

Sensitivity to glucocorticoids and bone metabolism in patients with endogenous hypercortisolism

Doctoral dissertation

Ágnes Szappanos MD

Semmelweis University
Clinical Medicine of PhD Studies



Tutor: Miklós Tóth, associate professor, MD, PhD

Opponents: Endre Nagy, professor, MD, PhD, DSc
Emese Kiss, associate professor, MD, PhD

Chairman of committee: Péter Lakatos, professor, MD, PhD, DSc

Members of committee: Péter Andréka, MD, PhD
Nyitrayné Erna Pap, associate professor, PhD

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INTRODUCTION

Endogenous hypercortisolism is caused in 70 percent of patients by ACTH-producing pituitary adenoma (CD), in 15 percent adrenal Cushing's syndrome (ACS) and in 15 percent ectopic ACTH excess syndrome (ECS). Hypercortisolism may cause serious symptoms and usually affects each organ system. The term of glucocorticoid-induced osteoporosis represents the whole spectrum of osseal abnormalities caused by endogenous glucocorticoid excess, ranging from mild biochemical abnormality of bone turnover to multiple fractures.

Prolonged exposure to excessively produced endogenous glucocorticoids markedly alters bone metabolism resulting in a disturbed correlation between bone formation and bone resorption. The restoration of coupled remodelling following the cure of endogenous Cushing's syndrome has been previously demonstrated. The sensitivity and cellular responses of various tissues to glucocorticoids are determined by the glucocorticoid receptor (GR) and the 11β -hydroxysteroid dehydrogenase (HSD11B) enzyme. The type 1 isoform of HSD11B enzyme (HSD11B1) primarily interconverts the hormonally inactive cortisone to hormonally active cortisol, while the type 2 isoform inactivates cortisol into cortisone.

It is well known that there is huge interindividual variability in the physiological and pharmacological responses to glucocorticoids. The altered physicochemical properties of the GR and the altered activity of the HSD11B1 enzyme may represent the most important mechanisms resulting in altered sensitivity to the glucocorticoids.

The genetic background of endogenous Cushing's syndrome is still unclear, however several lines of evidence suggest that the GR and the HSD11B1 enzyme modulating effect on the setpoint of the hypothalamic-pituitary-adrenal axis are at least partially genetically determined. More than a dozen polymorphisms of the *GR* gene have been published. The four most frequently investigated polymorphisms within the coding gene of the GR are the exonic N363S and ER22/23EK, the intronic BclI and the A3669G which is located within the transcriptionally inactive glucocorticoid receptor β splice variant. The BclI and the N363S variants have been associated with increased, while the ER22/23EK and the A3669G variants have been associated with reduced sensitivity to glucocorticoids. The rs846911 polymorphism of the *HSD11B1* gene is supposed to be related with Alzheimer's disease, while the rs846910 variant was associated with increased prevalence of type 2 diabetes mellitus and hypertension. The third extensively studied polymorphism of the *HSD11B1* gene is the 83,557insA, located at the third intronic region.

It has been suggested that the presence of the 83,557insA variant is associated with reduced enzyme expression.

OBJECTIVES

Bone metabolism was studied in the active phase of endogenous Cushing's syndrome and after the cure of the syndrome during a postoperative follow-up time for 4 years. Furthermore the importance of glucocorticoid sensitivity in relation with the genetic variants of the GR and HSD11B1 genes was also evaluated in patients as well as in healthy subjects. The clinical-, hormonal- and genetic evaluations were processed in patients with CD, ACS and ECS and in health control subjects, investigated at the Second Department of Medicine, Semmelweis University. I asked the following questions:

1. To assess bone formation (serum osteocalcin, sOC) and resorption markers (serum C-terminal cross-links of human collagen type I, sCTX) in patients in the active phase of endogenous Cushing's syndrome and to examine bone marker restoration after the cure of endogenous hypercortisolism. Furthermore, the potential usefulness of OC and CTX as a biomarker of endogenous Cushing's syndrome

during the active phase and after clinical cure was also evaluated.

2. To investigate whether polymorphisms of the *GR* gene, including the BclI, N363S, ER22/23EK and A3669G variants, could have an impact on clinical variability and severity of endogenous glucocorticoid excess syndromes, especially on altered bone turnover and mineral density.
3. To develop a rapid, simple and cost-effective multiplex allele-specific polymerase chain reaction (PCR) method for detection of the rs846910 and rs846911 genetic variants of the *HSD11B1* gene.
4. To explore presumed associations between the rs846910, rs846911 and the 83,557insA variant of the *HSD11B1* gene and circulating hormone concentrations, bone turnover and bone mineral density in patients with endogenous Cushing's syndrome.

MATERIALS AND METHODS

3.1. Patients

The clinical-, hormonal- and genetic evaluations were processed in patients with endogenous hypercortisolism, diagnosed at the Second Department of Medicine, Semmelweis University. Patients were grouped into the diagnostic categories of adrenocorticotrophic hormone (ACTH)-producing pituitary adenomas (CD, 49 patients), cortisol producing adrenal tumors (CS, 29 patients) and ectopic ACTH excess syndrome (ECS, 9 patients). One hundred and sixty-one healthy volunteers were included in the control group.

3.2. Endocrine investigations

All patients underwent a detailed clinical and hormonal evaluation. Plasma cortisol concentrations at 0800 and 2400 h as well as after a low dose dexamethasone suppression test (LDDST) were measured. The diagnosis of Cushing's disease and adrenal Cushing's syndrome was based on hormonal findings while pituitary and adrenal imaging studies were performed with magnetic resonance imaging and/or computed tomography.

3.3. Bone mineral density measurement and laboratory assessment of bone markers

BMD was determined by dual-energy X-ray absorptiometry using Hologic 4500C densitometer at the lumbar spine (L1-4), proximal total femur and femoral subregions.

Test kits from Roche Laboratory were used according to the manufacturer's instructions to measure sOC and sCTX.

3.4. Molecular genetic investigations

Total genomic DNA was isolated from peripheral blood lymphocytes using a QIAamp DNA Blood Mini Kit.

3.4.1. Detection of glucocorticoid receptor gene polymorphisms

Genotypes for the BclI and the N363S variants were determined by allele-specific PCR. Genotypes for the ER22/23EK polymorphism were investigated using PCR amplification followed by restriction fragment length analysis. The amplified fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Genotypes for the A3669G polymorphism were detected using a primer-probe set purchased as predesigned Taqman allelic discrimination assay. The assay was performed according to the manufacturer's instructions on a 7500 Fast Real Time PCR System.

3.4.2. Analysis of *HSD11B1* gene polymorphisms

The 83,557insA polymorphism was identified using restriction fragment length analysis with the XcmII restriction endonuclease. The amplified fragments were separated by agarose gel electrophoresis. For the detection of the of the rs846910 and rs846911 *HSD11B1* gene variants, a multiplex allele-specific PCR method was developed. Four allele specific and two generic inner primers were designed and agarose gel electrophoresis was used to separate the PCR fragments. Contrary to some literature data, we have found the rs846911 polymorphism to be very rare. I have generated a homozygous mutant genotype with cloning procedure, in order to get a positive control sample. Direct sequencing was used to verify the results obtained by the multiplex allele-specific PCR method.

3.5. Statistical analysis

All statistical analyses were performed using Statistica package (version 7.0, Statsoft Inc). A value of $p < 0.05$ was considered to be significant. Normality of data distribution was analysed by the Shapiro-Wilk's test. The relations between characteristic variables were examined with χ^2 -analysis and Fisher exact test. Means of

continuous variables were evaluated with Student's t test or Mann–Whitney rank sum test. Means of continuous variables were compared between multiple groups by analysis of variance (ANOVA) or Kruskal-Wallis ANOVA, while pair wise comparisons were evaluated using Bonferroni post hoc test. Analysis of covariance (ANCOVA) was used to analyze the effects of categorical independent variables, controlling for the effects of continuous predictor variables. Receiver-operating characteristic (ROC) curves were generated to estimate the discriminatory capability of bone markers for the separation of individuals with and without active hypercortisolism. Hardy–Weinberg equilibrium was calculated for each polymorphism where it was possible.

RESULTS

4.1. Bone turnover in patients with endogenous Cushing's syndrome before and after successful treatment

Serum OC showed a significant negative, while sCTX displayed significant positive linear correlation with serum cortisol in the morning, midnight, and after LDDST. Our study failed to document a statistically significant correlation between sCTX and sOC levels in patients with endogenous hypercortisolism in the active phase of the disease. However, analysis of the whole dataset of bone markers obtained from each time point after cure of endogenous hypercortisolism indicated a significant positive correlation between sCTX and sOC levels, similar to that observed in our healthy control group.

Our follow-up data demonstrate that sOC increases markedly within a few days or weeks after successful surgery, reaches its maximum at the sixth postoperative month, and it remained stable after the 24th postoperative month. Serum CTX levels failed to show statistically significant differences at baseline as compared to healthy controls or during the 4-year follow-up period.

Receiver-operator characteristics curves for sOC measurements for the discrimination between individuals with and

without endogenous hypercortisolism was only slightly lower (AUC 0.9227) than the accuracy of current diagnostic tests for the diagnosis of endogenous Cushing's syndrome.

4.2. Association between *GR* gene polymorphisms and clinical manifestations of endogenous glucocorticoid excess syndromes

No statistically significant differences were found in the allelic frequencies of the BclI, N363S, ER22/23EK and the A3669G variants between patients with CD, ACS and healthy controls.

Patients with the homozygous BclI polymorphic genotype had lower Z scores at femoral neck and at trochanteric region compared to patients with the wild-type (CC) genotype (femoral neck Z-scores: -1.44 ± 0.73 vs. -0.39 ± 0.91 ; trochanteric Z-scores: -1.89 ± 0.47 vs. -0.54 ± 0.98). In addition patients with endogenous hypercortisolism who had the polymorphic GG genotype variant of the BclI polymorphism showed significantly higher sCTX compared to patients with the CG and the CC variant (sCTX Z-score: $+4.42 \pm 2.37$ vs. $+0.79 \pm 1.67$ and $+0.11 \pm 1.47$).

4.3. New multiplex allele specific PCR method for the detection of two *HSD11B1* enzyme gene polymorphisms

A new multiplex allele specific PCR method was developed for the detection of the rs846911 and rs846910 polymorphisms of the *HSD11B1* gene. We have verified our method precision with direct DNA sequencing results, what showed 100% accuracy with our detection.

4.4. Association between *HSD11B1* enzyme gene polymorphisms and clinical manifestations of endogenous glucocorticoid excess syndromes

No statistically significant differences were found in the allelic frequencies of the rs846910, rs846911 and 83,557insA polymorphisms between patients with CD, ACS and healthy controls.

The polymorphic 83,557insA genotype was found to be associated with ACTH levels in patients with ACS; the 83,557insA heterozygotes had significantly higher plasma ACTH concentrations compared to patients with the wild-type variant (7.38 ± 4.05 pg/ml vs. 4.81 ± 8.25 pg/ml, $p=0.025$) Additionally, the 83,557insA carriers had smaller tumor size compared to non-carriers (28.44 mm \pm 9.91 vs. 51.05 ± 38.53 mm, $p=0.03$). Among all patients with endogenous

hypercortisolism carriers of the 83,557insA variant had significantly higher sOC as compared to non-carriers (15.88 ± 10.24 ng/ml vs. 10.24 ± 5.87 ng/ml, $p=0.027$).

DISCUSSION

1. In patients with endogenous hypercortisolism sOC concentrations were significantly decreased, whereas CTX indicated normal bone resorption. Despite the severely disturbed and uncoupled bone turnover in active hypercortisolism characterized by the lack of correlation between bone formation and bone resorption, serum OC showed a significant negative and serum CTX displayed a significant positive correlation with serum cortisol concentrations. Furthermore serum OC was found to be statistically significantly associated with some of the clinical consequences/complications (diabetes, myopathy) of endogenous Cushing's syndrome. Evaluating the potential usefulness of sOC measurements as a marker of endogenous hypercortisolism with ROC analysis, I have found that sOC has a remarkable sensitivity and specificity for the discrimination of patients with endogenous Cushing's syndrome from healthy subjects. Our results demonstrate that the bone formation marker sOC increases markedly within a few days or weeks after successful surgery. Serum OC concentration reached its maximum at the sixth postoperative month, and it remained stable after the 24th postoperative month. Contrary to the marked postoperative

changes in sOC concentrations, CTX levels failed to show statistically significant differences at baseline as compared to healthy controls or during the 4-year follow-up period.

2. The BclI, N363S, ER22/23EK and A3669G polymorphisms of the *GR* gene probably do not modify the risk for the development of CD or ACS. Patients with endogenous Cushing's syndrome carrying the polymorphic BclI allele in a homozygous form showed significantly reduced Z-scores in two femoral regions as well as an increase in bone resorption. The BclI polymorphism may modify the skeletal sensitivity to glucocorticoids in patients with endogenous glucocorticoid excess and increase glucocorticoid sensitivity in alignment with previously reported studies.

3. A new multiplex allele specific PCR method was developed for the detection of the rs846911 and rs846910 polymorphisms of the *HSD11B1* gene. It seems to have several advantages over previously used methods, since it needs only one PCR reaction, it is rapid and cost-effective. I have verified the method precision with direct DNA sequencing results, what showed 100% accuracy with results detected by the novel method. The

multiplex allele specific PCR.can be easily applied for screening of large sample sizes required for population-based studies.

4. The importance of the genetic variants of the *HSD11B1* gene has not been investigated previously in patients with endogenous hypercortisolism. The allele frequencies of the rs846910 the rs846911 and the 83,557insA polymorphisms in patients with endogenous hypercortisolism were not different from those observed in our healthy controls, which suggests that these polymorphisms do not modify the susceptibility of CD and ACS. However the association between the 83,557insA polymorphisms and serum OC, and ACTH levels and with tumor size are in accordance with previous findings suggesting that the presence of this variant leads to reduction of the HSD11B1 enzyme activity resulting in decreased glucocorticoid effect.

ORIGINAL PAPERS

Publications related to the dissertation

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