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Reduced DNA methylation of *FKBP5* in Cushing's syndrome

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Abstract

FKBP5 encodes a co-chaperone of HSP90 protein that regulates intracellular glucocorticoid receptor sensitivity. When it is bound to the glucocorticoid receptor complex, cortisol binds with lower affinity to glucocorticoid receptor. Cushing's syndrome is associated with memory deficits, smaller hippocampal volumes, and wide range of cognitive impairments. We aimed at evaluating blood DNA methylation of FKBP5 and its relationship with memory and hippocampal volumes in Cushing's syndrome patients. Polymorphism rs1360780 in FKBP5 has also been assessed to determine whether genetic variations can also govern CpG methylation. Thirty-two Cushing's syndrome patients and 32 matched controls underwent memory tests, 3-Tesla MRI of the brain, and DNA extraction from total leukocytes. DNA samples were bisulfite treated, PCR amplified, and pyrosequenced to assess a total of 41CpG-dinucleotides in the introns 1, 2, 5, and 7 of FKBP5. Significantly lower intronic FKBP5 DNA methylation in CS patients compared to controls was observed in ten CpG-dinucleotides. DNA methylation at these CpGs correlated with left and right HV (Intron-2-Region-2-CpG-3: LHV, $r = 0.73$, $p = 0.02$; RHV, $r = 0.58$, $p = 0.03$). Cured and active CS patients showed both lower methylation of intron 2 (92.37, 91.8, and 93.34 %, respectively, $p = 0.03$ for both) and of intron 7 (77.08, 73.74, and 79.71 %, respectively, $p = 0.02$ and $p < 0.01$) than controls. Twenty-two subjects had the CC genotype, 34 had the TC genotype, and eight had the TT genotype. Lower average DNA methylation in intron 7 was observed in the TT subjects compared to CC (72.5vs. 79.5 %, $p = 0.02$) and to TC (72.5 vs. 79.0 %, $p = 0.03$). Our data demonstrate, for the first time, a reduction of intronic DNA methylation of FKBP5 in CS patients.

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Conflict of interest

The authors declare that they have no conflicting interests.

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Keywords

3Tesla MRI; Cushing's syndrome; FKBP5 DNA methylation; FKBP5 polymorphism; Glucocorticoid resistance; Hypercortisolism

Introduction

The gene expressing the FK506 binding protein 51 (FKBP5) is involved in the regulation of glucocorticoid receptor (GR) sensitivity. FKBP5 encodes a co-chaperone of heat shock protein 90 (HSP90), which regulates GR sensitivity. When it is bound to the GR-HSP90 complex, cortisol binds with lower affinity to GR, and GR's nuclear translocation is less efficient, thereby reducing the effect of cortisol at the DNA level. FKBP5 is also induced by glucocorticoids (GC) and thus regulates GR sensitivity via an ultra-short feedback loop [1, 2].

Since FKBP5 is both a regulator and a target gene of cortisol signaling, variations in FKBP5 can influence the response to GC. In fact, enhanced expression of FKBP5 following GR activation has been associated with an increased GC resistance and with a decreased efficiency of the negative feedback system of the hypothalamic-pituitary-adrenal (HPA) axis, accompanied by prolonged cortisol release in healthy individuals [2]. In fact, an impaired negative feedback regulation and subsequent partial GC resistance appear to be one of the most important biological abnormalities observed in mood disorders [3]. There is also ample evidence that hypercortisolism plays a role in the generation of psychiatric symptoms and that epigenetic variations within HPA axis genes can mediate behavioral changes [2, 4, 5]. Specifically, the common single nucleotide polymorphism (SNP) rs1360780 (C/T) within FKBP5 has been reported to be associated with an altered GC response of the HPA axis and the development of stress-related psychiatric disorders such as post-traumatic stress disorder [6–8]. The minor T allele is associated with higher FKBP5 induction by cortisol compared with the C allele [8], and it is considered a risk factor for developing post-traumatic stress disorder and depression after trauma exposure [9, 10].

Cushing's syndrome (CS), a chronic endogenous hypercortisolism, is associated with deficits in memory and a wide range of cognitive impairments and mood disorders; therefore, it serves as a good human model to evaluate the effect of GC excess on the brain. The hippocampus, a subcortical brain area critical for learning and memory, is rich in GR and particularly vulnerable to GC excess [11, 12].

In particular, verbal and visual memory performance is worse in CS patients than controls, with CS patients exhibiting reduced hippocampal volumes (HV) [11], with incomplete reversibility after cure. Moreover, persistently abnormal levels of metabolites have been found in the head of both hippocampi of CS patients, despite endocrine cure of hypercortisolism [13].

While there is controversy on the reversibility of brain impairments, emerging evidence suggests that longer disease duration and older age are associated with a diminished recovery of brain function. The exact mechanisms involved in brain damage during chronic

hypercortisolism are poorly understood. However, persistent brain impairments should have a common origin related to the effects of GC exposure.

Epigenetics may be a mechanism through which GR signaling influences brain function and mood. Methylation of the cytosine base within the CpG dinucleotide is the most common epigenetic modification of DNA in mammals. DNA methylation plays a crucial role in the regulation of chromatin architecture, and therefore in the control of gene expression and chromosome structure [14].

Glucocorticoids alter gene expression in many regions of the brain, and these alterations are associated with epigenetic modifications in a mouse model [15].

Whereas expression and DNA methylation changes in the brain are obviously more relevant to GC-induced changes in behavior, comparable changes in blood might provide a clinically valuable surrogate, given the easy access to this tissue in patients. For instance, FKBP5 mRNA levels in blood have functional relevance because they have a positive correlation with plasma cortisol [8].

Recently, GC-induced methylation changes in Fkbp5 observed in blood DNA have been demonstrated to serve as a proxy to both methylation and expression changes in the mouse brain [15]. In particular, DNA methylation from blood can be used to assess GC-induced changes in Fkbp5 expression and methylation occurring in the hippocampus in a mouse model of hypercortisolism [15]. These findings in the mouse model prompted us to ask whether FKBP5 methylation in the human blood can be used to assess central nervous system changes caused by GC excess.

In particular, we aimed at evaluating DNA methylation of FKBP5 and its relationship with HV and memory in CS patients. Further, polymorphism rs1360780 in FKBP5 has also been assessed to determine whether genetic variations can also govern CpG methylation.

Methods

Subjects

In this cross-sectional study, 32 right-handed CS patients (5 males, 27 females, age 45 ± 12 years, 13 ± 4 years of education) clinically followed in Hospital Sant Pau and 32 right-handed healthy controls matched for age, sex, and years of education (6 males, 26 females, age 44 ± 11 years, 14 ± 4 years of education) were included. Control subjects were recruited among right-handed healthy volunteers who had previously participated in clinical studies in our institution; they had no history of GC exposure and were free of medications.

At the time of the study, nine CS patients were hypercortisolemic (active disease), seven were of pituitary origin, and two were of adrenal origin. All were on medical therapy, four on metyrapone and five on ketoconazole. Four of nine were awaiting surgery (two adrenal and two pituitary). The remaining five had previously undergone unsuccessful pituitary transsphenoidal neurosurgery.

Twenty-three CS patients were in remission (biochemically cured), 18 of pituitary, five of adrenal origin. All cured CS of pituitary origin had undergone transsphenoidal surgery and six also had pituitary radiotherapy. Unilateral adrenalectomy was performed in CS patients of adrenal origin. Four patients had adrenal insufficiency at the time of the study and required hydrocortisone replacement therapy (median of 20mg/day in two or three doses), whereas the remaining 19 had presented transient hypocortisolism after surgery but did not require substitution therapy.

Cushing's syndrome was considered in remission if patients achieved adrenal insufficiency or morning cortisol suppression (<50 nmol/l; <1.8 μg/dl) after 1 mg dexamethasone overnight and repeated normal 24-h urinary free cortisol (measured with a commercially available RIA kit after a previous urine extraction with an organic solvent; normal <280 nmol/24 h). Mean time of biochemical cure at study date was 7.1 ± 2.2 years.

The patients had verbal and visual memory impairments, as previously described [11]. Duration of hypercortisolism was considered as the time from symptom onset until remission of hypercortisolism after treatment and was assessed by the endocrinologist in charge. At diagnosis, the duration of hypercortisolism was estimated by personal interview and detailed review of medical records and photographs of patients. All information was written or kept in clinical records, together with data regarding the achievement of the biochemical cure. Mean duration of hypercortisolism was 5.1 ± 2.8 years.

Cushing's syndrome patients with diabetes mellitus and GH deficiency were excluded because cognitive deficits and hippocampal atrophy have been described in these conditions [16–19].

All participants were Caucasians; six CS patients and eight controls were smokers. Table 1 summarizes the clinical features of the patients and controls.

All patients and controls signed an informed consent after study approval by the Hospital Ethics Committee. None of the participants had a past medical history of head injury, cerebrovascular disease, mental illness or psychiatric disorders, or were taking tranquilizers.

Genomic DNA extraction from total leukocytes

Genomic DNA extraction from total leukocytes was carried out using an adapted proteinase K and Phenol protocol [20] in all subjects. Blood samples from the patients were collected in EDTA tubes to reduce blood clotting and DNA degradation. Genomic DNA was isolated from blood buffy coats. The buffy coat and white blood cell pellets were stored frozen at −80°C before processing. The white blood cell layers were harvested and digested with a buffer containing 0.1 M $MgCl_2$, 0.02 M EDTA, 0.5 % SDS, 0.01 M Tris, pH 8.0, and 1 mg/ml of proteinase K at 37 °C overnight. The lysates were homogenized by passing through a blunt 20-gauge needle (0.9mm diameter) at 4°C temperature and DNA was purified by phenol: chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Finally, genomic DNA was dissolved in Tris–EDTA buffer and quantified by spectrophotometric analysis. The quality of genomic DNA was checked for high molecular weight by 1% agarose gel electrophoresis.

Bisulfite PCR and pyrosequencing

DNA methylation was measured by pyrosequencing of the PCR products, which measures methylation variation at >90 % precision [21]. 250 ng of DNA was bisulfite-converted using the EZ DNA Methylation Gold kit, according to the manufacturer's protocol (Zymo Research, Irvine, CA, USA). Multiple sets of primers were designed to amplify several intronic regions of the human FKBP5. Genomic organization and average intronic methylation of the human FKBP5 are shown in Fig.1. The intronic regions have been selected based on their homology to conserved mouse glucocorticoid response elements (GRE) [4] and previous study implicating their CpG methylation to trauma-related HPA axis dysregulation [9]. In particular, CpGs within intron 2 have been divided into four PCR amplicon regions to cover most of the intron for determination of DNA methylation [9]. List of primer sets are provided in Supplementary Table 1. Thermocycling was carried out using the Veriti and SimpliAmp thermal cyclers (Life Technologies, Carlsbad, CA, USA), and 25ng of bisulfite treated DNA was used for each PCR reaction. An additional nested PCR was performed with 2μL of the previous PCR reaction and one biotinylated primer (other primer being unmodified). Amplification for both PCR steps consisted of 40 cycles (94 °C for 1 min, 53 °C for 30 s, 72 °C for 1 min). PCR products were confirmed on agarose gels. Pyro Gold reagents were used to prepare samples for pyrosequencing according to manufacturer's instructions (Qiagen, Germantown, MD, USA). For each sample, biotinylated PCR product was mixed with streptavidin-coated sepharose beads (GE Healthcare, Waukesha, WI, USA), binding buffer, and Milli-Q water, and shaken at room temperature. A vacuum preptool was used to isolate the sepharose bead-bound single-stranded PCR products. The attached DNAs were released into a PSQ HS 96-plate containing pyrosequencing primers in annealing buffer. Pyrosequencing reactions were performed on the PyroMark MD System (Qiagen). CpG methylation quantification was performed with the Pyro Q-CpGt 1.0.9 software (Qiagen). An internal quality-control step was employed to disqualify any assays that contained unconverted DNA. Percentage of methylation at each CpG as determined by pyrosequencing was compared among DNA from control and Cushing's patients.

Neuropsychological tests and MRI

Rey Auditory Verbal Learning Test (RAVLT) for verbal memory and Rey-Osterrieth Complex Figure memory test (ROCF) for visual memory were administered in all subjects as previously described [11].

Beck Depression Inventory-II (BDI-II) is a self-reported measure of the severity of depressive symptoms. It has 21 items with a four-point scale ranging from 0 to 3. The total score is the sum of each item-rating and can range from 0 to 63. The manual states that higher scores indicate more severe depressive symptoms. Scores 0–13 indicate minimal depression, 14–19 indicate mild depression, 20–28 indicate moderate depression, and 29–63 indicate severe depression. The BDI-II can be separated into affective and somatic dimensions.

State-Trait Anxiety Inventory (STAI) is a self-reported measure that includes two subscales to evaluate two types of anxiety: state anxiety (anxiety related to current events) and trait

anxiety (anxiety as a personal characteristic). Each subscale has 20 questions with a four-point scale ranging from 0 to 3. The total score for each subscale is the sum of each item-rating and can range from 0 to 60. Higher scores indicate higher levels of anxiety.

MRI was obtained using a 3Tesla Philips Achieva facility (software version 2.1.3.2) and hippocampal volumes were calculated in all subjects as previously described, with FreeSurfer version 4.3.1 image analysis software (/http://surfer.nmr.mgh.harvard.edu) [11].

Statistical analysis

Data were analyzed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk, NY, USA) with a level of significance of $p < 0.05$. Data distribution was analyzed by the Kolmogorov–Smirnov test.

Epigenetic analysis was performed using non-parametric tests, Mann Whitney U-test for two groups and Kruskal–Wallis for three groups. The p-values reflect correction for multiple testing by the number of CpGs interrogated. Data are expressed as median (range).

Correlations among variables were studied using Spearman's rho for non-parametric measures. Friedman test and Wilcoxon test have been used to compare the average DNA methylation between the four introns (1, 2, 5, and 7).

Results

DNA methylation

A primary analysis evaluated the methylation of a total of 41 CpG dinucleotides in introns 1, 2, 5, and 7 of the FKBP5 in active, cured CS patients, and controls. Ten significant differentially methylated CpG dinucleotides were identified and shown in Table 2. Figure 2 shows the CpG methylation of Intron 2 Region 2 CpG-3, which shows the greatest methylation difference, in active, cured CS patients, and controls. Correlations between these ten significant differentially methylated CpG dinucleotides and HV, memory tests, anxiety, depression or disease duration were evaluated. Intron 2 Region 2 CpG-3 had a positive correlation with both left ($r = 0.73$, $p = 0.02$) and right HV ($r = 0.58$, $p = 0.03$) in active CS patients (Fig. 3). Intron 2 Region 2 CpG-3 also exhibited a positive correlation with verbal memory scores ($r = 0.53$, $p = 0.01$) in cured CS patients. No correlations between the methylation of the other CpG dinucleotides and HV or memory tests were observed. No correlation between CpG dinucleotides methylation and disease duration, 24-h urinary free cortisol, anxiety, or depression was found.

Finally, differences in the average DNA methylation of each intron were evaluated between active, cured CS patients, and controls. Introns 2 and 7 exhibited lower methylation in CS patients compared to controls. Cured CS patients and active CS patients had lower methylation of intron 2 than controls (92.37, 91.8 and 93.34 %, respectively, with $p = 0.03$ for both comparisons), without differences between cured and active. Moreover, cured CS patients and active CS patients had lower methylation of intron 7 than controls (77.08, 73.74, and 79.71 %, respectively, with $p = 0.02$ and $p < 0.01$), without differences between cured and active (Fig. 4).

Genotyping

All of the subject samples were genotyped for a SNP in FKBP5 (rs1360780). The rs1360780 is a C/T SNP located in intron 2, where individuals that harbor the TT genotype are homozygous for the “risk allele” (= T). Moreover, it has been shown to influence interaction among the promoter, introns 2 and 7 [9]. Twenty-two subjects (8 cured, 3 active and 11 controls) had the CC genotype, 34 subjects (10 cured, 5 active, 19 controls) had the TC genotype, and 8 subjects (5 cured, 1 active, 2 controls) had the TT genotype.

A primary analysis included all subjects. Two CpG dinucleotides (Intron 2 Region 2 CpG-3 and Intron 7 CpG-5) were evidenced as significantly different between the genotypes. Significantly lower methylation in Intron 2 Region 2 CpG-3 was observed in the TT subjects compared to CC (median 66.6 vs. 71.0 %, $p = 0.01$) and compared to TC (66.6 vs. 70.8 %, $p = 0.02$), without differences between TC and CC genotypes. Similarly, significantly lower methylation in Intron 7 CpG-5 was observed in the TT subjects compared to CC (74.2 vs. 77.7 %, $p = 0.01$) and compared to TC (74.2 vs. 77.6 %, $p = 0.01$), without differences between TC and CC.

No differences in methylation between the three genotypes were observed in the other CpGs. Furthermore, there were no differences between the three genotypes regarding HV, memory, anxiety, and depression.

A secondary analysis was performed with only CS patients, divided into two groups: with the T allele (TT, CT, n = 21) and without the T allele (CC, n = 11). This division was decided due to the small number of the CS samples. Significantly lower methylation in Intron 2 region 1 CpG-5 was evidenced in the patients with the T allele compared to the patients without the T allele (median 93.5 vs. 95.0 %, respectively, $p = 0.04$). No differences in methylation between the two genotypes were evidenced in the other CpGs. There were no differences between the two groups regarding HV, memory, anxiety, and depression. Interestingly, both Intron 2 Region 2 CpG-3 and Intron 2 region 1 CpG-5 were two of the ten significantly low methylated CpG dinucleotides in CS patients compared to controls. Table 3 shows genotyping results in CS patients and controls.

Finally, an average DNA methylation of each intron was calculated in order to compare DNA methylation of each intron between the three genotypes, including all subjects. No differences in the average DNA methylation of introns 1, 2, 5 were observed among the three genotypes. However, significantly lower average DNA methylation in intron 7 was observed in the TT subjects compared to CC (72.5 vs. 79.5%, $p=0.02$) and to TC (72.5 vs. 79.0%, $p=0.03$) (Fig. 5).

Discussion

Our study demonstrates, for the first time, a reduction of intronic DNA methylation of FKBP5 in patients with CS.

These data are consistent with that observed in the mouse model of excess GC exposure: GC excess leads to loss of DNA methylation in FKBP5 [4]. The latter is an important regulator

of the GR complex that decreases cortisol binding to GR and prevents translocation of the receptor complex to the nucleus [9], through an intracellular, ultra-short negative feedback mechanism. Loss of methylation in the FKBP5 has been shown to lead to an increase in expression of this gene [9], increasing the ultra-short negative feedback and leading to GC resistance, while reducing the efficiency of the long negative feedback on the HPA axis [2]. This results in a prolonged HPA axis activation following exposure to stress [2].

Patients with CS suffer from prolonged hypercortisolism, and therefore our data suggest that the prolonged cortisol exposure would produce GC resistance, due to reduced CpG methylation of the FKBP5. We speculate that excessive cortisol release would lead to GR-mediated epigenetic changes in FKBP5, resulting in a disruption of the ultra-short feedback that balances FKBP5 and GR activity, finally dysregulating the HPA axis. This would contribute to a prolonged HPA axis activation following exposure to hypercortisolism.

Interestingly, there was also a difference in the methylation of some of the CpGs between active and cured CS patients. Even with a reduced number of patients (nine active) there were significant differences, all indicating lower methylation in active compared to cured CS patients. This observation leads us to speculate that an earlier diagnosis and successful treatment would likely minimize the progression of DNA methylation reduction and may potentially thwart the development of GC resistance.

On the other hand, the presence of reduced methylation in other CpGs in cured patients is also an intriguing point. One would assume that in the cured CS patients, the methylation would become normalized after they were cured of hypercortisolism; however, some of the GC-induced damage may be permanent, less severe than in the active phase, but still different from controls. These data suggest that the presence of hypercortisolism promotes lower methylation, which may improve with control of the disease without normalization of DNA methylation. However, the cross-sectional nature of the current study precludes understanding the longitudinal, complex process of methylation loss and restoration. Further, longitudinal studies are needed to clarify this point.

In the current study, we also asked whether GC-induced changes in DNA methylation assessed in the blood can be used as a proxy to detect GC-induced morphological and functional changes in the human hippocampus. More specifically, we compared DNA methylation signatures in FKBP5 in blood and compared them to HV. To our knowledge, our study is the first to use epigenetic signatures in blood of CS patients to assess GC-induced changes in the human hippocampus. Emerging studies demonstrate the utility of peripheral markers to assess activity of important processes occurring in the central nervous system [15, 22]. However, we found a subtle association between methylation and brain impairments, less than what we had hypothesized. In fact, we found few correlations between methylation in the differentially methylated CpG dinucleotides, namely one between Intron 2 Region 2 CpG-3 vs. HV and memory tests. In contrast, the mouse model showed numerous correlations [15], and different reasons could explain this difference. Mouse models often use inbred strains that are kept in carefully controlled environments to test for biochemical, epigenetic, or behavioral changes with limited translability to humans. Further, it is hard to imagine any epigenetic loci harboring markers that can predict a

complex parameter such as memory. Nevertheless, it is tempting to speculate whether other GC-induced peripheral targets can reflect those measures. In this study, we focused on intronic CpGs of FKBP5, some of which have been demonstrated to have an association with GC-induced epigenetic changes in a mouse model [15]; an unbiased, genome-wide assay might provide additional loci that may correlate with memory impairments observed in CS patients.

By modulating GR signaling, FKBP5 protein has the potential to modulate the actions of GC hormones with pleiotropic effects that can affect essentially every body tissue, including the brain. We speculate that GC resistance at the cellular level, a protective mechanism at the beginning, could lead to tissue impairments with the passing of the time and long-term exposure to GC. FKBP5 dysregulation, and in particular FKBP5 disinhibition, has been linked to a number of stress-related disorders [2, 4, 5, 23]. Postmortem studies have found FKBP5 overexpression in several brain regions in association with Alzheimer's disease and schizophrenia [23], and FKBP5 overexpression has also been implicated in other medical conditions including insulin resistance and immune dysregulation [23]. However, the translational implications are not so clear.

Our results have been adjusted for smoking, because it has been shown that nicotine can affect HV on one hand and FKBP5 methylation on the other [24, 25].

Finally, we performed an analysis comparing the average intronic methylation, in order to investigate whether specific intronic methylation would influence clinical findings and a genotyping analysis in order to determine whether genetic variations can also govern CpG methylation. We found lower methylation in introns 2 and 7 in both cured and active CS patients compared to controls. Moreover, the results of the genotyping analysis indicated that there was lower methylation of intronic CpGs located in introns 2 and 7 in risk allele carriers (subjects with T allele). In addition, the average intron 7 methylation was significantly lower in individuals that had the TT genotype. This is consistent with the results of another study that reported loss of DNA methylation in introns 2 and 7 of risk carriers in post-traumatic stress exposure [9].

As introns 7 and 2 are important for binding with the GR [9], and decreased methylation of these introns has been associated with higher induction of FKBP5 by GR activation especially in risk allele carriers [8, 9, 26], this would represent an enhancement of the ultra-short feedback loop leading to increased GC resistance. In other words, being TT subjects or Cushing's patients would imply having more GC resistance at the cellular level. However, this is only a speculation, due to the small number of samples, and further studies are needed in order to provide a global view of clinical implications for these molecular findings. There is also evidence that differences in methylation patterns of each intron may affect gene splicing in some way [22]. It is possible that changes in intronic CpG methylation, as observed in the current study, may affect splicing events.

We did not find correlation among intronic CpGs, genotype, and anxiety or depression. One reason could be due to the low number subjects who have the risk allele in the genotyping analysis (eight TT subjects: six patients and two controls). Another reason could be that our

CS patients had only minimal/mild depression and anxiety, not requiring drugs. Our data also did not show a significant genotype effect on HV. We do not have a clear explanation for this interesting observation. Once again, the low number of subjects with the “T” allele, particularly in the CS patients, may be an explanation. The literature data are heterogeneous, due to different population studies and different imaging techniques. Fani et al. examined the effect of rs1360780 on brain volumes and reported allele-specific functional and structural (but not volume) changes in the hippocampus. This is a notable study from the same group that first identified the significance of rs1360780 in the context of Major depressive disorder (MDD) and childhood trauma [27]. Fujii et al. performed MRI of non-clinical subjects and reported volume reduction in only the dorsal anterior cingulate cortex in the T-carriers [28]. Tozzi et al. found a correlation between the genotype at rs1360780 and HV, performing a functional magnetic resonance imaging, emotional attention task, and Diffusion tensor imaging, which studies white matter integrity, in MDD subjects and controls. However, in our study we performed for the first time a Freesurfer analysis in CS patients and controls; therefore, the studies are not comparable [29].

This study has several limitations. First, the sample size of our cohort is relatively small; however, this is difficult to avoid in a rare disease like CS. Our study population included patients with pituitary-dependent CS and adrenal CS, since they shared chronic exposure to endogenous hypercortisolism, which we hypothesized, is harmful to the brain regardless of its origin.

Second, the polymorphism rs1360780 in FKBP5 has also been assessed, even if the sample size was small, with the thought that this would complete our data and lead to additional avenues of investigation.

Third, it is not clear as to the extent in which cell composition in the blood is affected by hypercortisolism. It is possible that epigenetic changes observed in introns 2 and 7 may be a consequence of cortisol-induced changes in the cell-type composition of blood. However, we speculate that tissue- or cell-type specific CpG methylation occur over large swaths (>1 kb) of consecutive CpGs [30, 31], whereas methylation changes that occur due to hormones may alter only few CpGs adjacent to glucocorticoid response elements (or GREs), such as in our study and that of others [9]. Immunophenotyping and cell sorting in Cushing’s patients are needed to clearly address cortisol-induced changes in cell composition and DNA methylation.

Technical limitations should also be mentioned. We limited our assay design and analysis to CpG dinucleotides of intronic regions that were previously identified as showing epigenetic changes in childhood trauma exposure [9]. For that reason, we omitted determination of the FKBP5 promoter region. However, additional regions within FKBP5 may need to be assayed. Although the same intronic CpGs underwent epigenetic changes in Cushing’s (introns 2 and 7), it is possible that Cushing’s-specific regions that correspond to different intracellular signaling and transcription factor binding sites within FKBP5 may play a role. In addition, mRNA was not extracted during sample processing and thus precluded functional expression studies that could have supported the epigenetic findings. Finally, a

genome-wide investigation is necessary to identify epigenetic changes at other genes that may correlate with Cushing's related brain impairments.

In conclusion, our data demonstrate, for the first time, a reduction of intronic DNA methylation of the FKBP5 gene in patients with CS. Since lower intronic DNA methylation of FKBP5 has been associated with higher gene expression, this would imply subsequent GC resistance. We speculate that GC resistance at the cellular level, a protective mechanism at the beginning, could lead to tissue impairments with the passing of time and long-term exposure to GC. Future studies that incorporate measurement of FKBP5 protein levels and dexamethasone suppression tests are needed to clearly demonstrate the relationship among lower methylation, protein expression, and GC resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FKBP5	human protein
FKBP5	human gene
FKBP5	mouse protein
Fkbp5	mouse gene

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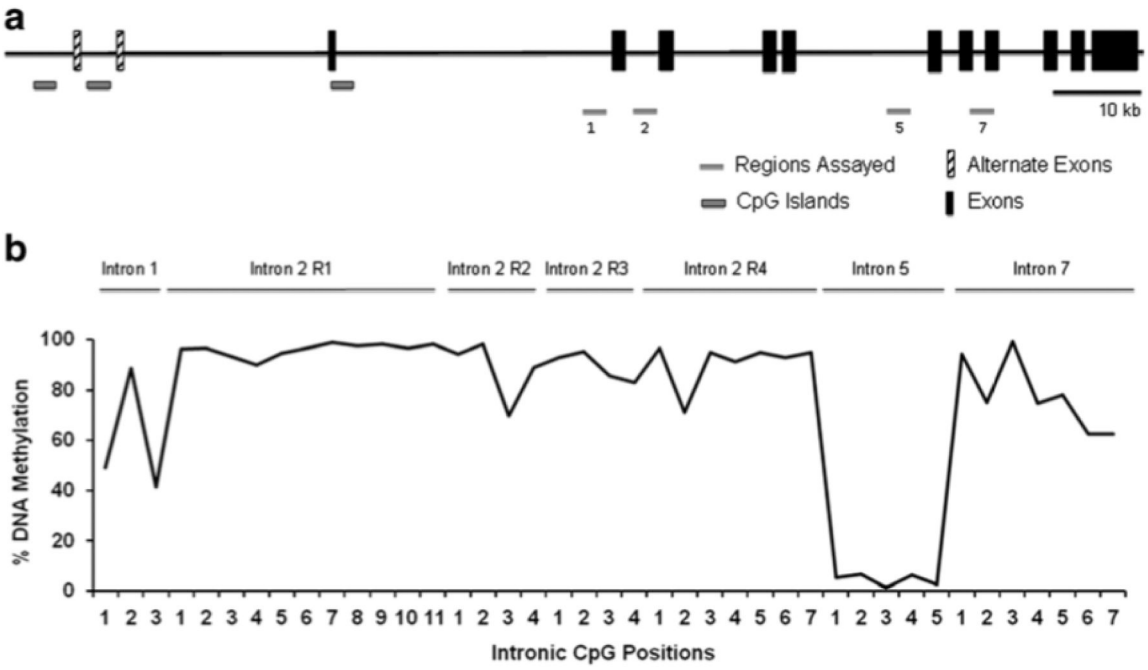

Fig. 1.

Genomic organization and average intronic methylation of the human FKBP5 gene. a FKBP5 is located on the negative strand on Chromosome 6: 35,541,362–35,696,360 (UCSC Genome Browser, hg19). It has 11 exons (black boxes) and two 5′ alternate exons (white striped boxes) that can serve as additional sites of transcription for alternative transcripts. Three 5′ CpG islands are represented by horizontal gray boxes. Four gray, numbered horizontal lines represent intronic regions assayed in our study by bisulfite pyrosequencing, and the numbers refer to the intron locations (e.g. 2 = intron 2). b DNA methylation levels across four intronic regions (1, 2, 5, and 7) reflect average percent methylation values (Y-axis) for both control and Cushing's samples. CpG positions are numbered from the first CpG for each intronic region. Intron 2 is comprised of four different sub-regions that correspond to four separate bisulfite pyrosequencing assays, and each of which starts with CpG-1. R = region.

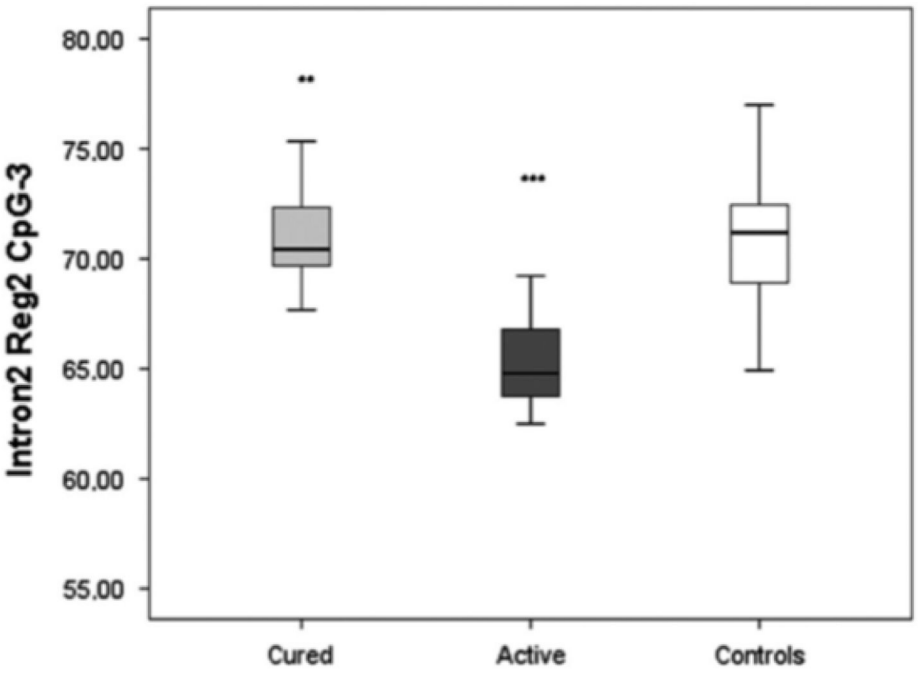

Fig. 2.

Box plot of percent DNA methylation levels for Intron 2 Region 2 CpG-3 in active, cured CS and controls. **p = 0.002 (significance between cured CS patients and active CS patients). ***p = 0.001 (significance between active CS patients and controls). Y-axis: % of DNA methylation.

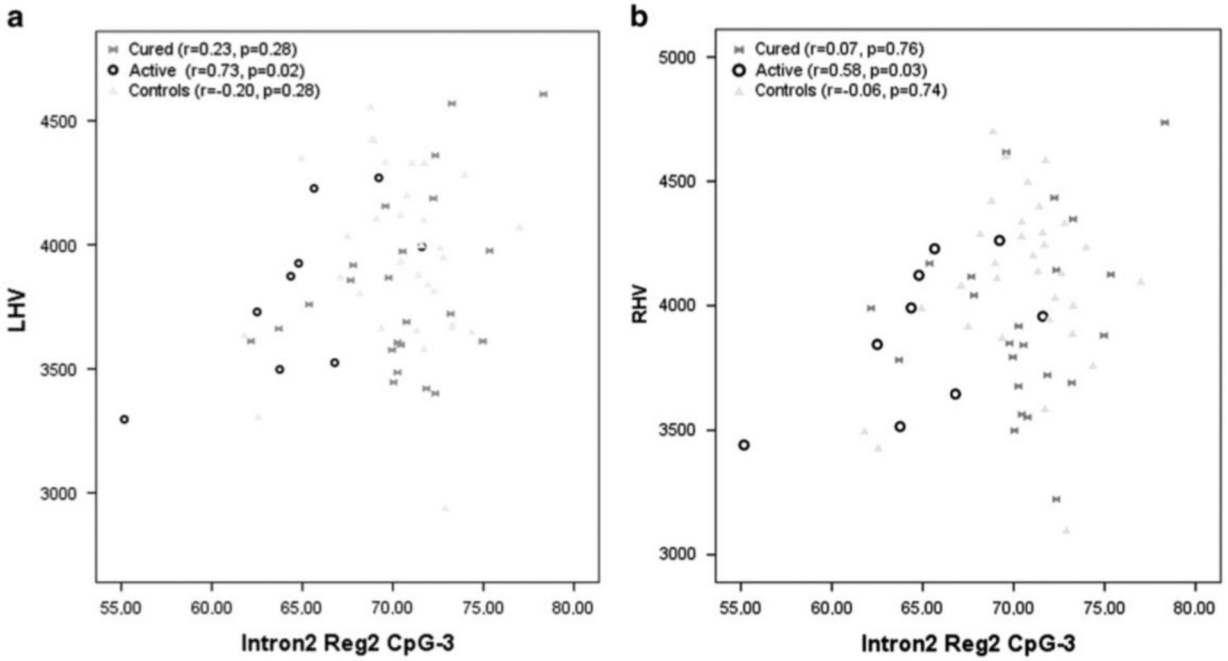

Fig. 3.

Correlations between intronic DNA methylation levels in Intron 2 Region 2 CpG-3 and hippocampal volumes in CS patients and controls. **a** Correlation between the left hippocampal volume (LHV) and DNA methylation at Intron 2 Regions 2 CpG-3 for CS patients and controls. There was a significant correlation between LHV and Intron 2 Region 2 CpG-3 ($r = 0.73$, $p = 0.02$) in active CS patients. **b** Correlation between the right hippocampal volume (RHV) and DNA methylation at Intron 2 Regions 2 CpG-3 for all Cushing's and Control samples are shown. A weaker but still significant correlation was observed in active Cushing's patients ($r = 0.58$, $p = 0.03$). X-axis: % of DNA methylation, Y-axis: hippocampal volumes, mm^3.

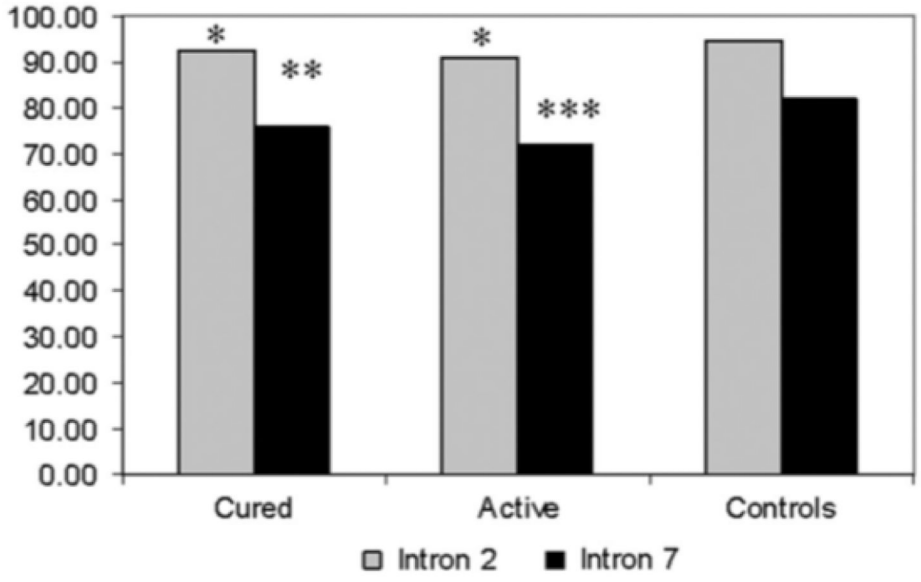

Fig. 4.

Average DNA methylation of introns 2 and 7 between active, cured CS patients and controls. *Cured CS patients and active CS patients had lower methylation of intron 2 than controls, $p = 0.03$ for both comparisons. **Cured CS patients had lower methylation of intron 7 than controls, $p = 0.02$. ***Active CS patients had lower methylation of intron 7 than controls, $p < 0.01$. X-axis: % of DNA methylation.

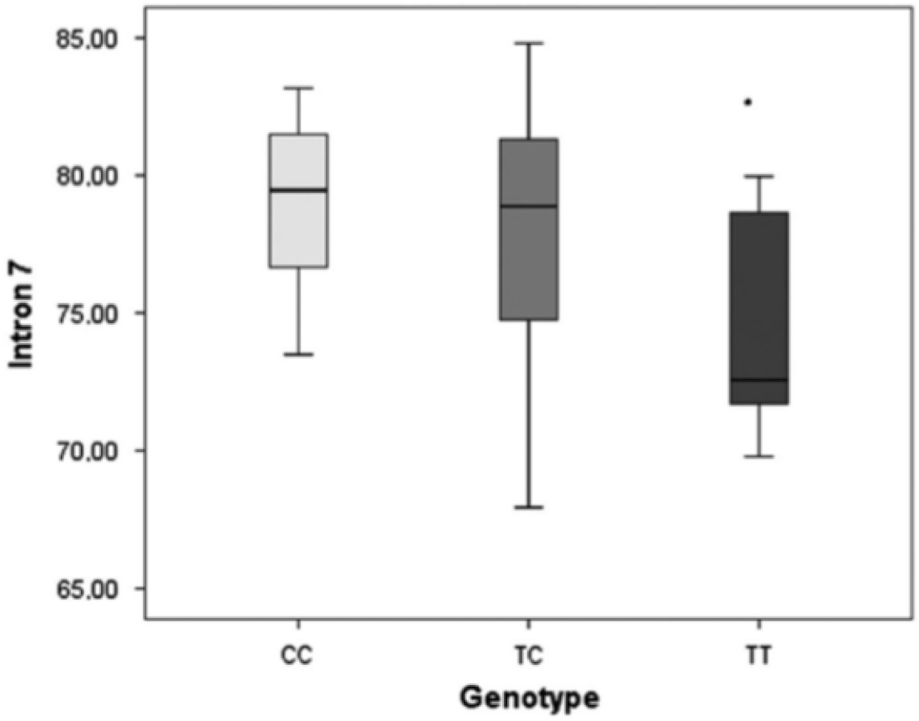

Fig. 5.

Box plot of average intron 7 methylation levels among the three rs1360780 genotypes (CC, CT, TT) in all subjects. DNA methylation levels were calculated from all of the seven pyrosequenced CpGs from intron 7. *p < 0.05.

Table 1

Clinical features of patients and controls

	CS patients (*n* = 32)	**Controls (*n* = 32)**
Gender (male/female)	5/27	6/26
Age (years)	45 ± 12	44 ± 11
Years of education	13 ± 4	14 ± 4
Duration of disease control (years)	7.1 ± 2.2	-
Medical treatment for hypercortisolism	*n* = 9	-
Metyrapone	4/9	
Ketoconazole	5/9	
Postoperative radiotherapy	6/32	-
CS origin		-
Pituitary	25/32	
Adrenal	7/32	
Hydrocortisone replacement therapy	4/32	
Smokers	6/32	8/32

Table 2

Ten significant differentially methylated CpG dinucleotides and average methylation of introns 2 and 7, in Cushing's syndrome (CS) patients compared to controls

	Cushing patients Cured (*n* = 23)	**Active (*n* = 9)**	**Controls (*n* = 32)**	**P**	**P^1**	**P^2**
Intron2reg1CpG-3	94.7 % (45.8–95.5)	94.8 % (94.5–95.4)	95.5 % (72.2–97.1)	0.014	0.001	ns
Intron2reg1CpG-4	91.2 % (35.4–93.8)	92.2 % (89.6–93.6)	94.1 % (65.4–96.0)	0.006	<0.001	ns
lntron2reg1CpG-5	93.7 % (82.5–96.1)	95.1 % (87.7–97.2)	95.5 % (89.7–100)	ns	0.002	ns
Intron2reg2CpG-3	70.4 % (62.7–78.3)	64.8 % (55.2–71.6)	72.2 % (61.8–77.0)	0.001	ns	0.002
Intron2reg3CpG-4	82.1 % (79.0–100)	82.2 % (80.8–82.8)	83.1 % (81.0–100)	0.012	0.001	ns
Intron5 CpG-1	4.8 % (3.03–7.5)	4 % (2.8–4.7)	5.2 % (3.8–7.9)	0.000	ns	0.001
Inlron7 CpG-1	91.88 % (73.0–100)	90.6 % (76.3–98.2)	98.5 % (86.3–100)	<0.001	0.001	ns
Intron7 CpG-2	74.4 % (53.1–83.7)	72.8 % (51.5–77.3)	79.0 % (54.4–87.1)	0.001	0.002	ns
Intron7 CpG-6	63.4 % (43.0–73.4)	53.5 % (43.8–63.3)	66.7 % (51.6–73.2)	<0.001	ns	0.007
Intron7 CpG-7	62.3 % (44.3–87.1)	56.3 % (48.8–60.0)	62.2 % (48.7–74.0)	0.002	ns	0.006
Average methylation of intron 2	92.37 %,	91.80 %	93.34 %	0.03	0.03	ns
Average methylation of intron 7	77.08 %	73.74 %	79.71 %	<0.01	0.02	ns

Values indicate the percent of *FKBP5* DNA methylation at the indicated CpG dinucleotides and are expressed as median (range). A CpG site (shorth and for C-phosphate-G) refers to a cytosine nucleotide followed by a guanine nucleotide in the linear sequence of bases along the length of DNA. Cytosines in CpG dinucleotides can be methylated to alter gene function in animals and plants, where lower CpG methylation generally correlates with higher gene expression

Intron 2 is divided into four regions that reflect four independent bisulfite pyrosequencing assays

Five significant differentially methylated CpG dinucleotides were located in intron 2, one in intron 5 and four in intron 7

Reg = region

ns= not significant

p = significance between active CS patients and controls

p^1 = significance between cured CS patients and controls

p^2 = significance between cured CS patients and active CS patients

Table 3

Percentage of *FKBP5* DNA methylation according to gcnotyping in CS patients and controls

All subjects (n = 64)	*CC genotype (n = 22)*	*TC genotype (n = 34)*	*TT genotype (n = 8)*	*p*	p^1	p^2	p^3
Intron2 Region2CpG-3	71.0 % (62.6–77)	70.8 % (55.2–78.3)	66.6 % (62.5–70.2)	0.01	0.02	ns	ns
Intron7 CpG-5	77.7 % (72.2–89.6)	77.6 % (68.9–87.6)	74.2 % (60.2–76.4)	0.01	0.01	ns	ns
CS patients (n = 32)	*CC genotype (n = 11)*	*TC+TT genotypes (n = 21)*					
Intron 2 region 1 CpG-5	95.0 % (92.3–97.2)	93.5 % (82.5–95.8)	-	-	-	-	0.04

All subjects

p = significance between TT genotypes compared to CC genotypes

p^1 = significance between TT genotypes compared to TC genotypes

p^2 = significance between TC genotypes compared to CC genotypes

CS patients

P^3 = significance between CS patients with the T allele (TTgenotypes + TC genotypes) compared to the patients without the T allele (CC genotypes)