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Linkage Analysis in a Large Primary Osteoporosis Family

[Geniş Primer Osteoporoz Ailesinde Bağlantı Analizi]

Burcu Balcı[1],
Bülent O. Yıldız[2],
Rivka Ofir[3],
Pervin Dinçer[1],
Miyase Bayraktar[2]

Hacettepe University, Faculty of Medicine,
[1]Department of Medical Biology,
[2]Department of Internal Medicine, Endocrinology and Metabolism Unit, Sıhhiye, Ankara, Turkey.
[3]Laboratory of Desert Plant Leads R&D Hazeva, Head Jacob Blaustein Institute for Desert Research & Research and Development Laboratory of Human Molecular Genetics, Department of Development Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Israel.

Yazışma Adresi
[Correspondence Address]

Prof. Dr. Pervin Dinçer

Hacettepe University Faculty of Medicine, Department of Medical Biology
06100, Sıhhiye, Ankara, TURKEY.
Tel: +90 312 305 2541
Fax: +90 312 309 6060
E-mail: pdincer@hacettepe.edu.tr

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ABSTRACT

Rationale and objectives: Genetic factors play an important role in regulating bone mineral density and in the development of osteoporosis. In order to identify the gene(s) contributing to bone mineral density, we have performed a linkage approach in the largest primary osteoporosis family in the literature.

Methods: This family was tested for a linkage to eight candidate genes: Bone gamma-carboxyglutamate protein, Chloride channel , Collagen type 1 alpha 1, Collagen type 1 alpha 2, Estrogen receptor alpha, Insulin like growth factor 1/somatomedin C, Low density lipoprotein receptor related protein 5 and Vitamine D receptor. The computations were performed with SuperLink v1.3.

Results: The LOD score calculation and haplotype results of our study showed that none of these genes are responsible for low bone mineral density in this family.

Conclusions: This study presents the largest primary osteoporosis family in the literature and suggests that Bone gamma-carboxyglutamate protein, Chloride channel 7 , Collagen type 1 alpha 1, Collagen type 1 alpha 2, Estrogen receptor alpha, Insulin like growth factor 1/somatomedin C, Low density lipoprotein receptor related protein 5 and Vitamine D receptor genes are not responsible for low bone mineral density in this family.

Key Words: Bone Mineral Density, candidate genes, linkage analysis, osteoporosis.

ÖZET

Amaç: Genetik faktörlerin kemik mineral dansitesinin regülasyonunda ve osteoporoz gelişiminde önemli rol oynadığı bilinmektedir. Çalışmamızda, literatürde şu ana kadar tanımlanmış olan en büyük primer osteoporoz ailesinde kemik mineral dansitesinden sorumlu olduğu düşünülen genler için bağlantı analizi yapılmıştır.

Yöntemler: Çalışmamız kapsamındaki aile sekiz aday gen için bağlantı analizine alınmıştır. Bunlar; Kemik gama-karboksiglutamat protein, Klorid kanal 7, Kollajen tip 1 alfa 1, Kollajen tip 1 alfa 2, Östrojen reseptör alfa, İnsülin benzeri büyüme faktörü 1, Düşük dansiteli lipoprotein reseptörü ile ilişkili protein 5 ve Vitamin D reseptör genleridir. İstatistiksel analizler SuperLink v1.3 yazılım programı ile gerçekleştirilmiştir.

Bulgular: LOD skor hesaplamaları ve haplotip analizleri sonucunda aday genlerin bu ailede düşük kemik mineral dansitesinden sorumlu olmadığı saptanmıştır.

Sonuç: Çalışmamız kapsamında, literatürde şu ana kadar rapor edilen en büyük primer osteoporoz ailesinde, Kemik gama-karboksiglutamat protein, Klorid kanal 7, Kollajen tip 1 alfa 1, Kollajen tip 1 alfa 2, Östrojen reseptör alfa, İnsülin benzeri büyüme faktörü 1, Düşük dansiteli lipoprotein reseptörü ile ilişkili protein 5 ve Vitamin D reseptör genlerinin ailedeki düşük kemik mineral dansitesinden sorumlu olmadığı sonucuna varılmıştır.

Anahtar Kelimeler: Kemik Mineral dansitesi, aday genler, bağlantı analizi, osteoporoz.

Introduction

Osteoporosis is a common metabolic bone disease characterized by reduced bone density and deterioration of bone tissue that leads to bone fragility and increase in fracture risk (1). It affects up to 40% of women and 12% of men at some point during life (2). Many factors contribute to the pathogenesis of osteoporosis including smoking, poor diet, lack of exercise and excessive alcohol intake. However, evidence from twin and family studies suggests that genetic factors have a major role in bone mineral density (BMD) and in the development of osteoporosis (3,4).

Decreased BMD, which is a major determinant of osteoporotic fracture risk is inherent in the definition of osteoporosis and is the most important susceptibility factor identified to date (5,6). Researchers are trying to determine which genes contribute to regulation of bone mass and how the effects of individual genes are mediated. Previous studies demonstrated that BMD is associated with several genes. They include genes that encodes cytokines, growth factors, calciotropic hormones and receptors and bone matrix proteins (7). Among these genes we have focused on Vitamin D receptor (VDR) (8), Estrogen receptor- α (ESRA) (9), Collagen type 1 alpha 1 (COLIA1) (10), Collagen type 1 alpha 2 (COLIA2) (11), Bone gamma-carboxyglutamate protein (BGLAP) (12), Insulin-like growth factor 1 (IGF-1) (13), Low density lipoprotein receptor related protein 5 (LRP5) (14) and Chloride channel 7 (CLCN7) (15).

Vitamin D and its receptor plays an important role in calcium homeostasis by regulating bone cell growth and differentiation. A number of Restriction Fragment Length Polymorphisms (RFLP) (BsmI, ApaI, TagI and FokI) in the VDR gene have been identified as related to biological variations in bone mass and there is evidence to suggest that these effects may be modified by dietary calcium and vitamin D intake (8,16). Recently, a novel binding site for transcription factor Cdx-2 in the promoter region of VDR gene was also associated with variation in BMD (17).

ESRA is another candidate for osteoporosis phenotype. Estrogens are important hormones for bone mass regulation in both genders because C to T transition in the coding region of the gene led to a partial estrogen resistance with low BMD in a young man (18). PvuII and XbaI polymorphisms in the first intron of the ESRA gene have been associated with low BMD (19). In a similar study, an association has been reported between a TA repeat polymorphism in the ESRA gene promoter and bone mass in some populations (9).

COLIA1 and COLIA2 genes which encodes collagen types Iα1 and Iα2 are also important candidates for the genetic regulation of reduced bone mass because mutations that affect the coding regions of these genes have been shown to cause osteogenesis imperfecta (20,21). Although a polymorphism at the first base of a binding site for the transcription factor Sp1 in the first intron of the COLIA1 gene was found to be associated with reduced bone density and predisposes women to osteoporotic fractures (10), two polymorphisms have been described in the promoter of COLIA1 gene that are in LD with the Sp1 polymorphism and are also associated with BMD (22).

BGLAP also known as Osteocalcin is an abundant, highly conserved bone specific protein that is synthesized by osteoblasts and determinant of bone formation. HindIII polymorphism in the BGLAP gene which encodes for BGP was found to be associated with reduced BMD and predisposes women to osteoporosis at the femoral neck (12). Raymond et al. suggests that genetic variation at the osteocalcin locus impacts BMD levels in the postmenopausal period and may predispose some women to osteoporosis (23).

IGF-1 is an attractive candidate gene for low BMD, because IGF-1 has marked effects on bone cells (24) and has been implicated in the pathogenesis of osteoporosis, also low IGF-1 levels are associated with an increased risk of osteoporotic fractures (25). The IGF-1 gene contains a microsatellite repeat polymorphism in 5'-flanking promoter region and investigators have found a higher prevalence of the 192/192 genotype of this polymorphism among men with idiopathic osteoporosis compared to controls (26). The presence of a 194bp allele has been related to either higher BMD or to increased levels of circulating IGF-1 (27).

LRP5 gene seems to be another candidate gene for reduced BMD and osteoporotic fractures. It encodes the transmembrane-spanning protein named low density lipoprotein receptor related protein 5, which has a role in the Wnt signalling pathway. Nonsense or missense mutations in the LRP5 gene caused autosomal recessive osteoporosis-pseudoglioma syndrome while heterozygous substitution in exon 3 was found in autosomal dominant high-bone-mass trait (14).

Also, CLCN7 gene which encodes an endosomal/lysosomal ion channel and highly expressed in the osteoclast ruffled border may be a candidate gene for BMD regulation. Inactivating mutations in the CLCN7 gene have been shown to cause severe infantile osteopetrosis, whereas missense mutations of CLCN7 gene have been found to cause a milder type of osteopetrosis, termed autosomal dominant osteopetrosis type II, or Albers Schonberg disease (15).

Therefore, all these genes may contribute to the decreased BMD phenotype. We have investigated the role of these eight candidate genes in the control of BMD by linkage analysis in a large consanguineous primary osteoporosis family.

Materials and Methods

Subjects

The study protocol was approved by the Ethical Committee of Hacettepe University and a written informed consent was obtained from all participants.

Our index patient (Figure 1, case V:1) was 66 years old man who came to the clinic because of back pain. Medical history of him revealed that he had no known systemic illnesses. He was a non-smoker, and was not consuming alcohol. Family history was positive for osteoporosis. Physical examination showed scoliosis and lower back tenderness. The remainder of the examination was normal. Laboratory studies disclosed that complete blood count, liver and kidney function tests, calcium, phosphorus, fasting and postprandial glucose levels, thyroid function tests, testosterone and intact parathyroid hormone levels were all in normal limits. Radiograph of the lumbar vertebrae showed a compression fracture at the level of L3. T score of the lumbar vertebrae and total hip were -4.32 and -2.32 respectively. Serum bone specific alkaline phosphatase, osteocalcin, urinary calcium excretion rate and urinary deoxypyridinoline adjusted for urinary creatinine were normal.

Because the patient had family history of osteoporosis, his relatives were invited to be evaluated for osteoporosis. The geneology of the family was questioned for three generations. A total of 32 close relatives out of more than 1000 individuals in our family had their BMD of the spine (L_2–L_4) and the hip (total femur, femur neck and trochanter) measured (18 DEXA-dual energy X-ray absorptiometry (Hologic QDR-4500A), 33 calcaneal QUS (Lunar Achilles express)) with an assessment of their risk factors for osteoporosis. The pedigree of the family is shown in Figure 1.

The patient's three brothers (69, 71, 78 years) and an 54 years old premenopausal sister were osteoporotic at the lumber spine and osteopenic at the total hip on BMD. His wife was 64 years old. She was osteoporotic at the lumber spine and osteopenic at the total hip on BMD. The patient had eight children; because one son and one daughter lived abroad we could studied three sons (32, 34, 41 years) and three daughters (31, 36, 46 years). Only the 31 years old daughter (case VI:7) was osteopenic at the lumber spine and normal at the total hip on BMD. All the others were osteoporotic on BMD of the lumber spine and osteopenic on BMD of the total hip. Later, BMD measurement of the 33 years old daughter living abroad was received. She was osteoporotic at the lumber spine and osteopenic at the total hip on BMD.

Six nephews (age range 29-51 y), 5 nieces (22-48 y) and 3 children (3 boys 17-22 y) had their bone density measured. Only 1 nephew (Figure 1, case VI:17) and 2 children (case VII:1 and VII:3) had normal BMD values. Three nieces (33,36,38 y) were osteoporotic (case VI:13, VI:15 and VI:16) and all the others were osteopenic. One osteoporotic men was heavy smoker (>20 pack-years) and drinker. One osteoporotic woman had congenital dislocation of the hip and the others did not have risk factors for osteoporosis.

Linkage Analysis

Linkage analysis was performed on 30 individuals in this family (Figure 1). The genomic DNA from 23 affected individuals (12 male, 11 female) with low BMD and 7 unaffected individuals (3 male, 4 female) with normal BMD was extracted from peripheral blood samples according to the standart protocols (after written informed consent). A total of 24 microsatellite markers and RFLPs located close to or within 8 candidate genes were selected. Microsatellites and/or RFLPs selected for each

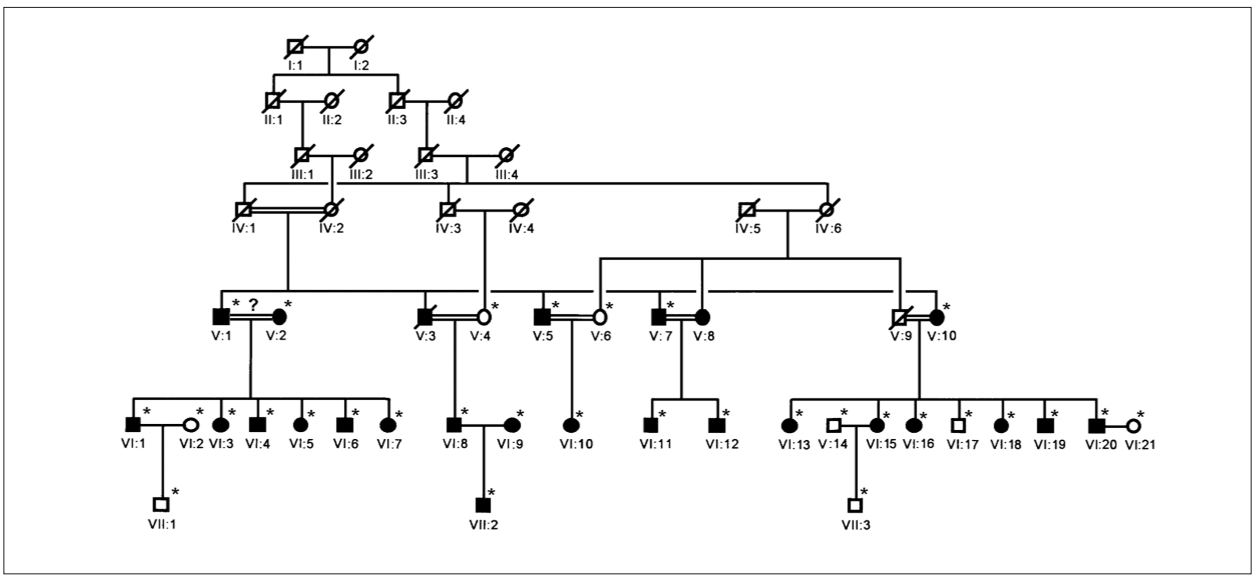

Figure 1. A small part of primary osteoporosis family with analyzed individuals: (*)analyzed individuals, (?) no information about the level of consanquinity)

gene were: VDR (FokI, ApaI, TaqI, D12S398), ESRA (D6S440, D6S2436, D6S473), COLIA1 (D17S588, RsaI), COLIA2 (COL1A2, MspI), BGLAP (D1S303, HindIII, D1S2624, D1S194), IGF-1 (D12S1074, D12S2023, D12S1030), LRP5 (D11S1337, D11S4178), CLCN7 (SmaI, intron 8 VNTR-50 bp. repeat, MslI, D16S3024). Chromosomal localization of the selected genes and markers are shown in Table 1.

PCR products of microsatellite markers were then seperated by 6% Polyacrylamide Gel Electrophoresis (PAGE) and stained with silver nitrate. The alleles of the individuals were numerated after comparison with CEPH control DNA (1347-02) and evaluated together to form the haplotypes.

For each RFLP marker, genomic DNA were amplified by PCR in a total volume 25 µl using specific primers described by Morrision et al., 1994 (8) for the ApaI and TaqI, by Gross et al., 1996 (16) for the FokI, by Sokolov et al., 1991 (28) for the RsaI, by Baker et al., 1991 (29) for the MspI and by Dohi et al., 1998 (12) for the HindIII. SmaI, MslI and 50 bp. repeat polymorphisms in the in CLCN7 gene were amplified by using exon1, exon15 and exon8/9 primers, respectively.

After sequence-specific digestion with 10 units of restriction enzymes (New England Biolabs), the samples were electrophoresed through a 2% agarose gel containing ethidium bromide and scored for genotypes.

Table 1. Chromosomal localization of the selected genes and markers

GENE	MARKER	Marshfield Map (cM)	Chromosomal Position
BGLAP Chr. 1 154449397 – 154479747 bp.	D1S303	161.05	-
	Hind III	-	Inrtagenic (5'UTR-Exon 1)
	D1S2624	162.57	154897940 - 154898142 bp.
	D1S194	178.42	163703895 - 163704127 bp.
CLCN7 Chr. 16 1435346-1465582	Sma I	-	Intragenic (Exon 1)
	50 bp repeat	-	Intragenic (Intron 8)
	Msl I	-	Intragenic (Exon 15)
	D16S 3024	7.05	1594204 - 1594430 bp.
COL1A1 Chr. 17 45616456 – 45633992 bp.	D17S588	-	45570495 - 45570653 bp.
	Rsa I	-	Intragenic (5'UTR)
COL1A2 Chr. 7 93861809 – 93898480 bp.	Msp I	-	Inrtagenic (3'UTR)
	COL1A2	-	93854267 - 93854422 bp.
ESRA Chr. 6 152170379 - 152466099 bp.	D6S440	154.80	152374791 - 152375061 bp.
	D6S2436	154.64	-
	D6S473	155.17	155287394 - 155287577 bp.
IGF-1 Chr. 12 101313806 – 101398454 bp.	D12S1074	-	101333066 - 101333234 bp.
	D12S2023	-	Intragenic (WI-YAC Map: Chr 12 / Position: 386 (ordinal))
	D12S1030	109.47	101448937 - 101449195 bp.
LRP5 Chr. 11 67836711 – 67836711 bp.	D11S1337	68.55	67888234 - 67888517 bp.
	D11S4178	67.48	67945684 - 67945935 bp.
VDR Chr. 12 46521587 – 46585081 bp.	Fok I	-	Intragenic (Exon 2)
	Apa I	-	Intragenic (Exon 9)
	Taq I	-	Intragenic (Exon 9)
	D12S398	68.16	51483354 - 51483480 bp.

Lod-score calculations

LOD score calculations were carried out under the assumptions of autosomal dominant inheritance, using a software called SuperLink v1.3 that enables extended two-point linkage analysis on large kindred in its entirety (30). Based on some or all markers on a chromosome, computer programs such as SuperLink v1.3 can compute this likelihood for any assumed position of the disease locus. Pedfiles of the family members and their alleles were submitted for calculation at the bioinformatics site at the Technion, Haifa [http://bioifo.cs.technion.ac.il/pedtool].

The possible mode of inheritance (MOI) and the frequency of the penetrance in this family were analyzed by using the same software, comparing the hypothesis "recessive inheritance" versus "dominant inheritance". The likelihood of the data was computed without marker information by taking into account only affection status and family relationships for both dominant and recessive inheritance models by using various penetrance frequencies.

The most likely combination of parameters for two-point LOD score analysis of these eight candidate genes were: a dominant inheritance model using a penetrance of q=0.8, p=1 and a mutant allele frequency of 0.01.

Results

According to the WHO criteria (World Health Organisation, 1994) this family was diagnosed as the first primary osteoporosis family with ~3000 individuals, who live in the Samandağı-Hatay, Turkey. A total of 30 patients in this large and ethnically isolated family were recruited for our study (Figure 1). We were not able to analyse other family members due to the conservative nature of the family. 23 affected (13 osteoporotic [T score is -2.5 or lower] / 10 osteopenic [T score is between -1.0 and -2.5]) individuals (12 male, 11 female) with a frequency of 76.6% had low BMD and 7 unaffected individuals (3 male,4 female) with a frequency of 23.3% had a BMD T score ≥-1.0. Also, we should clearly indicate that most of the affected individuals were young (either young men or premenopausal women) meaning that their Z scores are pretty much the same with their T scores.

Segregation analysis rejected a recessive model of inheritance and supported a dominant inheritance model in this family (data not shown). As such, LOD score calculation was carried out under the assumptions of autosomal dominant inheritance for all of the polymorphic markers located close to or within candidate genes.

For the 7 candidate genes (IGF-1, ESRA, COLIA1, COLIA2, BGLAP, LRP5, CLCN7) we found no evidence for a linkage (Table 2). The maximum two-point LOD score of 0.90 (θ= 0) was calculated for ApaI polymorphism in the VDR gene but three point analysis using markers FokI and TaqI did not support linkage to VDR (data not shown).

Discussion

Genetic factors play an important role in determining BMD and may also influence its treatment (3). Low BMD is a major risk factor for the development of osteoporosis (5,6). Although BMD and other osteoporosis-related phenotypes are usually determined by the effects of several genes, occasionally osteoporosis or high bone mass may occur as a result of mutation in a single gene such as osteoporosis-pseudoglioma syndrome (31). Most work in the field of osteoporosis and low BMD genetics has focused on responsible gene studies in populations including linkage studies, candidate gene studies, gene association studies, family studies, genome scans in sibling pairs and family-based association studies (32-34). Family based studies that use linkage analysis such as pedigree studies are generally more informative than association studies. The limited heterogenity within families, combined with detailed knowledge of pedigree is a key factor (35).

In order to identify potential genetic determinants of low BMD we have investigated the role of 8 candidate genes by linkage approach in the large osteoporosis family. To the best of our knowledge, this family is the largest family in the literature which is diagnosed as primary osteoporosis. Segragation analysis rejected recessive mode of inheritance and as such the mode of inheritance in this family is likely to be autosomal dominant. Using the software SuperLink v1.3 that is suitable for lod score calculation of families with complex relationship, we were able to calculate lod score for each suspected gene/loci inspite of the limited number of individuals that could have been studied due to the conservative nature of the family. The results of our preliminary study suggested that VDR, ESRA, COLIA1, COLIA2, BGLAP, LRP5, IGF1 and CLCN7 genes are not responsible for low BMD in this family. No evidence of linkage was observed between low BMD and these 8 candidate genes according to the two-point linkage analysis. Although our results suggest that neither of these potentially genes are responsible for low BMD in this family, our preliminary findings and this large primary osteoporosis family will be the initiator of our subsequent studies. Further work will be required to screen other potential candidate genes and to identify responsible locus / loci by genome-wide scan using single nucleotide polymorphism (SNP) arrays in this family. Consanguinity in this family will allow homozygosity mapping to detect responsible genes. Also, gene-gene and gene-environment interactions could have a potential role in the development of clinical phenotype in this family which should be addressed in future studies. The identification of the genes which are responsible for low BMD may represent a major advance in understanding of pathways that regulate bone density.

Table 2. Two point LOD score results of the polymorphic microsatellites/ RFLPs covering 8 candidate genes

GENE	MARKER	RECOMBINATION FRACTION							
		0	0.01	0.05	0.1	0.2	0.3	0.4	maximum
BGLAP	D1S303	-6.595363	-1.578384	-0.605514	-0.222278	0.010527	0.040435	0.026216	0.040435
BGLAP	Hind III	-0.452575	-0.394487	-0.246703	-0.152193	-0.071845	-0.041778	-0.023129	-0.023129
BGLAP	D1S2624	-8.401130	-3.517166	-1.250819	-0.359164	0.216139	0.235817	0.055805	0.235817
BGLAP	D1S194	-7.390258	-3.652541	-1.796166	-1.036070	-0.399096	-0.129793	-0.011908	-0.011908
CLCN7	Sma I	-6.331163	-1.247278	-0.528446	-0.231756	-0.007170	0.039158	0.019606	0.019606
CLCN7	50 bp repeat	-19.8853	-10.0588	-5.809050	-3.823619	-1.824477	-0.819349	-0.278090	-0.278090
CLCN7	Msl I	-6.808098	-4.170940	-2.798012	-1.820588	-0.737434	-0.249329	-0.046817	-0.046817
CLCN7	D16S 3024	-6.872104	-2.454089	-1.136522	-0.625073	-0.219349	-0.068441	-0.011710	-0.011710
COL1A1	D17S588	-14.88470	-5.679952	-2.907653	-1.699669	-0.629035	-0.213136	-0.073568	-0.073568
COL1A1	Rsa I	-6.378455	-2.281785	-0.915568	-0.380047	0.019997	0.100153	0.047661	0.019997
COL1A2	Msp I	-0.412400	-0.425478	-0.464867	-0.450389	-0.264790	-0.090456	0.000357	0.000357
COL1A2	COL1A2	-8.567177	-2.296404	-1.556238	-1.043592	-0.419230	-0.122856	0.009523	0.009523
ESRA	D6S440	-1.98013	-1.469106	-0.891901	-0.596838	-0.279377	-0.110804	-0.028484	-0.028484
ESRA	D6S2436	-7.473524	-6.347153	-3.567233	-2.188486	-0.896553	-0.311938	-0.056535	-0.056535
ESRA	D6S473	-8.982073	-2.106609	-0.882308	-0.462477	-0.175418	-0.065378	-0.011837	-0.011837
IGF-1	D12S1074	-6.633864	-3.642493	-2.139065	-1.296411	-0.513641	-0.171934	-0.025616	-0.025616
IGF-1	D12S2023	-4.665676	-3.459979	-1.757705	-0.946463	-0.265726	-0.029007	0.023117	0.023117
IGF-1	D12S1030	-0.438757	-0.374313	-0.230781	-0.158487	-0.105494	-0.052080	-0.004123	-0.004123
LRP5	D11S1337	-7.677588	-7.268064	-4.205905	-2.440473	-0.913193	-0.282141	-0.039848	-0.039848
LRP5	D11S4178	-10.33683	-7.017144	-3.859004	-2.359285	-0.988139	-0.362937	-0.077832	-0.077832
VDR	Fok I	0.337273	0.319217	0.248879	0.166461	0.030948	-0.042416	-0.043046	0.337273
VDR	Apa I	0.904880	0.868817	0.726196	0.555619	0.264910	0.071042	-0.012208	0.904880
VDR	Taq I	-1.602874	-1.021787	-0.468310	-0.238445	-0.057644	0.003408	0.015091	0.015091
VDR	D12S398	-6.823183	-5.393646	-3.092398	-1.928151	-0.839187	-0.335408	-0.089427	-0.089427

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