

DNA methylation of the POMC gene promoter is associated with craving in alcohol dependence

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Abstract Hypothalamic–pituitary–adrenal (HPA) axis dysfunction has been implicated in the pathogenesis of addictive behaviour and especially in alcohol craving. The pro-opiomelanocortin gene (POMC), encoding a 241 amino acids stretching polypeptide hormone precursor, plays an important role in the regulation of the HPA, and is prone to epigenetic regulation due to promoter-related DNA methylation. Aim of the present study therefore was to investigate possible differences in promoter-related DNA methylation in patients suffering from alcohol dependence compared to healthy controls. We analysed the DNA methylation of the 5' promoter of the POMC gene that is embedded in a CpG island using bisulfite sequencing in 145 alcohol-dependent patients and 37 healthy controls taken from the Franconian Alcoholism Research Studies. We found only marginal, hence significant differences at single CpG sites between patients and controls. We identified a cluster of CpGs showing a significant association with alcohol craving in the patients group. These results implicate that epigenetic changes possibly due to alcohol intake may contribute to craving via promoting HPA-axis

dysfunction. Further studies should more closely investigate the impact of these changes on the several derivatives of the POMC gene.

Keywords Alcohol · Craving · DNA methylation · POMC · HPA axis

Introduction

The hormone precursor polypeptide pro-opiomelanocortin (POMC), mainly expressed and processed in neurons of the pituitary gland, is modified post-translationally into several active hormones. The primary representative adrenocorticotrophic hormone (ACTH), stimulating the release of corticosterone, the primary glucocorticoid, acts therefore as a modulator of the stress response. The regulation of the hypothalamic–pituitary–adrenal axis (HPA) underlies a negative feedback (Fig. 1). A modulation of any actuator of this system thus leads to an alteration in this feedback mechanism. There is a wide variety of subsequent consequences.

Recent studies showed that ethanol influences HPA-axis regulation (Richardson et al. 2008) and that several signs of this dysregulation are present in patients suffering from alcohol dependence (Rasmussen et al. 1998; Junghanns et al. 2003). To date only little is known about the way ethanol interferes with HPA-axis regulation. Only a few studies showed that POMC expression is controlled by epigenetic mechanisms, mainly by the methylation at the 5' POMC promoter region, where hypermethylation leads to silencing of the POMC expression (Newell-Price et al. 2001; Newell-Price 2003; Ye et al. 2005). Disturbances in DNA methylation have been described recently to be present in patients suffering from alcohol dependence

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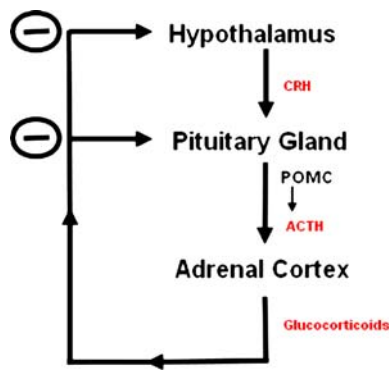


Fig. 1 Negative feedback regulation of HPA axis

(Bleich et al. 2006) and are linked to typical psychopathology such as alcohol craving (Hillemacher et al. 2009). A close link between alcohol craving as well as withdrawal severity and HPA-axis dysregulation has already been established (Rasmussen et al. 2002). Various studies showed that craving for alcohol is associated with alterations of the hypothalamic–pituitary–adrenal (HPA) axis (Kiefer and Wiedemann 2004). Specifically, higher craving was found to be associated with lower corticotropin releasing hormone (CRH) (Fahlke et al. 1994; Olive et al. 2003) and also with lowered cortisol- and ACTH-serum levels (Kiefer et al. 2002; Junghanns et al. 2003).

The aim of the present study therefore was to test the hypothesis that DNA methylation at the POMC gene promoter differs between patients suffering from alcohol dependence and healthy controls, and that these differences show a connection to the extent of alcohol craving.

Materials and methods

Patients and design

The present study was part of the Franconian Alcoholism Research Study (FARS) (Bleich et al. 2005). The work was approved by the local Ethics Committee of the University of Erlangen-Nuremberg. After complete description of the study to the subjects, written informed consent was obtained.

For bisulfite sequencing of the POMC promoter, 145 blood samples were taken from the FARS sample archive. Among these samples were 35 from early abstinent patients and 110 who were acutely intoxicated at admission. The data of OCDS and its subscales were taken on day of admission. The period of time of craving that was evaluated refers to the week before admission. The blood samples used for our study were taken on the day of admission (day 0). Further 37 samples, obtained from healthy students, served as a control group. The data of all subjects

are composed as follows. Patients: mean age 43.9 years; standard deviation (SD 9.2); gender 79.3% male, 20.7% female, with a mean duration of alcohol consumption of 17.39 years (SD 10.5); controls: mean age 22.4 years (SD 4.8); gender 44.7% male, 55.3% female. The patients BMI was calculated with 24.4 (SD 4.3), the controls had a mean BMI of 22.8 (SD 2.7). Mean blood alcohol level at admission was 134 mg/dl (SD 116). Mean daily ethanol intake was 293 g (SD 172), the mean years of drinking were 17.39 (SD 10.5), mean clomethiazole dose for treatment of withdrawal syndromes was 1,727 mg (SD 1146), mean amount of carbamazepine applied to patients was 2.051 mg (SD 237) (Table 1). Concomitant psychiatric illnesses, other substance abuse apart from alcohol or nicotine and existence of severe somatic illness were regarded as exclusion criteria. Study characteristics of the patients such as sociodemographic data and medical history were taken using a semi-structured interview. Alcohol craving was assessed at admission using the Obsessive Compulsive Drinking Scale (Anton et al. 1995), which provides a well validated and reliable measurement, distinguishing a total score with obsessive and compulsive subscales (Anton et al. 1995). The severity of alcohol withdrawal was assessed by the withdrawal syndrome scale for alcohol and related psychoactive drugs (WSA) (Kristensen et al. 1986) and by the necessity of clomethiazole treatment. There was no certain duration of clomethiazole application. The medication was applied according to the individual patients necessity. The patients were treated according to the actual guidelines for withdrawal treatment. The duration of treatment differed from patient to patient.

As indicator for acute intoxication we used the blood alcohol concentration (BAC) which was measured with the alcohol-dehydrogenase (ADH) automated standard procedure (Leithoff 1963).

Bisulfite sequencing of the promoter of POMC

Fasting blood samples for DNA extraction were drawn on admission. Genomic DNA was extracted from whole frozen EDTA–blood with the QIAamp[®] DNA Blood Mini Kit (QIAGEN AG, Hilden, Germany) according to the manufacturer's protocol. Afterwards, 500 ng of genomic DNA were modified by sodium-bisulfite using the EpiTect[®] Bisulfite Kit (QIAGEN AG, Hilden, Germany). Whereas sodium-bisulfite deaminates cytosines in CpG dinucleotides to uracils, while methylated cytosines are protected from alteration (Frommer et al. 1992; Clark et al. 1994).

Primers were designed to amplify a region covering a specific fragment of 766 base pairs (from –486 to +280 bp prior to the starting base pair of the first exon of POMC)

Table 1 Sociodemographic data of the study population, clinical variables and results

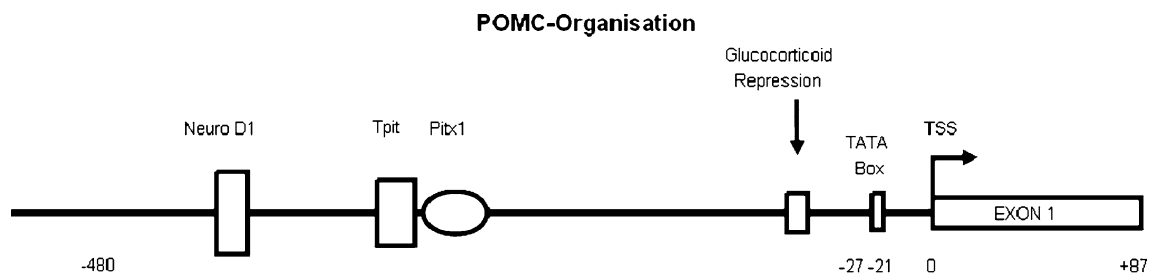
	Patients (<i>n</i> = 145)		Controls (<i>n</i> = 37)
Gender: male, female (%)	20.7, 79.3		55.3, 44.7
Age (mean/SD)	43.9/9.2		22.4/4.8
BMI (kg/m ²) (mean/SD)	24.4/4.3		22.8/2.7
Blood alcohol concentration (mg/dl) (mean/SD)	134/116 ^a		0/0
Daily ethanol intake (g) (mean/SD)	293/172		Not available
Years of drinking (years)	17.39/10.5		Not available
Clomethiazole (mg) (mean/SD)	1,727/1,146 ^b		Not available
Carbamazepine (mg) (mean/SD)	2,051/237 ^c		Not available
OCDS total day 0 (mean/SD)	22.33/7.4		Not available
OCDS obsess day 0 (mean/SD)	8.22/4.7		Not available
OCDS compuls day 0 (mean/SD)	12.1/3.8		Not available
Clomethiazole	Yes (103)	No (42)	Not available
Mean methylation at cluster 1 (%) (mean/SD)	32.0/6.9	29.3/5.8	Not available

OCDS obsessive compulsive drinking scale, BMI body mass index

^a Measured from 103 patients

^b 103 patients received clomethiazole

^c 103 patients received carbamazepine

**Fig. 2** POMC TFBS within the displayed CpG dinucleotides

containing 68 CpG sites within the 5' upstream promoter region of the POMC gene. The amplified region contains transcription factor binding sites TFBS for E2, Neuro-D1, TPIT and PTX-1 (Fig. 2) (Kraus et al. 1993; Lamolet et al. 2001). MatinspectorTM from the Genomatix suite (accessible at <http://www.genomatix.com>) was used to identify possible transcription factor binding sites (TFBS) within the analysed promoter region. MatinspectorTM identifies TFBS using a large library of weight matrices.

To amplify a highly specific product we performed a semi-nested PCR using a hotstart taq (QIAGEN AG, Hilden) with 30 cycles each. For both PCR-passages a standard PCR temperature protocol was programmed on the thermal cycler (initial taq activation step: 15 min at 95°C, denaturation: 30 s at 95°C; primer annealing: 90 s at 54°C, and elongation at 72°C for 120 s).

The primers for the first PCR were (annealing temperature 54°C):

POMC-bsfFna: (5'-AGGTTTTTGGAGAGTAGGTTTT AGG-3');

OMC-bsfRn: (5'-TAAACCCTCCAACCTCAACTACTA AC-3');

Product size: 861 bp.

For the second, semi-nested, PCR (annealing temperature 54°C):

POMC-seminesF: (5'-GTGGAATAGAGAGAATATGATTT-3'),

POMC-bsfFRn: (5'-TAAACCCTCCAACCTCAACTACTAAC-3').

Product size: 766 bp including 68 CpG dinucleotides.

Subsequently, 10 µl of each PCR product were visualized on a standard 2.0% agarose gel and the remaining 40 µl were purified using the Qiaquick[®] PCR Purification Kit (QIAGEN AG, Hilden, Germany). Sequencing, both forward and reverse direction, was performed using a BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems,

Foster City, CA, USA) and Applied Biosystems® 3730xl DNA Analyser (Applied Biosystems) according to the manufacturer's instructions. 50 ng of purified PCR product were applied for the extension reaction. The oligo-nucleotides of the second PCR run served as sequencing primers. The obtained sequences were analysed using a software called Epigenetic Sequencing Methylation analysis Software (ESME) designed especially for artificially generated SNP's (C/T and G/A) that determines the DNA methylation levels from the sequence trace files. ESME performs quality control, normalizes the signals, corrects for incomplete bisulfite conversion, aligns generated bisulfite sequence and reference sequence to compare C to T values (forward sequence) and G to A values (reverse sequence) peaks at CpG sites (Lewin et al. 2004).

Statistical analysis

The analysed variables were normally distributed according to the Kolmogorov–Smirnov test; therefore, parametric methods were applied. All data are given as mean (SD). All statistical tests were two-tailed, a significance level of $\alpha = 0.05$ or less was considered significant.

Between group comparisons were done either by *t* test (age, mean promoter methylation, methylation of CpG clusters), chi-square (gender) or two-way analysis of variance [single CpG positions \times group (patients vs. controls)]. Afterwards we performed several multivariate general linear models including all CpG sites as dependent variables and computing group and gender as fixed factors and age as covariate incorporating also possible two-way interactions between group and gender as well as group and age.

For further analysis, a cluster selection algorithm was performed to discover regions of interest in the CpG island, including 62 CpG that we were able to sequence. The definition of the cluster was as follows:

Criterion 1 Median methylation $100\% > X > 0\%$ and mean methylation $90\% \geq X \geq 10\%$ for each single CpG site.

Criterion 2 A cluster should comprise at least three CpG sites.

Criterion 3 All of the CpG sites have to be positively and significantly correlated.

Criterion 4 All of the CpG sites have to be positively and significantly correlated with the cluster's mean methylation.

From 62 of the analysed CpG sites only 15 were in accordance with criterion 1 and only 10 with criteria 2–4. These 10 CpGs grouped into two clusters: Cluster 1: (CpG –399; –328) and Cluster 2: (CpG –6; 10). These clusters

were regarded as relevant and implemented in further multiple regression analyses. For further analysis of possible associations between clinical variables and promoter methylation a set of *t* tests, oneway analyses of variance and correlation analyses (Pearson's) with subsequent multiple linear regression analyses was performed.

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS™) for Windows 16.0.2 (SPSS Inc., Chicago, IL) and Graph Pad Prism V for Windows (Graph Pad Inc. San Diego, CA).

Results

In the two-way analysis of variance computing group and CpG position as independent factors we observed a significant effect of CpG position ($F = 194.5$, $P < 0.0001$ explaining 51.30% of variance of the promoter methylation) and a significant interaction between CpG position and group ($F = 2.671$, $P < 0.0001$ explaining 0.70% of total variation) while groups had no significant effect ($F = 0.3583$, $P = 0.55$). To further analyse the observed interaction, we computed a multivariate general linear model with every CpG position as dependent variable observing a significant effect of the group on overall methylation (Pillai-Spur = 0.511, $F = 1.705$, $P = 0.013$), that was not detectable anymore after adjusting for age but not affected by gender. Neither age nor gender had a significant effect on their own. Figure 3 shows the mean methylation at each CpG site.

Association with clinical variable

The results show no differences in mean methylation of the identified clusters when comparing acutely intoxicated with early abstinent patients. We found no correlation with withdrawal severity measured using the WSA score; however, patients needing clomethiazole for treatment of withdrawal symptoms had a slightly but significantly higher mean methylation of cluster 1 [*t* test: clomethiazole yes, $N = 103$; %mC = 32.0 (6.9) vs. no, $N = 42$; %mC = 29.3 (5.8), $T = -2.29$, $P = 0.023$]. Treatment with carbamazepine had no significant effect on methylation of the clusters. Furthermore, we did not find any significant correlation between mean methylation or the identified clusters and daily intake, lifetime drinking, years of drinking or the number of previous alcohol withdrawals (data not shown).

We found a significant correlation of craving as measured with the OCDS on day 0 (day of admission) with the mean methylation at cluster 1 (Fig. 4). We confirmed this finding using stepwise multiple linear regression analysis

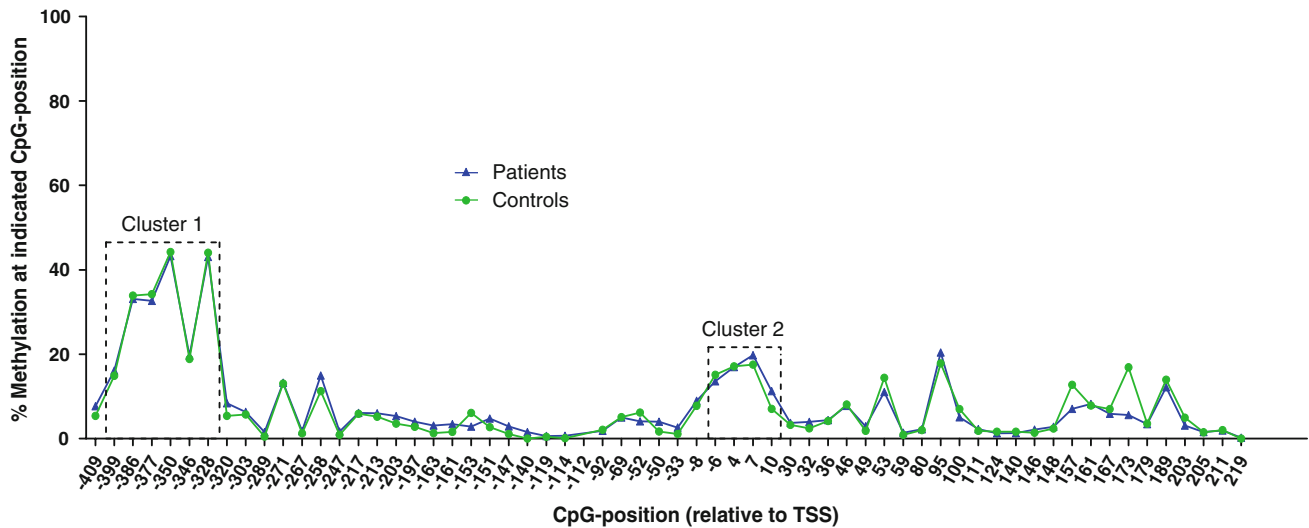


Fig. 3 Mean methylation at any CpG position between patients and controls. Cluster 1 and Cluster 2 are highlighted by rectangles

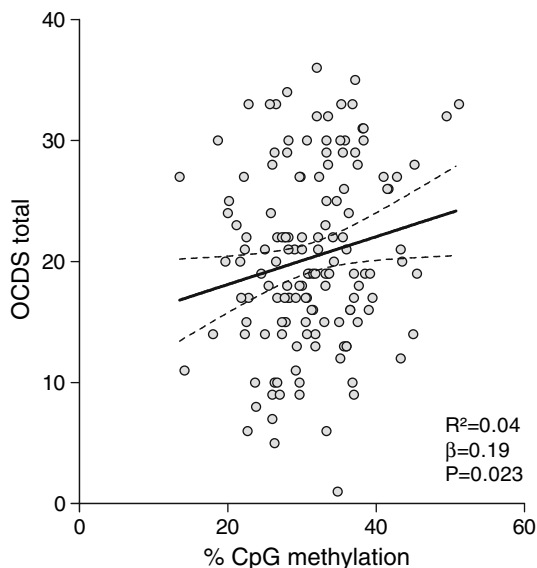


Fig. 4 Showing significant correlation of craving and mean methylation at cluster 1

computing the OCDS score as dependent and methylation of cluster 1 as well as age, gender, body mass index, years of drinking, and daily intake as independent variables using the BACKWARD method. In the model with the OCDS total score at admission, only the methylation of cluster 1 showed a significant association with the craving ($R^2 = 0.036$, $\beta = 0.19$, $P = 0.023$). Similar results were obtained for the obsessive and compulsive subscales of the OCDS. Results of OCDS total and its subscale are presented in Table 1.

In silico analysis of the POMC promoter

Using the genomatrix suite, several putative transcription factor binding sites within the identified cluster 1 were found, among them the previously described TF-module containing TFBS for Neuro-D1, TPIT and PTX-1. Among the putative TFBS within cluster 2, no additional evidence for functional relevant TF modules could be mapped.

Discussion

The present study is the first that focused on determination of an association between methylation status of the 5' upstream POMC promoter and craving in patients in early alcohol withdrawal. Various studies mention a close relation between an alteration in HPA-axis-mediated stress response, such as expression of ACTH and subsequently cortisol, and patients suffering from alcohol dependence (Rasmussen et al. 1998; Kiefer et al. 2002; Rasmussen et al. 2002; Olive et al. 2003; Kiefer and Wiedemann 2004). The idea was to find a possible epigenetically regulated mechanism for the altered stress response regarding POMC regulation.

In our study, we found no relevant differences of the methylation pattern in both groups, patients and healthy controls. Additionally, there was no difference at any CpG position detectable. While these findings point towards an absence of epigenetic mechanisms in the regulation of POMC expression in alcohol-dependent patients, results of our study show a significant correlation of the methylation pattern of a specific cluster of CpG islands (cluster 1) with craving, measured by OCDS. This finding, taking into

account the limitation of the present study, has to be interpreted with caution. However, this may be a first hint towards a pathophysiologic role of epigenetic mechanisms in the regulation of POMC in alcohol-dependent patients, which should be evaluated in further investigations. Furthermore, these results may be regarded as an important step towards a more specific view on epigenetic regulation of POMC, as possibly only specific regions (clusters) of the POMC gene may be involved in the pathogenesis of alcohol craving, which should be taken into account in further studies.

Another finding of our investigation was the mentioned association of cluster 1 with the use of clomethiazole as withdrawal medication. It is important to mention that all patients received their medication (clomethiazole) after taking blood samples. These blood samples were used as templates for the presented methylation analysis. So we can definitely say that clomethiazole cannot have any influence on the results of our methylation analysis. In contrast, clomethiazole medication can be regarded as a marker for severe alcohol withdrawal symptoms. Hence, our findings regarding the association with clomethiazole medication hint towards a possible involvement of epigenetic mechanisms in the regulation of POMC (cluster 1) in alcohol withdrawal syndrome. Evidentially, these findings are limited by the lack of significant findings regarding the withdrawal scale (WSA).

In general, the study suffers from several limitations: first, POMC, mainly transcribed in neurons of the pituitary gland, is only weakly expressed in peripheral blood cells. The amount of transcript that can be found in neurons will not be reached in blood cells. This could be an explanation for the methylation pattern that is associated with the low level of peripheral POMC expression found in patients and healthy controls. As it is difficult to obtain cerebral cells in humans, we decided to use peripheral blood mononuclear cells in this pilot study. Therefore, further investigations should try to replicate these findings using cerebral cells, e.g. from animal experiments.

Second, the composition of the control group is significantly different in age and gender than the patients group. However, we corrected for age and gender differences in the statistical analysis and did not find a direct influence of both parameters on DNA methylation of POMC. However, this may be regarded as a limitation and a replication with gender- and age-matched controls should be performed to verify these results.

In conclusion, our results show little evidence for an important role of epigenetic mechanisms regulation POMC expression in alcohol-dependent patients. However, we found significant association of a specific cluster of CpG island with the use of clomethiazole as withdrawal medication and with alcohol craving. These results, while being

interpreted with caution, may be regarded as a first hint towards a role of epigenetic mechanisms in specific regions of the POMC gene in alcohol dependence, at least in association with specific features like craving and severity of withdrawal.

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