Gene expression analysis in epileptic hippocampi reveals a promoter haplotype conferring reduced aldehyde dehydrogenase 5a1 expression and responsiveness

Despina Tsportouktzidis | Herbert Schulz | Motaz Hamed | Hartmut Vatter | Rainer Surges | Susanne Schoch | Thomas Sander | Albert J. Becker | Karen M. J. van Loo

Abstract

Increasing evidence indicates the pathogenetic relevance of regulatory genomic motifs for variability in the manifestation of brain disorders. In this context, cis-regulatory effects of single nucleotide polymorphisms (SNPs) on gene expression can contribute to changing transcript levels of excitability-relevant molecules and episodic seizure manifestation in epilepsy. Biopsy specimens of patients undergoing epilepsy surgery for seizure relief provide unique insights into the impact of promoter SNPs on corresponding mRNA expression. Here, we have scrutinized whether two linked regulatory SNPs (rs2744575; 4779C > G and rs4646830; 4854C > G) located in the aldehyde dehydrogenase 5a1 (succinic semialdehyde dehydrogenase; ALDH5A1) gene promoter are associated with expression of corresponding mRNAs in epileptic hippocampi (n = 43). The minor ALDH5A1-GG haplotype associates with significantly lower ALDH5A1 transcript abundance. Complementary in vitro analyses in neural cell cultures confirm this difference and further reveal a significantly constricted range for the minor ALDH5A1 haplotype of promoter activity regulation through the key epileptogenesis transcription factor Egr1 (early growth response 1). The present data suggest systematic analyses in human hippocampal tissue as a useful approach to unravel the impact of epilepsy candidate SNPs on associated gene expression. Aberrant ALDH5A1 promoter regulation in functional terms can contribute to impaired γ-aminobutyric acid homeostasis and thereby network excitability and seizure propensity.

KEYWORDS

ALDH5A1, early growth response 1, rSNPs, temporal lobe epilepsy
FIGURE 1  

ALDH5A1 promoter haplotypes correlate with ALDH5A1 expression levels in hippocampi of temporal lobe epilepsy (TLE) patients and in vitro, and are potentially regulated by Egr1. (A) Schematic representation of ALDH5A1 promoter haplotypes. The ALDH5A1-CC haplotype represents the rs2744575/C and rs4646830/C genotype, whereas ALDH5A1-GG refers to rs2744575/G and rs4646830/G (single nucleotide polymorphism [SNP] positions are indicated in red). (B) Violin plots show the expression levels of ALDH5A1 in hippocampi of TLE patients stratified by haplotype. Patients homozygous for ALDH5A1-CC haplotype have higher ALDH5A1 expression levels than patients homozygous for the ALDH5A1-GG haplotype (ALDH5A1-CC: \( n = 33, 8.652 \pm 0.03716 \); ALDH5A1-GG: \( n = 10, 8.467 \pm 0.05144 \); \( t \)-test, \( p = .0160 \)). Dashed and dotted lines in the violin plot show median and quartiles (median: ALDH5A1-CC = 8.690, ALDH5A1-GG = 8.452). (C) Schematic representation of the location of the ALDH5A1 promoter SNPs relative to the ATG. The SNPs rs2744575 and rs4646830 are located 250 bp and 175 bp upstream of the start-ATG, respectively. (D) Luciferase activity of NG108-15 cells transfected with ALDH5A1-CC, ALDH5A1-GG, and the pGL3 empty vector. Both haplotypes have higher affinity than cells transfected with the pGL3 vector, and the ALDH5A1-CC haplotype has a stronger promoter activity compared to the ALDH5A1-GG haplotype (\( N = 9, n = 3 \), data normalized to pGL3 and represented as mean \( \pm \) SEM, pGL3 1.00 \( \pm \) 0.2024, ALDH5A1-CC 4.938 \( \pm \) 0.8484, one-way analysis of variance [ANOVA] of repeated measures, Tukey multiple comparisons test, *\( p \leq .05 \), **\( p \leq .01 \)). (E) Schematic representation of Egr1 binding affinity to allelic variants of SNP rs2744575 shows higher affinity for variant rs2744575/C. (F) Relative luciferase activity of the ALDH5A1 promoter haplotypes after overexpression with Egr1. Both haplotypes show increased promoter activity when exposed to 100 ng of Egr1 (\( N = 5, n = 3 \), data normalized to ALDH5A1-CC basal and represented as mean \( \pm \) SEM, two-way ANOVA, Sidak multiple comparisons test, **\( p \leq .01 \), ****\( p \leq .0001 \)). (G) Luciferase activity of NG108-15 cells transfected with the ALDH5A1-CC and ALDH5A1-GG promoter haplotypes and Egr1dN. The ALDH5A1-CC haplotype shows a decreased luciferase activity after exposure to Egr1dN, whereas the ALDH5A1-GG haplotype remains constant (data normalized to ALDH5A1-CC basal and represented as mean \( \pm \) SEM, two-way ANOVA, Dunnett multiple comparisons test; \( p = .0497 \), \( N = 4, n = 3 \)). ns, not significant.
1 | INTRODUCTION

Transient neuronal network hyperexcitability manifested by seizures represents the shared leading symptom of both acquired focal and genetic generalized epilepsies. Genome-wide association studies have identified a varying number of susceptibility loci for different forms of epilepsies. Many associated single nucleotide polymorphisms (SNPs) are located in noncoding genomic regions. Functionally, their effects often stay unresolved. However, SNP variants located in promoter regions can differentially impact the dynamic expression of corresponding genes, which can have relevance in epilepsy syndromes with very distinct genetic components. These can include (1) acquired epilepsies, including temporal lobe epilepsy (TLE) with only minor genetic contribution; (2) idiopathic generalized epilepsies with a complex pattern of inheritance suggesting an interaction of several susceptibility genes; (3) monogenic epileptic encephalopathies; and (4) rare genetic disorders affecting aspects of neurotransmission. The latter includes deficiency in the succinic semialdehyde dehydrogenase (SSADH) resulting in impaired γ-aminobutyric acid (GABA) degradation. This epilepsy syndrome shows differences in the clinical manifestation albeit the presence of the same mutational event. Here, we have analyzed two linked regulatory (r)SNPs (rs2744575; 4779 > G and rs4646830; 4854 > G) located in the promoter of ALDH5A1, the gene mutated in SSADH, by using a unique tissue repository consisting of hippocampal biopsies of patients with chronic epilepsies undergoing neurosurgery for seizure relief and complemented the approach by in vitro promoter analyses.

2 | MATERIALS AND METHODS

2.1 | TLE patients, SNP genotyping, and expression analysis

Biopsies of hippocampal tissue from pharmacoresistant TLE patients with hippocampal sclerosis (HS) were included in this study (N = 74). Patients underwent surgical treatment in the Epilepsy Surgery Program at the University of Bonn Medical Center. SNP genotyping using Human660W SNP array (Illumina) and gene expression using the HumanHT-12 v3 Expression BeadChip (Illumina) were performed and analyzed as before.

2.2 | Plasmids

ALDH5A1-GG was made by amplifying a fragment of 566 bp (chr6:24494417-24494982, GRCh38/hg38) located upstream of the ALDH5A1 start-ATG (Figure 1C) and cloning it into the pGL3-basic vector (Promega). Subsequent mutagenesis using QuickChange Site-Directed Mutagenesis (Agilent) resulted in ALDH5A1-CC.

2.3 | Cell cultures, transient transfection, and luciferase reporter assays

NG108-15 cells were maintained and transfected as described previously, using 100 ng of luciferase reporter plasmid (pGL3, ALDH5A1-CC, or ALDH5A1-GG), 25 ng of Renilla luciferase vector (Promega), and early growth response 1 (Egr1)/Egr1dN as indicated in the single experiments. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s specifications. Renilla and firefly luciferase activities were determined using the Glomax Luminometer (Promega).

2.4 | Statistical analyses

Statistical analyses were performed with GraphPad Prism software. Student t-test, one-way analysis of variance (ANOVA), and two-way ANOVA followed by multiple comparison tests were used to evaluate the statistical significance of the results. All in vitro experiments were independently repeated at least four times (N), each with three technical replicates (n). Values were considered significant at p < .05. Results are plotted as mean ± SEM.

3 | RESULTS

We found the two SNPs present in the ALDH5A1 promoter region to be associated with differential ALDH5A1 expression in the present TLE cohort. Patients with the ALDH5A1-CC haplotype (rs2744575; 4779C > G and rs4646830; 4854C > G) showed an augmented hippocampal ALDH5A1 expression compared to patients with the ALDH5A1-GG haplotype (rs2744575; 4779G > C and rs4646830; 4854G > C), indicating that the ALDH5A1 promoter SNPs could function as regulatory (r)SNPs in TLE pathogenesis.

In line with the data available in the linkage disequilibrium database (LDLink database; https://ldlink.nci.nih.gov/), we observed for both rSNPs identical genotype and allele frequencies; the homozygous CC variant was observed in 33 (44.6%) patients, the heterozygous CG variant in 31 (41.9%) patients, and the homozygous GG variant in 10 (13.5%) patients, resulting in an allele frequency of 65.5% and 34.5% for the C-allele and G-allele, respectively. No differences in genotype and allele distribution were observed between our HS cohort and a control group (p = .80). Because the two rSNPs were in complete linkage
dis-equilibrium, we now refer to the two SNPs as one haplotype, consisting of two variants, that is, the \( ALDH5A1\)-CC haplotype block (both SNPs harbor the C-allele) and the \( ALDH5A1\)-GG haplotype block (both SNPs harbor the G-allele; Figure 1A).

To examine whether the two \( ALDH5A1\) haplotypes can lead to differential \( ALDH5A1\) promoter activity, we tested them in neuronal NG108-15 cells. Using a luciferase reporter assay with \( ALDH5A1\) reporter fragments harboring both rSNPs (Figure 1C), we found that both haplotypes had a stronger luciferase activity compared to the pGL3 empty control vector, indicating that the 566-bp \( ALDH5A1\) promoter fragment harbors promoter activity. Furthermore, we observed the \( ALDH5A1\)-CC haplotype to have a stronger basal promoter activity compared to the \( ALDH5A1\)-GG haplotype (Figure 1D).

To unravel the underlying molecular mechanisms of the observed differential expression of the two haplotype blocks, we compared the \( ALDH5A1\)-CC and \( ALDH5A1\)-GG haplotypes for differential transcription factor (TF) binding affinities. Bioinformatic analysis using GWAS4D revealed the strongest difference in TF binding affinity for the allelic variants of SNP rs2744575 (effect \( p\)-value = 9.6252E-04). In line with GWAS4D, another bioinformatic tool, EPOSSUM2/Jaspar2018, predicted a higher binding affinity for EGR1 to allelic variant rs2744575-C in comparison to rs2744575-G (76.3% and 40.7%, respectively; Figure 1E). A key role of Egr1 in epileptogenic processes as well as in absence epilepsy has been described before.10,12

To determine whether Egr1 differentially regulates the \( ALDH5A1\)-CC and \( ALDH5A1\)-GG haplotypes, we transfected an expression vector for Egr110 into NG108-15 cells and determined the luciferase activity of the two haplotype blocks. Applying increasing concentrations of Egr1 resulted in significant \( ALDH5A1\) promoter activation for both haplotypes (Figure 1F), indicating that the \( ALDH5A1\) promoter is under the control of Egr1. Intriguingly, stepwise increased concentrations of Egr1 exposure were reflected by a significant increase of promoter activity only in the case of the \( ALDH5A1\)-GG variant, whereas for the \( ALDH5A1\)-CC haplotype only the maximal Egr1 concentration exposure resulted in significant activation of the promoter (Figure 1F). We extrapolated on the regulation of the \( ALDH5A1\) promoter through Egr1 by applying a dominant-negative variant of Egr1 (Egr1dN).12 This modified TF contains the DNA-binding domain, but lacks the activating domain. Upon binding of Egr1dN to the DNA, endogenous Egr1 can no longer bind to the DNA. By this approach, we observed a reduction of promoter activity only for the \( ALDH5A1\)-CC haplotype at the highest concentrations of Egr1dN, whereas exposure of the \( ALDH5A1\)-GG haplotype promoter to increasing concentrations of Egr1dN did not exert significant effects with respect to activity as determined by luciferase reporter assays (Figure 1G).

4 DISCUSSION

Here, we have detected a minor haplotype in the \( ALDH5A1\) promoter that is associated with reduced expression of the corresponding mRNA in human epileptic hippocampal tissue. In contrast to a previous study on another regulatory motif in the context of \( ALDH5A1\) mRNA expression,13 we have now confined the present analysis to hippocampal biopsies with the damage pattern of HS, where all patients had a clinical history of epileptogenesis. Furthermore, we now report on rSNPs located in the functional core promoter of the \( ALDH5A1\) gene.14

Intriguingly, the present haplotype consists of two expression quantitative trait locus SNPs. Of note, there was no significant abundance of the minor haplotype with functional impairment of gene expression in the TLE patient cohort. \( ALDH5A1\) encodes a protein with critical importance for GABA recycling and thus inhibition. Functionally, impairment of GABA homeostasis has been shown to be a key pathogenetic aspect of TLE.15,16 Egr1 represents a key TF in epileptogenesis.12 The present in vitro data suggest that under basal conditions, a higher amount of Egr1 is already bound to the Egr1 binding sites in the \( ALDH5A1\)-CC variant. This is reflected by a stronger activity of the \( ALDH5A1\)-CC promoter under basal conditions and lack of activation by only slightly increased concentrations of Egr1 (50 ng). In contrast, the \( ALDH5A1\)-GG variant has a lower binding affinity for Egr1, that is, 41% for the \( ALDH5A1\)-GG variant compared to 76% for the \( ALDH5A1\)-CC variant, and can become activated upon slight Egr1 (50 ng) increases. However, after exposure to maximal concentrations of Egr1, reflecting, for example, post-status epilepticus conditions, the \( ALDH5A1\) promoter activation status is indistinguishable for both haplotype variants. Thus, under stress conditions, both haplotypes can confer virtually indistinguishably strong promoter activation putatively leading to the production of high amounts of the corresponding \( ALDH5A1\) protein. However, under basal conditions, the dominant-negative variant for Egr1 can only reduce the \( ALDH5A1\)-CC promoter activity, which means that (1) under normal in vitro conditions, the \( ALDH5A1\)-GG variant already lacks binding of Egr1; and (2) the "regulatory range" of varying Egr1 concentrations is larger for the \( ALDH5A1\)-CC variant. As a functional consequence, the \( ALDH5A1\)-GG variant may contribute to functionally impaired GABA recycling in TLE.

Furthermore, mutations of \( ALDH5A1\) have been related to several forms of epilepsy.2,11,17 However, the clinical manifestation of individual \( ALDH5A1\) mutations has been
extremely diverse, and only approximately 50% of patients with SSADH deficiency suffer from seizures. Missense mutations considered to be causative of SSADH deficiency have been demonstrated to reduce the SSADH activity to less than 5% of the normal activity in in vitro expression systems. Therefore, differences in the activity and the regulatory range of the ALDH5A1 promoter dependent on the presence of distinct rSNPs in individual patients may either confer a higher propensity for the availability of the mutated ALDH5A1 protein variant in neurons of patients with SSADH deficiency or induce compensatory effects.

The present data may be paradigmatic for the relevance of distinct SNPs in the regulation of neurotransmitter homeostasis-related gene promoters in human epilepsies, an aspect that appears important to be considered for an improved understanding of the phenotypic diversity of epilepsies.

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