, Asians and Caucasians. Allele frequencies in the *TGFB*, *COL1A1* and *CSR* genes were extremely low (<0.04) in the Sudanese. Frequencies of microsatellite alleles in four genes were significantly different among Sudanese, Asians and Caucasians. SNPs in the *VDR* and *ER $\alpha$*  genes were associated with BMD and/or BMC (bone mineral content) at several bone sites.

**Conclusions:** Genetic difference may play a role in the ethnic diversity in BMD and/or BMC.

**Key words:** Africans ■ Sudanese ■ bone ■ genetics ■ polymorphism

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## INTRODUCTION

We have recently reported that bone mineral density (BMD) of the spine in recent sub-Saharan Sudanese immigrants in the United States was significantly lower compared with the normative values of African Americans and Caucasians.<sup>1</sup> Their total body and hip BMDs were positively correlated with their length of stay in the United States. In addition, hip BMD was significantly correlated with milk intake and marginally with their length of stay in the United States, independent of body weight.<sup>1</sup> These findings suggest a potential role of environmental factors in the ethnic diversity of BMD. Racial/ethnic difference in allele frequencies of BMD-affecting genes may exist and play an important part, which has not been adequately assessed.

Studies have shown that polymorphisms at >30 candidate gene loci are associated with BMD in Asians and/or Caucasians, suggesting genetic effect on BMD.<sup>2,3</sup> Therefore, a potential ethnic difference in allele frequencies in these genes is likely to contribute to ethnic difference in BMD. However, few have reported allele frequencies of BMD-affecting genes in Africans living in the African continent.<sup>2–4</sup> Although certain polymorphisms of BMD-affecting genes have been investigated among African Americans,<sup>5–12</sup> their allele frequencies may not represent true frequencies in Africans living in the African continent because native African Americans have 7–26% of Caucasian genetic background.<sup>13</sup> Also, previously reported phenotype-genotype association in native African Americans might be a spurious one due to racial admixture.<sup>2</sup> Thus, it is desirable to determine gene allele frequencies and genotype-phenotype association in more homogeneous populations from Africa.

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We have recently demonstrated that an association between polymorphisms and BMD in the three major races (Africans, Caucasians and Asians) would suggest that the linkage disequilibrium had occurred before their divergence.<sup>2</sup> This would further imply that these BMD-affecting loci are likely to be within the candidate genes rather than in genes far away.<sup>2</sup> Thus, from this perspective, it is also important to perform association studies in Africans from the African continent. Because of civil war, many Sudanese from southern Sudan (sub-Saharan) have migrated to the United States recently, which provides a convenient sample to study. Based on allelic distribution pattern of several genes, studies have shown that Africa is not only the homeland of populations of the recent African

diaspora but also the homeland of all modern humans.<sup>14</sup> It would be interesting to see whether bone-affecting gene alleles follow the same pattern.

## METHODS

### Subjects

The subjects and their demographic information and BMD data have been reported previously.<sup>1</sup> In summary, a total of 143 recent Sudanese immigrants from southern Sudan (88 women and 55 men, aged between 18–51 years) to the United States were studied. Women were all premenopausal with normal menstrual cycles. Creighton University's institutional review board approved this study.

**Table 1. Allele frequencies of SNPs at 11 candidate genes in sub-Saharan Africans (Sudanese) versus Asians and Caucasians**

Gene	Enzyme	Primer	Sudanese				Asian			Caucasian				
			Ref	Freq.	Freq.	n	p	Ref	Freq.	n	p	Ref		
COL	MscI	TAACTTCTGGACTATTTGCGGACTTTTGG	49	0.032	0.000	583	0.001	15	16	17	0.188	966	0.001	27
		CAACCTCAGCCCATTGGCGCTG												
PTH	BstBI	CATTCTGTACTATAGTTTG	28	0.843	0.859	804	0.470	18	0.620	91	0.001	28		
		GAGCTTTGAATTAGCAGCATG												
GLA	HindIII	CCGCAGCTCCCAACCACAATAAGCT	19	0.762	0.800	160	0.155	19	0.799	630	0.164	29		
		CAATAGGGCGAGGAGT												
CSR	BsaH	CTGAGCTTTGATGAGCCTCAGAAGGAC	50	0.979	0.970	767	0.240	20	0.866	353	0.001	20		
		CACTGATGACCAAGCTCTGTGAAGTGA												
CTR	AluI	CTCAGTGATCACGATACTGTG	21	0.399	0.115	152	0.001	21	0.625	307	0.001	30		
		ATTCAGTGAACCAGCGTTGG												
ER	PvuII	CTGCCACCCTATCTGTATCTTTTCTATTCTCC	51	0.715	0.635	767	0.011	20	0.590	353	0.001	20		
		TCTTCTCTGCCACCCTGGCGTGCATTATCTGA												
	Xba	Same as for PvuII	51	0.821	0.760	767	0.023	20	0.690	353	0.001	20		
VDR	FokI	AGCTGGCCCTGGCACTGACTCTGCTCT	11	0.161	0.375	1433	0.001	22	0.341	405	0.001	31		
		ATGGAACACCTTGCTTCTTCCCTC												
		CAGAGCATGGACAGGGAGCAAG												
	Taq	GCAACTCCTCATGGCTGAGGTCTCA	52	0.395	0.115	1433	0.001	22	0.434	114	0.371	33		
	Apal	Same as for Taq	52	0.378	0.731	417	0.001	23	0.380	353	0.950	20		
TGFB	BstUI	GCTAGCCAGCTGGTGTIAT	24	0.997	0.589	287	0.001	24	0.608	3075	0.001	33		
		ACCACTCTGGGAGAAGGGTA												
AHSG	Sacl	GTAAGGCAACACTCAGTGA	53	0.291	0.260	767	0.239	20	0.370	353	0.017	20		
		TCATCTCTGCCATGTCTAG												
IL-6	BsrBI	GAGACGCCTTGAAGTAACTG	25	0.750	0.826	470	0.004	25	0.566	3376	0.001	34		
		AACCAAAGATGTTCTGAACTGA												
OPG	RsaI	TTCATGCTAAGATGATGCC	54	0.872	0.730	259	0.001	26	0.845	310	0.310	35		
		ATCCTAATTAATTTGCTGCAC												

n=143 for Sudanese; p values were derived when comparing the frequency with that of the Sudanese; COL: collagen type 1 $\alpha$ 1 (COL1A1); PTH: parathyroid hormone; GLA: osteocalcin; CSR: calcium-sensing receptor; CTR: calcitonin receptor; ER: estrogen receptor  $\alpha$ ; VDR: vitamin D receptor; TGFB: transforming growth factor- $\beta$ 1; OPG: osteoprotegerin; AHSG:  $\alpha$ 2HS-glycoprotein; IL-6: interleukin-6

## Genotyping

Genomic DNA was extracted from leukocytes with a Puregene kit (Gentra System) according to manufacturer's instructions. Polymerase chain reaction (PCR) was performed with primers specific for 18 polymorphic sites [among them, 14 were single nucleotide polymorphisms (SNPs)] in 13 genes that have been reported to be associated with BMD in Asians and/or Caucasians. PCR primers for these candidate genes, PCR conditions and endonucleases used are described in the references cited in Tables 1 and 2.<sup>11,19,21,24,25,41,42,49-56</sup> Polymorphisms in most of these genes had been reported to be associated with BMD by  $\geq 2$  independent articles.<sup>2,3,5-12,15-56</sup> PCR products were digested with appropriate enzymes that distinguish different SNP alleles. Microsatellite marker alleles were determined by the size of the nucleotides. Gel electrophoresis was performed to determine the genotypes for each individual. Genotype based on microsatellite alleles was determined by the presence or absence of a particular allele that had been reported to be significantly associated with BMD in other ethnic groups.<sup>36-39,41-43,55,56</sup>

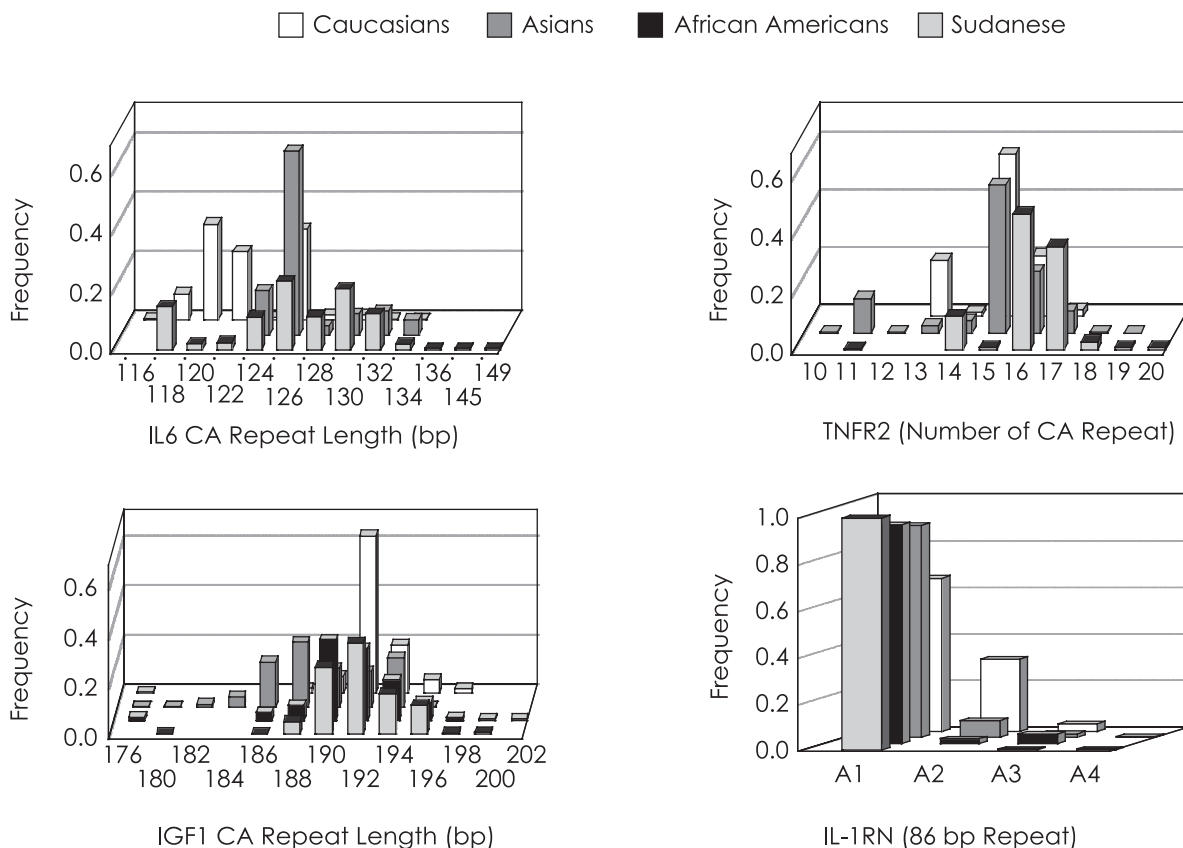
## Sequencing

To determine the age of most recent common ancestors based on polymorphisms of vitamin-D receptor and estrogen receptor- $\alpha$  genes, the most extensively studied genes, we sequenced bidirectionally a fragment of approximately 800 bp (*VDR*) and 1,300 bp (*ER $\alpha$* ) in 10 individuals from each of the four ethnic groups: Sudanese, Native Americans, Chinese and Caucasians. PCR products were purified with Qiagen column. The PCR products were sequenced directly (Lark Technology, Houston, TX) with primers that amplify the DNA segments used for genotyping that contain the *Taq* and *PvuII* and *XbaI* polymorphic sites for *VDR* and *ER $\alpha$* , respectively.

## Statistical Analysis

The significance of difference in allele frequency (SNPs or microsatellite markers) among ethnic groups was determined by Chi-squared test or Fisher's exact test. Hardy-Weinberg Equilibrium test (including exact HWE test) was performed. Bonferroni correction was used to determine the critical  $\alpha$  values for statistical significance. We used multiple regression analysis to determine the proportion of variance in BMD and BMC explained by individual SNPs after controlling for

**Figure 1. Microsatellite allele frequencies in sub-Saharan Africans (Sudanese) versus Asians, Caucasians and Native African Americans**



covariates. Specifically, each genotype was dummy coded as a categorical variable, where a homozygote genotype was coded as the reference group. In the first step, for each of the 12 BMD and BMC variables we entered the covariates of age, weight and height into a linear regression model. In the second step, for each polymorphism we entered codes for genotypes and computed the proportion of variance ( $R^2$ ) in BMD and BMC explained by the genotypes in the regression model. Those genes that had extremely low allele frequency (*TGF $\beta$* , *COL1A1*, *CSR* and *IL-1RN*; see Results) were excluded from the analysis. The age of the most recent common ancestors of *VDR* and *ER $\alpha$*  polymorphisms was determined with the methods of Fu et al.<sup>57</sup>

## RESULTS

All the 14 SNPs were in Hardy-Weinberg linkage equilibrium. Among the 14 SNPs, 10 were significantly different in allele frequency between Sudanese and Asians, and 10 between Sudanese and Caucasians (Table 1). Only one SNP (of osteocalcin gene) was *not* significantly different between Sudanese and Asians or Caucasians. Notably, there was only one minor allele at the transforming growth factor- $\beta$  gene, six minor alleles at the calcium-sensing receptor gene, and nine minor alleles at the collagen 1 $\alpha$ 1 gene out of a total of 286 alleles in each gene in the Sudanese immigrant population. Allele frequency in each of these three SNPs was <0.04 (Table 1).

Frequencies in most microsatellite marker alleles in the four genes were significantly different between Sudanese and Asians or between Sudanese and Caucasians (Table 2 and Figure 1). There were only one A3 and one A4 alleles in the *IL-1RN* gene in the Sudanese. However, frequency of microsatellite markers of the *IGF1* gene was more similar between Sudanese and African Americans, although there was a significant difference in one of the 11 common alleles. To appreciate the anthropologic implication of racial difference in allele frequency, we also compared allele frequency of vitamin-D receptor gene among different ethnic groups of East Asians based on published data (Table 3). The frequency of the *f* allele was significantly different between Chinese and Korean and between Chinese and Japanese, while the *t* allele frequency was significantly different between Japanese and Koreans and between Japanese and Chinese (Table 3). There was no difference in the *a* allele frequency among the three ethnic groups. Based on Fu's method,<sup>57</sup> we calculated that the age of SNPs in the vitamin-D receptor gene and estrogen receptor gene was between 124,000–1 million years.

Table 4 provides the proportion of variance ( $R^2$ ) in bone variables explained by each polymorphism after adjustment for age, body weight and height. Six SNPs at *ER $\alpha$* (*PvuII*), *ER $\alpha$* (*Xba*), *VDR*(*Taq*), *VDR*(*Fok*), *PTH* and *CTR* were significantly ( $p < 0.05$  or  $p < 0.01$ ) associated with BMD and/or BMC at several bone sites after adjustment for age, body weight and height. Notably,

**Table 2. Statistical analysis of difference in allele frequencies of microsatellite markers in Asians, Caucasians and African Americans as compared with Sudanese**

Gene	Primer	Genotyping				Asian				Caucasian				African American			
		Ref	n	NAS	p	Ref	n	NAS	p	Ref	n	NAS	p	Ref			
<i>IL-1RN</i>	CTCAGCAACACTCCTAT																
	TCCTGGTCTGCAGGTAA	41	714	1	<0.0001	37	108	1	<0.0001	41	72	2	<0.006	44			
<i>IL-6</i>	TTCTACATGACAGCAGAACAC																
	TCTGTGGGAAAGTATATGTGC	42	165	2	<0.0083	38	636	9	<0.0006	42	–	–	–	–			
<i>IGF1</i>	GCTAGCCAGCTGGTGTATT																
	ATGGGAAGAGGGGTCTCACCA	55	300	5	<0.0001	39	646	4	<0.003	43	165	1	0.008	43			
<i>TNFR2</i>	GTGATCTGCAAGATGAACTCAC																
	ACACCACGTCTGATGTTCA	56	1263	7	<0.0015	36	357	6	<0.0001	40	–	–	–	–			

Allele frequencies are shown in Figure 1; n: number of subjects; nas: number of alleles with a significant difference in frequency compared with the Sudanese; "–": not available; p values are the largest of significant p values (with Bonferroni correction) when comparing allele frequencies with the Sudanese; *TNFR2*: tumor necrosis factor receptor super-family, member 1B gene (also known as osteoprotegerin); *IL-1RN*: interleukin -1 receptor antagonist gene; *IL-6*: interleukin-6 gene; *IGF1*: insulin growth factor 1 gene.

**Table 3. Ethnic variation in allele frequency of VDR gene among Asians**

Allele	Frequency					
	Chinese		Korean		Japanese	
	Ref.		Ref.		Ref.	
<i>f</i>	0.52 (n=223)	45	0.389 (n=229)	46	0.375 (n=1433)	22
<i>t</i>	0.05 (n=223)	45	0.055 (n=417)	23	0.115 (n=1431)	22
<i>a</i>	0.705 (n=767)	20	0.731 (n=417)	23	0.706 (n=1430)	22

*f* allele, Chinese versus Korean and Chinese versus Japanese:  $p < 0.0001$ ; Japanese versus Korean:  $p = 0.604$ ; *t* allele, Japanese versus Koreans, Japanese versus Chinese ( $p < 0.0001$ ), Chinese versus Korean:  $p = 0.690$ ; *a* allele, Koreans versus Japanese,  $p = 0.945$ ; Chinese versus Korean,  $p = 0.164$ . Japanese versus Chinese,  $p = 0.183$

*ERα(Xba1)* was associated with 9 of the 12 bone variables ( $p < 0.05$  or  $p < 0.01$ ). A total of 16 phenotype-genotype associations with significance levels below 0.05 were found (Table 4).

### DISCUSSION

Results of the present study show that among 18 polymorphisms in 13 genes that have been reported to be associated with BMD in Asian and/or Caucasian populations,<sup>2,3,5-12,15-56</sup> 17 were significantly different in allele frequency between the Sudanese and Asians or Caucasians. Notably, allele frequency in only one (the *HindIII* restriction site in the osteocalcin gene) of the 18 polymorphisms was not different among the three ethnic groups. Since the effects of these polymorphisms on BMD have been established previously, the genetic difference in allele frequency of these bone-affecting genes may conceivably be responsible for the ethnic difference in BMD and/or BMC. It is not surprising that allele frequencies in some genes (e.g., *IGF1*) were more similar between the Sudanese and native African Americans, although there was still a significant (after Bonferroni correction) difference in the frequency of one (the 186-bp allele) out of 11 alleles. We also showed that the frequency in certain alleles of the vitamin-D receptor gene was also different among the Chinese, Koreans and Japanese. Recent studies show that allele frequency of the vitamin-D receptor gene is also significantly different among different ethnic groups of the Chinese people.<sup>47</sup> In addition to the ethnic difference, sampling and/or genotyping errors might also be in part responsible for the observed statistically significant difference.<sup>48</sup> However, allele frequency of certain genes was strikingly different between the Sudanese and Asians and between the Sudanese and Caucasians. Notably, in three

genes, the SNP allele frequencies were <4% (0.3% for calcium-sensing receptor gene, 2.1% for transforming growth factor-β1 gene and 3.2% for collagen type-1α1 gene) in the Sudanese, while they were >13% in any of these genes in Caucasians. Interestingly, Asians lack polymorphism in the *COL1A1* gene,<sup>2</sup> while the Sudanese do not. Although frequencies of certain minor alleles were very low in the Sudanese, they, nonetheless, have polymorphisms in all these genes studied. This is consistent with previous findings that sub-Saharan Africans tend to have all of the polymorphisms in other ethnic groups, which is the genetic basis of the “Out of Africa” theory.<sup>14</sup> Recent evidence suggests that the “Out of Africa” process might have started 150,000 years ago in Ethiopia.<sup>58</sup> The present studies show that all human populations had shared the SNPs in the vitamin-D receptor gene and estrogen receptor gene 124,000–1 million years ago.

Our studies have provided frequencies of BMD-affecting genes in Africans from the African continent, which are crucial for designs in future association (linkage disequilibrium) studies (e.g., in the power and sample size calculations). Those genes that have higher polymorphism information content (such as the vitamin-D receptor and estrogen receptor genes) will receive high priority in future association studies in Africans. In our analysis based on the limited sample size, polymorphisms in these two genes did show promise of association with BMD or BMC at several bone sites, consistent with findings in Caucasian and Asian populations.<sup>2,3</sup> It should be noted that although we used Bonferroni correction in pairwise comparison of allele frequencies among ethnic groups, we did not use it in the association study, because it would be too conservative considering the large number of comparisons

**Table 4. Proportion of variance (R<sup>2</sup>) in BMD and BMC explained by each polymorphism after adjusting for age, weight and height**

Gene	BMD							BMC				
	Total Body	Total Hip	Spine (L1-4)	TC	Wards' Triangle	Z-Spine	Z-Hip	Total Body	Hip	Spine (L1-4)	TC	Wards' Triangle
CTR	0.007	0.004	<b>0.039*</b>	0.003	0.001	<b>0.035†</b>	0.007	0.006	<b>0.039*</b>	0.001	0.003	0.000
<i>ERα(PvuII)</i>	<b>0.029†</b>	0.011	0.022	0.010	0.016	0.025	0.009	0.011	0.013	<b>0.033*</b>	0.004	0.010
<i>ERα(Xba1)</i>	<b>0.066*</b>	<b>0.112**</b>	0.024	<b>0.133**</b>	<b>0.080*</b>	0.026	<b>0.133**</b>	<b>0.039*</b>	<b>0.060**</b>	<b>0.037†</b>	<b>0.073**</b>	<b>0.080*</b>
PTH	0.004	0.010	0.008	0.008	<b>0.026†</b>	0.009	0.022	0.002	0.005	0.003	0.004	<b>0.034*</b>
GLA	0.008	0.008	0.002	0.010	0.001	0.002	0.009	0.004	0.006	0.007	0.009	0.006
OPG	0.000	0.001	0.001	0.001	0.000	0.000	0.001	0.003	0.001	0.000	0.000	0.000
<i>VDR(Apa1)</i>	0.004	0.008	0.006	0.007	0.000	0.003	0.005	0.000	0.012	0.007	0.007	0.015
<i>VDR(Fok)</i>	0.001	0.005	0.006	0.003	<b>0.049†</b>	0.009	0.006	0.002	0.005	0.024	0.006	<b>0.049†</b>
<i>VDR(Taq)</i>	0.023	0.035	<b>0.050†</b>	0.042	<b>0.080*</b>	<b>0.050†</b>	0.037	0.012	0.022	<b>0.080*</b>	<b>0.050*</b>	0.016
AHSG	0.005	0.004	0.004	0.009	0.004	0.001	0.007	0.001	0.001	0.014	0.001	0.002
IL6	0.001	0.013	0.001	0.010	0.007	0.001	0.013	0.004	0.011	0.007	0.001	0.001
<i>IL6-micro</i>	0.000	0.011	0.008	0.010	0.008	0.009	0.002	0.004	0.012	0.013	<b>0.018†</b>	0.019
<i>TNFR2-micro</i>	0.032	0.018	0.019	0.008	0.029	0.023	0.013	0.017	0.013	0.017	0.019	0.015
<i>IGF-1-micro</i>	0.003	0.001	0.011	0.001	0.013	0.008	0.004	0.000	0.000	0.015	0.003	<b>0.027†</b>

TC: trochanter; †  $p < 0.10$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; Z-spine and Z-hip are Z scores of BMD; spine (L1-4): lumbar spine vertebrae 1–4; micro: microsatellite marker

involved. Therefore, some of the significant findings in the association study may be due to chance error. However, while we would expect <1 significant finding for each polymorphism among 12 BMD (or BMC) association results at a level of 0.05 by chance alone, nine *ER $\alpha$ (Xba1)* associations and three *VDR(Taq)* associations with BMD and/or BMC were found to be significant at a level of 0.05. Therefore, we strongly believe that *ER $\alpha$ (Xba1)* and *VDR(Taq)* are associated with BMD in the Sudanese.

In summary, allele frequencies in most of these genes examined are significantly different in sub-Saharan Africans as compared with Asians or Caucasians. These genetic differences might be in part responsible for the ethnic differences in BMD.

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