GH receptor isoforms and skeletal fragility in acromegaly

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Abstract

Objective: Acromegaly is associated with an increased prevalence of vertebral fractures (VFs) in close relationship with GH hypersecretion. Two isoforms of the GH receptor (GHR) have been identified; the two isoforms differ or not by the expression of the protein fragment encoded by exon 3 of the GHR gene. Deletion of the exon 3 may influence the functional properties of the GHR and affect fracture risk in acromegalic patients.

Design: A cross-sectional study was designed to investigate the association between the d3-GHR isoform and the prevalence of VFs in patients with acromegaly.

Methods: In this study, 109 acromegalic patients were included (M/F, 48/61): 73 with controlled/cured acromegaly and 36 with active disease. GHR genotype was assessed in each patient. All patients were evaluated for VFs and bone mineral density at lumbar spine and hip. Serum IGF1 levels and bone metabolism markers were measured. A multivariate analysis was performed to establish risk factors for VFs in our population.

Results: d3-GHR carriers showed an increased prevalence of VFs when compared with patients expressing full-length GHR (35/55 vs 12/54; \( P < 0.001 \)). The association between GHR deletion and VFs was demonstrated both in patients with active disease and in those with controlled/cured disease. Out of 35 patients who were prospectively evaluated, 13 (37.1%) developed incident VFs. The incidence of VFs was significantly higher in patients for whom the GHR gene has been deleted when compared with those harboring the fl gene (\( P = 0.04 \)). In multivariate analysis, male sex (odds ratio (OR), 3.250; \( P = 0.041 \)), IGF1 levels (OR, 1.183; \( P = 0.031 \)), length of active diseases (OR, 1.038; \( P = 0.001 \)), and d3-GHR genotype (OR, 3.060; \( P = 0.015 \)) were all confirmed as risk factors of VFs in our population.

Conclusions: This study suggests for the first time that exon 3 deletion of GHR may predispose patients with active and controlled acromegaly to a higher risk of VFs.

Introduction

Growth hormone (GH) and insulin-like growth factor 1 (IGF1) are important regulators of the bone remodeling process. Although GH may act directly on skeletal cells, most of its effects are mediated by IGF1 that exerts its action as a systemic hormone synthesized in the liver under GH stimulation or as a local growth factor synthesized in peripheral tissues (1). Circulating GH binds to a single-chain transmembrane glycoprotein receptor (GH receptor (GHR)). The GHR in its final form consists of an extracellular, a transmembrane, and an intracellular domain, whereas the GHR gene consists of nine exons encoding the receptor protein and several additional untranslated exons (2). To date, two isoforms of human GHR have been identified: a full-length (fl-GHR) isofrom retaining the protein fragment encoded by exon 3 and an exon 3-deficient (d3-GHR) isoform excluding this
fragment (3). Exon 3 encodes a segment in the extracellular domain of the receptor; although both the isoforms retain the capacity of binding GH with a high affinity, there may well be significantly functional differences between the two isoforms (4, 5). In fact, GH-deficient patients expressing d3-GHR isoform are known to be more responsive to GH replacement therapy than fl-GHR patients (5, 6, 7). Acromegalic patients with exon 3 being deleted in the GHR gene consistently express higher serum IGFI levels even after treatment with somatostatin analogs or pegvisomant (8, 9, 10, 11). Moreover, exon 3 deletion in acromegalic patients has been associated with a higher incidence of long-term complications of the disease, such as diabetes, osteoarthritis, and colon polyps (12, 13).

Over the recent past years, an expanding body of knowledge is supporting the idea that patients with acromegaly suffer from skeletal fragility and are at an increased risk for vertebral fractures (VFs). Fracture risk correlates with the activity of the disease and the presence of concomitant additional risk factors, such as hypogonadism (14, 15, 16). Nonetheless, it is still largely unclear as to whether GHR polymorphisms are implicated in this phenomenon and have an impact on skeletal fragility in acromegaly. Recently, Wassenaar et al. (13) did not find any significant association between the deletion of GHR exon 3 and VFs. However, the authors restricted their analysis to patients with longstanding controlled acromegaly. In our opinion, active acromegaly may well provide a better model to study the potential impact of GHR polymorphisms on skeletal fragility. Therefore, the aim of our study is to investigate the effects of exon 3 deletion of GHR on bone metabolism, bone mineral density (BMD), and incidence of VFs in patients with active and controlled/cured acromegaly.

Subjects and methods

Subjects

A cross-sectional study was designed and a total of 109 patients consecutively treated at our Institution (Pituitary Unit of Endocrinology Department, Gemelli Hospital, Rome) were enrolled. All patients were diagnosed with acromegaly or had a previous diagnosis of acromegaly. Exclusion criteria from the study were: i) diagnosis of active neoplastic disease; ii) ongoing treatment with anti-osteoporotic drugs with the exception of calcium and vitamin D supplements; iii) treatment with drugs causing osteoporosis with the exception of glucocorticoid replacement therapy in hypopituitary patients; iv) clear history of moderate or high-energy VFs; v) history of surgical intervention on the spine; and vi) prolonged immobilization (more than 6 weeks). Out of the 109 enrolled patients, 84 had been already evaluated in previously published studies from our research group (14, 15, 16). Both surgically treated and medically treated patients were included.

Active acromegaly was defined as the failure of suppression of GH plasma levels ≤1 ng/ml after a 75 g oral glucose load and fasting plasma IGFI levels above the normal ranges for sex and age (17). Patients undergoing somatostatin analog treatment were evaluated by measurement of serum random GH and IGFI, and those undergoing pegvisomant were evaluated by serum IGFI alone, whereas patients treated by neurosurgery alone were evaluated also by serum GH after a 75 g oral glucose load, according to the current guidelines (18). Acromegaly was defined as controlled if the IGFI values were within the reference ranges for age and, in patients undergoing treatment with somatostatin analogs and after neurosurgery, random GH was below 1.0 ng/ml. When the 75 g oral glucose load was performed, the GH values of 0.4 ng/ml or below were considered as expression of cured disease. Disease activity was defined as ‘cured’ if, after a previous treatment for acromegaly, patients had normal biochemical parameters at follow-up with no ongoing medical therapy. Patients’ gonadal status was also assessed: hypogonadism was defined as absence of or irregular menstrual cycles in women and low total testosterone associated with symptoms and signs of sexual dysfunction in men. Hypogonadic patients undergoing replacement therapy with sex steroids were assumed to be eugonadal only if treatment was started at least 12 months before study entry. All patients signed an informed consent form after full explanation of the purpose and nature of all procedures used. The study was also approved by our Institutional Ethical Committee.

Biochemical measurements

All biochemical measurements were performed within 6 months of lumbar and femoral neck dual energy X-ray absorptiometry (DXA) scan and vertebral X-ray assessment. Blood samples were collected in fasting conditions. GH and IGFI levels were measured using the Immulite 2000 immunoassay system (Siemens, Diagnostic Product Corp., Los Angeles, CA, USA). The inter-assay coefficient of variation was 5.5–6.2% for GH assay, and 6.4–11.5% for IGFI assay; detection lower limits were 0.01 and 0.2 μg/l.

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respectively. IGF1 levels were reported as absolute concentrations and SDS relative to normal age-adjusted adult values (normal range from −2 to +2 SDS). The formula used for SDS calculation is, as reported previously, \((\text{IGF1}_\text{p} - \text{IGF1}_{\text{adj-value}})/\text{IGF1}_{\text{n.d.}}\), where IGF1\(_p\) is the absolute level of IGF1 of the patient, and IGF1\(_{\text{adj-value}}\) and IGF1\(_{\text{n.d.}}\) are the average and the S.D. of the age-matched IGF1 levels in healthy patients respectively (19). Serum 25-hydroxy vitamin D was measured using a chemiluminescent assay (Liaison Instrument, DiaSorin Inc, Stillwater, MN, USA). Measurements were made at the time of the spinal X-ray. BMD is expressed as absolute values and Z-scores using a standard Caucasian European reference population. Fractured vertebrae, defined as described below, were excluded from the lumbar BMD analysis. A quantitative morphometrical assessment of VFs in the T4–L4 region was performed using a dedicated morphometrical software (Spine-X Analyzer, ICAM Diagnostics, Milan, Italy) (20). Briefly, using a translucent digitizer and a cursor, six points were marked on the borders of each vertebral body. Anterior (Ha), middle (Hm), and posterior (Hp) vertebral heights were measured and height ratios were calculated for each vertebra from T4 to L4; fractures were defined as mild, moderate, or severe based on a height ratio decrease of 20–25, 25–40, and more than 40% respectively (21). All VFs identified by the morphometrical scan were independently and blindly reviewed by two experienced physicians (M Mormando and L A Nasto). Moreover, a spinal deformity index (SDI) was calculated for each patient. Each VF was assigned a score according to the fracture severity (one for mild, two for moderate, and three for severe); SDI was defined as the sum of all scores in a single patient (22).

After 3 years of follow-up, 35 patients without pre-existing VFs were evaluated for incident VFs. Incident VFs were defined as a decrease of 20% or more and at least 4 mm in length in any of the three vertebral heights (Ha, Hm, or Hp) compared with baseline radiograph (16).

**Genetic testing**

DNA was extracted from 100–200 µl peripheral blood using the Illustra blood genomicPrep Mini Spin Kit (GE Healthcare, Little Chalfont, UK) for the detection of the GHR gene polymorphism. Polymorphisms were studied through standard PCR amplification by Eppendorf Master Mix (2.5×) (Eppendorf, Hamburg, Germany). The following primers were used for amplification, 5'-TGTGTGCTGGTCTGTGTGCTC-3', 5'-AGTCGGTTCC-TGGGACGAGA-3', and 5'-CCTGGAATACACTTTGCA-GACTC-3' (23). The reaction was carried out with one cycle at 94 °C for 5 min (denaturing), 35 cycles at 94 °C for 30 s (denaturing), 60 °C for 30 s (annealing), 72 °C for 60 s (extension), and one cycle at 72 °C for 7 min (last extension). The amplification products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide labeling. The full-length allele (fl-GHR) is represented by a 935-bp fragment and the exon 3-deficient allele (d3-GHR) by a 532-bp fragment.

**Statistical analysis**

Data are expressed as mean ± s.d., unless otherwise stated. Continuous variables were tested using the t-test, whereas frequencies were compared using the χ²-test with the Fisher correction, when appropriate. Correlation between variables was sought using Pearson’s correlation coefficient. A multivariate logistic regression model was used for statistical analysis of risk factors for occurrence of VFs. Statistical significance was assumed when P ≤ 0.05. Data were analyzed using the SPSS Software, version 17.

**Results**

A total of 48 men and 61 women were included in the study (mean age 47 years, ±19–79). Of them, 58 patients had controlled disease (nine of them received medical therapy as first-line treatment, whereas 49 were treated with somatostatin analogs or pegvisomant after unsuccessful neurosurgery) and 15 patients were cured by neurosurgery. The remaining 36 patients had active disease; of them, 31 were on medical therapy after unsuccessful neurosurgery and radiotherapy and five were waiting for surgery or refused it at the time of this study. Of the total number of patients, 61 patients were eugonadal (35 men and 26 women), 20 patients were
hypogonadal (13 men and seven women), and the remaining 28 women were post-menopausal and were assigned to the hypogonadic group as well.

We found 54 patients (49.5%) to be homozygous for the fl-GHR isoform and 55 patients (50.5%) to be carriers of at least one allele of the d3-GHR isoform. A total of 18 patients were homozygous for the d3-GHR isoform. Patients were assigned to two groups: i) homozygous carriers of the WT allele (flfl-GHR, referred as fl patients hereafter) and ii) homozygous or heterozygous carriers of the d3-GHR isoform and d3d3-GHR, referred as d3 patients hereafter). We decided to apply a dominant model because we did not observe any significant difference in terms of VFs between carriers of one d3 allele and carriers of two d3 alleles; we did not observe any gene-dosage effect on fractures. The two groups did not differ in sex, lumbar BMD values, serum IGF1 levels, and bone metabolism parameters (PTH, vitamin D, osteocalcin, and β-CTx). BMD and Z-score of the lumbar spine were not different between fl patients and d3 patients even after adjusting values for gender and BMI (Table 1). A significantly positive linear correlation between IGF1–SDS levels and β-CTx was observed both in d3 patients (Pearson’s $R^2$, 0.552; $P=0.001$) and in fl patients (Pearson’s $R^2$, 0.194; $P=0.022$), whereas only a positive trend was observed between IGF1–SDSs and osteocalcin in d3 patients (Pearson’s $R^2$, 0.0433; $P=0.407$). No correlation was found between IGF1–SDS and osteocalcin levels in fl patients (Pearson’s $R^2$, 0.060; $P=0.192$; Fig. 1).

Of the total number of patients, 47 (43.1%) had at least one VF, with a total of 53 mild, 13 moderate, and eight severe fractures. The average SDI of fractured patients was 2.19 ($\pm$ 1.48). Fractured patients did not differ from non-fractured patients with regard to age (48.49 vs 45.32 years, $P=0.213$), lumbar Z-score ($-0.57$ vs $-0.10$, $P=0.100$), femoral Z-score (0.153 vs 0.280, $P=0.527$), and duration of total disease (91.43 vs 72.17 months, $P=0.151$). Patients with fractures were more frequently male (27/47 males, 57.4% vs 21/62 females, 33.8%) ($P=0.012$). Moreover, fractured patients showed significantly higher IGF1 levels (SDS 3.45 vs 2.10; $P=0.022$), and had longer duration of active disease (52.43 vs 23.31 months; $P<0.001$) than non-fractured patients. PTH and vitamin D levels did not differ significantly between the two groups. Osteocalcin levels did not differ significantly either ($P=0.769$), whereas β-CTx levels were higher in fractured patients although not significantly so (0.61 vs 0.45 ng/ml; $P=0.071$) (Table 2).

Prevalence of VFs was significantly higher in d3 carriers than fl patients (63.6 vs 22.2%; $P<0.001$) and this association was confirmed in patients with controlled/cured and active disease (Fig. 2). The mean SDI was significantly higher in fractured d3 carriers (1.24 $\pm$ 1.44)

![Table 1](image-url)
than in fractured fl carriers (0.65 ± 1.43) (P = 0.035). In 35 patients prospectively evaluated, 13 (37.1%) developed incident VFs. The incidence of VFs was significantly higher in patients for whom the GHR gene has been deleted when compared with those harboring the fl gene (52.6 vs 18.8%; χ², 4.3; P = 0.04).

Multivariate analysis

Variables associated with VFs were entered in a multivariate logistic regression model in order to assess the fracture risk associated with each variable. GHR isoform, IGF1 levels, length of active disease, and male sex were all significantly associated with an increased risk of VFs. Odds ratio (OR) of having a VF in patients who were carriers of one or two d3 alleles was 3.060 (P = 0.015). Each 1 SDS level of IGF1 was associated with a 1.183 OR (P = 0.031), whereas length of active disease each month had a 1.038 OR (P = 0.001). Male sex was associated with an increased risk of VFs with an OR of 3.250 (P = 0.041; Table 3).

Discussion

Data from our study demonstrate for the first time, to the best of our knowledge, that the deletion of the exon 3 of GHR (d3-GHR) in at least one allele is associated with a higher prevalence of VFs in acromegalic patients. This is the first report examining the effects of GHR isoforms on bone fragility and risk of VFs in a heterogeneous population of patients (active acromegaly, controlled/cured disease).

The d3-GHR polymorphism enhances GHR signal transduction by altering the structure of the extracellular domain of the receptor. The increased intracellular signal transduction may enhance sensitivity to GH as demonstrated by studies conducted on GH-deficient children.
At the time of our study, immediately evident in active acromegaly because of the complications of the disease. Nonetheless, the effect of d3-GHR polymorphism on bone metabolism markers and their association with fractures. Previous studies demonstrated that active acromegaly was associated with increased calcium and phosphate serum levels, increased calciuria and markers of bone formation, and bone resorption (26, 27, 28, 29, 30, 31). Bonadonna et al. (14) found that fractured acromegalic women had a significantly increased prevalence of osteoarthritis, adenomatous polyps, and dolicoocolon but anthropometric parameters, cardiovascular risk factors, and GHR isoforms. In a selected population of patients with long-term remission of acromegaly, Wassenaar et al. demonstrated that d3 carriers had an increased prevalence of osteoarthritis, adenomatous polyps, and dolicoocolon but anthropometric parameters, cardiovascular risk factors, BMD, and (non) VFs did not differ between fl homozygous patients and d3 patients.

In our population, comprising patients with active and controlled/cured acromegaly, we confirmed some of the previous results concerning BMD not significantly correlated with fractures (14, 15). However, in our cohort, a significant difference in terms of prevalence of VFs was observed between fl homozygous patients and d3 carriers. In fact, patients carrying at least one d3 allele had a significantly increased prevalence of VFs (Fig. 2). Another original observation of our study was concerned with bone metabolism markers and their association with fractures. The authors also studied the prevalence of other comorbidities such as adenomatous polyps, dolicocolon, and the association between anthropometric parameters, cardiovascular risk factors, and GHR isoforms. In a selected population of patients with long-term remission of acromegaly, Wassenaar et al. (13) investigated the impact of d3 deletion of GHR on BMD, osteoarthritis, and (non) VFs. The authors also studied the prevalence of other comorbidities such as adenomatous polyps, dolicoocolon, and the association between anthropometric parameters, cardiovascular risk factors, and GHR isoforms. In a selected population of patients with long-term remission of acromegaly, Wassenaar et al. demonstrated that d3 carriers had an increased prevalence of osteoarthritis, adenomatous polyps, and dolicoocolon but anthropometric parameters, cardiovascular risk factors, BMD, and (non) VFs did not differ between fl homozygous patients and d3 patients.

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higher serum bone alkaline phosphatase and urinary deoxypiridinoline and lower serum levels of vitamin D compared with non-fractured patients, but the study population was too small (36 women) to draw any real final conclusion. In our population, β-CTx (a bone catabolism marker) correlated with a higher prevalence of VFs and, although not significant (P=0.07), this assessment may be helpful to estimate bone fragility in acromegalic patients. In addition, we found a positive linear correlation between IGFI1 levels (increased in active disease) and β-CTx in both fl and d3 patients, although the correlation was stronger for d3 patients (Fig. 1). Taking the stronger linear correlation between IGFI1–SDS and β-CTx in d3 carriers into consideration, we can hypothesize that in these patients when acromegaly is active, the increased sensitivity to GH may increase bone remodeling and consequently plasma levels of bone catabolism markers.

Our study confirmed previous cross-sectional observations in which acromegalic patients developed VFs independent of normal BMD and there were no significant differences in terms of BMD values between fractured and non-fractured patients (14, 15, 32). For this reason, X-ray assessment is of paramount importance in establishing the real fracture risk in patients affected by acromegaly (33, 34) as well as in all patients affected by other forms of secondary osteoporosis (35). In accordance with previous studies (14, 15), fracture rate in our population was 43% and the biochemical control of acromegaly appeared effective in reducing the risk of fractures.

We also found an increased prevalence of VFs in men, which has not been previously reported in other studies (14, 15, 26, 32). In other previous cross-sectional studies, acromegalic men and only post-menopausal women were analyzed separately. Conversely, two recent studies have demonstrated a higher prevalence of VFs in acromegalic males (36, 37), and also in the recently published prospective study (16), the percentage of incident fractures was slightly, but not significantly, higher in males (45%) than in females (36%). The authors explained this finding by assuming a bone protective effect of estrogens in pre-menopausal women included in this study. Alternatively, an increased bone tissue sensitivity to GH stimulation in males may be hypothesized as already demonstrated for other clinical end-points of GH (38). However, this effect appeared to be independent of the polymorphisms of GHR.

Although the lack of sex steroids was traditionally considered as a risk factor for bone loss at lumbar spine in patients with acromegaly (39), data on association between VFs and hypogonadism have been inconclusive (15, 32, 40, 41), probably because the negative effects of hypogonadism seem to be variable related to the activity of acromegaly (16). In fact, hypogonadism was shown to be associated with the high risk of VFs in patients with controlled/cured disease but not in those with active disease (16). Consistent with these findings, in this study performed on a mixed population of acromegaly patients (i.e. with active and controlled/cured disease), we did not find a significant association between hypogonadism and VFs.

The real novelty of this work is the strong correlation found between d3-GHR isoform and high prevalence of VFs in patients with acromegaly. Interestingly, we also observed a significant correlation between d3-GHR isoform and incident VFs in a small subgroup of acromegalic patients followed up for 3 years. Notably, all these patients did not have a high prevalence of VFs at baseline and this feature provides further evidence that deletion of GHR might have been involved in the pathogenesis of skeletal fragility in acromegaly regardless of coexistent risk factors for fractures (16). Very few contrasting data are currently available on the relationship between GHR polymorphisms and osteoporosis in healthy patients. A progressive decrease in GH secretion has been hypothesized; however, recent experimental evidences have suggested that, besides the age-dependent decline of GH and IGFI1 serum levels, dysregulation of GH and IGFI1 action may be due to an impairment of the post-receptor signaling of GHR (42). In rodents, complete inhibition of GH action by disrupting or knocking out of the GHR gene (which resulted in GH resistance, elevated GH levels, and markedly reduced IGFI1 levels) led to reduced muscle mass and BMD (43). In line with these observations, some authors reported that subjects receiving GH replacement therapy showed no significant changes in BMD (44, 45), probably because of other mechanisms being involved in elderly subjects affected by pituitary disease (i.e. impaired post-receptor GH signaling). The effects of GHR exon 3 isoform expression on osteoporosis has been studied in healthy patients previously. Kenth et al. (46) studied the possible influence of GHR exon 3 isoform on final adult height, quantitative ultrasound (QUS) of the heel, and BMD (spine and femoral neck) in a healthy population of 368 white women; there was no correlation between GHR exon 3 genotype and final height, QUS, and BMD. In acromegalic patients, Waassen et al. (13) did not find a significant difference in terms of BMD and prevalence of VFs between carriers of exon 3 deletion and patients carrying the WT allele. In our population, on the other hand, which consists also of patients affected by active acromegaly, a stronger impact of GHR polymorphism on the risk of fracture due to GH excess can be expected.
In conclusion, our study demonstrates for the first time that another important risk factor for vertebral fragility fractures in acromegalic patients does exist, i.e. the presence of d3 deletion of GHR gene. Genetic testing may represent an additional tool for stratification of risk of VFs in acromegalic patients and help in devising an ordered plan and adequate management in each patient.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
L De Marinis designed this study and M Mormando conducted the study. M Mormando and A Giampietro collected data. L A Nasto, A Bianchi, and G Mazzotti conducted data analysis and interpretation. M Mormando drafted the manuscript. L A Nasto, A Giustina, and E Pola revised the manuscript content. A Pontecorvi, E Pola, and L De Marinis approved the final version of the manuscript. M Mormando and L De Marinis were responsible for the integrity of the data.

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