Effects of polymorphisms of the *HSD11B1* gene on bone metabolism in women with osteoporosis and non-functioning adrenal adenomas

Ph.D. thesis

Karolina Feldman-Kovács, MD

Semmelweis University
Clinical Medicine PhD School

Supervisor: Attila Patócs, associate professor, MD, Ph.D.

Reviewers: Artúr Beke, MD, Ph.D.
Gábor Kovács, MD, Ph.D.

Final Exam Committee:
Head: Ida Gerendai†, D.Sc.
Members: Miklós Garami associate professor, MD, Ph.D.
Sándor Spisák, Ph.D.

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1. Introduction

Glucocorticoids play a key role in many processes of the human body including but not limited to the carbohydrate and amino acid metabolism, regulating stress and immune responses. Beside their apparent physiological importance, glucocorticoids also have a major role in the pathophysiology or therapy of many conditions and diseases ranging from endocrinology to bone metabolism and allergic disorders. A major complication of either endogenous or therapeutic glucocorticoid excess is osteoporosis which affects many patients and therefore draws great scientific interest.

The effects of glucocorticoids mainly depend on the local bioavailability of the active hormone: cortisol which is regulated according to our current understanding at least at two different levels. General, system-wide regulation is organized by the hypothalamic-pituitary-adrenal axis setting the level of blood cortisol and cortisone. Another much poorer understood level of regulation happens locally in the glucocorticoid target tissues. This tissue- and cell-type-specific local regulation can fine-tune the global supply of glucocorticoids at the prereceptor level setting the hormone levels available to the receptors and thus adjusting glucocorticoid effects on the target tissue. Alterations in the local regulation may therefore lead to changes in glucocorticoid effector responses without an apparent hypo- or hypercortisolism.

The key components of this mechanism are the 11β-hydroxysteroid-dehydrogenase (11β-HSD1 & 2) isoenzymes, which convert the inactive cortisone to the hormonally active cortisol (11β-HSD1) and vice versa (11β-HSD2). The direction of the enzyme activity is mainly determined by the NADPH-level in the endoplasmic reticulum depending on the activity of another enzyme hexose-6-dehidrogenase (H6PDH). 11β-HSD1 is expressed highly in brain, liver, gonads, adipose tissue and bone. It is also expressed in osteoblasts and an increased activity of this enzyme is implicated in age-related bone-loss. Better understanding about the function and mechanism of action of this enzyme could prove invaluable in developing better strategies to treat the various pathological conditions of these organs.

Polymorphisms of *HSD11B1* gene have been previously shown to affect the function of 11β-HSD1 enzyme and thus have been linked with some (patho)physiological conditions such as obesity (high BMI), insulin resistance, diabetes mellitus, hypertension, polycystic ovary syndrome (PCOS) or Alzheimer's disease. The 83,557insA genetic variant of the *HSD11B1* gene, located to the third intron, have been extensively studied and several associations have been described. The rs12086634 genetic variant is in complete linkage disequilibrium with the 83,557insA.

The aim of our present work was to identify and analyze the polymorphisms of the *HSD11B1* gene in relation with postmenopausal osteoporosis. Better understanding the effects of the polymorphisms of this gene may lead to better knowledge on the genetics of osteoporosis and may even contribute to better diagnostic and therapeutic approaches in the future.

2. Objectives

Hypo- and hyperactivity of 11β-HSD1 enzyme may contribute to or may even be the key factor in the pathophysiology of several conditions. According to our current understanding the primary regulation of its action beside the cofactor supply is the expression level and endogenous activity of the enzyme mainly depending on the structure and transcriptional activity of the *HSD11B1* gene.

The aim of my PhD work was to systematically explore the previously identified but functionally not yet described genetic variants of the *HSD11B1* gene in relation of bone metabolism, and further analyze those with a phenotypic feature.

1. To identify the genetic variants of the *HSD11B1* promoter *in silico* using online available databases (NCBI, Hapmap).

2. To determine the allele frequencies of the previously identified polymorphisms in a group of healthy Hungarian women.

3. To evaluate the effect of these polymorphisms on the bone turnover of healthy postmenopausal women.

4. To asses these phenotypic effects in women:

    a. with postmenopausal osteoporosis and

    b. with non-functioning adrenal adenoma

5. *In vitro* analysis of the functional mechanism of a polymorphism associating with the most significant phenotype.

3. Materials and Methods

3.1. Patients and healthy subjects

Genotyping of the polymorphisms was performed in a patient group consisting of 154 women with postmenopausal osteoporosis and 71 with non-functioning adrenal incidentaloma diagnosed at the 2nd Department of Internal Medicine, Semmelweis University, Budapest and compared to a control group formed from 209 healthy women

3.1.1. Endocrine investigations in patients with non-functioning adrenal incidentalomas

The diagnosis of adrenocortical adenoma was based on the imaging results Detailed endocrine evaluation was carried out in all patients. Concentrations of serum cortisol were measured at 08:00 and at 24:00 hours as well as after low-dose dexamethasone suppression test. Serum markers of bone metabolism, osteocalcin and C-terminal crosslinks (beta crosslaps, CTX) of human collagen type I (C-telopeptide) were also determined using electrochemiluminescence immunoassay.

Serum cortisone, urine metanephrine, serum dehydroepiandrosterone, 17-hydroxyprogesterone, testosterone, plasma ACTH levels, serum potassium, sodium were measured in all patients and serum renin/aldosterone levels in patients with hypertension.

3.2. Bone mineral density measurement

Bone mineral density (BMD) of the lumbar spine (L1-4), proximal total femur and femoral neck, intertrochanteric and trochanteric subregions were measured by dual-energy X-ray absorptiometry.

3.3. In silico analysis of the tagging HSD11B1 gene polymorphisms

The *in silico* selection of the *HSD11B1* polymorphisms was based on data retrieved from online databases.

3.4. Molecular biology methods

3.4.1. Genotyping of the *HSD11B1* polymorphisms

Genomic DNA was isolated from peripheral blood leukocytes with commercially available kits. Genotyping of the polymorphisms were performed using predesigned Taqman SNP Assays and on Real-Time PCR and direct DNA sequencing.

3.4.1.1. Real Time-PCR.

Genotyping of the polymorphisms (rs4393158, rs11576775, rs17389016, rs760951, rs4844880, rs3753519, rs12086634, rs11807619, rs2884090, rs4844488) were performed using Taqman SNP Assays (Life Technologies).

3.4.2. In vitro functional methods

3.4.2.1. Luciferase reporter plasmid constructs

Polymerase chain reaction (PCR) primers were designed for the sites of *HSD11B1* promoter. The amplified product was cloned into the pGL3 basic vector. All plasmid constructs were verified by direct DNA sequencing.

3.4.2.2. Cell culture

Human epitheloid cervix carcinoma cells (HeLa) were used for transient transfection.

3.4.2.3. Transient transfection

HeLa cells were plated at 10^4 cells per well on 96-well plates in antibiotic-free medium. Cells were transfected with Lipofectamine 2000 according to the manufacturer's protocol.

3.4.2.4. Dual-luciferase assay

After 24 h luciferase assay was performed using Dual-Glo luciferase assay system according to the manufacturer's protocol. Each experiment was carried out in six replicates and repeated five times.

3.5. Statistical analysis

Hardy–Weinberg equilibrium was calculated for all SNPs. Haploview (release 4.1) was used to determine the LD blocks and the r2 values.

Shapiro-Wilks normality test was used for controlling data distribution. Then the data were evaluated with Student's T-test or Mann–Whitney rank.

Results obtained from luciferase assays were compared using one-way ANOVA for repeated measurements followed by Bonferroni posthoc test.

Statistical analysis was performed using SPSS. A value of $p < 0.05$ was considered to be significant.

4. Results

4.1. Identification of the promoter polymorphisms of HSD11B1

Using *in silico* methods I indentified the polymorphisms of the *HSD11B1*. The initial search was performed on the 28 kb region upstream from the start codon. The number of polymorphims in this region was too high though for the further detailed analysis and particularly for the intended genotyping so the results were further filtered in multiple steps. First, the search was narrowed to the tagging single nucleotide polymorphisms (SNPs) only. Since many tagging SNPs of some haplotypes are not residing in the promoter region but in one of the introns instead, the search was eventually extended by including those intronic tagging SNPs also. Then all the tagging SNPs were excluded that are reported as less frequent than 5% in the general population. Taken these criteria together haplotypes and tagging SNPs were analysed of the proximal 38 kb region of the *HSD11B1*.

4.2. Allele fequencies of HSD11B1 polymorphisms

Genotyping the 10 tagging SNPs identified in the previous step did not show any significant difference in allele frequencies between the online available international data and our group of Hungarian healthy individuals.

4.3. Effects of HSD11B1 polymorphisms on the bone turnover in healthy women

Next, I explored any possible correlations between the genotype of the formerly identified promoter tagging SNPs and the phenotype of a major glucocorticoid target tissue, the bone.

The biochemistry parameters more or less describing bone turnover are independent of the genotype of the analyzed polymorphisms. However, more relevant to the patient are the physical features of the bones mainly determined by the mineral content and density. From the clinical parameters used to evaluate bone mineral content, L1-4 bone mineral density (BMD) showed a significant association with rs4844880 and femur neck BMD with rs3753519. Of theses associations the one between rs4844880 and L1-4 BMD was statistically stronger therefore rs4844880 was analyzed further in detail.

4.4. HSD11B1 polymorphisms and bone turnover in diseases

Next, I investigated whether the beneficial effects of *HSD11B1* polymorphisms to bone mineral density in healthy individuals is also present in women with a manifest bone disease, ie. postmenopausal osteoporosis and in women with non-functioning adrenal adenoma. Two polymorphisms were analyzed in detail: the rs4844880 just identified above and the rs12086634 which was found to be associated with better bone turnover previously by our group.

4.4.1. Associations between rs4844880 and clinical features

4.4.1.1. Postmenopausal osteoporosis

Analysis of data from healthy postmenopausal women revealed that carriers of the minor allele of the rs4844880 had higher BMD, T-score and Z-score at the lumbar spine (L1-4) compared to women who carried the major allele. These associations were statistically significant after adjustment for age and BMI.

To evaluate the potential role of the rs4844880 in postmenopausal osteoporosis, 154 patients with this condition was evaluated. The allele frequency was similar in the osteoporotic and healthy postmenopauasal women (0.128 vs. 0.136). However, in osteoporotic postmenopausal women a strong association was observed at the femoral neck in carriers vs. non-carriers. In addition a significant association was observed between the rs4844880 and biochemical markers of bone metabolism. Higher serum osteocalcin and lower serum CTX were detected in carriers than in non-carriers.

4.4.1.2. Non-functioning adrenal adenoma

The prevalence of the rs4844880 polymorphic allele in women with NFAA was similar to those found in healthy postmenopausal women and to those reported in databases for Caucasian population

The rs4844880 genotype showed a significant association with the lumbar spine BMD, T-score and with femoral neck Z-score in our patients. The association between the rs4844880 variant and these osteodensitometric parameters at lumbar spine remained statistically significant after adjustment for age, gender and BMI.

4.4.2. Effect of rs12086634 polymorphism on bone turnover in women with non-functioning adrenal adenoma

The rs12086634 polymorphism carriers had significantly higher lumbar spine BMD, T-score and Z-score, as well as femoral neck T-score and Z-score. These associations were statistically significant after adjustment for age and BMI.

4.5. Functional analysis of the effect of rs4844880 polymorphism on the transcriptional activity of HSD11B1

Rs4844880 has been shown above to be beneficial for bone turnover in both healthy and osteoporotic individuals. The next goal of my PhD work was the identification of the molecular mechanism behind this phenotype. Being a promoter polymorphism, rs4844880 is supposed to act through affecting the transcriptional activity of the *HSD11B1* promoter and thus altering the level of the active enzyme. Therefore an in vitro transcriptional activity assay was planed and set up to investigate this SNP.

4.5.1. Luciferase reporter vector construct

Rs4844880 resides -7372 bp from the start codon of the *HSD11B1* gene making it technically impossible to ligate the transcription start site and the polymorphism in one piece into a bacterial vector. Thus these 2 regions needed to be cloned separately. Because of this 3 different constructs were made: HSD11B1-control containing only the transcription initiation site; HSD11B1-rs4844880W having the transcriptional initiation site and a 851bp region (-7820 – -6969) around the site of rs4844880 containing the wild type version of the SNP; and HSD11B1-rs4844880M with transcription initiation site and the mutant version of rs4844880.

4.5.2. Effect of rs4844880 polymorphism on the transcriptional activity of *HSD11B1*

In our in vitro model both the pHSD11B1-rs4844880W and pHSD11B1–rs4844880M constructs showed significantly lower luciferase activity compared to the pHSD11B1-control construct suggesting a possible repressive role of this 851 bp-long sequence residing between nucleotides -7820 and -6969 upstream of the start codon. In addition, the pHSD11B1-rs4844880M construct had a significantly lower luciferase activity than the wild type pHSD11B1-rs4844880W.

5. Conclusions

During my PhD thesis work I investigated the proximal region of the *HSD11B1* gene searching for tagging SNPs to identify any connections between the genetic structure of the *HSD11B1* promoter and the clinical and laboratory markers of a major glucocorticoid target tissue, the bone.

1. The proximal 38 kb region of the *HSD11B1* contains 10 tagging SNPs .The allele frequencies of all SNPs were the same in our Hungarian group as in the Caucasian population found in online databases.

2. Of these polymorphisms rs4844880 and rs3753519 showed significant association with bone density parameters.

3. In postmenopausal healthy women the beneficial effect of rs4844880 is more apparent; carriers of this SNP have significantly higher BMD, Z- and T-score in the L1-4 region. Postmenopausal osteoporotic women also benefit from carrying the rs4844880 by having significantly better bone turnover as indicated by higher femur neck BMD, T-score, Z-score, higher serum osteocalcin and lower serum β-crosslaps.

4. Rs4844880 is associated with better bone features (L1-4 BMD, T- and Z-score, femur neck T- and Z-score) in women patients with non-functioning adrenal adenoma (NFAA).

   Another tagging SNP of *HSD11B1*, rs12086634 is in 100% linkage disequilibrium with insA and is also associated with better bone turnover in NFAA.

5. The region of rs4844880 proved to contain a functional repressor site, and the mutant (polymorph) allele of rs4844880 acted as a stronger repressor than the wild type allele.

6. List of publications

6.1. Publications related to the PhD thesis

1. Feldman K, Szappanos A, Butz H, Grolmusz V, Majnik J, Likó I, Kriszt B, Lakatos P, Tóth M, Rácz K, Patócs. A. (2012). "The rs4844880 polymorphism in the promoter region of the *HSD11B1* gene associates with bone mineral density in healthy and postmenopausal osteoporotic women." **Steroids**. 77(13):1345-1351. **IF:2.83**

2. Feldman K, Likó I, Nagy Zs, Szappanos A, Grolmusz V, Tóth M, Rácz K, Patócs A. (2013) A 11-β-hidroxi-szteroid-dehidrogenázenzim jelentősége klinikai kórképekben. **Orvosi hetilap** 2013, 154, 283–293.

3. Szappanos A, Patócs A, Gergics P, Bertalan R, Kerti A, Ács B, Feldmann K, Rácz K, Tóth M. (2011) The 83557insA variant of the gene coding 11β-hydroxysteroid dehydrogenase type 1 enzyme associates with serum osteocalcin in patients with endogenous Cushing's syndrome. **J Steroid Biochem Mol Biol** 123: 79–84. **IF:3.053**

6.2. Other publications

1 Boyle B, Butz H, Liko I, Zalatnai A, Toth M, Feldman K, Horanyi J, Igaz P, Racz K, Patocs A.(2010) Expression of glucocorticoid receptor isoforms in human adrenocortical adenomas. **Steroids.** 75(10):695-700. **IF:3.106**

2 Beko G, Varga I, Glaz E, Sereg M, Feldman K, Toth M, Racz K, Patocs A. (2010) Cutoff values of midnight salivary cortisol for the diagnosis of overt hypercortisolism are highly influenced by methods. **Clin Chim Acta**.411(5-6):364-7. **IF:2.389**

4. Sereg M, Szappanos A, Toke J, Karlinger K, Feldman K, Kaszper E, Varga I, GlázE, Rácz K, Tóth M. (2009) Atherosclerotic risk factors and complications in patients with non-functioning adrenal adenomas treated with or without adrenalectomy: a long-term follow-up study. **Eur J Endocrinol**. 160(4):647-55. **IF:3.539**