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Association of status of acetylcholinesterase and *ACHE* gene 3' UTR variants (rs17228602, rs17228616) with drug addiction vulnerability in pakistani population

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ABSTRACT

Substance addiction is a chronic, relapsing mental disorder Characterized by compulsive drug seeking, and loss of control over drug intake and relapse after prolonged abstinence. Genetics has been shown to contribute towards an individual's vulnerability to addiction. Acetylecholine (ACh), a cholinergic neurotransmitter hydrolyzed by acetylcholinesterase (AChE), is an essential neurotransmitter and neuromodulator in central and peripheral nervous system and has regulatory influence on numerous neuronal functions including addiction. The present study was carried out to investigate the role of acetylcholinesterase (AChE) in addiction through measurement of enzyme activity and to find potential association of *ACHE* gene 3'UTR variants rs17228602 and rs17228616 in heroin, hashish and poly drug addicts. Both SNPs are located within microRNA (miRNA) recognition sites with potential to affect miRNA/transcript interaction. A total of 122 addicts of heroin, hashish and polydrug were recruited from local rehabilitation centers to participate in this study. AChE activity was measured in blood by Ellman's method. SNP genotyping was performed by restriction fragment length polymorphism (PCR-RFLP) and Sanger sequencing. The AChE activity was found significantly higher ($p \leq 0.005$) in addicted cohort (mean ± standard error of mean 0.020 ± 0.001 μmol/L/min; 95% confidence interval (CI) 0.018–0.022) in comparison to non-addicted healthy subjects (0.011 ± 0.001 μmol/L/min; 95% confidence interval CI 0.010–0.013). A statistically significant association of ACHE rs17228602 SNP with addiction vulnerability in dominant (DM: Odd's ratio OR = 2.095, 95% CI = 1.157–3.807 p = 0.009) and allelic genetic models (OR = 1.854 95% CI = 1.082–3.187, p = 0.016) was observed. However, no statistically significant association of rs17228616 SNP with substance abuse disorder was found. The data presented here shows that AChE could play significant role in substance addiction. Further studies with larger sample size and other variants of AChE are recommended to identify novel therapeutic approaches for cholinergic based treatment of addiction.

1. Introduction

Drug addiction is a persistent relapsing mental disorder encompassing uncontrollable drug use and severe emotional impairment. Repeated intake of illicit drugs changes several brain regions making drug seeking a compulsive and sole purpose of addict's life, eventually harming the physiological and physical well-being of the addicted individual [1,2]. There are many substances that can lead to addiction if used excessively including but not limited to heroin, alcohol, cocaine, cannabis, ice and opioids [3]. Worldwide estimates put the number of addicts to an estimated 27.8 million according to United Nations Office of Drugs and Crime (UNODC). In Pakistan, around 6.7 million people have been reported to abuse drugs at least once in their life and, 4.25 million of them became heroin dependent according to 2013 UNODC report. Due to considerable morbidity and mortality, drug addiction is a serious socioeconomic concern besides spreading many infectious diseases like hepatitis B and C, HIV etc. It is also associated with psychiatric disorders like anxiety and depression.

Drug addiction is a multifactorial disorder involving genetics, epigenetics, environment, type of illicit drugs, route of drug administration, physiological and socioeconomic factors [4]. Males are more involved in drug addiction than females so gender differences play a role

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in addictive behavior [5]. Genetics contributes 50% to addiction vulnerability [2]. The children of addicted parents are eight times more likely to develop drug dependence [6].

Drugs of abuse modulate the expression of genes involved in neuroplasticity via epigenetic and microRNA modifications, eventually disrupting intracellular signal pathways and the neuronal circuits whose dysfunction induces long-lasting changes associated with state of addiction [2].

Acetylcholine (ACh) is an essential component of cholinergic system. It functions as a neurotransmitter and neuromodulator and, is involved in various central cognitive functions. Acetylcholinesterase (EC 3.1.1.7) terminates the neural impulse transmission by rapidly hydrolyzing acetylcholine in different cholinergic pathways in the peripheral and central nervous system [7]. Studies have shown that acetylcholine plays significant role in development and progression of addiction [8]. Elevated AChE in hippocampus and cortex region causes decline in glutamatergic synapse by altering expression of neuroligin and neurexin *trans*-synaptic proteins, which control synaptic stability [9]. Almost 40% of AChE activity has been observed to be lost in heroin abusers which could be restored after the treatment. Hence, AChE activity can be used as a probe to diagnose addiction disorders [10]. AChE inhibitors have been shown to treat many types of neuropathological conditions including the substance use disorders [11].

ACHE gene is highly conserved among populations. Mutations are present in ACHE gene but deleterious variants are rare, usually occur in heterozygous form and there are no loss of function mutations in homozygous state [12,13]. So far, according to Exome Aggregation Consortium (ExAC) (http://exac. broadinstitute.org/gene/ENSG00000114200), 137 missense variants and 141 synonymous mutations have been reported for ACHE gene.

The enzymatic activity of acetylcholinesterase can be modified by coding and non-coding variations of ACHE gene [14]. Single nucleotide polymorphisms (SNP) located in 3′-UTR of ACHE gene can affect recognition sequences of microRNAs, usually present there. MicroRNAs are non-coding small RNA molecules which regulates many molecular pathways by post-transcriptional gene-silencing. Cholinergic signal pathways are subjected to regulatory control by means of miRNA network. Any disruption of miRNA/cholinergic transcripts interaction might perturb the regulatory network leading to development of diseased state such as addiction.

The aim of the present study was to measure circulating AChE enzymatic activity and to detect any potential association of ACHE 3′-UTR SNPs (rs17228602 and rs17228616) with addiction vulnerability in addicted cohort especially of heroin, hashish and polydrug. This is the first study investigating selected ACHE 3′-UTR variants in addicted cohort of Pakistani descent to the best of our knowledge.

2. Material and methods

2.1. Sampling of study subjects

In the present study, the sampling of addicted individuals was conducted from three addiction rehabilitation centers located in Rawalpindi and Islamabad, Pakistan. The study was approved from authorities of the centers and by ethics review committee of the Department of Biosciences, COMSATS University Islamabad. The study conformed to tenets of 1964 Declaration of Helsinki and its later amendments. The participants in the study provided written informed consents. One hundred and twenty two addicted individuals were included in the study. Exclusion criteria included presence of any viral infections and any chronic condition like hypertension and diabetes. Although according to our selection criteria, females were not excluded but most of participants were males. This may be due to the fact that our social and cultural issues hinder the females to seek professional help from rehabilitation centers. The average age of males was 30.12 ± 9.79. Among total, 53 were heroin addicts, 32 were hashish addicts and 37 were polydrug users (using combination of different drugs at a time). One hundred and thirty two healthy non-addicted individuals were recruited as a controls that were ethnic and age matched. Demographic characteristics of addicted subjects are described in Ref. [15]. 3-ml venous blood sample was collected from every participant in EDTA-k vacutainers tubes (Atlas –Labovac Italiano, FL Medical, Torreglia PD, Italy) for biochemical and molecular analysis. The vacutainer tubes containing blood samples were stored at 4 °C until further use.

Table 1
Primer Sequences of Acetylcholinesterase gene *ACHE* SNPs rs17228602 and rs17228616.

PRIMER SEQUENCES	PRODUCT SIZE (bp)
rs17228602:	365bp
F: 5′-GAGGAGGAGAAAAGAATGACC-3′	
R: 5′-TCCTCTAATGAGTGGTCGGAC-3′	
rs17228616:	337bp
F: 5′-ATTCCGGCGTTCTGCCTCTAC-3′	
R: 5′CTACATGGTGCACTGGAAGAAC-3′	

F forward, R reverse.

2.2. Primer designing and chemicals

Primer 3 version 0.4.0 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) was utilized to design primers for selected ACHE SNPs. Specificity of primers was confirmed by NCBI Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) software and further checked on UCSC genome browser In-silico-PCR tool (https://genome.ucsc.edu./cgi-bin/hgpcr). Primers were made by macrogen Inc. (Rockville, MD, USA). Primer sequences of both SNPs (rs17228602 and rs17228616) are given in Table 1.

Chemicals that were utilized in extraction of DNA,PCR, RFLP analysis, Gel electrophoresis and AChE estimation were obtained from Thermo Fisher Scientific (Waltham, MA USA) and Sigma-Aldrich (St Louis, MO,USA)

2.3. AChE measurement

Ellamn's method modified by Worek et al. [16] was used for the measurement of acetylcholinesterase. The measurement was carried out in the presence of selective butyrylcholinesterase inhibitor, ethopropazine. at 436 nm and 37 °C by using 3 ml polystyrol cuvettes (Thomas Scientific, Swedesboro, NJ, USA; catalog number 1218871). All reagents (2 ml 0.1M phosphate buffer, pH 7.4, 100 µl DTNB (10 mM), 10 µl Ethopropazine) and 1 ml blood dilution were mixed and incubated at 37 °C for 20 min. Then 50 µl acetylthiocholine (28.3 mmol/l) was added to the reaction mixture and mixed. Absorbance of reaction mixture was noted by using spectrophotometer (model “Specord 50 plus” Number; 233H1280C manufactured by Analytic Jena, Germany). Detailed procedure is given in Worek et al. [16].

2.4. Genomic DNA extraction and SNP genotyping

Genomic DNA was extracted from 1 ml blood using salting out method as described by Ref. [17]. The method includes the cell lysis followed by protein digestion and then DNA precipitation with ethanol. Quantification of DNA was done by nanodrop (Implen Pearl Nano-Photometer; Thomas Scientific). The DNA suspended in TE buffer was stored at −20 °C until further use.

The genotyping of ACHE variant (rs17228602) was performed by PCR-RFLP method by using forward and reverse primers shown in Table 1. Quantities used for PCR amplification are illustrated in Table 2. The PCR products were incubated at 37 °C for 16 h with restriction enzyme Psp5II (PpuMI) (Catalog # ER0761, Thermofisher Scientific).

Table 2
Procedures and Reagents Quantity used for Genotyping of Single Nucleotide Polymorphisms (SNP) rs17228602 and rs17228616.

Reagents and procedures	Quantity of reagents for rs17228602 (μL, Conc.)	Quantity of reagents for rs17228616 (μL)
Reagents (total Volume 25 μL)		
Taq buffer	2.5 (1X)	2.5(1X)
$MgCl_2$	2.5 (1.5 mM)	2.5 (1.5 mM)
dNTPs	0.5 (2.5 mM)	0.5(2.5 mM)
Primers	F = 0.5 (10 pmol)	F = 0.5(10 pmol)
	R = 0.5 (10 pmol)	R = 0.5(10 pmol)
DNA sample	2.0 (40 ng/μl)	2.0 (40 ng/μl)
Taq polymerase	0.5 (5 Units)	0.5(5 Units)
PCR water	15.5	15.5
Thermal profile		
Denaturation	95 °C for 5 min; 95 °C for 1 min	95 °C for 5 min; 95 °C for 1 min
Annealing	62 °C for 45 s	58 °C for 45 s
Extension	72 °C for 1 min; 72 °C for 7 min	72 °C for 1 min; 72 °C for 7 min
Total cycles	35	35

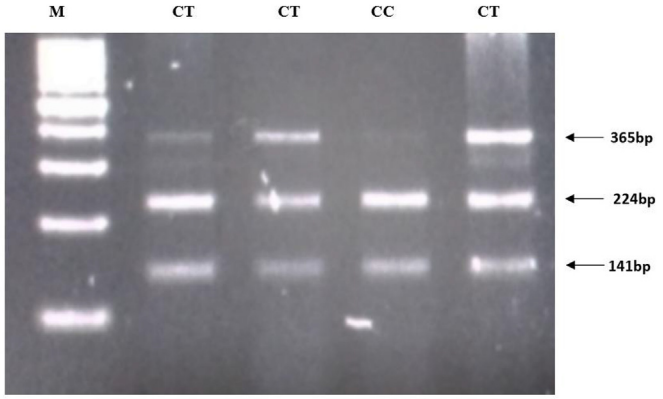

Fig. 1. Identification of SNP rs17228602 (c.*172G > A) in acetylcholinesterase (ACHE) gene. Visualization of PCR product after digestion with restriction enzyme Psp5II (PpuMI) are shown on 2% ethidium bromide-stained agarose gel. Genotypes are shown above the lane and fragment size are indicated by arrow to right of figure.

Psp5II cleaves in presence of major C allele producing fragments of 224bp and 141bp size while in presence of T allele the 365bp fragment remains uncut as shown in Fig. 1. The restriction products were visualized on 2% agarose gel in mini horizontal gel electrophoresis system (Cleaver Scientific, Rugby, UK; catalog number MSMINI10). Genotyping of ACHE variant rs17228616 was conducted by Saner Sequencing (Fig. 2) by Molecular cloning laboratories (South San Francisco, CA, USA).

2.5. Statistical analysis

Mann-Whitney order rank test was used to determine the significance of AChE between addicts and non-addicts groups. Alpha ≤ 0.05 was considered as statistical significance. The SPSS statistic software version 20.0 (IBM Corp., Armonk, NY, USA) was used for data analysis. Genotypes and allelic frequencies between addicts and non-addicts were analyzed by chi-square and Fisher exact test. The Odd ratio with 95% Confidence Interval for both SNPs was calculated to determine the association effect in different inheritance models. Genotype frequencies were evaluated for deviations from Hardy-Weinberg Equilibrium (HWE) in addicted and non-addicted groups by means of goodness of fit Chi-square test (http://www.had2know.com/academics/hardy-weinberg-equillibriumcalculator-2-alleles.html). Data was analyzed by using Graphpad Prism 7.0 (Graphpad Software Inc., La Jolla, CA, USA).

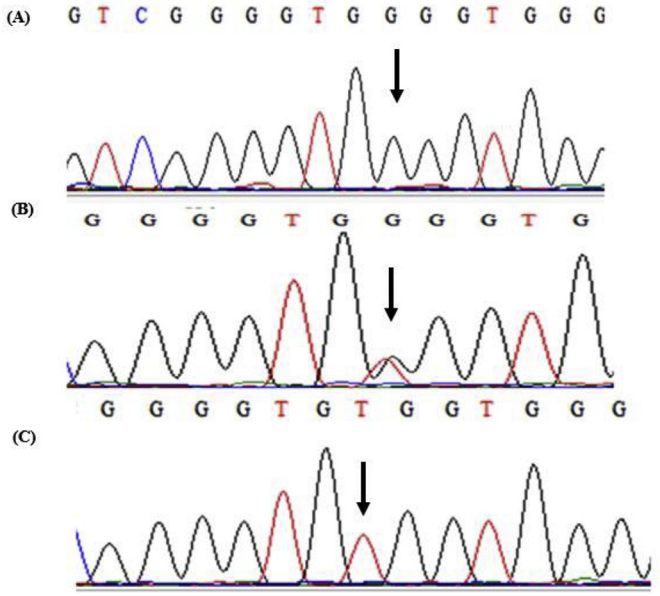

Fig. 2. Sanger sequencing chromatogram for ACHE SNP rs17228616 (c.*114C > A). The chromatograms are showing, (A) homozygous GG (B) heterozygous GT and (C) TT genotypes of *ACHE* rs17228616 SNP (sequencing position shown by arrow).

3. Results

3.1. Status of AChE in addicted and non-addicted subjects

AChE activity in addicted and non-addicted cohort is shown in Table 3 and Fig. 3a and b. Overall, a significantly higher levels of AChE activity was observed in addicted groups (0.020 μmol/L/min) in comparison to non-addicted control group (0.011 μmol/L/min). The mean enzyme activity was found to be more in Heroin addicts (0.023 μmol/L/min) followed by Hashish (0.017 μmol/L/min) and polydrug addicts (0.017 μmol/L/min)) which was still higher compared to healthy control (HC) (0.011 μmol/L/min).

3.2. Distribution of allele and genotypes frequencies of ACHE SNPs rs17228602 and rs17228616

The genotype distribution of ACHE rs17228602 in control was according to Hardy Weinberg Equilibrium (HWE) ($\chi 2 = 1.858$, $P = 0.172$), while deviation from HWE was observed in addicted cases ($\chi 2 = 5.90$, $p = 0.015$). For rs17228616 SNP, distribution of genotypes in both control and addicted groups fitted with HWE (Controls: $\chi 2 = 2.287$, p = 0.130; Cases: $\chi 2 = 2.609$, p = 0.106).

The genotype and allele frequencies of rs17228602 (c.*172G > A) in addicted and non addicted individuals is summarized in Table 4. A significant difference in genotype distribution between addicts and HC was observed ($\chi^2 = 6.89$, $p = 0.031$) as shown in Fig. 4. Genotype frequencies of *ACHE* rs17228602 CT genotype was higher in addicted cohort in comparison to non addicts (36.06%, 21.21%) respectively (Fig. 4), CT genotype was in particular higher in polydrug and hashish users followed by heroin consumers compared to HC (Fig. 3a). But no minor allele TT homozygotes were present in both addicts and non addicts that's why no recessive model was assessed. Fig. 5 shows genotype frequency of ACHE rs17228602 in different drug users. Allele frequency of major C allele was 81.97% in addicted and 89.40% in HC whereas frequency of minor T allele was slightly higher in addicted cohort (18.30%) in comparison to non-addicted healthy controls

Table 3
Acetylcholinesterase Activity in Addicted and Non addicted Groups.

Groups	N	Mean ± SEM (μmol/L/min)	95% CI	*P* value
Heroin addicts (A)	44	0.023 ± 0.002	0.020-0.026	0.000**
Hashish addicts (B)	20	0.017 ± 0.009	0.015-0.019	0.002*
Polydrug addicts (C)	26	0.017 ± 0.002	0.014-0.021	0.002*
Overall addicted cohort (A + B + C)	90	0.020 ± 0.001	0.018-0.022	0.000**
Non addicts	131	0.011 ± 0.001	0.010-0.013	——

SEM Standard error of mean, CI confidence interval.

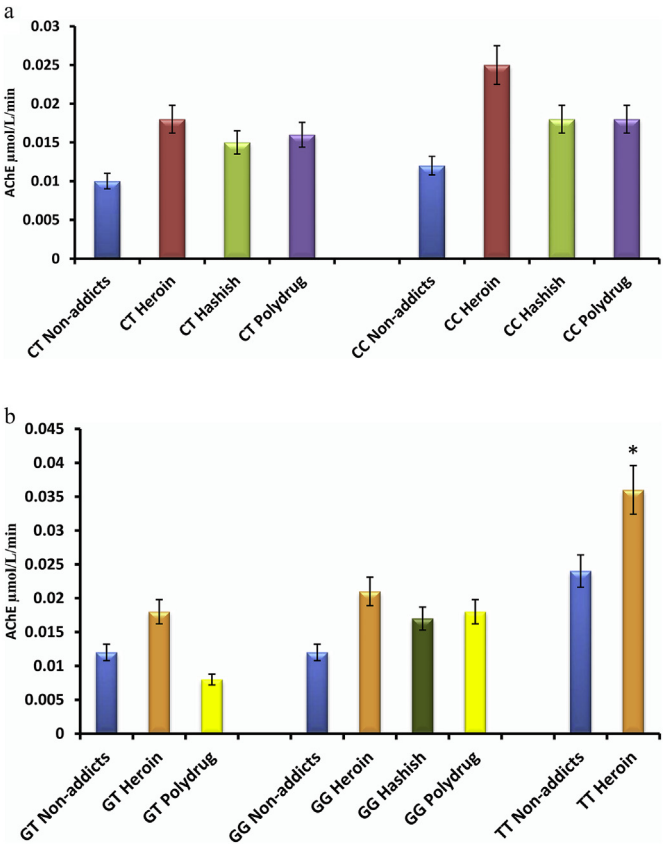

Fig. 3. a AChE activity (μmol/L/min) in specific addicted group according to distribution of rs17228602 Genotypes (CC, CT)., b: AChE activity (μmol/L/min) in specific addiction group according to distribution of rs17228616 Genotypes (GG, GT, TT).

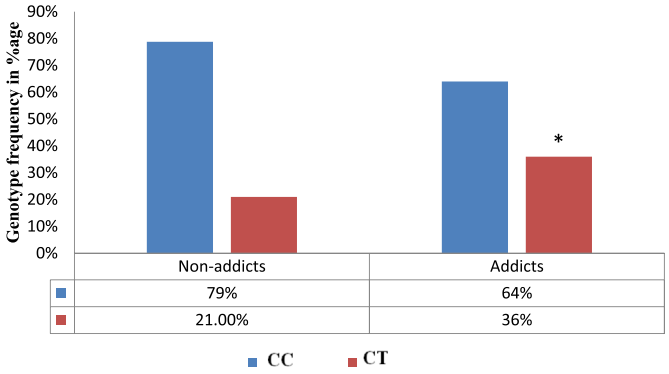

Fig. 4. Distribution of genotype frequencies in %age (CC, CT) of ACHE rs17228602 SNP in addicted and non addicted cohort.

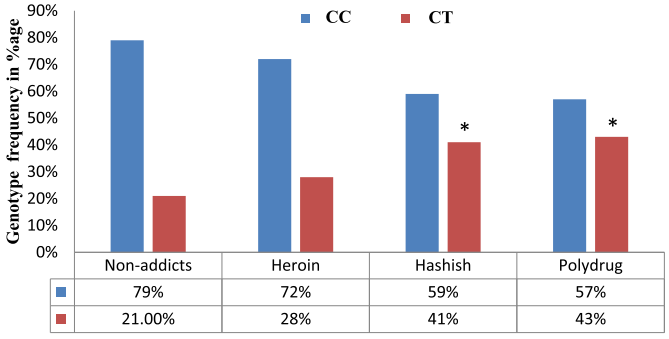

Fig. 5. Genotype frequencies in % age of ACHE rs17228602 SNP in non-addicted and addicted individuals stratified to drug type usage (heroin, hashish and polydrugs).

(10.60%) respectively. In addition, statistically significant association of rs17228602 ACHE variant with addiction was found in dominant and allelic genetic models (DM: OR = 2.095, 95%CI = 1.157–3.807 p = 0.009; Allele: OR = 1.854 95% CI = 1.082–3.187, p = 0.016).

Analysis of r17228602 genotype distribution in addicts based on drugs abused (heroin, hashish, polydrug) and healthy controls also showed significant statistical association with different genetic models as is detailed in Table 5. A significant association was found for hashish and polydrug users both in dominant and allelic models (DM:OR 2.541 95% CI(1.120–5.768), p = 0.0035; Allele: OR 2.148,

Table 4
Genotype and Allele frequencies of ACHE rs17228602 in Addicted and Non Addicted Cohorts.

Genotype and allele frequencies	Addicts, N (%)	Non-addicts, N (%)	Odd ratio (95% CI)	Chi-square (χ^2)	P Value
Genotype					
CC	78 (63.93)	104 (78.78)		6.89	0.031[a]
CT	44 (36.06)	28 (21.21)			
TT	0 (0%)	0 (0%)			
DM: CT + TT vs CC			2.095 (1.157-3.807)	6.887	0.009[b]
Allele					
C	200(81.97)	236(89.40)	1.854 (1.082-3.187)	5.749	0.016[b]
T	44(18.03)	28(10.60)			

DM Dominant model.
[a] by 2 × 3 Contingency table.
[b] Statistical Significance.

Table 5
Association analysis of *ACHE* rs17228602 SNP according to drugs used in addicts.

Models Groups	Dominant model (DM)		Allele	
	Odd ratio(95% CI)	χ2 (p value)	Odd ratio(95% CI)	χ2 (p value)
Heroin	1.466 (0.707-3.039)	1.065 (0.302)	1.389 (0.709-2.721)	0.925 (0.336)
Hashish	2.541 (1.120-5.768)	5.177 (0.023)[a]	2.148 (1.042-4.432)	4.437 (0.035)[a]
Polydrug	2.830 (1.307-6.129)	7.284 (0.007)[a]	2.325 (1.180-4.581)	6.194 (0.013)[a]

[a] Statistical Significance.

Table 6
Genotype and allele frequencies of single nucleotide polymorphism rs17228616 in Addicted and Non addicted cohorts.

Genotype and allele frequencies	Addicts, N (%)	Non-addicts, N (%)	Odd ratio (95% CI)	Chi-square (χ^2)	P Value
Genotype					
GG	43 (87.75)	39 (86.67)		0.025	0.988[a]
GT	5 (10.20)	5 (11.12)			
TT	1(2.04)	1 (2.23)			
DM: GT + TT vs GG			1.103(0.3281-3.705)	0.025	0.875
Allele					
G	91(92.85)	83(92.23)	1.096 (0.369-3.259)	0.028	0.868
T	7(7.14)	7(7.78)			

DM Dominant model.
[a] by 2×3 Contingency table.

95%CI = 1.042–4.432 p = 0.035) (DM: OR 2.830,95% CI1.307-6.129, p = 0.007; Allele: OR 2.325, 95%CI 1.180–4.581, p = 0.013).

An apparent increase in AChE enzyme activity was observed in all three different drug users compared to healthy individuals regardless of the rs17228602 genotypes. Hence a clear correlation between the CT genotype and increased AChE enzyme activity in addicted subjects could not be established in the current study. Enzyme activity was particularly higher in heroin users followed by polydrug and hashish users (Fig. 3a).

Allele and genotype frequencies of ACHE rs17228616 (c.*114C > A) are summarized in Table 6. There was no statistically significant difference in allele and genotype frequencies of ACHE rs17228616 between addicted and non-addicted cohort (χ^2 = 0.025, p = 0.988). Therefore no association of rs17228616 SNP with addiction vulnerability was identified in addicts in any of the three inheritance models. The overall frequencies of rs17228616 GT and TT genotypes in both groups were strikingly lower as can be seen in Table 6 and Fig. 6. Comparison of genotype frequencies in addicted cohort based on type of drug compared to healthy controls also did not show significant association for rs17228616 SNP with addiction (Table 7). TT genotype for

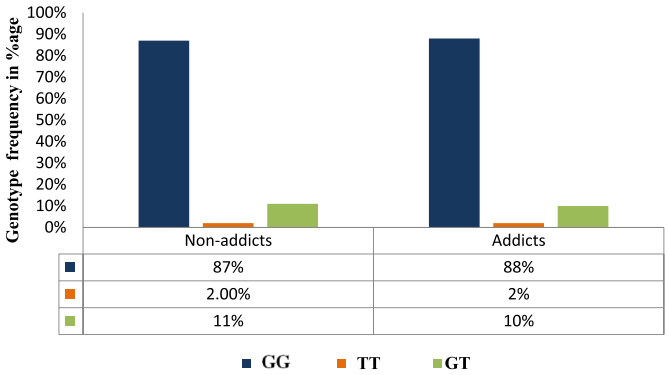

Fig. 6. Distribution of genotype frequencies in %age (GG,TT, GT) of ACHE rs17228616 SNP in Addicted and Non addicted cohort.

rs1722861 in heroin addicted users was the only observed genotype. TT and GT genotypes were not found in hashish group and no TT homozygote was observed in polydrug group (Fig. 7; Table 7). Even though, there was obvious increase in AChE enzyme activity in addicts compared to non-addicts independent of the genotype. The enzyme activity was higher in heroin users followed by polydrug and hashish users (Fig. 3b).

4. Discussion

Drug addiction or substance use disorder is a complex chronic neurological disorder characterized by uncontrollable compulsive drug seeking and relapse after withdrawal despite severe negative consequences for the addicted individual [2]. The cellular and molecular underpinnings of development of addictive state are so far not completely elucidated. Studies have established role of cholinergic system in pathophysiology of drug addiction including cocaine dependence [8]. AChE, an acetylcholine hydrolyzing enzyme, regulates ACh signaling by serving to terminate neurotransmission at cholinergic neurons which are involved in various vital cognitive functions including learning, attention, memory, stress response and reward [7]. These processes are engaged in development and maintenance of addictive behavior to drug of abuse. Due to the interaction of ACh with the dopaminergic reward system primarily in specific brain regions i.e. nucleus accumbens (NAc), ventral tegmental area (VTA), and prefrontal cortex (PFC), it is suggested to play a considerable role in substance abuse disorder [8,18].

Despite the functional significance of cholinergic genes in addiction pathology, limited studies have investigated association of *ACHE* gene variants and circulating AChE activity with addiction vulnerability in drug addicts (cannabis, heroin,and polydrugs) in different populations. Hence, the goal of the present study was to investigate association of status of circulating AChE enzymatic activity and 3′UTR variants (rs17228602, rs17228616) in *ACHE* gene with vulnerability to drug addiction in Pakistani addicted cohort.

The results showed a significantly higher circulating AChE levels in addicted cohort than in healthy controls (Table 3, Fig. 3a and b), anticipating lower ACh in the examined addicted subjects. Heroin users showed higher AChE enzymatic activity followed by polydrug and hashish users (Table 3). A significant association of ACHE rs17228602 SNP minor T allele and CT genotype with risk of addiction in examined samples was identified (Table 4, Fig. 4). This is the first report of rs17228602 SNP association with vulnerability to addiction to the best of our knowledge. However, no significant association of rs17228616 and addiction risk in studied addicted cohort was found (Table 6, Fig. 6).

The two SNPs lie in the 3′ UTR region of ACHE gene. The 3′ UTR region of cholinergic genes harbor recognition sequences for microRNAs (miRs), that act as key regulators for cholinergic signaling and for modulating neuronal and non-neuronal functions [7]. SNPs located within miRNAs recognition sites in 3′UTR alter miRNA/target mRNA binding affinities affecting not only the expression of target transcripts but also potentially disrupting the complex miRNA regulatory network and contribute to neurological pathologies including addiction. For example, the presence of minor allele of rs17228616 SNP

Table 7
Association analysis of rs17228616 SNP genotype according to drugs type in addicted groups.

Models Groups	Dominant model (DM)		Recessive model (RM)		Allele	
	Odd ratio (95% CI)	Chi square (p value)	Odd ratio (95% CI)	Chi square (p value)	Odd ratio (95% CI)	Chi square (p value)
Heroin	3.250 (0.883–11.967)	3.335 (0.068)	2.933 (0.173–49.860)	0.604 (0.437)	2.196 (0.644–7.490)	1.639 (0.200)
Hashish	0.000————	2.366 (0.124)	0.000——	0.361 (0.548)	0.000——	2.640 (0.104)
Polydrug	0.867 (0.157–4.780)	0.027 (0.869)	0.000——	0.384 (0.535)	0.741 (0.146–3.758)	0.132 (0.717)

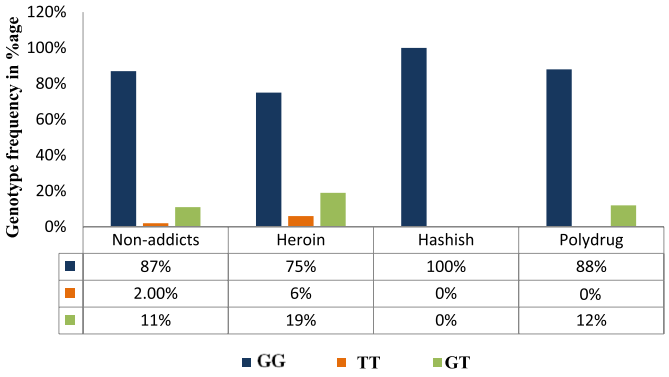

Fig. 7. Genotype frequencies (%age) of *ACHE* rs17228616 in non-addicted and addicts of heroin, hashish and polydrugs.

in ACHE 3′ UTR, weakens hsa-miR-608/ACHE interaction and regulation, resulting in increased AChE activity in brain and reduction of other hsa-miR-608 targets including CDC42, IL-6, NACC1 and CD44 possibly due to 'free' miR-608s [19]. The SNP induced changes have been associated with increased anxiety, inflammation and hypertension and relative resilience to post-traumatic stress in homozygous carriers [20]. Although in our study, no association of rs17228616 with addiction was observed, probably because of size of sampling population employed in the study or because of very low prevalence of minor allele. The other investigated ACHE 3′UTR SNP rs17228602 is located in the binding site for miR-125b in proximity to miR-608 binding site. The presence of minor rs17228602 T allele diminishes interaction between miR-125b and ACHE transcripts. miR-125b can predictably target both soluble ACHE-R, suppression of which will decrease ACh inactivation and vesicular ACh transporter, inactivation of which inversely diminishes ACh production. This suggests a bidirectional regulatory control on cholinergic signaling by miR-125b [14,21]. Defective interaction of miR-125b/ACHE as result of rs17228602 may impair regulation of cholinergic signaling, impacting homeostasis/balance of cholinergic pathways which can lead to complex pathological consequences. By preventing binding with ACHE transcripts, the rs17228602 SNP might cause 'unemployed' miR-125b molecules to target other miR-125b target transcripts reducing their levels, in a manner similar to hsa-miR-608/ACHE interfering SNP [19], changing neurological pathways and synaptic plasticity. Interestingly, mice lacking 3′ UTR sequence in ACHE display stress induced cognitive deficits [22], indicating significance of 3′ UTR miRNA binding sites.

Notably, elevated AChE enzymatic activity in addicts was observed in our study but associative correlation with both the SNP genotypes (rs17228616, rs17228602) could not be established in currently examined samples. However, elevation of AChE activity in addicted individuals regardless of the present genotypes does show its role in etiology of the addiction.

Abdel-Salam et al. [23] found an increased AChE activity in brain of rats when treated with cannabis resin. Zhou et al. [24] concluded their study based on experiments on rats that cholinergic transmission affects heroin self-administration and reinstatement. It is obvious that inhibition of AChE at synapse causes the accumulation of ACh, cholinergic neurotransmitter.

An increased understanding of the relative importance of AChE in addiction will help in treatment decisions for addiction. It is well established that Inhibitors of AChE like donepezil, and galantamine are the drugs of choice for Alzheimer disease. However, these clinically available drugs have been investigated in preclinical and clinical trials for stimulant addiction [25,26]. AChE inhibitor has shown a promising treatment option for stimulant addiction in human studies [27]. AChE inhibitors were also investigated for cocaine addiction [28]. Diehl et al. [29] reported that galantamine, an AChE inhibitor reduces smoking frequency in alcoholic people. In short, AChE inhibitors have been shown to be promising treatment option for stimulant addiction in human studies [27] and investigated in some other addictions, However, detailed study with different types of substance abuse is warranted.

Moreover, further studies with larger sample size are recommended for tangible conclusion and to establish the roles of these SNPs toward drug abuse vulnerability. Also, our study cohort consisted of addicted individuals undergoing treatment, so effects of medications on elevated AChE activity could not be discounted. Future work comparing the AChE enzyme activity between treated and non-treated addicts should be done to ensure that treatment is not an influencing factor.

5. Conclusion

In conclusion, elevated circulating AChE activity was observed in addicts compared to healthy controls, where highest AChE activity was detected in Heroin users. Moreover, we identified significant association of minor allele of 3' UTR SNP rs17228602 in *ACHE* with addiction vulnerability in addicts. These results confirm the role of AChE in pathophysiology of addiction. Further studies are required to find the causative role of rs17228602 SNP and miR-125b/ACHE interaction on cholinergic signaling in pathogenesis of addiction. Study with larger sample size focusing on other AChE enzyme forms and SNPs is also recommended to identify genetic influences on AChE activity, and its implication in the development of addiction and usefulness in therapeutic interventions for addiction.

Conflicts of interest

None.

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Appendix A. Supplementary data

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Transparency document

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