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DAT1 methylation changes in alcohol-dependent individuals vs. controls

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ABSTRACT

Introduction: The dopaminergic system plays a crucial role in the development of alcohol dependence. Regulation of the extracellular dopamine concentration is driven by dopamine transporter. Both, the expression and function of dopamine transporter, are influenced by chronic alcohol intake. Dopamine transporter *(DAT)* gene is also supposed to be differentially methylated in alcohol-dependent patients than in controls.

Material and methods: DNA was extracted from peripheral blood leukocytes. We analyzed the methylation status in 23 CpG islands of *DAT* gene promoter in alcohol dependent subjects (n = 171) and control (n = 160) group.

Results: No statistical differences in the general frequency of *DAT* CpG islands was revealed between patients (altogether 175 methylated islands) and control subjects (170 methylated islands (p = 0.86). However it was revealed that one of analyzed positions is significantly more often methylated in control subjects than in alcohol dependent individuals (p = 0.0296).

Conclusion: Further subsequent studies are necessary to determine whether the methylation change of one (out of 23) CpG site results in DAT expression changes.

1. Introduction

Epigenetic processes are crucial for cellular development and tissue differentiation. It also enables the long–term regulation of gene function by non–mutagenic mechanisms (Henikoff and Matzeke, 1997). Inversely to the DNA sequence which is stable and conserved, epigenetic processes are developmentally dynamic, and are known to be influenced by many factors including the environment (Dolinoy et al., 2007), DNA sequence variation (Kerkel et al., 2008; Schalkwyk et al., 2010) and stochastic events in the cell (Ushijima et al., 2003). This processes are also possibly reversible.

DNA methylation is the best understood epigenetic modification modulating transcriptional plasticity. It refers to binding a methyl group to a CpG islands in the genomic sequence. CpG islands, mainly in promoter regions of genes, are usually less frequently methylated than CpG sequences that are spread throughout the genome. In most cases, a higher methylation leads to a repression of the gene, and less methylation usually leads to the induction. Methyl groups bound to the genomic sequence reduce the DNA–binding capacity for transcription factors (Doerfler, 1983; Egger et al., 2004; Holliday, 1987). However, in some cases methyl groups are able to enhance transcription factors attachment to promoter regions (Lopez – Serra et al., 2006).

One of the genes reported to be differentially methylated in alcohol-dependent patients is *DAT*. Furthermore, the hypermethylation of *DAT* was negatively associated with the severity of craving, which may be explained by the elevated dopamine levels in patients with higher methylation levels of *DAT* (Hillemacher et al. 2009).

The dopaminergic system plays a crucial role in the development of alcohol dependence (AD) (Gorzkowska et al., 2014; Jasiewicz et al., 2014; Samochowiec et al., 2014; Soderpalm and Erickson, 2013). Modification in this system is a predisposing factor for AD (Blum et al., 1996) and a consequence of a chronic alcohol intake (Gardner, 2011; Melis et al., 2005). Chronic alcohol consumption is often associated with a decrease in dopaminergic

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function (Volkov et al., 2007). Regulation of the extracellular dopamine concentration is driven by dopamine transporter (DAT) (Gainetdinov et al., 1998). Both, the expression and function of DAT, are influenced by chronic alcohol intake (Barbier et al., 2008; Szot et al., 1999; Tiihonen et al., 1995). Genetic polymorphism of *DAT* gene associated with altered transporter availability has been linked to alcohol dependence (Grzywacz and Samochowiec, 2008; Heinz et al., 2004; Samochowiec et al., 2006). *DAT* gene is also supposed to be differentially methylated in alcohol–dependent patients than in controls (Hillemacher et al., 2009). In the same study, the authors presented a negative association of *DAT* hypermethylation with severity of craving. In contrast to above mentioned data, Nieratschker et al. (2014) found no significant differences in *DAT* methylation between alcohol dependent patients and controls. Thus, the methylation status and its contribution to alcohol addiction remains the subject of intensive studies showing no consistent conclusions. The aim of this study was to determine the methylation status of the *DAT* promoter region in control subjects and AD patients. In order to realize this plan we analyzed the methylation status in 23 CpG islands of *DAT* promoter in both studied groups, that gave us much broader perspective than in any other studies performed so far.

2. Material and methods

2.1. Samples

The study group consisted of 171 men diagnosed with alcohol dependence syndrome based on ICD 10 Criteria. All patients were recruited from the Department of Psychiatry at the Medical University. The control group comprised 160 healthy volunteers and blood donors (Table 1) matched for age and sex. The patients and controls were Caucasians from the same region of Poland. The study protocol was approved by the Ethics Committee (No. BN – 001/75/07) and written consent was provided by the participating individuals.

2.2. Genotyping

DNA was extracted from peripheral blood leukocytes using a DNA isolation kit (A&A Biotechnology, Gdynia, Poland) as previously described (Rubiś et al., 2012) and stored at −20 °C. Bisulfite modification of 250 ng DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA), following manufacturer's instructions. Methylation-specific PCR assay was carried out in a Mastercycler epgradient S (Eppendorf, Germany). Primer oligonucleotides were obtained from Genomed.pl (Warsaw, Poland). Primer sequences were designed using methprimer (http://www.urogene.orgbin/methprimer/.cgi). The status of DAT promoter (http://www.ncbi.nlm.nih.gov/nuccore/13661994?report=fasta) was assessed by PCR using primers specific to a fragment of the gene, i.e., DATF: 5′-GGTTTTTGTTTTTTTTATTGTT-GAG-3'; DATR: 5′-AAATCCCCTAAACCTAATCCC-3'. The PCR conditions in order to amplify the 447-bp fragment covering 23 CpG islands in DAT gene promoter were as follows: initial denaturation (94 °C/5 min), followed by 35 cycles (94 °C/61 °C/72 °C, 25 s each step) with final elongation at 72 °C for 5 min. The concentration of magnesium chloride ions was 2.5 mM. After amplification assay, the PCR products were subjected to sequencing as previously described (Rubiś et al., 2012). Briefly, samples were verified by sequencing using the BigDye v3.1 kit (Applied Biosystems, Darmstadt, Germany) and separation by ethanol extraction using the ABI Prism 3130XL (Applied Biosystems, Darmstadt, Germany) in a 36 cm capillary in a POP7 polymer (both polymorphisms), using the reverse primer.

Table 1
Patients and control groups characteristics.

Group[a]	n	Mean age	SD	Age range
ADS	171	33	8.75	19–61
Lesch typology type I	36	33	7.32	18–48
Lesch typology type II	44	33	9.95	19–54
Lesch typology type III	50	32	6.99	19–43
Lesch typology type IV	27	34	10.67	20–61
Cloninger's typology type I	39	40	8.31	22–61
Cloninger's typology type II	131	31	7.68	19–54
Delirium and/or seizures	49	34	7.84	20–58
Delirium	31	35	9.20	20–58
Seizures	29	34	6.47	20–50
Early onset of drinking (<26. years)	122	31	7.47	19–54
Control	160	39	16.1	18–80

[a] For analysis were included only men.

2.3. Statistical analysis

Data were analyzed using Fisher's exact test, with $p \leq 0.05$ being considered as statistically significant (GraphPad Prism 5.0).

3. Results

No statistical differences in the general frequency of *DAT* CpG islands was revealed between patients (altogether 175 methylated islands) and control subjects (170 methylated islands ($p = 0.86$). Interestingly, considering the islands that are most often analyzed in literature (positions no. 9 and 18 according to our numbering system, see Fig. 1), we also did not find any significant difference in methylation status. However, the analysis of other CpG locations indicated that there is a significant methylation status frequency difference in position 12 (Fig. 1, Fig. 2). It was revealed that this position is significantly more often methylated in control subjects than in alcohol dependent individuals ($p = 0.0296$) (Fig. 1, Fig. 2).

Metylation of island 12 did not correlate with age. Generally, metylation of DAT promotor is correlated with age, but not in our study in the case of island 12.

4. Discussion

Gene expression may be regulated by methylation and this process in turn may affect the ability of transcription factors to access and bind specific regions in promoter sequence. Several factors regulating *DAT* transcription have been identified, including NURR1, HEY1/HESR, Sp1 and Sp3 [Zhao et al., 2013]. The Sp1-binding sites on promoters of genes are conserved among mice, rats and humans but some of them have atypical Sp1-binding motif i.e. GGGCGT or GGGCGA [Dhar et al., 2013]. Such an atypical Sp1 binding site is located in the promoter sequence that we studied, and covers exactly CpG at position 12 i.e. **GGGGCGACG.** Sp1 is involved in many cellular processes, including cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling. Post-translational modifications such as phosphorylation, acetylation, glycosylation, and proteolytic processing significantly affect the activity of this protein, which can be an activator or a repressor [Hwang et al., 2001]. The ability of alcohol consumption to affect Sp1-binding activity (followed by alteration in hsp70 induction) was already shown [Mandrekar et al., 2008]. Molecular studies on ethanol impact on human cells revealed that DNA binding activity of Sp1 is modulated that consequently altered ethanol-induced p21 expression, cell cycle arrest, and apoptosis [Do et al., 2013]. We still do not know if that is a possible mechanism of ethanol intake or dependence