Impaired Regulation of ALDH2 Protein Expression Revealing a Yet Unknown Epigenetic Impact of rs886205 on Specific Methylation of a Negative Regulatory Promoter Region in Alcohol-Dependent Patients

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Key Words

Alcohol dependence · Epigenetics · ALDH2 · rs886205

Abstract

Acetaldehyde, the carcinogenic metabolite of ethanol known to provoke aversive symptoms of alcohol consumption, is predominantly eliminated by aldehyde dehydrogenase 2 (ALDH2). Reduced ALDH2 activity correlates with low alcohol tolerance and low risk for alcohol dependence. The ALDH2 promoter polymorphism rs886205 (A>G) is associated with decreased promoter activity, but a molecular mechanism and allele-dependent ALDH2 protein expression has not been described yet. On the basis of allele-dependent epigenetic effects, we analyzed the rs886205 genotype, methylation rates of cytosine-phosphatidyl-guanine (CpG)-sites within a regulatory promoter region and ALDH2 protein levels in 82 alcohol-dependent patients during a 2-week withdrawal and compared them to 34 matched controls. Patients without the G-allele of rs886205 showed higher methylation of the promoter region than controls and

readily adapted epigenetically as well as on protein level during withdrawal, while patients with the G-allele displayed retarded methylation readjustment and no change in ALDH2 protein levels. Our data provide novel insights into an unknown genetic-epigenetic interaction, revealing impaired ALDH2 protein expression in patients with the G-allele of rs886205. Additionally, we checked for an association between rs886205 and protection against alcohol dependence and found a trend association between the G-allele and protection against alcohol dependence that needs replication in a larger Caucasian cohort.

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Introduction

Aldehyde dehydrogenase 2 (ALDH2) plays a crucial role in ethanol metabolism, as it eliminates toxic acetaldehyde (AcH), the main metabolite of ethanol, in a rate-limiting reaction. Blood levels of AcH have been shown to inversely correlate with blood and hepatic ALDH2 ac-

tivity and to mainly affect AcH levels during alcohol consumption, even though polymorphisms of the alcohol dehydrogenase gene are also known to influence AcH levels [1–5]. The loss of ALDH2 activity has been reported to influence susceptibility not only to acute adverse effects of alcohol consumption like nausea and headache, but also to severe alcohol-related health risks, particularly esophageal squamous cell carcinoma (ESCC) [6].

Genetic association studies have identified rs671, a functional single nucleotide polymorphism (SNP) of the ALDH2 gene, which decreases the risk for alcohol dependence. This SNP causes complete loss of activity of the encoded ALDH2 protein and therefore high accumulation of AcH after drinking, but is predominantly prevalent in East-Asian populations [7].

Only recently, rs886205 (A>G), an SNP in the promoter region of the ALDH2 gene has been identified in several Western populations as a risk marker for ESCC in individuals with the G-allele and moderate to heavy alcohol consumption (>3 times per week) [8, 9]. Furthermore, the G-allele is known to decrease basal levels of ALDH2 promoter activity and gene transcription both in vitro and in vivo. Interestingly, a specific promoter region upstream the ATG codon of the ALDH2 gene exerts a negative regulatory function on basal ALDH2 promoter activity and mRNA levels in the co-presence of the G-allele of rs886205, while no decrease of basal ALDH2 gene transcription can be assessed in the presence of the A-allele [10].

Yet, the precise molecular mechanism of this interaction between rs886205 and the negative regulatory ALDH2 promoter region remains elusive, as transcription factors known to bind around the polymorphic site did not show binding preferences to either of the 2 promoter alleles [11]. Moreover, no study could link genetic predisposition via rs886205 and chronic alcohol consumption to impaired regulation of ALDH2 protein expression.

Epigenetic effects of chronic alcohol exposition on regulation of different genes in vivo and in vitro have been extensively described earlier [12–14].

The main goal of this investigation was to check if rs886205 was associated with altered methylation levels of the negative regulatory promoter fragment and corresponding ALDH2 protein levels in alcohol-dependent patients' blood during withdrawal.

Assuming that genetic disposition by rs886205 causes accumulation of AcH and impaired alcohol tolerance, we then examined the association between the G-allele and protection against alcohol dependence in a second step, as most association studies were performed in East-Asian

populations with contradictory outcome and biasing effects of strong linkage disequilibrium with rs671 [15, 16]. The primary outcome of the study was the occurrence of CpG-methylation at 11 specific sites in the negative regulatory ALDH2 promoter fragment in alcohol-dependent patients on days 1, 7 and 14 of withdrawal and healthy matched controls of Caucasian descent, whereas secondary outcomes were the presence of ALDH2 protein levels and the rs886205 genotype.

Materials and Methods

Alcohol-Dependent Patients and Healthy Controls

This investigation is part of a large prospective research project on neurobiological mechanisms in alcohol dependence (NENA: Studies in Neuroendocrinology and Neurogenetics in Alcoholism) approved by the local Ethics Committee of the University of Erlangen-Nuremberg [17]. All 82 patients included in the study were Caucasian males and they gave written informed consent. Each patient suffered from alcohol dependence, as defined by the International Classification of Diseases-10 and Diagnostic and Statistical Manual of Mental Disorders-IV and was admitted for detoxification treatment to the Hospital for Psychiatry, Psychotherapy and Psychosomatics, Obermain, Germany. All patients underwent a detailed physical examination, routine laboratory testing and urine drug screening. Additional information about the previous daily intake of ethanol and years of drinking was collected using a structured interview as previously described [18]. Exclusion criteria were concomitant psychiatric illnesses, substance abuse apart from alcohol or nicotine, cerebral ischemia, cerebral hemorrhage, epilepsy, cardiovascular and renal diseases. Carbamazepine and clomethiazole were used as detoxification medication during withdrawal; in particular, 70 patients received carbamazepine and 50 received clomethiazole. The group of healthy controls consisted of 34 Caucasian males who gave written informed consent while not displaying severe somatic or psychiatric diseases or any evidence for harmful alcohol use as confirmed by the Alcohol Use Disorders Identification Test (mean = 4.4, SD = 6.4) [19]. While age or BMI did not differ between the two groups, alcohol-dependent patients displayed a significantly higher number of smokers than controls. Sociodemographic data for both alcohol-dependent patients and healthy controls are summarized in table 1.

Molecular Analysis

Analysis of DNA Methylation

Fasting blood samples were drawn from alcohol-dependent patients on the day of admission between 8:00 and 10:00 am. Ethylenediaminetetraacetic acid (EDTA)-blood samples were aliquoted and stored at –80°C promptly after collection. The following preparation steps for the analysis of cytosine-phosphatidyl-guanine (CpG)-methylation were the same for all blood samples. Extraction from blood and cleanup of genomic deoxyribonucleic acid (DNA) was performed using the NucleoMag® Blood 200 µl DNA Kit (Macherey-Nagel, Cat. No. 744501.1).

Bisulfite-conversion of genomic DNA and subsequent amplification of ALDH2 target region DNA samples were then bisulfite-converted using the EpiTect® 96 Bisulfite Kit (QIAGEN AG, Cat.

Table 1. Sociodemographic data for 82 alcohol-dependent patients and 34 healthy controls included for methylation analysis

	Alcohol-dependent patients (n = 82)	Healthy controls (n = 34)	Student's t test/chi-square test, p
Age in years, mean $\pm$ SD	42.24 $\pm$ 7.97	44.56 $\pm$ 11.6	0.217
BMI, mean $\pm$ SD	24.58 $\pm$ 3.62	25.10 $\pm$ 2.77	0.454
Number of smokers	82	9	<0.0001
Years of drinking, mean $\pm$ SD	9.56 $\pm$ 7.72	–	–
Daily intake of ethanol in g/day, mean $\pm$ SD	189.39 $\pm$ 76.22	–	–
Total OCDS score, mean $\pm$ SD	19.69 $\pm$ 6.79	–	–
Total CIWA-A score, mean $\pm$ SD	15.65 $\pm$ 4.29	–	–

BMI = Body mass index; OCDS = Obsessive Compulsive Drinking Scale; CIWA-A = Clinical Institute Withdrawal Assessment for Alcohol.

No significant differences regarding mean age and BMI, but number of smokers, were found for healthy controls and alcohol-dependent patients (Student's t test: age: $T = 1.24$, $p = 0.217$; BMI: $T = 0.75$, $p = 0.454$ and chi-square test: number of smokers: $\chi^2 = 76.86$; $p < 0.0001$; total OCDS score: total score for alcohol craving that was assessed at admission to detoxification treatment with the OCDS; total CIWA-A score: total score for the degree of withdrawal symptoms that was assessed at admission to detoxification treatment with the CIWA-A).

No. 59720) according to the manufacturer's protocol. Applying a semi-nested PCR approach, the amplified ALDH2 product covered a region of 254 basepairs (–1,020 to –766 before the ATG codon of the first exon of ALDH2) containing 11 CpG loci within the promoter region of the ALDH2 gene: 5'-TCTGGTGTGAAATCAGAGTCCCACCGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGGCCGAGGCAGGCAGGATCATGAGGTGAGGAGATCGAGCATCCTGGCTAACACCGTGAAACCCCGTCTCTACTGAAAATACAAAAATAGCCGGGCGTGGTGGCAGGCGCCTGTGGTCCCAGCTACTCCGGAGGCGGAGGCAGGAGACTGGTGTGAACCCGGGAGGCAGAGCTTCTGGTGTGAAATCAGAGTCTGCAGTGAGC-3'. The 11 CpG loci were located at 948, 939, 918, 898, 887, 858, 854, 842, 821, 815 and 790 bp before the ATG codon. In order to assess methylation rates of individual 11 CpG loci, we applied the Sanger sequencing technique. Detailed information regarding methylation analysis of the ALDH2 target region is provided in online supplementary material (for all online suppl. material, see www.karger.com/doi/10.1159/000381018).

Genotyping of rs886205

Genomic DNA was gained from blood samples as described earlier. Amplification of rs886205 promoter region was done through touchdown PCR. Sequences of oligonucleotides used as primers for PCR as well as reaction components and protocols can be found in the online supplementary material.

Allele-Specific Digestion of the ALDH2 Promoter Target Sequence Containing the rs886205 Polymorphism

Restriction enzyme digestion of the amplified ALDH2 promoter region was performed according to the modified manufacturer's protocol using HpyAV restriction enzyme (New England BioLabs, Cat. No. R0621S; see online suppl. material, fig. S1). For the purpose of validation of specific restriction enzyme digestion by HpyAV, we also confirmed rs886205 genotyping results of ran-

dom samples by additional sequence analysis, including sequencing PCR and purification of PCR products as described earlier for CpG-methylation analysis of bisulfite-converted DNA. Primer ALDH2-rs886205-RC was used for reverse sequencing of the respective region. The following ENSEMBL accession number refers to the primary nucleotide sequences of the ALDH2 gene: ENSG00000111275.

Western Blot Analysis of Relative ALDH2 Protein Quantification in Blood Samples from Alcohol-Dependent Patients during Withdrawal and Healthy Controls

Out of the groups of alcohol-dependent patients and healthy controls, we subjected blood samples from 16 alcohol-dependent patients, 8 patients without (A/A) and 8 patients with (A/G + G/G) the G-allele of rs886205 and 16 healthy, age- and genotype-matched controls to ALDH2 protein expression analysis. Alcohol-dependent patients' and matched controls' sociodemographic data selected for western blot analysis are listed in table 2.

We generated blood protein lysates that were subjected to western blot analysis and subsequent densitometric assessment of antibody-detected protein bands. 'HeLa Whole Cell Lysate' was used as positive control lysate, while detection of the abundantly expressed loading control vinculin was applied [20, 21] to calculate relative ALDH2 expression in blood samples. Detailed information regarding the generation of protein lysates from frozen whole blood samples, relative ALDH2 protein quantification and validation of precise detection of ALDH2 by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) as described earlier [22], can be found in the online supplementary material.

Statistics

Deviance from normal distribution was checked according to Kolmogorov-Smirnov. In case of normally distributed variables, parametric methods were applied; for all other cases, non-parametric methods were used.

Table 2. Sociodemographic data for 16 selected alcohol-dependent patients and matched healthy controls included for western blot analysis

Alcohol-dependent patients					Healthy controls				
rs886205 genotype	code	age	BMI	smoking	rs886205 genotype	code	age	BMI	smoking
A/A	84	46	32.3	yes	A/A	MK040	49	25.3	no
A/A	50	45	20.5	yes	A/A	MK051	40	30.6	yes
A/A	12	47	21.3	yes	A/A	MK040	49	30.6	no
A/A	59	48	25.8	yes	A/A	MK040	49	30.6	no
A/A	2	43	27.1	yes	A/A	MK051	40	30.6	yes
A/A	76	41	25.5	yes	A/A	MK051	40	30.6	yes
A/A	94	39	27.4	yes	A/A	MK051	40	30.6	yes
A/A	53	52	24.2	yes	A/A	MK065	53	27.3	no
A/G	27	55	20.1	yes	A/G	MK070	51	23.8	no
G/G	56	32	20.7	yes	A/G	MK025	33	23.7	yes
A/G	1	45	28.4	yes	A/G	MK061	45	27.0	yes
A/G	17	46	24.8	yes	A/G	MK061	45	27.0	yes
G/G	11	34	34.6	yes	A/G	MK050	33	21.0	yes
A/G	93	40	22.5	yes	A/G	MK059	40	24.5	no
A/G	40	47	25.1	yes	A/G	MK061	45	27.0	yes
A/G	52	49	24.9	yes	A/G	MK061	45	27.0	yes

BMI = Body mass index; A/A = individual with 2 A-alleles; A/G = individual with 1 G-allele; G/G = individual with 2 G-alleles).

We matched each of the 16 alcohol-dependent patients from the NENA study, described in the Materials and Methods section, individually by age and rs886205 genotype with healthy controls (rs886206: polymorphism referring to the G-allele of the ALDH2 promoter).

Using the Student's t test, we analyzed single ALDH2 CpG-methylation between alcohol-dependent patients on day 1 of withdrawal and healthy controls. To compare the mean methylation of all 11 CpG-sites in patients on days 1, 7 and 14 with healthy controls, the one-way analysis of variance (ANOVA) method was used. Student's t test was used to find possible differences between single and mean CpG-methylation rates of medicated and non-medicated alcohol-dependent patients during detoxification treatment. Furthermore, we applied the Student's t test to check for differences in mean methylation between smoking and non-smoking healthy controls. In order to search the respective promoter regions for possible transcription factor binding sites, we used MatInspector® (Genomatix Software GmbH).

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS™) for Windows 20.0.0 (SPSS Inc.). We used GraphPad Prism for Windows 5.03 for data illustration (GraphPad Inc.).

Deviance of allele and genotype frequencies in alcohol-dependent patients and healthy controls from Hardy-Weinberg equilibrium as well as differences of allele and genotype frequencies between the groups were tested using the online tool from the Institute for Human Genetics (Helmholtz Center Munich, Germany) available at <http://ihg.gsf.de/ihg/snps.html> (accessed March 1, 2013).

We performed repeated measures-ANOVA to compare changes of CpG-methylation and relative ALDH2 protein levels in patients during withdrawal between different rs886205 genotypes. The Stu-

dent's t test was used to check for differences of CpG-methylation and relative ALDH2 protein level between rs886205 homozygous A-allele individuals and those carrying at least one G-allele. One sample Student's t test was applied to assess the deviance of the mean protein level in patients from the respective matched control level.

Bivariate correlations tests were performed to find possible associations between the rs886205 genotype, CpG-methylation rates and clinical parameters using the Pearson's correlation coefficient.

When performing multiple testing, p values were corrected using the Bonferroni's correction method and are designated as pc.

Results

Methylation of the Negative Regulatory ALDH2 Promoter Region in Alcohol-Dependent Patients and Healthy Controls

Significant differences regarding single ALDH2 CpG-methylation in alcohol-dependent patients on day 1 of withdrawal and healthy controls were found for all CpG-sites in the negative regulatory ALDH2 promoter region, except for CpG-939 and CpG-821 (Student's t test: d.f. = 124; CpG-948: T = 2.281, p = 0.024; CpG-939: T = 1.275, p = 0.205; CpG-918: T = 3.387, p = 0.002; CpG-898: T =

4.651, $p < 0.0001$; CpG-887: $T = 4.493$, $p < 0.0001$; CpG-858: $T = 2.192$, $p = 0.034$; CpG-854: $T = -2.587$, $p = 0.011$; CpG-842: $T = -2.808$, $p = 0.006$; CpG-821: $T = -0.078$, $p = 0.938$; CpG-815: $T = 4.326$, $p < 0.0001$; CpG-790: $p < 0.0001$; $T = 4.114$; fig. 1a).

Mean methylation rates of the 11 CpG-sites were significantly higher in patients than in controls on day 1, but decreased significantly from days 1 to 14 of withdrawal to normal levels (one-way analysis of variance; $F = 19.37$; d.f. = 3; $pc < 0.0001$; fig. 1b).

We could not detect significant differences in methylation between medicated and non-medicated patients during withdrawal (day 1, $p = 0.263$; day 7, $p = 0.543$ and day 14, $p = 0.069$). Furthermore, no different methylation could be assessed between smoking and non-smoking healthy controls ($p = 0.360$).

Methylation of the Negative Regulatory ALDH2 Promoter Region in Alcohol-Dependent Patients and Healthy Controls with and without the G-Allele of rs886205

Further methylation analysis in dependence of the rs886205 genotype revealed that only patients without, but not with the G-allele displayed significant hypermethylation of the regulatory ALDH2 promoter region on day 1 of withdrawal, compared to controls with the respective allele (Student's t test; A/A patients on day 1 vs. A/A controls: d.f. = 65, $T = 3.325$, $p = 0.001$; A/G and G/G patients on day 1 vs. A/G and G/G controls: d.f. = 49, $T = 1.139$, $p = 0.218$).

All patients displayed a significant decrease in mean methylation from days 1 to 14, but only patients without the G-allele showed a significant decrease from days 1 to 7 of withdrawal. In contrast, patients with 1 or 2 G-alleles displayed a significant decrease in mean methylation from days 7 to 14 of withdrawal (repeated measures-one-way analysis of variance; A/A patients day 1 vs. 14: $F = 22.58$, d.f. = 2, $pc < 0.0001$, A/A patients day 1 vs. 7: $pc < 0.0001$, A/A patients day 7 vs. 14: $pc = 1.00$ and A/G + G/G patients day 1 vs. 14: $F = 10.29$, d.f. = 2, $pc = 0.001$, A/G + G/G patients day 1 vs. 7: $pc = 0.610$, A/G + G/G patients day 7 vs. 14: $pc = 0.030$).

However, mean methylation rates did not differ significantly between patients with and without the G-allele of rs886205 on days 1, 7 and 14 of withdrawal (Student's t test; day 1: A/A = 0.9290; SD = 0.0183; A/G + G/G = 0.9230; SD = 0.01412; d.f. = 81, $T = 1.501$, $p = 0.138$; day 7: A/A = 0.9113; SD = 0.021; A/G + G/G = 0.9200; SD = 0.0165; d.f. = 81, $T = -1.962$, $p = 0.053$ and day 14: A/A = 0.9116; SD = 0.021; A/G + G/G = 0.9018; SD = 0.0272; d.f. = 81, $T = 3.233$, $p = 0.076$; fig. 1c).

Relative Levels of ALDH2 Protein in Alcohol-Dependent Patients with and without G-Allele

Patients without the G-allele (A/A, $n = 8$) showed significant higher relative ALDH2 protein levels on day 1, but not on day 7 or 14 of withdrawal, compared to patients with the G-allele (A/G + G/G, $n = 8$; Student's t test; day 1: $T = 2.195$, $p = 0.050$; day 7: $p = 0.772$ and day 14: $p = 0.873$).

Furthermore, only patients without the G-allele displayed a significant reduction of relative protein levels from days 1 to 7 and days 1 to 14 of withdrawal, while patients with 1 or 2 G-alleles did not show any alteration of relative protein levels during withdrawal (repeated measures-one-way analysis of variance; relative protein levels in A/A patients day 1 vs. 7: $F = 23.3$; d.f. = 2; $pc < 0.0001$; A/A patients day 1 vs. 14: $pc < 0.0001$, A/A patients day 7 vs. 14: $pc = 1.00$ and A/G + G/G patients day 1 vs. 7: $F = 0.071$; d.f. = 2; $pc = 1.00$, A/G + G/G patients day 1 vs. 14: $p = 1.00$, A/G + G/G patients day 7 vs. 14: $pc = 1.00$). However, relative ALDH2 protein levels did not significantly differ between patients with and without the G-allele and matched controls on day 1 of withdrawal (one-sample Student's t test; A/A-patients on day 1 vs. matched controls: $T = 2.003$, $p = 0.085$ and A/G + G/G-patients vs. controls: $T = -0.919$, $p = 0.389$; fig. 2a).

Moreover, we could reveal a significant positive correlation between a decrease in the mean CpG-methylation of the respective ALDH2 promoter region from days 1 to 7 of withdrawal with resulting relative ALDH2 protein levels on day 7 of withdrawal only in alcohol-dependent patients without the G-allele. In contrast, patients carrying 1 or 2 G-alleles did not show any correlation with ALDH2 promoter methylation (Pearson's correlation coefficient; A/A: $R^2 = 0.728$, $p = 0.041$ and A/G + G/G: $R^2 = -0.067$, $p = 0.875$; fig. 2b).

Analysis of Genotype and Allele Frequencies

Frequencies of the A-allele and G-allele of rs886205 were in agreement with the Hardy-Weinberg equilibrium in alcohol-dependent patients ($p = 0.540$) and healthy controls ($p = 0.446$). No significant differences in the rs886205 genotype or allele frequencies between healthy controls and alcohol-dependent patients were observed, even though we could detect a trend association between the G-allele and healthy controls, as assessed by the online tool from the Institute for Human Genetics, Helmholtz Center Munich, Germany, available at <http://ihg.gsf.de/ihg/snps.html> (difference in G-allele frequency between patients and controls: 0.23 vs. 0.34; $\chi^2 = 3.18$; $p = 0.074$ and overall difference in genotype frequency between pa-

tients and controls: $\chi^2 = 2.89$; $p = 0.089$; table 3). To replicate a genetic effect of this size, the following association studies should consist of at least 300 Caucasian patients and 300 Caucasian controls ($\alpha = 0.05$; $1 - \beta = 0.83$), as assessed by the online tool available at <http://homepage.stat.uiowa.edu/~rlenth/Power/>.

Discussion

We are here reporting findings of epigenetic implications of the ALDH2 promoter polymorphism rs886205, which is strongly related to a change in methylation kinetics in a negative regulator, thereby influencing the adaptation of ALDH2 protein levels during detoxification.

More precisely, we observed that ALDH2 promoter methylation was increased only in A-homozygous patients on day 1 of withdrawal, but not in those with the G-allele, compared to matched controls. Furthermore, ALDH2 protein expression was lower in patients with the G-allele of rs886205, compared to patients without it on day 1 of withdrawal. As known from previous studies, G-allele-directed impairment of basal ALDH2 gene transcription was shown to be tightly linked to the presence of the respective promoter region upstream of the polymorphic site, as promoter constructs lacking G-allele and the upstream region did not cause decreased promoter activity [10]. With the data presented here, we provide novel information concerning a putative molecular mechanism for the modulation of ALDH2 protein expression involving epigenetic mechanisms contributing to reported health risks due to genetic disposition by rs886205 and combined heavy alcohol consumption [8, 9].

Our findings indicate a trend association between the G-allele of rs886205 and protection against alcohol dependence in Caucasian populations. However, future studies, including larger numbers of Caucasian individu-

als should be performed to confirm this preliminary finding, as previous studies did not find significant associations [16].

Allele-Dependent Methylation Kinetics during Withdrawal from Alcohol

Deletion construct studies previously demonstrated that the ALDH2 promoter contains a specific region between -981 and -744 bp upstream of the first exon that negatively affects ALDH2 promoter activity and gene transcription only in the presence of the G-allele of rs886205 [10]. Also, the investigation of rs886205 showed no altered binding affinity of transcription factors known to bind around the polymorphic promoter site [11].

Interestingly, induction of hyper- and hypomethylation of CpG-sites at multiple gene loci have already been widely described in the context of chronic alcohol consumption [12, 13]. Thus, we searched the negative regulatory ALDH2 promoter for possible epigenetic targets and could detect that the mean methylation of the 11 CpG-sites was increased only in alcohol-dependent patients homozygous (A/A) for rs886205. Also, kinetics of methylation readjustment during detoxification within the first week of withdrawal was present only in these patients. In terms of epigenetic adaptation to normal physiological conditions after withdrawal from alcohol, we observed that the readjustment of mean ALDH2 methylation rates in homo- and heterozygous G-allele carriers took 1 week longer, compared to those without the G-allele (fig. 1b).

Impact of rs886205 on Regulation of ALDH2 Protein Expression during Withdrawal

We show for the first time that the G-allele of rs886205 is associated with a lower expression of ALDH2 protein before a 2-week detoxification treatment in alcohol-dependent patients' blood, compared to patients with two

Fig. 1. a Significant differences regarding methylation rates were found for all 11 single CpG-sites (relative to ATG codon) in the regulatory ALDH2 promoter region, except for CpG-939 and CpG-821 in male alcohol-dependent patients on day 1 of withdrawal, compared to male, healthy controls. **b** Mean methylation rate of all 11 CpG-sites in the ALDH2 promoter region was significantly higher on day 1, but not on day 7 and 14 of withdrawal in alcohol-dependent patients, compared to healthy controls ($p < 0.0001$). **c** Mean methylation rate of all 11 CpG-sites on day 1 of withdrawal was significantly higher only in patients without, but not in patients with at least one G-allele, compared to, healthy matched controls (day 1: A/A patients vs. A/A healthy controls:

$p = 0.001$; A/G and G/G patients vs. A/G and G/G healthy controls: $p = 0.218$). A significant decrease of mean methylation of the ALDH2 promoter region from days 1 to 14 of withdrawal could be observed in patients with and without the G-allele, but only patients without the G-allele displayed a significant decrease from days 1 to 7 of withdrawal towards control levels. Patients with 1 or 2 G-alleles displayed a significant reduction in mean methylation from days 7 to 14 of withdrawal (A/A patients: day 1 vs. 14: $p < 0.0001$; day 1 vs. 7: $p < 0.0001$; day 7 vs. 14: $p = 1.00$ and A/G + G/G patients: day 1 vs. 14: $p = 0.001$; day 1 vs. 7: $p = 0.610$; day 7 vs. 14: $p = 0.030$). Methylation rates (decimal numbers) are presented as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

(For figure see next page.)

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A-alleles. Moreover, a reduction of relative ALDH2 protein levels during withdrawal was present only in patients homozygous for the A-allele, while levels were in the range of the control group in G-allele patients (fig. 2a). Specific methylation changes of CpG-rich islands in promoter regions have been proposed to affect the binding of positive

and negative elements to regulatory regions. In line with this, previous studies reported both positive and negative correlation between CpG-methylation and gene expression proposing that the impact of DNA-methylation on gene activity strongly depended on the genetic context and functional nature of the analyzed region [23, 24].

Fig. 2. a The relative ALDH2 protein levels in blood samples was significantly higher in patients without the G-allele, compared to patients with at least one G-allele on day 1, but not 7 and 14 of withdrawal (day 1: $p = 0.050$, day 7: $p = 0.772$ and day 14: $p = 0.873$). Only patients without the G-allele displayed a significant reduction of relative ALDH2 protein levels from days 1 to 7 and days 1 to 14 of withdrawal, while patients with at least one G-allele did not show any alterations of protein levels during withdrawal (A/A patients: day 1 vs. 7: $pc < 0.0001$; day 1 vs. 14: $pc < 0.0001$; day 7 vs. 14: $pc = 1.00$ and A/G + G/G patients: day 1 vs. day 7: $pc = 1.00$; day 1 vs. 14: $pc = 1.00$; day 7 vs. 14: $pc = 1.00$). **b** A positive correlation between change in mean ALDH2 methylation rate of

the regulatory promoter region from days 1 to 7 and relative ALDH2 protein levels on day 7 of withdrawal could be observed for patients without the G-allele, but not for patients with at least one G-allele (A/A patients: $R^2 = 0.728$, $p = 0.041$ and A/G + G/G patients: $R^2 = -0.067$, $p = 0.875$). **c** Quantification of relative ALDH2 protein levels in blood samples was assessed by densitometric volume analysis of normalized ALDH2 expression in relation to ALDH2 expression in matched controls. The ALDH2/vinculin ratio of each band was calculated and divided by the ALDH2/vinculin ratio of the respective healthy matched control to assess the relative ALDH2 expression. Methylation rates (decimal numbers) are presented as mean $\pm$ SD. * $pc < 0.05$; *** $pc < 0.0001$.

Correlation of Allele-Dependent Methylation Kinetics with Protein Levels in Patients with Two A-Alleles

Interestingly, the analyzed ALDH2 promoter region from -981 to -744 bp upstream the ATG start codon has been demonstrated to exert repressive functions on ALDH2 gene transcription in presence of the G-allele of

rs886205 [24]. We observed a positive correlation between the decrease of mean methylation from days 1 to 7 in the repressive ALDH2 promoter region and protein levels on day 7 of withdrawal in A-homozygous patients. Decreasing blood levels of ALDH2 protein in alcohol-dependent patients without the G-allele upon withdrawal

Table 3. Genotype and allele frequencies of rs886205 in alcohol-dependent patients and healthy controls

rs886205	Alcohol-dependent patients (n = 82)	Healthy controls (n = 34)	χ^2 test
Allele frequency			allele frequency difference
A-allele	0.77 (n = 127)	0.66 (n = 45)	$\chi^2 = 3.18$; p = 0.074
G-allele	0.23 (n = 37)	0.34 (n = 23)	
Genotype frequency			Armitage's trend test
A/A	0.61 (n = 50)	0.47 (n = 16)	$\chi^2 = 2.89$; p = 0.089
A/G	0.33 (n = 27)	0.38 (n = 13)	
G/G	0.06 (n = 5)	0.15 (n = 5)	

Frequencies of rs886205 genotype and allele did not deviate from the Hardy–Weinberg equilibrium in alcohol dependent patients (p = 0.540) and healthy controls (p = 0.446). Genotype and allele frequencies did not significantly differ between the 2 groups, yet, a trend association between the G-allele and healthy controls could be observed (difference in allele frequency between patients and controls: 0.23 vs. 0.34; $\chi^2 = 3.18$; p = 0.074 and overall difference in genotype frequency between patients and controls: $\chi^2 = 2.89$; p = 0.089).

might hence be caused via lowered epigenetic repression of negative elements binding to the specific region of the ALDH2 gene. Thus, these findings strengthen the concept of preceding methylation changes of the negative regulatory promoter region, affecting ALDH2 protein expression in dependence of the respective rs886205 promoter allele.

Limitations of Our Study

While analysis of CpG-methylation was performed using blood samples from 82 patients and 34 healthy controls, we used blood samples from 16 patients on days 1, 7 and 14 of withdrawal and 16 matched controls for western blot analysis and could reveal allele-dependent differences of relative ALDH2 protein expression in patients. Due to a relatively small number of control samples, we were not able to compare allele-dependent protein expression of ALDH2 in healthy controls and could not demonstrate significant alterations between mean ALDH2 protein levels in alcohol-dependent patients, compared to healthy matched controls.

Another facet yet unclear is why the alteration of protein expression in the cohort bearing the G-allele seemed to be missing in spite of respective regulatory methylation effects. Protein levels remained consistently low across the whole period of investigation (fig. 2a), a phenomenon possibly caused by a yet elusive regulatory mechanism not considered by the approaches applied. Future studies might overcome these limitations by protein expression analysis of a greater number of healthy, male and female individuals in order to assess the genetic disposition by rs886205 for basal ALDH2 protein ex-

pression before exposition to ethanol. We were able to assess methylation changes in the negative regulatory ALDH2 promoter fragment and correlating protein levels in the dependence of the rs886205 genotype in alcohol-dependent patients during withdrawal. As an important next step, future in vitro gene expression studies using methylated and non-methylated ALDH2 promoter constructs might reveal binding of methylation- and allele-specific regulatory factors involved in the epigenetic regulation of ALDH2 gene expression. Even though no study has yet demonstrated an impact of smoking behavior on ALDH2 methylation, an association between smoking and methylation status of different genes has been described earlier in vivo and in vitro [25, 26]. Due to the significantly higher number of smokers in the group of alcohol-dependent patients (100%), compared to healthy controls (26.4%), we are not able to rule out possible co-effects of smoking on allele-dependent ALDH2 methylation and protein expression before withdrawal. This is also a limitation for the comparison with healthy controls, even though we did not find differences in the mean methylation between smoking and non-smoking healthy controls in our cohort. To rule out smoking-related epigenetic effects on specific ALDH2 methylation, future studies including larger numbers of smoking non-drinkers will be needed.

In spite of these limitations, we are the first to describe the alterations of relative ALDH2 protein expression in alcohol-dependent patients with and without the G-allele of rs886205 during withdrawal, compared to matched controls. Based on our new molecular findings, we might be able to connect previous observations about

genetic predisposition by rs886205 and environmental risk factors such as heavy alcohol consumption [8, 9], reduced gene transcription found in vivo and in vitro [10] with impaired expression and regulation of ALDH2 protein in alcohol-dependent patients. Increased susceptibility to carcinogenic effects of chronic alcohol consumption in dependence of rs886205 might therefore be caused by the impaired elimination of toxic AcH during chronic alcohol consumption as a consequence of maladaptation of ALDH2 protein regulation in G-allele carriers. Confirmation of our findings about the epigenetic impact of rs886205 on impairment of ALDH2 protein expression might highlight the possible benefits of rs886205 genotyping and assessment of ALDH2 methylation status in easily accessible blood cells for early prevention of alcohol-induced physiologic damage, as induction of promoter methylation has been detected already at early stages of tumorigenesis before the manifestation of ESCC in carcinogen-exposed individuals [27].

Interestingly, ALDH2 protein levels were not induced in patients with the G-allele of rs886205 before withdrawal from chronic exposure to alcohol. Moreover, when comparing allele frequencies between patients and non-drinking healthy controls, an association between the G-allele of rs886205 and protection against alcohol dependence could be assessed.

Even though future studies will be needed to confirm our results, we were able to reveal the association between the G-allele of rs886205 and reduction of ALDH2 protein levels previously reported to decrease risk for alcohol dependence [3, 4].

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Disclosure Statement

All authors report no biomedical financial interests or potential personal conflicts of interest.

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