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Acetylcholinesterase and Association of *ACHE* 3' UTR SNP rs17228602 with Psychiatric Disorders

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Abstract

Psychiatric disorders are complex mental conditions that cause significant emotional distress and impairment in a person's ability to function normally. Globally, there is an alarming rise in the prevalence of psychiatric conditions. Genetic and environmental factors are involved in the pathophysiology of these disorders, but molecular underpinnings are still elusive. Cholinergic dysregulation is one of the etiology of psychiatric disorders. This study was aimed to assess the status of hydrolyzing enzyme of the cholinergic neurotransmitter acetylcholinesterase (AChE) from blood and investigate the possible association of a single nucleotide polymorphism (rs17228602) in the 3'UTR region of *the ACHE* gene with a predisposition to psychiatric disorder. Ninety-five confirmed psychiatric and one hundred thirty healthy individuals were recruited for the study with due consent. AChE was determined by Elman's method. SNP was studied by polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) method and Sanger sequencing of DNA samples. The results showed notably reduced AChE levels in psychiatric cohorts with statistical significance ($p \leq 0.05$). Genotype and allelic association of the examined SNP revealed a risk of the psychiatric condition in patients. It is concluded that AChE activity and *the ACHE* gene's 3′-UTR variant have a significant role in developing psychiatric disorders. Further studies will open a new direction of the investigation and therapeutic interventions for psychiatric disorders. However, further study with more study participants of homogenous composition in terms of psychiatric disorder is recommended to conclusively understand the role of *ACHE* gene variants in the development of psychiatric diseases.

Keywords: Acetylcholinesterase; *ACHE*; Cholinergic; MDD; Psychosis; rs17228602; 3' UTR SNP.

1. Introduction

Psychiatric disorders are a group of mental illnesses with multiple presentations characterized by changes in cognition, emotion, mood, perception, and behavioral patterns causing considerable distress or impairment to an individual's functioning. Psychiatric conditions afflict 25% of the population annually and are among the foremost reasons for disability and 90% of suicides globally (Brådvik, 2018). A worldwide increase in the prevalence of psychiatric diseases has been observed with substantial impacts on life's health, social and economic aspects. Psychiatric disorders are complex multifactorial conditions influenced by environmental and genetic factors (Moffitt, 2005; Schmitt *et al*., 2014). The different types of psychiatric disorders can be broadly categorized into mood disorders (depression, bipolar disorders), psychotic disorders (Schizophrenia, psychoses), and anxiety disorders (panic disorder, phobias, obsessive-compulsive disorder OCD, posttraumatic stress disorder) (Rakofsky & Rapaport, 2018; Vigne *et al*., 2019). According to a WHO report, MDD is one of the fourth leading causes of disability worldwide (Kessler & Bromet, 2013). Similarly, a bipolar disorder affecting almost 45 million people worldwide includes abnormally elevated or pressured mood states, alternating with normal or depressed moods (Bymaster & Felder, 2002).

Schizophrenia is a mental disorder characterized by hallucinations and delusions marked by the distorted perception of thinking, emotions, reality, and behavior of a person suffering from it. Schizophrenia typically starts in late adolescence or early adulthood and afflicts 20 million people worldwide (Bhugra, 2005; Charlson *et al.*, 2018).

Despite enormous research progress over the decades in understanding psychiatric disorders, the causal neuropathogenetic mechanisms of most psychiatric illnesses remain unclear. Although imbalanced neurotransmitter systems, particularly dysregulated cholinergic systems, are implicated in the pathophysiology of neuropsychiatric disorders such as Schizophrenia, bipolar disorder, and mood disorders (Shi *et al*., 2008; Scarr *et al*., 2013; Higley & Picciotto, 2014).

Acetylcholine (ACh) is the main component of the cholinergic system that operates as a neurotransmitter and neuromodulator in diverse cognitive functions (Simchovitz *et al*., 2017). Acetylcholinesterase is the enzyme that hydrolyzes acetylcholine to terminate the neural transmission in different cholinergic pathways involved in the central and peripheral nervous system (Picciotto *et al*., 2012; Colović *et al*., 2013; Shahaf, 2016). Measurement of cholinergic enzymes from the blood of psychiatric patients and investigation on the association with psychotic illness is decades-old practice (Randall & Jellinek, 1939; Plum, 1960; Lucas *et al.*, 1971) and showed cholinergic imbalance in certain psychotic disorders (Janowsky *et al*., 1972; Davis & Berger, 1978). Though no concrete conclusions could be drawn from these earlier studies, cholinergic implications in some psychiatric disorders are well endorsed. However, research has been chiefly diverted and limited to the AChE inhibitor-based treatment for dementia, Alzheimer's, and cognitive-related psychiatric problems in recent years. However, some of the recent studies (Ullas Kamath *et al.*, 2019) reported an increased level of RBC-AChE in moderate depressive patients. On the contrary, several epidemiological reports and reviews of exposure to

anticholinesterase pesticides found decreased AChE with depression and other neuropsychological disorders (Stallones and Beseler, 2016; Serrano-Medina *et al*., 2019; Suarez-Lopez *et al*., 2019; Guignet *et al*., 2020).

ACHE gene is highly conserved among populations, with most mutations being heterozygous and no homozygous loss of function mutations (Hasin *et al*., 2005; Lockridge *et al.*, 2016). One of the most prominent *ACHE* polymorphisms is a missense mutation (His353Asn) that defines the Cartwright Yt blood group antigen. This variation does not affect the enzyme's catalytic activity but decreases its half-life by 38% (Lockridge *et al*., 2016). The acetylcholinesterase function can be altered by coding and non-coding variations in *the ACHE* gene. Notably, variants located within the 3′ UTR of *the ACHE* gene can change recognition sequences of microRNAs, affecting their binding efficiency. MicroRNAs are non-coding small RNA molecules that regulate post-transcriptional gene silencing in several molecular pathways. Cholinergic signal pathways are subjected to multilevel control through the miRNA regulatory network. Any disturbance in the interaction of miRNA/cholinergic transcripts dynamics might upset the regulatory network and pave the way for developing disorders such as psychiatric conditions (Simchovitz *et al*., 2017).

The present study aimed to find the status of acetylcholinesterase in the blood of different psychiatric groups compared to healthy non-psychiatric individuals. In addition, to identify any potential association of 3′-UTR polymorphism in *ACHE* gene (rs17228602) with selected Psychiatric disorders in Pakistani patients. The basis of the selection of this SNP is its being located within a miRNA recognition site. The overall global minor allele frequency (T allele) for rs17228602 is reported to be 1%, and the highest MAF written for the European population is 5 % according to NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/snp/). The study will provide insight into the mechanism of psychiatric conditions and the basis for pharmacogenetics. Moreover, identifying an increased or decreased state of cholinergic enzymes in a specific mental illness may lead to novel treatment strategies.

2. Materials and Methods

2.1 Sampling of Study Subjects

Psychiatric patients for the study were recruited from rehabilitation centers and hospitals in the metropolitan areas of Rawalpindi and Islamabad. Ethical approval (CIIT/BIO/ERB/19/97) for the study was obtained from the ethics review board of Biosciences Department, COMSATS University Islamabad (CUI), and Islamabad, Pakistan. The study conformed to ethical guidelines postulated by tenets of the Declaration of Helsinki and CUI. Consent was taken from the participants. The Psychiatrists performed diagnoses of psychiatric conditions in the relevant clinics. If an actual psychiatric disorder could not be assigned to a psychiatric patient during the sampling period, they were classified as an undiagnosed psychiatric condition. The total number of recruited psychiatric individuals was 95.

The mean age of the cases was 34.32 ± 9.34, with 77 being males and 18 females. Most of the patients were males in our study, although females were not excluded. The smaller number of females can be attributed to our social and cultural stigma issues which prevent the females from seeking professional help. In addition, 130 healthy individuals were included in the study as control, of which 108 were males and 22 were females. The average age of rules was 35.26 ±9.37. The study included the healthy individuals after extensive face-to-face interviews confirming the absence of any mental illness history in them and their respective families. For case-control association study design, at statistical power of 80%, and significance level of 5% with minor allele frequencies of 15% and 7% in cases and controls, the expected sample size was estimated to be 239 cases and 239 controls, respectively (http://osse.bii.astar.edu.sg/calculation1.php. For the biochemical and molecular analysis, about three milliliters of venous blood sample was collected from every patient and controlled in EDTA-k vacutainers tubes. These vacutainer tubes with blood samples were stored at 4°C until further use.

2.3 Acetylcholinesterase Determination

Ellman's method, altered by Worek et al., 1999 was used for acetylcholinesterase determination in psychiatric and non-psychiatric controls. The procedure was carried out according to the process described by (Javed *et al*., 2019).

2.4 Primer designing and chemicals

For designing primers for the *ACHE* SNPs, Primer 3 version 0.4.0 software (http://bioinfo.ut.ee/primer 3-0.4. 0/primer3/) was used. In addition, NCBI Blast (https://www.ncbi. nlm.nih.gov/tools/primer-blast/) and UCSC genome browser In-silico-PCR tool (https://genome.ucsc.edu./ CGI-bin/hgpcr) were used to confirm the specificity of primers. Macrogen Inc (Rockville, MD, USA) made the primers.

2.5 Genomic DNA Extraction and SNP Genotyping

One ml of blood extracted the genomic DNA by the salting-out method (Lahiri *et al*., 1992). The DNA suspended in the TE buffer was stored at −20°C until further use. *ACHE* variant (rs17228602) genotyping was performed by PCR-RFLP method using primers (F: 5′-GAGGAGGAGAAAAGAATGACC-3, R:5′-TCCTCTAATGAGTGGTCGGAC-3′). PCR amplification was performed in 25µl reaction volume containing: 40ng/ µl genomic DNA, 1X Taq buffer, 2mM MgCl2, 2.5mM dNTPs (Fermentas, Thermo Scientific), 10pmol each of forward and reverse primers, and 5units Taq polymerase (Fermentas, Thermo Scientific). The thermal cycler condition was performed with an initial denaturation at 95^{o}C for 5min, followed by 36 cycles: denaturation for 1 min at 95^{o}C, annealing at 62^{o}C for 1min, extension at 72^{o}C for 1 min, and final extension at 72^{o}C for 7min.

Restriction assay of PCR products by enzyme Psp5II (PpuMI) (Catalog # ER0761, Thermofisher Scientific) was performed by incubating at 37°C for 16h. Fragments of 224bp and 141bp size are produced when Psp5II cleaves in a significant C allele, while 365bp element

remains uncut in the presence of T allele. 3% agarose gel was used to visualize the restriction products (Figure 1).

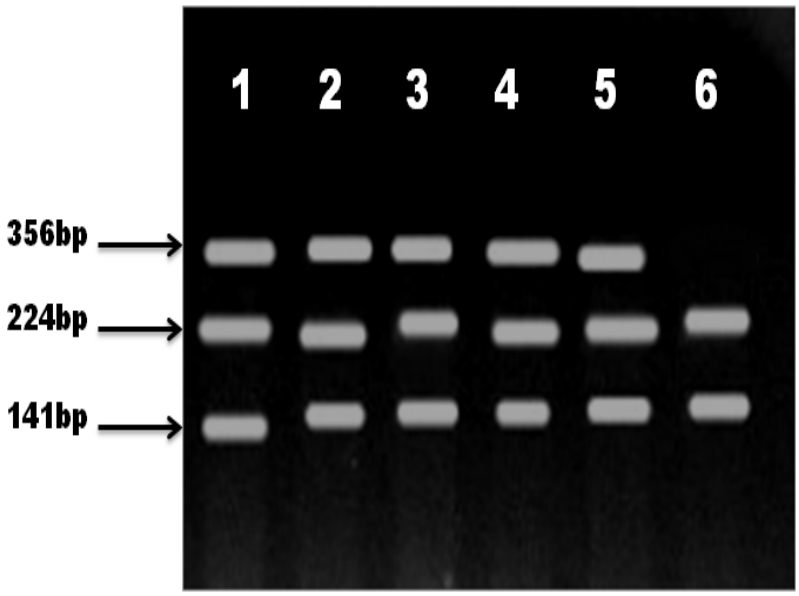

Fig. 1. RFLP image of *ACHE* rs17228602. Lane 1-5 shows three fragments of 356bp, 224bp, and 141bp containing heterozygous CT genotype. Lane 6 shows two fragments of 224bp and 141bp containing homozygous CC genotype.

2.6 Statistical Analysis

To determine the significance of AChE, the Mann-Whitney order rank test was used between psychiatric and non-psychiatric groups. In addition, statistical significance of Alpha ≤ 0.05 was considered. The SPSS statistic software version 20.0 (IBM Corp., Armonk, NY, USA) was used for data analysis. Chi-square and Fisher exact tests analyzed genotypes and allelic frequencies between psychiatric and non-psychiatric subjects. To determine the association effect in different inheritance models, the odds ratio with 95% Confidence Interval for the SNP association was calculated. Genotype frequencies were evaluated for deviations from Hardy Weinberg Equilibrium (HWE) in psychiatric and non-psychiatric groups using the goodness of fit Chi-square test (http://www.had2know.com/ academics/hardy-Weinberg-equillibriumcalculator-2-alleles.html). GraphPad Prism 7.0 was used for genotype analysis.

3. Results

3.1 Acetylcholinesterase in Psychiatric and Non-psychiatric Groups

Overall, the AChE activity decreased in psychiatric patients compared to healthy controls (Figure 2). Significantly low mean AChE was noted in schizophrenia patients 0.195 µmol/l/min, MDD 0.237µmol/l/min followed by undiagnosed psychiatric patients 0.241µmol/l/min and drug-induced psychosis; 0.316µmol/l/min (Table 1).

Table 1. Acetylcholinesterase activity in psychiatric and non-psychiatric groups

Groups	N	Mean±SEM (µmol/l/min)	SD	95% CI	Significance (p ≤0.05)
Psychiatric (overall)	94	0.274±0.017	0.168	0.239-0.308	0.000
Drug-induced Psychosis	40	0.316±0.031	0.197	0.252 - 0.378	0.057
Bipolar Disorder	7	0.311±0.042	0.113	0.207 - 0.415	0.384
Major Depressive Disorder	10	0.237±0.039	0.124	0.148 - 0.326	0.027
Undiagnosed Psychiatric condition	29	0.241±0.025	0.135	0.189 -.0291	0.001
Schizophrenia	8	0.195±0.061	0.173	0.050 - 0.338	0.015
Non-psychiatric	130	0.389± 0.019	0.224	0.350 - 0.428	-

SEM Standard error of the mean, SD Standard deviation, 95%CI Confidence Interval

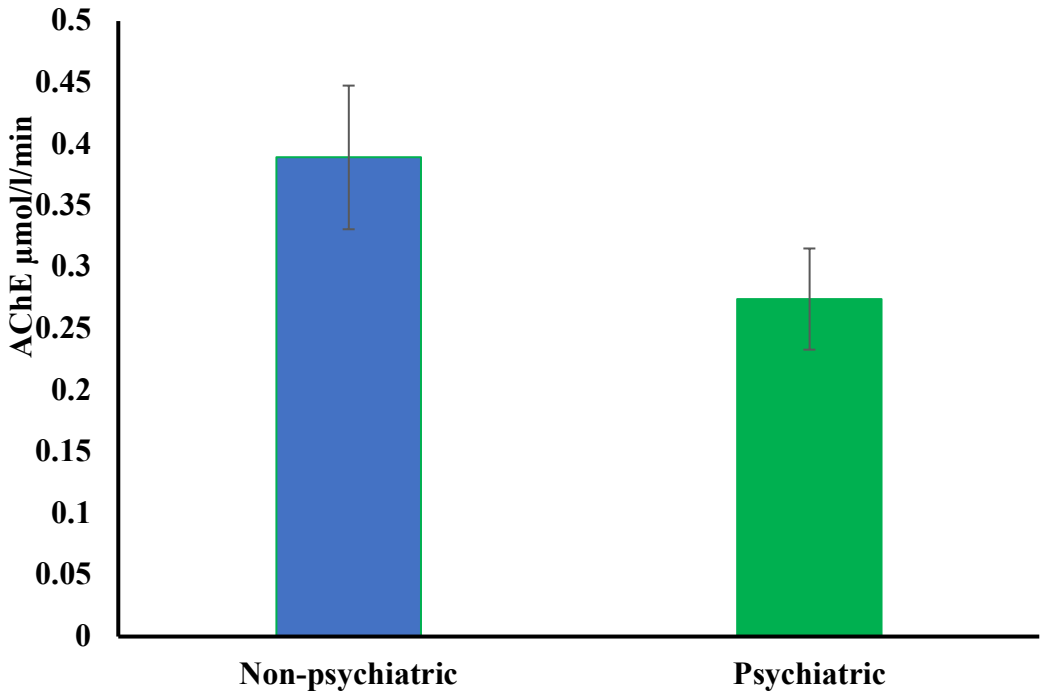

Fig. 2. Distribution of Acetylcholinesterase activity in non-psychiatric and Psychiatric affected Subjects.

3.2 Association Analysis of *ACHE* Polymorphism rs1722860

The genotype frequencies of *ACHE* SNPs rs17228602 were in concordance with HWE in both cases and controls ($\chi2$= 0.00117, p= 0.973; $\chi2$= 0.483, p= 0.487).

The genotype and allelic frequencies of *ACHE SNP* rs17228602 in patients and controls are summarized in Table 2 and Figure 3 to 6. Differences in genotype and allele frequencies of rs17228602 between the cases and controls were significantly apparent ($\chi2$= 24.9, p= <0.0001; $\chi2$=26.00, p <0.0001). The CT genotype was observed to be higher in patients than in healthy controls (37.85% vs. 13%), while the TT genotype was present only in patients (6.31%) (Table 2), respectively. The allele frequencies of major allele C were 74.73% in cases and 93.5% in controls, whereas the minor allele T frequencies were increased in cases (25.26%) compared to HC (6.5%).

Overall, a statistical significant association was revealed for rs17228602 SNP with risk of Psychiatric condition in dominant and allelic modals (DM:OR=5.3, CI = 2.61 to 10.8 p=<0.0001; AM: OR= 4.86 , CI=2.54 to 9.32 , p= <0.0001) (Table 2).

Regarding the correlation of rs17228602 genotype with ACHE enzyme activity, no statistically significant relation was detected between (CT, TT) genotypes and enzyme activity in psychiatric patients (Figures 5, 6). A marked decreased enzyme activity was noted in patients with no distinct differences regarding any genotype compared to controls.

Table 2. Genotype and Allele frequencies of ACHE SNP rs17228602 in Psychiatric and Healthy Cohort

PC Psychiatric cases, HC Healthy control, OR Odd's Ratio, DM Dominant Model

Genotype	PC (95) n (%)	HC (100) n (%)	$\chi2$	OR (95% CI)	P-Value
CC	53(55.78)	87 (87)			
CT	36(37.89)	13 (13)	24.9		<0.0001
TT	6 (6.31)	0 (0)			
DM: CT+TT vs. CC			23.4	5.3 (2.61 to 10.8)	<0.0001
Allele					
C	142(74.73)	187(93.5)	26.00	4.86 (2.54 to 9.32)	<0.0001
T	48(25.26)	13(6.5)			

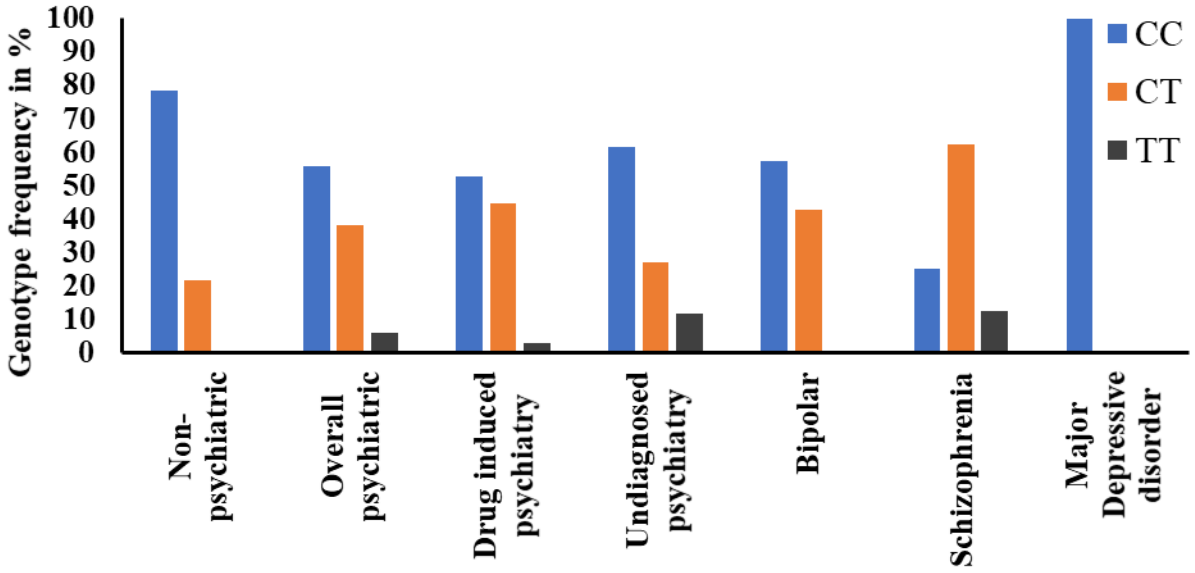

Fig. 3. Genotype frequency in non-psychiatric and different psychiatric cohorts. Variation trends in psychiatric groups may be noted.

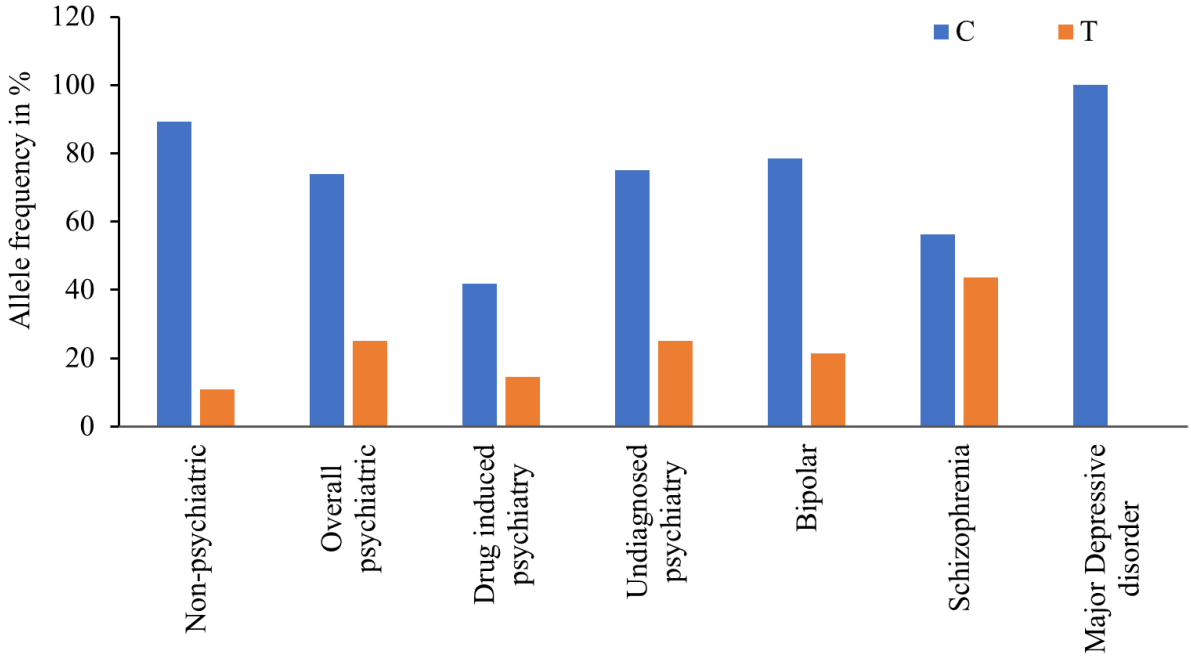

Fig. 4. Allelic frequency in different Psychiatric cohorts. Variation trends in allelic frequency may be observed in other groups.

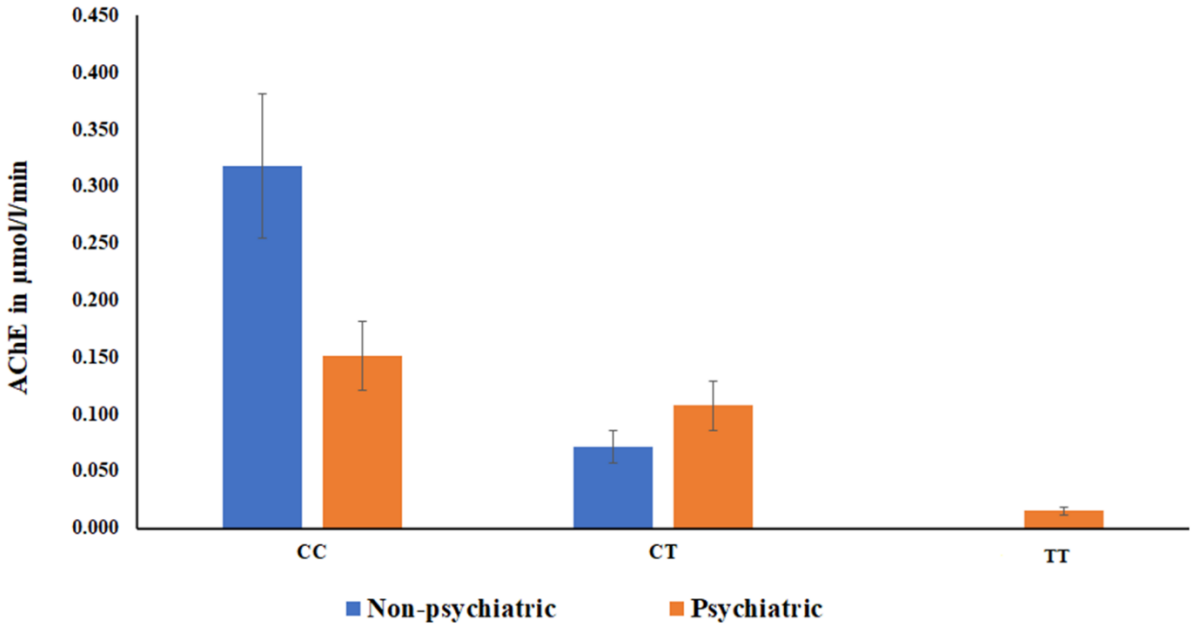

Fig. 5. Distribution of Acetylcholinesterase activity with regards to genotypes in Psychiatric Cohort.

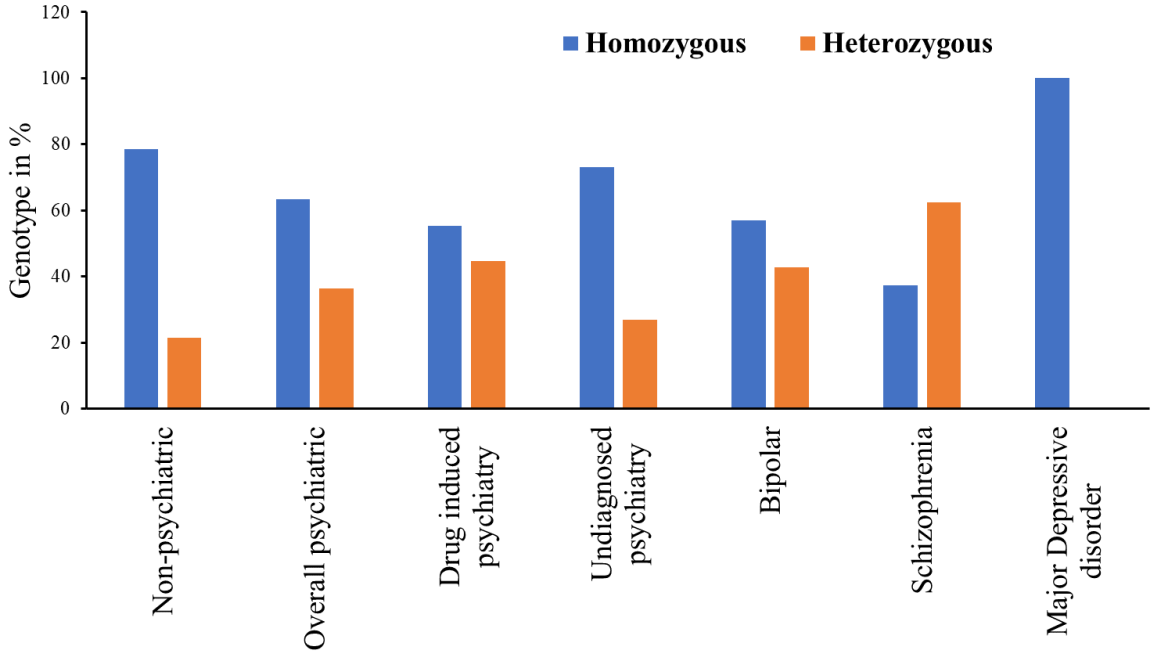

Fig. 6. Distribution of Homozygous and Heterozygous Genotypes in Patients of different Psychiatric conditions.

4. Discussion

Due to genetic and environmental factors (Sullivan & Geschwind, 2019). Acetylcholine (ACh) signaling is involved in diverse cognitive functions and suppresses inflammation (Hori, 2017). AChE, an acetylcholine hydrolyzing enzyme, directly relates to the activity of ACh. Aberrations in ACh signaling contribute to cognitive dysfunction and increase inflammation, involved in different neurological disorders (Simchovitz *et al*., 2017). The possible role of the dysregulated cholinergic system and AChE enzyme levels in the blood of psychiatric patients have been attributed for more than three decades (Plum, 1960; Lucas *et al*., 1971; Davis & Berger, 1978; Davis, 1979), but no conclusive association could be established yet. However, a literature search reveals a research gap on this aspect of the study, though the involvement of the cholinergic system, directly or indirectly, in psychiatric conditions has been established (Fritze & Beckmann, 1987; Dulawa & Janowsky, 2019).

In the present study, the status of AChE in different psychiatric groups was assessed and attempted to find an association with *ACHE* SNP rs17228602 with a predisposition to psychiatric disorder. Our finding shows that AChE was decreased in all psychiatric patients. The results are comparable with the previous studies of different psychiatric conditions. For instance, decreased RBC-AChE were found in patients with depression and Schizophrenia but not in mania by Jope *et al.* (1985). Low AChE activity levels were reported in adolescents with depressive symptoms (Suarez-Lopez *et al*., 2019) and in MDD patients with suicidal tendencies exposed to pesticides (Altinyazar *et al*., 2016). AChE levels were found to decline in blood and brain under stress (Chakrabarty *et al*., 2012).

Additionally, acetylcholinesterase inhibitors like Physostigmine have been shown to increase depression and anxiety symptoms in humans (Risch *et al*., 1981). Then, Mineur *et al.*, 2013 noted that Physostigmine induced anxiety and depressive behaviors in mice were reversed by administration of acetylcholine receptor antagonists (both nicotinic and muscarinic). AChE is the primary enzyme that regulates acetylcholine (ACh) levels in the extracellular space; hence low AChE leads to decrease hydrolysis and elevation of ACh, resulting in overstimulation of ACh receptor signaling (Mineur *et al*., 2013; Higley & Picciotto, 2014), perturbing the delicate balance of cholinergic system dynamics in brain areas involved in psychiatric disorders. Taken together, the results point out that aberrant hyperactive ACh signaling has a role in the etiology of psychiatric illness.

Several gene SNPs other than *ACHE* have been reviewed and discussed for the association with the risk of psychiatric conditions (Szczepankiewicz, 2013; Shadrina *et al.*, 2018). However, despite the significance of the cholinergic system in mental disorders, few studies explore the association of *ACHE* gene variants with different neuropsychiatric disorders. Hence the present study was focused on the SNP of the *ACHE* gene (rs17228602). The SNP is present in the 3′-UTR region of *the ACHE* gene and contains a binding site for microRNA. MicroRNA network acts as a critical modulator of cholinergic signaling, thus maintaining different neuronal and non-neuronal functions (Soreq, 2015). SNPs present within miRNAs recognition sites can change miRNA/target

binding affinities affecting the expression of target transcripts. This altered binding can upset the multi-component miRNA regulatory networks and contribute to neurological pathologies, including psychiatric disorders.

Interestingly, we found a significant association of rs17228602 (CT genotype and minor T allele) with the risk of psychiatric condition. Recently, a notable association of this *ACHE* variant with vulnerability to drug addiction was identified (Javed *et al*., 2019). However, in another study, no association was detected between this SNP and the risk of cannabis addiction (Furqan *et al*., 2020). Interestingly, Lin *et al*. (2016) studied another nearby located 3' UTR SNP (rs17228616) that resided within miRNA-608 recognition site in connection with effects on posttraumatic stress disorder and proved interrupted miRNA-608- AChE interaction to be involved in stress linked neural reactions.

The *ACHE* rs17228602 SNP is found within the binding sequence of miR-125b. The presence of minor rs17228602T allele within the recognition site weakens the interaction between miR-125b and ACHE transcripts. Soluble AChE-R and vesicular ACh are both predicted targets of miR-125b, and their suppression will result in decreased ACh hydrolysis and inversely diminish ACh production. Thus miR-125b exerts a bidirectional regulatory control on ACh signaling (Nadorp and Soreq, 2014; Simchovitz *et al.*, 2017). Defects in the interaction of miR-125b/ACHE transcript due to the presence of T variant may dysregulate cholinergic signaling, alter the homeostatic balance of cholinergic pathways, and produce complex pathological consequences. This variant will prevent the binding of miR-125b with ACHE transcripts causing free miR-125b molecules to bind with other free transcripts targeted by this miRNA, thus decreasing their levels (Hanin *et al.*, 2014) altering neurological pathways and synaptic plasticity.

Moreover, stress-induced cognitive deficits have been observed in mice with an absent 3′-UTR sequence in *ACHE* (Shaltiel *et al*., 2013). We understand that the association of rs17228602 minor T allele with susceptibility to selected psychiatric disorders, studied here, has not been reported earlier. Despite some limitations like smaller sample size, non-homogenous samples, and unavailability of data for the undergoing treatment of recruited patients, further studies with overcoming the limitations may provide a different concrete conclusion for the causative role of rs17228602 SNP and miR-125b/ACHE interaction on cholinergic signaling in etiology of psychiatric disorders.

5. Conclusion

The present study identified a decreased AChE activity in psychiatric cases compared to healthy individuals. In addition, a statistically significant association of minor allele and genotype of *ACHE* 3'-UTR SNP (rs 17228602) was noted with the studied psychiatric disorders. Therefore, further studies with a larger sample size focusing on other *ACHE* gene SNPs and SNPs in another component of the cholinergic system are also recommended to detect genetic influences of the cholinergic system and its implication in its pathogenesis and therapeutic potential in interventions for psychiatric disorders.

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Conflict of interest

The authors declare no conflict of interest of any kind.

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