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Complete List of Authors:	Riancho, Javier; Hospital U.M. Valdecilla, Internal Medicine García-Ibarbia, Carmen; Hospital U.M. Valdecilla-IFIMAV-University of Cantabria, Department of Internal Medicine Pérez-Núñez, María; Hospital U.M. Valdecilla-Universidad de Cantabria, Traumatology and Orthopedic Surgery Alonso, Maria; Hospital U.M. Valdecilla-Universidad de Cantabria, Traumatology and Orthopedic Surgery Díaz, Teresa; Hospital U.M. Valdecilla-IFIMAV-University of Cantabria, Department of Internal Medicine Perez-Castrillon, Jose; Hospital U. Rio Hortega, Universidad de Valladolid, Internal Medicine Riancho, Jose; Hospital U.M. Valdecilla, Internal Medicine; University Cantabria, Medicine
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GENETIC POLYMORPHISMS OF THE WNT RECEPTOR LRP5 ARE DIFFERENTIALLY ASSOCIATED WITH TROCHANTERIC AND CERVICAL HIP FRACTURES

Javier Riancho[1], Carmen García-Ibarbia[1], María I. Pérez-Núñez[2], María A. Alonso[2], Teresa Díaz[1], José L. Pérez-Castrillón and José A. Riancho[1]

[1]Department of Internal Medicine. Hospital U.M. Valdecilla-IFIMAV, Universidad de Cantabria. RETICEF. Santander, Spain.
[2]Service of Traumatology and Orthopedic Surgery. Hospital U.M. Valdecilla-IFIMAV, Universidad de Cantabria. Santander, Spain.
[3]Service of Internal Medicine. Hospital U. Río Hortega, Universidad de Valladolid. RETICEF. Valladolid, Spain.

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Correspondence and reprint requests:
José A. Riancho
Department of Internal Medicine
Hospital U.M. Valdecilla, University of Cantabria
Santander 39008, Spain
Phone: 34-942201990
Fax: 34-942201695
Email: rianchoj@unican.es

ABSTRACT

Purpose. Epidemiological studies suggest that cervical and trochanteric hip fractures have different pathogenesis. We planned to test the hypothesis that genetic factors have different influences on both types of fractures.

Methods. Ten polymorphisms of genes known to play an important role in skeletal homeostasis (estrogen receptor alpha [ESR1], aromatase [CYP19A1], type I collagen [COL1A1], and lipoprotein receptor-related protein 5 [LRP5]) were analyzed in 471 Spanish patients with fragility hip fractures.

Results. Two polymorphisms of the LRP5 gene (rs7116604 and rs3781600) were associated with the type of fracture (p-value 0.0085 and 0.0047, respectively). The presence of rare alleles at each locus was associated with trochanteric fractures over cervical fractures (OR 1.7 in individuals with at least one rare allele at rs7116604 or rs3781600 loci, in comparison with the common homozygotes). Considering individuals bearing the four common alleles as reference, the OR for trochanteric fractures was 1.6 in those with 1 or 2 rare alleles, and 7.5 in those with 3 or 4 rare alleles (p-value for trend 0.0074), which is consistent with an allele-dosage effect. There were no significant differences in the frequency distributions of the ESR1, CYP19A1 and COL1A1 genotypes between trochanteric and cervical fractures in either the original group or in an extended group of 818 patients.

Conclusions. These results suggest LRP5 alleles influence the type of hip fractures. They support the view that different genetic factors are involved in cervical and trochanteric fractures, which should be taken into consideration in future genetic association studies.

KEYWORDS: Osteoporosis, lipoprotein receptor-related protein, estrogens, aromatase, collagen, polymorphisms

INTRODUCTION

Hip fractures represent the most devastating consequence of osteoporosis and cause significant morbidity and mortality in old women and men. Several risk factors have been identified, including low body mass index (BMI), physical inactivity, propensity to fall, etc. [1]. On the other hand, some treatments, such as thiazide diuretics may exert a protective effect [2]. Bone mineral density (BMD) has a strong hereditary component [3]. The propensity to suffer osteoporotic fractures also has an hereditary influence, which is revealed by the increased fracture risk in relatives of patients with hip fractures [4]. However, the influence of genetic factors on fracture risk is often more difficult to demonstrate than their influence on BMD, particularly in elderly individuals. This is due to the confounding influence of other variables, including comorbid conditions and falls. Nevertheless, hereditary factors have been estimated to account for up to 68% of the risk of hip fractures in patients under the age of 69 and 47% in those between 69 and 79 years old [5]. A few studies have found significant associations between certain genetic polymorphisms and the global risk of hip fractures [6-9]. Hip fractures can be divided into three main types according to their location: fractures of the femoral neck (cervical fractures, fractures of the trochanter region (trochanteric), and fractures below the lesser trochanter (subtrochanteric). Several epidemiological investigations suggest that hip fractures constitute a heterogeneous group whose risk factors only partially overlap. For instance, it has been suggested that bone geometry may be more important in determining the risk of cervical fractures, whereas low BMD may be a stronger risk factor for trochanteric fractures [10]. These results led us to hypothesize that the influence of genetic factors may not be the same for all types of hip fractures. Thus, the aim of this study was to explore the differential association of several polymorphisms of genes known to play an important role in skeletal homeostasis with cervical and trochanteric hip fractures.

SUBJECTS AND METHODS

Subjects

Caucasian patients with hip fractures admitted to hospitals Marques de Valdecilla and Rio Hortega, located 300 km apart in Northern and central Spain, respectively, were included in the study. They were part of an ongoing effort to study the genetic determinants of osteoporosis which includes the collection of DNA samples from patients admitted to hospitals with osteoporotic fractures since 2003. Patients with fractures until March 2010 were included. Due to logistical reasons, not all patients admitted can be recruited, though they are otherwise unselected (except for the exclusion criteria mentioned below). Patients were excluded if they had diseases causing secondary osteoporosis (cancer, liver or kidney insufficiency, organ transplantation, malabsorption, hyperthyroidism, hyperparathyroidism), had received drugs impairing bone metabolism (glucocorticoids, antiepileptics, immunosupressors) or were of non-Spanish ancestry. Fractures due to high impact trauma (traffic accidents or falls from a height), or having occurred in relation with a prosthesis were excluded. Current alcohol consumption was defined as a daily intake of more than 10 g ethanol, while current smoking was defined as more than 5 cigarettes per day. Calcium intake from dairy products was estimated using a questionnaire. The clinical charts and x-rays were reviewed and the types of fractures (cervical, trochanteric or subtrochanteric) were recorded. However, given the small number of patients with subtrochanteric fractures, they were excluded from the analyses. Patients with two fractures of different types were also excluded. In a small subset of patients (14 with cervical fractures and 6 with trochanteric fractures), BMD was measured by DXA with an Hologic densitometer, with a coefficient of variation of 1.4% in normal subjects after repositioning.

Informed consent was obtained from each patient (or their close relatives in case of dementia). The study was part of a project approved by the Institutional Ethical Committee.

SNP selection and genotyping

We analyzed 10 single nucleotide polymorphisms (SNP) of genes known to play an important role in bone metabolism, including genes related to type I collagen, the Wnt pathway, and the estrogen pathway. All polymorphisms were located at genes which have been previously associated with bone mass or osteoporotic fractures and had a minor allele frequency of at least 0.1. They included:

- Four SNPs in the estrogen receptor alpha gene (ESR1) that have been associated with hip fractures [6;7;11].
- Four SNP in the proximal region of the Wnt co-receptor lipoprotein receptor-related protein 5 (LRP5), which has been associated with hip fractures [12].
- One SNP in the CYP19A1 gene, which encodes aromatase, the enzyme that converts androgens into estrogens in peripheral tissues. It has also been previously associated with BMD and osteoporotic fractures, including hip fractures [11;13].
- One SNP in the proximal region of the gene coding the alpha 1 chain of type I collagen (COL1A1), which is located in a SP1 binding site and has been extensively explored in genetic association studies of osteoporosis [14;15].

DNA was isolated from peripheral blood or buccal swabs using commercially available kits (Qiagen or GE Healthcare) and quantified with the Qubit procedure (Invitrogen). The SNP in the COL1A1 gene was genotyped de novo for this study; data for other SNPs were available in part from patients included in previous reports exploring their association with osteoporotic fractures [7;11;12]. SNPs in COL1A1, ESR1,and CYP19A1 genes were analyzed using specific primers and Taqman probes included in Taqman assays (Applied Biosystems). SNPs in LRP5 gene were analyzed on a mass-array Sequenom platform at the National Genotyping Center (Santiago de Compostela, Spain). Patients with trochanteric and cervical fractures were recruited throughout the inclusion period and were genotyped at the same time. The genotype concordance in replicated samples was >99%.

Statistical analysis

The Hardy-Weinberg equilibrium was tested with Plink software [16]. For each gene, the association of genotypes with fractures was tested assuming additive genetic models and the statistical significance was analyzed by the Cochran-Armitage trend test, implemented in Plink. The odd ratios (OR) for the association of genotypes combining 2 loci with the type of fracture was estimated with Epidat v3.1 (available at http://www.sergas.es/MostrarContidos_N3_T01.aspx?IdPaxina=62715), and the statistical significance was determined by a chi-square test for trend. The study power was estimated with QUANTO v1.2.3 (software available at http://hydra.usc.edu/gxe/). Polymorphisms showing significant associations were also explored after stratification by sex and after adjustment by age in logistic models. Nominal p-values are usually given in the text, but in cases of nominal $p<0.05$ multiple test-adjusted values are also shown. They were estimated by multiplying the nominal p-values by 8, which was the number of independent assays, estimated as proposed by Li and Ji [17], taking into consideration the between-loci linkage disequilibrium. This was done using the SNPSpD web tool developed by Nyholt [18].

RESULTS

The main characteristics of the 471 patients included in the study are shown in table 1. Cervical fractures were somewhat more frequent than trochanteric fractures and represented 56% of the total cases (265 patients aged 54-95 yr; vs. 206 patients aged 49-95). The average age was 3 years older and menopause was 1 year earlier in patients with trochanteric fractures. Otherwise both groups showed similar characteristics. In the small subset with DXA scans, BMD was about 1 SD smaller in those with trochanteric fractures, a difference almost reaching statistical significance ($p=0.08$).

The location and allelic frequencies of the SNPs analyzed are shown in table 2.

Two SNPs of the LRP5 gene showed differences in their genotypic frequencies in patients with cervical and trochanteric fractures, with nominal $p<0.05$ (table 3). After correcting p-values for multiple tests, one SNP remained significant (rs3781600, corrected p=0.037) and another was close to the significance threshold (rs7116604, corrected p=0.068). As shown in figure 1, the proportion of trochanteric fractures increased with the presence of A alleles at the rs7116604 locus: there were 142 trochanteric fractures out of 351 hip fractures (41%) in patients with the common GG genotype, 55 out of 110 (50%) in those with AG genotype, and 5 out of 5 (100%) in those with AA genotype. Likewise, trochanteric fractures were associated with the rare allele C at the rs3781600 locus; the proportions of trochanteric fractures were 40% (146 of 362), 51% (51 of 99) and 86% (6 of 7) in patients with GG, CG, and CC genotypes, respectively, at the rs3781600 locus. There was a small yet significant difference in age according to the type of fracture. However, including age as a co-variable produced very similar results: both rs7116604 and rs3781600 were associated with the type of fracture (nominal p-values 0.005 and 0.0037, respectively, and multiple test-adjusted p-values 0.040 and 0.030, respectively), whereas other polymorphisms remained non-associated with the type of fracture.

Similar but somewhat more significant results were observed when the analysis was restricted to the subgroup of female patients (multiple test-adjusted p-values 0.014 for both loci). The results were not significant in the male subgroup, probably due to the smaller number of individuals (supplementary table S1). In fact, there was no evidence for interaction between sex and the genotypes (p>0,20).

When both loci were considered together, the presence of the rare alleles at any locus was associated with trochanteric fractures over cervical fractures. Thus the OR was 1.7 (95% confidence interval 1.1-2.5; p=0.022) in individuals with at least one rare allele at rs7116604 or rs3781600 loci (A and C, respectively) in comparison with the homozygotes for common alleles at both loci. An allele-dosage effect appeared to exist. Thus, considering individuals bearing the

four common alleles as reference, the OR for trochanteric fractures was 1.6 in those with 1 or 2 rare alleles, and 7.5 in those with 3 or 4 rare alleles (p-value for trend 0.0074, table 4).

The analysis of other gene variations in the 471 patients with LRP5 genotypes available revealed no significant differences in the distributions of the ESR1, CYP19A1, and COL1A1 genotypes between trochanteric and cervical fractures (not shown). Therefore, we decided to extend the genotyping to an additional group of 347 patients with hip fractures, recruited from the same population. The clinical characteristics of these patients were comparable to those of the initial group (supplementary table S2). As in the initial analysis, no association of ESR1, CYP19A1 and COL1A1 genotypes with fracture type was observed in this extended group of 818 patients, either in the whole group (table 3) or in the sex-stratified analysis (supplementary table S1).

DISCUSSION

Osteoporosis has a strong hereditary component. However, little data is available about the genetic factors related to hip fractures, the most devastating consequence of osteoporotic bone fragility. Even less is known about the specific factors determining the risk of cervical versus trochanteric fractures, the two most common types of hip fractures. Nevertheless, epidemiological studies suggest the existence of etiologic differences between both fracture types [19;20]. For instance, the ratio of trochanteric to cervical fractures tends to increase with age in a sex-specific way, with the proportion of trochanteric fractures increasing in women, but not in men [21;22]. BMD has been reported to be lower in trochanteric than in cervical fractures in several studies, and consequently BMD appears to be a better predictor of trochanteric fractures [10;23-25], though this has not been a constant finding [26].

Several investigators reported that anthropometric parameters and the geometry of the pelvis and femur may specifically influence the risk of a particular type of hip fractures. For instance, patients with cervical fractures tend to be taller than those with trochanteric fractures [27;28].

Duboeuf et al. found a longer hip axis length in patients with cervical fractures than in controls, but this was not found in trochanteric fractures [28;29]. The neck shaft angle also appears to be related to the type of fracture, with a wider angle predisposing to cervical fractures [26;27;30], though this has not been confirmed in some studies [31].

BMD and body height are quantitative traits with an important hereditary influence. Therefore, we hypothesized that the allelic variations of some genes known to be involved in skeletal homeostasis and the risk of fractures could also specifically influence the susceptibility to trochanteric or cervical fractures. We checked this hypothesis by comparing the genotypic frequencies in patients with both fracture types. Estrogens are known to play a critical role in bone acquisition and maintenance in women and in men [32]. We and others have found an association between alleles of the aromatase and estrogen receptor alpha genes and hip fractures [6;11]. However, in this study we did not find significant differences in the genotype distribution between patients with trochanteric and cervical fractures. Type I collagen is a major component of bone matrix and has been widely studied as an osteoporosis candidate gene. A polymorphism in the promoter region influencing Sp1 binding has received the greatest attention and, according to a recent meta-analysis, it appears to be associated with BMD and fracture risk [15]. Nevertheless, our results suggest that it does not predispose to a specific type of hip fracture.

The Wnt pathway has emerged as a critical player in skeletal homeostasis. Wnt ligands bind to complex membrane receptors, which are composed of several proteins, including LRP5. Rare gain-of-function mutations of LRP5 cause an abnormal phenotype with high bone mass, while loss-of-function mutations induce osteoporosis [33]. Some common LRP5 polymorphisms, although with smaller influence on the skeleton, have been associated with BMD or osteoporotic fractures in several candidate gene studies and genome-wide studies including individuals from the general population [12;34-40]. In the present study, we show that SNPs in the proximal region of the LRP5 gene are specifically associated with the type of hip fractures. The

mechanisms by which LRP5 variations influence the fracture type are unclear. Patients with trochanteric fractures appear to have lower BMD than those with femoral neck fractures [10;20;25]. Therefore, genetic variants might act through an influence on BMD. However, we only have BMD data in a very small subset of patients and for this reason cannot confirm this hypothesis. In support of this view, as mentioned above, several investigators found an association between LRP5 variants and BMD in elderly populations, thus raising the possibility that BMD differences could influence the fracture type distribution across LRP5 genotypes. Alternatively, LRP5 variants might influence bone geometry. In fact, LRP5 polymorphisms appear to have a strong association with peak BMD, suggesting that they are particularly important during the growth period [38;40]. In line with this notion, LRP5 variants have been reported to be associated with body height and other anthropometric parameters [12]. Also in line with this, an association of polymorphisms of DKK1 (a Wnt inhibitor) with hip axis length has been reported [41]. Patient's heights, and/or other related geometric characteristics of the skeleton, appear to influence differentially the risk of trochanteric or cervical fractures [10;26-28]. Hence, LRP5 variants might also influence the predisposition to a particular type of hip fractures through their influence on skeletal size and geometry. Future studies combining genetic, bone geometry, and fracture data could confirm or refute this contention.

This study has several limitations. On the one hand, the sample size limited the power to identify polymorphisms with a small influence on the fracture type. We estimated that our extended group analysis had a 90% power to detect polymorphisms associated with a particular type of fracture with an OR≥1.5 (assuming a co-dominant model and the allelic frequencies actually found). However, it was underpowered to detect SNPs with smaller influences. Since we only included Spanish patients, it is unclear whether the results can be extended to Non-Caucasian patients. Only a minority of patients had BMD, height and weight measured and we do not have

bone geometry data. Therefore, we cannot elucidate if those variables mediate the association of LRP5 polymorphisms with the type of fractures.

In conclusion, we report an association of some common polymorphisms of the LRP5 gene with the type of hip fractures. To our knowledge, this is the first report showing that genetic background may have an influence on the type of fractures. The specific mechanisms involved remain to be established, but the study gives further support for the role of allelic variations of Wnt pathway genes in the susceptibility to osteoporotic fractures. On the other hand, our study reinforces the notion that different factors are involved in cervical and trochanteric fractures and suggests that it should be taken into consideration in future genetic association studies of hip fractures.

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CONFLICTS OF INTEREST

Authors do not have conflicts of interest to declare.

Table 1. Characteristics of patients with hip fractures (percentages or mean±SD).

	Cervical (n=265)	Trochanteric (n=206)	p
Female sex, %	80.6	83.7	ns
Age, yr	80±7	83±6	0.001
Age at menopause, yr [a]	50±4	49±5	0.027
Calcium intake, mg	604±331	618±365	ns
Total hip BMD, g/cm^2 [b]	0.739±0.121	0.628±0.109	0.08
Alcohol, %	5.7	4.6	ns
Tobacco, %	3.5	4.6	ns
Other non-vertebral fracture, %	24.8	24.6	ns
Family history of hip fracture, %	10.0	10.1	ns
Dementia, %	32.3	33.5	ns
Stroke or transient ischemic attack, %	11.2	12.2	ns
Type 2 diabetes, %	24.6	24.9	ns
Thiazides, %	21.2	17.7	ns

a) Data from 206 patients
b) Data from 20 patients

Table 2. Location and allele frequencies of the polymorphisms studied

SNP	Chromosome	Gene	Minor allele	Frequency, %	p (HWE)
rs2234693	6	ESR1	C	46.8	0.10
rs1801132	6	ESR1	G	18.5	0.56
rs3020314	6	ESR1	C	26.9	0.91
rs1884051	6	ESR1	G	24.1	0.44
rs7116604	11	LRP5	A	13.0	0.48
rs312014	11	LRP5	C	38.7	0.39
rs4988300	11	LRP5	G	47.4	0.53
rs3781600	11	LRP5	C	11.5	0.09
rs1062033	15	CYP19A1	C	40.5	0.66
rs1800012	17	COL1A1	T	22.3	0.93

Table 3. Genotype frequencies according to hip fracture type, number of individuals with genotypes available and unadjusted p-values for the comparison between trochanteric and cervical fractures (the multiple test-adjusted threshold for significance was 0.0062).

SNP	Gene	Genotypes	n	Frequency in trochanteric fractures	Frequency in cervical fractures	p
rs2234693	ESR1	CC/CT/TT	807	73/174/105	104/209/142	0.59
rs1801132	ESR1	GG/GC/CC	797	16/94/238	8/132/309	0.062
rs3020314	ESR1	CC/CT/TT	795	27/140/182	25/184/237	0.48
rs1884051	ESR1	GG/GA/AA	803	24/123/204	23/174/255	0.41
rs7116604	LRP5	AA/AG/GG	466	5/55/142	0/55/209	0.0085
rs312014	LRP5	CC/CG/GG	455	29/100/73	39/123/91	0.85
rs4988300	LRP5	GG/GT/TT	452	48/99/55	59/127/64	0.83
rs3781600	LRP5	CC/CG/GG	468	6/51/146	1/48/216	0.0047
rs1062033	CYP19A1	CC/CG/GG	788	59/157/138	79/229/126	0.25
rs1800012	COL1A1	TT/TG/GG	754	18/117/191	22/139/267	0.34

Table 4. Association of LRP5 polymorphisms with the type of fractures: combined analysis of the rs7116604 and rs3781600 loci. The table represents the number of individuals divided according to the number of rare alleles at those loci (A at rs7116604, C at rs3781600) and the type of fracture. The odds ratios (and 95% confidence intervals) for trochanteric fractures over cervical fractures are also shown, considering patients homozygous for common alleles at both loci as reference.

	Number of rare alleles			
	0 (reference)	1-2	3-4	≥ 1
Trochanteric	137	56	5	61
Cervical	206	54	1	55
OR	1	1.6 (1.0-2.4)	7.5 (0.9-65.0)	1.7 (1.1-2.5)
p		0.007 (trend)		0.022

FIGURE LEGEND

Figure 1. Relative distribution of cervical and trochanteric fractures among patients with different LRP5 genotypes at the rs7116604 and rs3781600 loci (p-values 0.0085 and 0.0047, respectively, for the association between genotypes and the type of hip fracture). The number of patients in each category is also shown.

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Supplementary online materials

Table S1. Genotype frequencies according to the fracture type in males and in females.

			---------- Females ----------			---------- Males ----------		
SNP	Gene	Genotypes	Frequency in Trochanteric	Frequency in Cervical	p-value	Frequency in Trochanteric	Frequency in Cervical	p-value
rs2234693	ESR1	CC/CT/TT	62/148/89	86/166/118	0.94	11/26/16	18/43/24	0.84
rs1801132	ESR1	GG/GC/CC	12/81/203	6/106/252	0.45	4/13/35	2/26/57	0.61
rs3020314	ESR1	CC/CT/TT	22/120/155	20/147/194	0.47	5/20/27	5/37/43	0.82
rs1884051	ESR1	GG/GA/AA	19/107/173	19/140/209	0.97	5/16/31	4/34/46	0.99
rs7116604	LRP5	AA/AG/GG	5/46/118	0/39/174	0.0018	0/9/24	0/16/34	0.64
rs312014	LRP5	CC/CG/GG	22/87/61	32/101/71	0.58	7/13/12	7/21/20	0.48
rs4988300	LRP5	GG/GT/TT	43/81/46	50/106/45	0.56	5/18/9	9/20/19	0.60
rs3781600	LRP5	CC/CG/GG	6/43/121	1/35/177	0.0018	0/8/25	0/13/38	0.89
rs1062033	CYP19A1	CC/CG/GG	52/129/116	65/178/124	0.32	7/23/22	14/43/28	0.31
rs1800012	COL1A1	TT/TG/GG	14/100/160	17/116/210	0.53	4/17/31	5/23/57	0.39

Supplementary online materials

Table S2. Characteristics of the extended group of patients with hip fractures (percentages or mean±SD).

	Cervical (n=455)	Trochanteric (n=363)	p
Female sex, %	81.2	85.0	ns
Age, yr	82±8	84±7	ns
Age at menopause, yr	50±4	49±5	ns
Calcium intake, mg	621±320	615±340	ns
Alcohol, %	3.2	3.1	ns
Tobacco, %	3.0	3.7	ns
Other non-vertebral fracture, %	26.2	27.7	ns
Family history of hip fracture, %	11.9	8.9	ns
Dementia, %	32.4	33.5	ns
Stroke or transient ischemic attack, %	11.2	12.2	ns
Type 2 diabetes, %	24.7	24.9	ns
Thiazides, %	22.4	18.1	ns

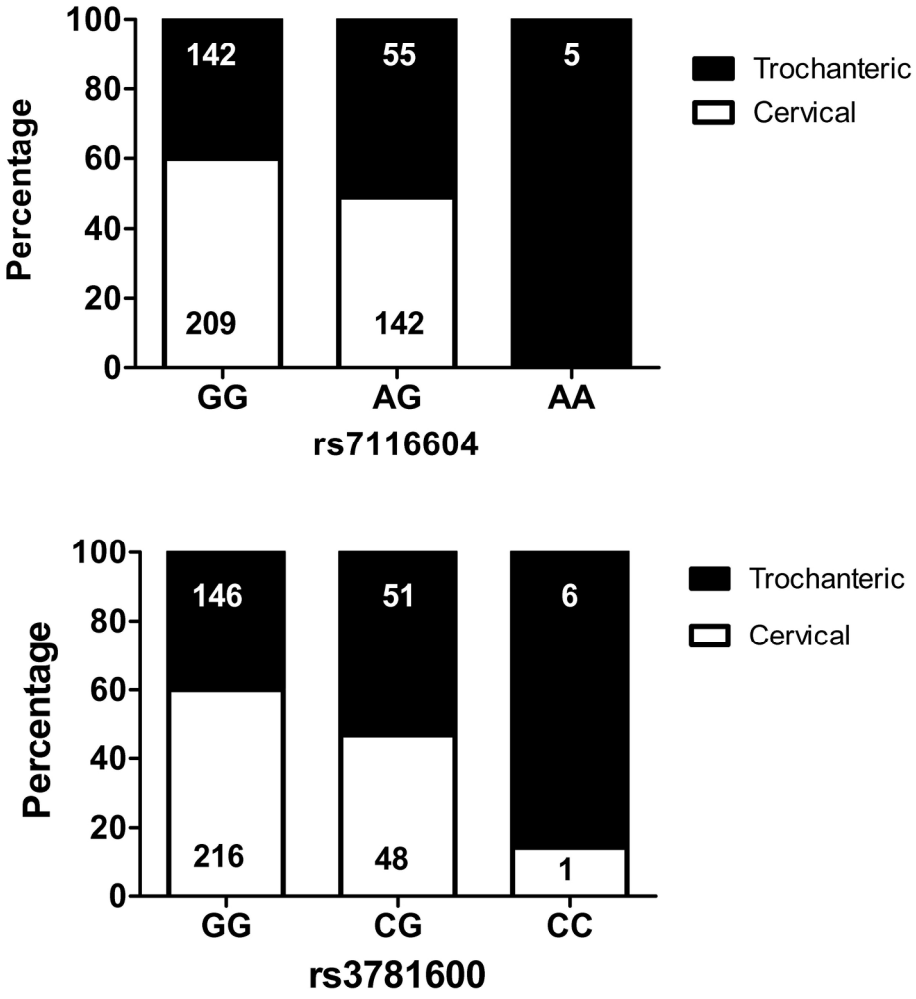

Figure 1. Relative distribution of cervical and trochanteric fractures among patients with different LRP5 genotypes at the rs7116604 and rs3781600 loci (p-values 0.0085 and 0.0047, respectively, for the association between genotypes and the type of hip fracture). The number of patients in each category is also shown.
196x215mm (300 x 300 DPI)