Mutational and Polymorphic Analysis of the Estradiol Receptor-α Gene in Men with Symptomatic Vertebral Fractures


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Abstract. In view of the importance of estrogens in the maintenance of the skeleton in men, we have carried out mutational analysis of all the exons of the estrogen-receptor-α (ER-α) gene in 64 men (36 patients with symptomatic vertebral crush fractures and 28 control subjects). Initial screening of the ER-α gene, carried out by single-strand conformation polymorphism analysis followed by sequencing, showed conservative mutations in exon 4 which resulted in a single base substitutions producing GGC→GGC transition in codon 274. We also carried out polymorphic analysis of the ER-α gene at the PvuII restriction site in 82 men with a range of bone density measurements (53 with symptomatic vertebral fractures and 29 controls). The frequencies of PP, Pp, and pp genotypes were 20.7%, 48.8%, and 30.5%, respectively. The distribution of the alleles was similar in the patients with symptomatic vertebral crush fractures and male control subjects. There was no association between ER-α genotypes and bone mineral density or arthropometric parameters. This relatively small study suggests that mutations in the ER-α gene are unlikely to be a common cause of osteoporosis in men with vertebral fractures. Furthermore, polymorphic variation of the ER-α gene appears to have little effect on the pathogenesis of osteoporosis in men.

Key words: Male osteoporosis — Estrogen receptor — Mutational analysis — PvuII polymorphism — Bone mineral density

Osteoporosis in men is a poorly understood disorder. Recent studies have suggested that genetic factors play an important role in determining peak bone mass and may also influence the subsequent rate of bone loss in both men and women [1]. Attempts have made to identify genes that have a predominant effect on bone mass and that may predict the risk of developing osteoporosis. Most of these studies have employed a candidate gene approach, where inherited variability in bone mass is related to polymorphism in a candidate gene. The first of these studies suggested that alleles of the vitamin D receptor (VDR) gene were responsible for the major part of the heritable component of bone mineral density (BMD) in women [2]. However, the association between allelic variation in the VDR gene and bone density remains unclear [3–5]. These studies have now been extended to other candidate genes, such as estrogen-receptor (ER), interleukin-1 receptor antagonist, and collagen1α genes, but the association between gene allele polymorphisms and bone density remains contentious [6–10].

The importance of estrogens in the maintenance of skeletal bone mass in women is now well established, as demonstrated by the central role that estrogen deficiency plays in the pathogenesis, of postmenopausal osteoporosis [11]. This role is further underlined by the therapeutic value of hormone replacement therapy in the prevention and treatment of postmenopausal osteoporosis [12]. There is now evidence that the action of testosterone on the male skeleton may be mediated in part by aromatization to estradiol [13]. Individual cases of male osteoporosis have been reported with mutation in the ER-α gene or aromatase gene. In the case of a 28-year-old man with a mutation in the aromatase gene, testosterone treatment produced no benefit, whereas transdermal estradiol resulted in skeletal maturation, rapid increase in spine BMD, and closure of the epiphyses [14]. A recent study in men, where endogenous sex steroid production was abolished, showed that estradiol was more important than testosterone in regulating bone turnover [15]. The Framingham study demonstrated a closer relationship between BMD and serum estradiol than the testosterone in men [16]. Another epidemiological study showed an inverse relationship between vertebral fracture and serum estradiol in men, which was not seen with testosterone [17].

The relative importance of ER gene subtypes in the growth and maturation of the male skeleton has been studied in transgenic mice, where ER-α and not ER-β
has been found to mediate important effects of estrogens [18]. There have been a number of studies of PvulII polymorphism of the ER-α gene and its relation to BMD, biochemical markers of bone turnover, and risk of fracture [7, 19–28], but only two of these were performed in men [27, 28]. Some studies demonstrated a relationship between ER-α gene polymorphism and BMD or other aspects of skeletal growth [19–24, 27, 28], while others found no association [25, 26]. We have therefore performed the first study of mutations in the ER-α gene in a group of men with symptomatic vertebral fractures and male control subjects. We have also examined the effect of ER-α PvulII polymorphism on the risk of fracture and BMD in these two groups of men.

### Methods

#### Selection of Subjects

ER-α gene mutational analysis was performed in 36 men with symptomatic vertebral crush fractures and a femoral BMD T-score of ≤-2.0 and in 28 male control subjects. We also examined the effect of estrogen-receptor PvulII genotype in 82 men, median age 64 and age range 27–77 years. These men comprised 33 patients with symptomatic vertebral crush fractures and 29 control subjects from Northeast England. Underlying secondary causes of osteoporosis, such as malignancy, corticosteroid therapy, and endocrine disorders, were excluded in men with vertebral fractures by medical history, physical examination, and laboratory investigations.

#### Laboratory Investigations

To exclude causes of secondary osteoporosis, full blood count, ESR, biochemical profile, random blood glucose, thyroid function tests, serum testosterone, estradiol, sex steroid binding globulin, gonadotrophins and serum, and urine electrophoresis were performed in men with symptomatic vertebral fractures.

### Bone Density Measurement

BMD measurements were performed by dual energy X-ray absorptiometry (DXA) using a QDR 2000 (Hologic). In vivo precision for measurement with this system is 1.0% for the lumbar spine (L1–L4) and 1.5% for the femoral neck. BMD results were obtained as an areal density in g/cm², but have also been expressed as T- and Z-scores. The T-score is the number of standard deviation units above or below the mean value for normal young men and the Z-score is the number of SD units above or below the mean value for normal men of the same age.

#### Molecular Analysis of the Estrogen Receptor Gene

All eight exons of the estrogen receptor gene were independently amplified by polymerase chain reaction (PCR) using exon (1–8) forward and reverse primers. The thermocycling parameters of PCR and the sequences of the primers used are given in Table 1. The PCR products from the patients with symptomatic vertebral crush fractures and normal subjects serving as controls were subjected to single-strand conformational polymorphism (SSCP) analysis. SSCP analysis was carried out on 6% polyacrylamide gels, which consisted of 2X MDE gel solution (Bio-Rad) (25 ml) and 10X TBE buffer (0.9 M Tris, 0.9 M borate, 2 mM EDTA) (3 ml) mixed by gentle swirling with an appropriate volume of distilled water (up to 50 ml). Ammonium persulfate (10%) freshly prepared solution (300 µl) and TEMED (30 µl) were added and mixed by inversion. The gels were allowed to polymerize for 60 min at room temperature. The samples were run overnight for 16 hours at 175 volts, and the gels were silver stained and photographed. Samples showing mobility shift during single-strand conformational analysis were selected for DNA sequencing. The sequencing reactions were carried out using a Big-Dye Terminator Cycle Sequencing kit (PE Applied Biosystems) with AmpliTaq® DNA polymerase, FS (fluorescent sequencing) and the products were analyzed in an automated ABI PRISM™ 377 sequencer (PE Applied Biosystems) which uses a laser beam to read through the fluorescently labelled nucleotides.

#### RFLP Analysis of the Estrogen Receptor Gene

Genomic DNA was isolated from blood samples obtained from all the subjects by a standard method. Analysis of the