ORIGINAL RESEARCH

Global DNA and RNA Methylation Signature in Response to Antipsychotic Treatment in First-Episode Schizophrenia Patients

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Background: Schizophrenia is a heterogeneous chronic psychiatric disorder influenced by genetic and environmental factors. Environmental factors can alter epigenetic marks, which regulate gene expression and cause an array of systemic changes. Several studies have demonstrated the association of epigenetic modulations in schizophrenia, which can influence clinical course, symptoms, and even treatment. Based on this, we have examined the global DNA methylation patterns, namely the 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC); and the global RNA modification N6-methyladenosine (m6A) RNA methylation status in peripheral blood cells. First-Episode Psychosis (FEP) patients who were diagnosed with Schizophrenia (SCZ) and undergoing treatment were stratified as Treatment-Responsive (TR) and Treatment-Non-Responsive (TNR). Age- and sex-matched healthy subjects served as controls.

Results: The methylation pattern of 5mC and 5hmC showed significant increases in patients in comparison to controls. Further, when patients were classified based on their response to treatment, there was a statistically significant increase in methylation patterns in the treatment non-responder group. 5fC and m6A levels did not show any statistical significance across the groups. Further, gender-based stratification did not yield any significant difference for the markers.

Conclusion: The study highlights the increased global methylation pattern in SCZ patients and a significant difference between the TR versus TNR groups. Global 5mC and 5hmC epigenetic marks suggest their potential roles in schizophrenia pathology, and also in the treatment response to antipsychotics. Since not many studies were available on the treatment response, further validation and the use of more sensitive techniques to study methylation status could unravel the potential of these epigenetic modifications as biomarkers for SCZ as well as distinguishing the antipsychotic treatment response in patients.

Keywords: schizophrenia, global methylation, treatment response, 5mC, 5hmC, m6A

Background

The flow of genetic information is modulated by several regulatory components that ensure homeostasis of the biological system. In addition to this regulatory network, several heritable chemical modifications impact gene expression without altering the DNA sequence, forming the basis of epigenetics.[1–4] The epigenetic marks/signatures primarily include nucleic acid methylation, nucleosomal histone modifications, chromatin remodelling, and non-coding RNA regulation.[2,3,5] These epigenetic pathways, which can be influenced by environmental factors, operate independently to regulate the genome. Several studies have depicted the impacts of early life epigenetic stressors on different aspects of the central nervous system (CNS), viz. neurodevelopment, signalling and plasticity, regeneration, and higher-order

functions of behaviour, memory, and cognition. However, the underlying molecular mechanisms linking epigenetic alterations and brain functions are yet to be completely discerned. Also, epigenetic changes as associative factors providing an interface between genotype and phenotype, for the development of complex neuropsychiatric disorders, are not understood completely.[6,7]

Schizophrenia (SCZ) is one such chronic psychiatric disorder with a complex multimodal etiology, and affects an estimated 20 million people globally.[8] This heterogeneous debilitating disease is characterized by positive symptoms such as delusion and hallucinations, and negative symptoms such as anhedonia, apathy, and cognitive impairment.[6] Although lacking a clear etiology, familial studies on twins, adopted individuals, and genetic studies including genome wide association studies (GWAS) suggest that SCZ exhibits an intricate heritability and polygenic nature, with variable effect size leading to variations in the severity of symptoms across patients.[5,7,9] Several lines of evidences point to an interplay between multiple factors that could increase the risk of development and progression of SCZ, including susceptibility genes with de novo mutations, exposure to environmental perturbations during prenatal neurodevelopment, and dysregulation of different epigenetic mechanisms.[6,7]

Antipsychotics are provided as the first-line treatment for the disorder, and to date are the only effective means of treatment. It is interesting to note that, although antipsychotics provide signs of improvement in many patients, others show significant resistance to them. Moreover, they may compromisethe social and/or cognitive functioning of subjects, indicating the need for a thorough understanding of the pathogenic mechanisms of SCZ to discover suitable bio-targets and device optimal therapeutic drugs against them.[5,7,10] Though the influence of polymorphic genes in the inter-individual drug response is well established in many diseases, the recent pharmaco-epigenetics (omics) studies indicate the influences of epigenetic markers on the responses of therapeutic drugs. These include the alteration of epigenetic modifications in response to drugs as well as the effect of drugs in modulating epigenetic markers that could address the interpersonal variations among clinical subjects in response to antipsychotic drugs.[1,5,11–13]

In this regard, antipsychotics such as clozapine and haloperidol have been shown to target either directly or indirectly, key epigenetic mechanisms such as DNA methylation, histone modification, and non-coding RNA expression.[7,12,13] Of these, DNA methylation which involves the addition of a methyl group via methyltransferases is a crucial regulator of gene expression/silencing, influencing the development and progression of a plethora of diseases, including SCZ.[1,7,14] Interestingly, stressful experiences from external or internal stimuli have been shown to alter the DNA methylation patterns globally as well as at the levels of specific genes known to be critical for brain plasticity and behaviour.[4] Several antipsychotics affect DNA methylation machinery and alter epigenetic homeostasis by inducing DNA methylation changes.[5,13] These alterations in DNA methylation patterns, which can be conveniently assessed in peripheral blood, influence the pathology and drug response of psychiatric disorders,[12] suggesting that DNA methylation could be a predictor for antipsychotic drug response in SCZ patients.

DNA methylation involves the covalent addition of methyl group at the 5'position of cytosine mediated by DNA methyltransferases (DNMT1, DNMT3A, DNMT3B), resulting in the formation of 5-methylcytosine (5mC). In addition to the DNA methyltransferases, the methylation pattern is controlled by other proteins such as methyl CpG-binding proteins (Methyl CpG binding Protein 2; MECP2), Methyl-CpG Binding Domain Protein 2; MBD2), and Ten-Eleven Translocations (TET1, TET2, TET3). For instance, sequential oxidation of 5mC via TET enzymes leads to the formation of demethylated products such as 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and then 5-carboxylcytosine (5caC), respectively.[15,16]

One of the earlier studies in the peripheral blood samples by Kinoshita et al,[17] assessed the whole-genome DNA methylation signatures of drug-naïve first-episode-psychosis (FEP) patients using microarray. Parallelly, they also studied and compared the DNA methylation pattern in monozygotic twins discordant for schizophrenia. The study identified dysregulation in methylation patterns in FEP patients compared to control. A similar whole-genome DNA methylation study also observed site-specific, especially CpG island abundant dysregulation in methylation patterns between FEP and control.[18] Whole-genome DNA methylation patterns were assessed in post-mortem cortical brain tissues of SCZ patients in comparison with controls which exhibited dysregulation in various regions, although it did not yield significant distinguishing methylation marks.[19] Murata et al[14] observed that there was a significant decrease in DNA methylation

levels in the peripheral blood samples of FEP patients compared to controls. In contrast, a study reported no significant difference in the LINE-1 DNA methylation level in FEP patients in comparison to controls.[20]

Analogous to DNA modifications, transcribed RNA also undergoes diverse post-transcriptional modifications, the most predominant being RNA methylation carried out by RNA methyltransferases. N7-methylguanosine (m7G), 2′-O-methylation (Nm), 5-methylcytosine (m5C), N1-methyladenosine (m1A), N6-methyladenosine (m6A) and 3-methylcytidine (m3C) are some of the commonly reported RNA methylation modifications which impact translation efficiency, mRNA metabolism, RNA stability and so on. Such alterations in epitranscriptome constitute a new pathway of gene regulation in the pathogenesis of psychiatric disorders.[21,22]

Among the epitranscriptomic alterations, m6A RNA methylation, which adds a methyl group to the sixth position of the adenine residues in RNA, is of great interest owing to its abundance and its reversible nature, the latter of which is not observed in most other covalent modifications in RNA bases.[3,23] The reversible regulation of m6A depends on the combined action of methyltransferases such as METTL3, METTL14, Wilms tumor 1-associating protein, and demethy-lases like AlkB homolog 5 and FTO.[24] Being present in all classes of RNA and possessing flexible reversibility, m6A epitranscriptomic modification has emerged as a significant modulator of post-transcriptional gene regulation. Moreover, emerging evidences suggest high abundance of m6A in the brain, indicating its role in the development and function of the nervous system, including in behaviour and learning and memory.[3,24] Reports in recent years have studied the role of m6A in neuropsychiatric disorders including SCZ and the results indicate that m6A impacts key genes such as *ALKBH5* involved in neuronal plasticity.[25–27]

Given the above rationale, epigenetic dynamics can be a key player in the cause and treatment response to antipsychotic in SCZ. Through the present study, we have examined the global DNA methylation pattern, namely the 5mC, 5hmC, 5fC, and global RNA modification m6A RNA methylation in the peripheral blood cells of patients with SCZ to understand their role in the treatment response to atypical antipsychotic drugs.

Methods
Subject Groups
All the participants in the study were recruited from the Schizophrenia Research Foundation (SCARF), a not-for-profit, tertiary care mental health service center in Chennai, India, based on International Classification of Diseases, Tenth Revision (ICD-10) guidelines. The inclusion criteria were as follows: (i) classification as first-episode psychosis upon the first psychotic symptoms; (ii) consensus diagnosis of SCZ based on the ICD 10 by experienced psychiatrists; (iii) ages between 18 and 60 years; and (iv) inclusions of both genders, while the exclusion criteria were as follows: (i) age <18 years; (ii) chronic SCZ cases; (iii) inability to give written informed consent; (iv) psychosis secondary to substance use; (v) severe mental retardation; (vi) history of significant head injury; and (vii) any history/presence of significant neurological disorder such as epilepsy.

After diagnosis, which was based on detailed medical history assessment, clinical and psychiatric examinations, the severity of the illness, social and occupational functions were assessed by appropriate standardized scales. A comprehensive treatment plan was devised by psychiatrists based on the standard treatment guidelines. In brief, a biopsychosocial model of treatment plan was devised for all participants with SCZ. All FEP participants were treated with second generation antipsychotic medications such as risperidone or olanzapine as the first choice of medications. The treatment responsiveness in individuals with SCZ was defined according to the Remission in Schizophrenia Working Group criteria for treatment remission.[28] Participants responding to the treatment with antipsychotic medications (measured by >20% changes in the severity and functioning scales; with a score of ≤3 on all items of the Positive and Negative Syndrome Scale (PANSS)) were categorized as treatment responders (TR). Inadequate response to 2 different antipsychotics, (after ≥ 12 weeks for positive symptoms [2 trials of ≥ 6 weeks]), each taken with adequate dose and duration, was classified as treatment non-responders (TNR) to antipsychotics. TNR patients were treated with clozapine as alternative pharmacologic treatment along with other non-pharmacologic treatments.

Based on the criteria mentioned above, a total of 30 FEP patients who were under pharmacological treatment with second generation atypical antipsychotics were recruited for the study, which included 15 each of TR and

Table 1 Biodemographic Data of Subjects Recruited for the Study

Characteristic	Schizophrenia Patients (SCZ)	Healthy Control
Subject Recruited	30	11
Gender (M:F)	(16:14)	(5:6)
Mean age (years) ± S.D.	40.3±8.6	42.1±8.3

TNR clinical cases of SCZ. Eleven healthy control subjects were also recruited with written informed consent from the individuals. Demographics of the patients are provided in Table 1. Ethical approval for the study was obtained from the Institutional Ethics Committee of SCARF (Ref. No. SRF-CR/09/MAR-2020).

Collection and Extraction of Nucleic Acids from Blood Samples

Two mL of peripheral blood samples were collected in EDTA-coated vacutainers from the patients as well as healthy controls. DNA was extracted using a standard phenol-chloroform procedure.[29] Similarly, another 2 mL of peripheral blood samples were collected in heparin-coated vacutainers from the study subjects. About 650 µL each of these samples were cultured in 5 mL of HiKaryoXL™ RPMI Medium (Cat# AL165A) supplemented with L-glutamine, Foetal Bovine Serum (FBS), Phytohemagglutinin M (PHA-M), penicillin, streptomycin and sodium bicarbonate at 37°C for 67 hr for subsequent harvest. Total RNA was isolated from the harvested cells using the Trizol method.[30]

Quantification of Global DNA Methylation Markers

The global DNA methylation in the study subjects was quantified by determining the amount of 5mC using the Methyl Flash Global DNA Methylation 5mC ELISA Easy Kit (Epigentek Group, USA) as per the specifications of the manufacturer. Similarly, DNA demethylation intermediates were quantified by estimating 5hmC and 5fC levels using Methyl Flash Global DNA Hydroxymethylation 5hmC ELISA Easy Kit and Methyl Flash 5-Formylcytosine (5fC) DNA Quantification Kit (Epigentek Group, USA), respectively. 100 ng genomic DNA was used as input for each sample, and the colorimetric assessment was performed in duplicates on a microplate reader at 450 nm for estimating 5mC and 5hmC percentage while 300 ng of DNA was used as input for 5fC estimation. Following the assessment, a standard curve was generated by plotting the optical density (OD) values versus the known positive control (PC) concentration from which the slope was calculated through linear regression. The percentages of 5mC, 5hmC and 5fC were calculated using the formula mentioned below:

$$5mC \: or \: 5hmC \: or \: 5fC \, \% = \frac{Sample \: OD - NC \: OD}{Slope \times S} \times 100 \, \%$$

where NC is the Negative Control and S is the amount of input sample DNA in ng.

Quantification of RNA m6A Methylation

Global m6A methylation in total RNA was quantified by the EpiQuik m6A/mRNA Methylation Quantification Kit (Epigentek Group, USA) following the manufacturer's specifications and using 200 ng input in duplicates. The amount of m6A methylation was quantified calorimetrically at 450 nm on a microplate reader. The standard curve was generated, and calculations for m6A% were performed as mentioned above for the DNA methylation assays.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software Inc., CA, USA). Continuous variables were analysed using two-tailed Mann–Whitney U-test, or Kruskal–Wallis test, as appropriate. The correlation between 5mC%, 5hmC% and 5fC% was assessed using non-parametric Spearman correlation. Two-way ANOVA

was performed to observe gender-dependent variation. Statistical significance level was set at p < 0.05 for all the analyses.

Results

Increase in DNA Methylation in Patients, Predominantly in TNRs

The levels of 5mC, 5hmC, and 5fC were quantified in the genomic DNA obtained from the peripheral blood samples of SCZ patients and healthy controls (Figure 1A–C). The levels of 5mC DNA methylation and its oxidized derivative, 5hmC DNA were significantly elevated in the SCZ group over the control group (p-value of 0.0094 and 0.0114, respectively). However, no significant differences in the levels of 5fC could be detected because of their low levels in our study subjects. 5mC, 5hmC, and 5fC levels were further compared between the TR and TNR subjects, and the controls (Figure 1D–F). 5mC and 5hmC levels were higher for the TNR group compared to the TR group, and least in controls (Kruskal Wallis Test, p-value of 0.0322 and 0.03, respectively). There was no significant difference in the levels of 5fC among the three groups (p-value 0.702). Benjamini, Krieger and Yekutieli's false discovery rate of multiple comparison tests shows significant differences amongst TR vs control, and TNR vs control for 5-mC (p-value of 0.0440 and 0.0113, respectively). For 5hmC, p-value was 0.0686 for TR vs control; while comparative tests between TNR and control groups showed significant differences with a p-value of 0.0088.

m6A RNA Methylation

The m6A RNA methylation levels were quantified for RNA isolated from peripheral blood samples of SCZ patients and controls (Figure 2A). No statistically significant differences were observed between the SCZ and control groups. The level of m6A RNA methylation was also compared amongst the TR, TNR, and control groups (Figure 2B). The level of m6A RNA methylation was slightly elevated in the TNR group, compared to TR and control; however, the difference
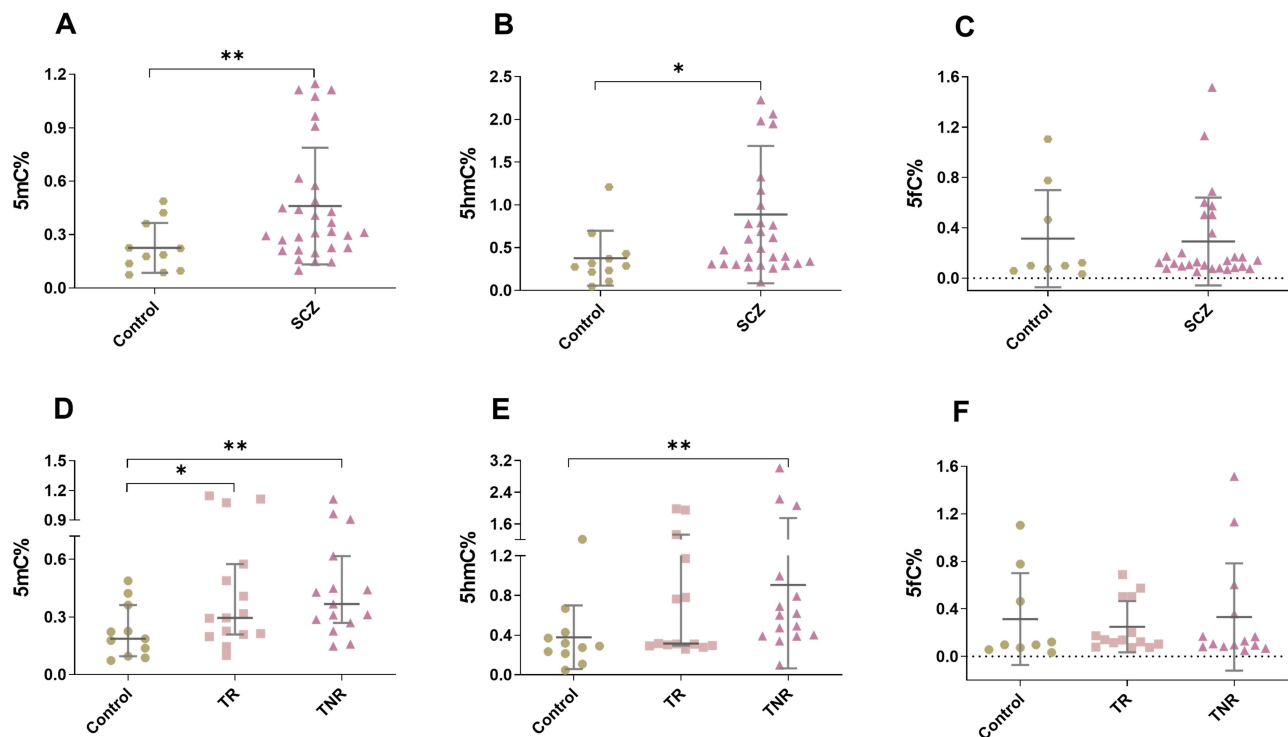
Figure 1 Percentage of DNA methylation markers among various groups. Levels of (**A**) 5-methylcytosine (5mC), (**B**) 5-hydroxymethylcytosine (5hmC), and (**C**) 5-formylcytosine (5fC) in SCZ and healthy control. Levels of (**D**) 5-methylcytosine (5mC), (**E**) 5-hydroxymethylcytosine (5hmC), and (**F**) 5-formylcytosine (5fC) in Treatment-Responders (TR), Treatment Non-Responders (TNR) and healthy controls. (Graph represented as Mean ± SD; *p < 0.05; **p < 0.01).

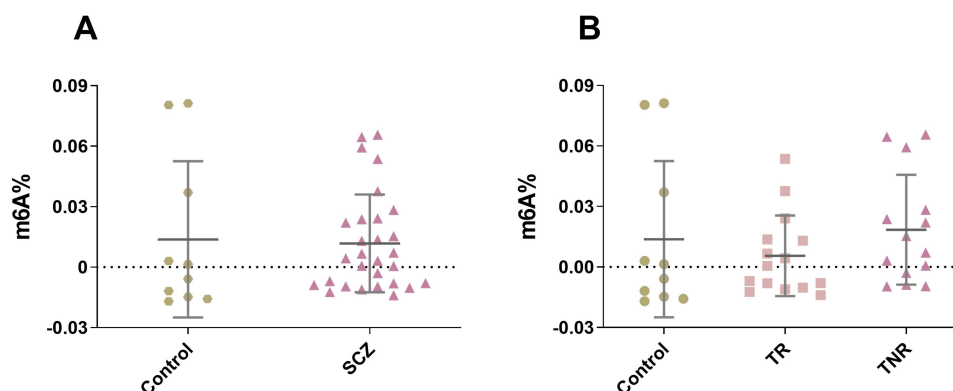**Figure 2** Percentage of m6A RNA modification markers among various groups. Levels of N6-methyladenosine (m6A) RNA methylation, (**A**) in SCZ and healthy control and (**B**) in Treatment-Responders (TR), Treatment Non-Responders (TNR) and healthy control. (Graph represented as Mean ± SD).

remained statistically non-significant. It should be noted that the levels of m6A was too low in many of the study subjects.

Significant Correlation Between 5-mC and 5-hmC Levels in Schizophrenia Cases

Since DNA demethylation is an active sequential process, the levels of 5hmC and 5fC depend on 5mC level, and the preferential activity of the ten eleven translocation (TET) family of enzymes.[31] Indeed, correlation analysis shows 5hmC is highly correlated with 5mC with a correlation coefficient (r) of 0.848 and a p-value of $3.3614e^{-009}$ in cases, compared to the control which attains a correlation coefficient (r) of 0.682 and a p-value of 0.025. The level of 5fC was negatively correlated with both 5mC (r = −0.241; p = 0.217) and 5hmC (r = −0.229; p = 0.24) in cases but was not statistically significant. However, a similar trend was not observed among controls.

No Gender Bias in 5mC and 5hmC Levels

To understand the gender bias in the 5mC, 5hmC among SCZ and control group, we stratified the samples and analysed the differences by a two-way ANOVA analysis. Both 5mC and 5hmC showed no significant difference among genders with a p-value of 0.8493 and 0.6304 (Figure 3). We did not perform the analyses for 5fC and m6A because of their low expression levels in our study cases and controls.

Discussion

In this study, we have determined the global DNA methylation 5mC, 5hmC and 5fC levels in the peripheral blood samples of first-episode psychosis patients stratified as TR and TNR following a treatment of second-generation antipsychotic medications and healthy controls using enzyme-linked immunosorbent assay. The global 5mC and 5hmC methylation levels were significantly higher in the FEP patients compared to the controls in this study. The same was not observed for 5fC levels.

Global methylation analysis in SCZ patients for 5mC and 5hmC has shown increased 5mC levels previously.[32] The study also showed that the 5hmC levels were higher in SCZ males compared to control cases, and lower than control among the female patients. One of the early studies by Shimabukuro et al reported lower methylation of peripheral blood DNA in male patients diagnosed with SCZ.[33] This is also supported in another study in the peripheral blood samples of FEP patients, who showed significant global hypomethylation compared to control.[14] A study on the methylation status in the frontal cortex of SCZ and autistic individuals has implied that the differential 5mC, 5hmC, and 5fC patterns influence proteins and enzymes involved in aerobic metabolism.[34] On the contrary, global DNA methylation studied in peripheral leukocytes did not yield any significant difference between SCZ patients and healthy controls. Interestingly, further analysis revealed that global DNA methylation was higher in non-smoking females compared to control females.[35] Although 5hmC and 5fC are DNA demethylation intermediates, studies have shown that 5hmC is comparatively more stable than its precursor DNA methylation
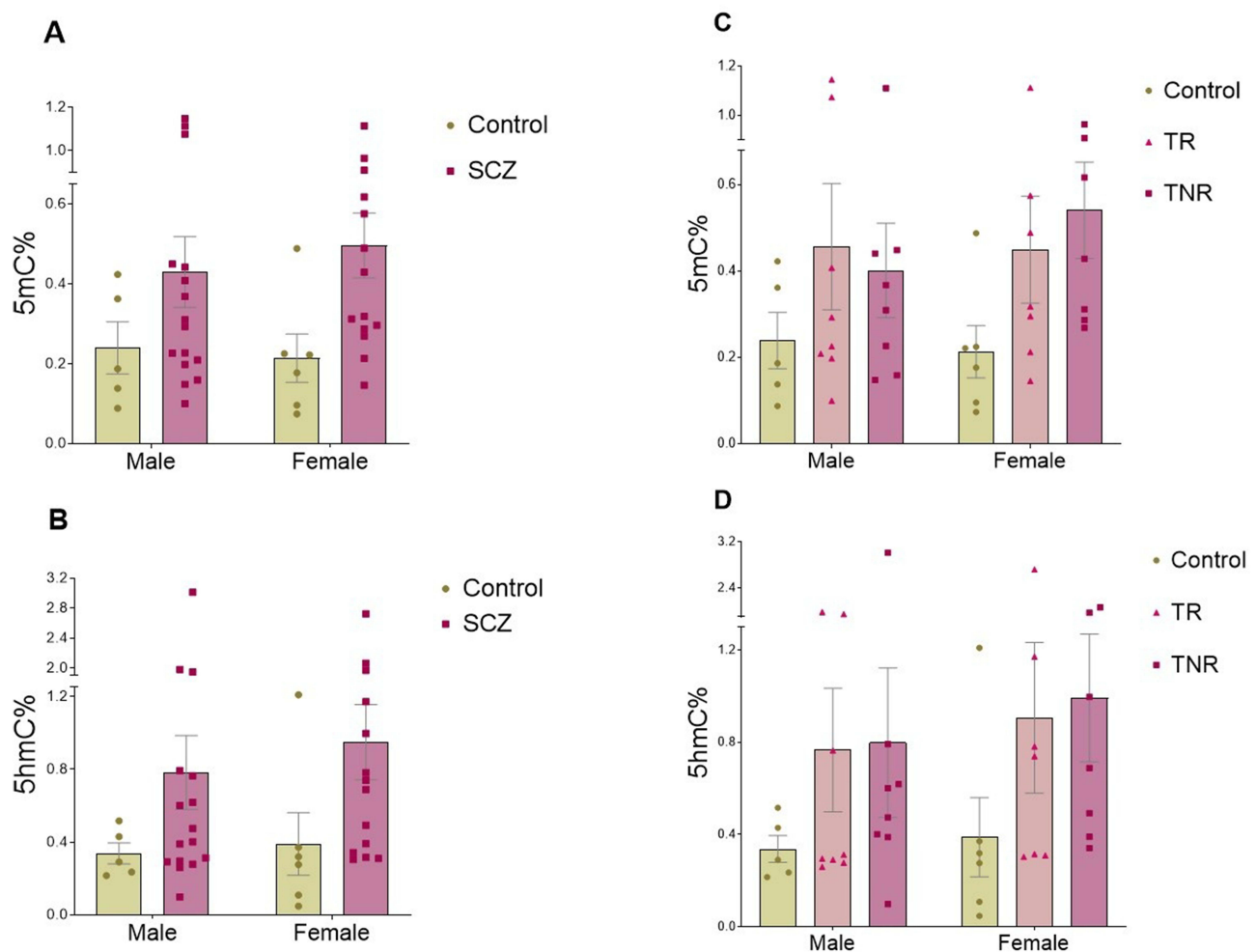
Figure 3 Gender-based stratification of methylation markers. Level of (**A**) 5-methylcytosine (5mC), (**B**) 5-hydroxymethylcytosine (5hmC), among SCZ and Control and (**C** and **D**) gender stratified among Control, TR, and TNR in 5-methylcytosine and 5-hydroxymethylcytosine (5hmC) respectively. (Graph represented as Mean ± SEM).

mark, 5mC, and also plays an active role in transcription regulation. However, it has been observed that 5fC levels are generally low across tissues and cells since they tend to be oxidized and removed immediately to complete the demethylation process.[16] This could probably be the reason for the low insignificant levels of 5fC in the study subjects. However, till date there are no reports regarding the 5fC levels in SCZ cases, and further studies are required. This study is the first attempt to understand the global 5fC and m6A RNA methylation in SCZ cases and controls.

In this study, the FEP group who were on standard treatment for second-generation antipsychotic medications were stratified based on their response to treatment as TR and TNR. The 5mC, 5hmC, and 5fC levels were further analysed in the TR, TNR, and control groups. The levels of 5mC were significantly elevated in the TR and TNR groups compared to the control. Among the FEP group, 5mC level was higher among the TNR subjects compared to the TR group. Similar to the 5mC pattern of methylation, the level of 5hmC was elevated in the TNR group compared to the controls, with the highest level in the TNR clinical cases. There was no significant difference in the 5fC levels among the three groups.

Though no studies have specifically reported on the global methylation levels (5mC, 5hmC, and 5fC) with regard to the treatment response in SCZ, some whole-genome methylation studies have been reported. Rukova et al[36] observed dysregulation in the methylated regions of male SCZ patients upon receiving treatment with antipsychotic drugs. Blood methylomes in patients prior to treatment and 4-weeks post treatment with risperidone revealed that response to medication is influenced based on the region of methylation marks, and correspondingly, antipsychotics cause

dysregulation in the methylome.[37] Similarly, Marques et al[38] evaluated the LINE-1 DNA methylation levels in FEP patients prior to and post treatment with risperidone. It was observed that there was a significant LINE-1 hypomethylation in FEP cases compared to controls, and this influenced the response to risperidone treatment. On the contrary, the pilot study conducted by De Luca et al[39] did not observe any significant association between the genome-wide methylation mark and treatment resistance in patients; although they suggested a larger sample size with stringent variables could help distinguish the dysregulation in methylation pattern.

Although there have been very few region-specific studies on the role of m6A RNA methylation in neuro-psychiatric disorders and the transcriptome-wide profiling of m6A in brain tissue;[25,26] there is much to be studied on the actual mechanism and potential role in SCZ pathology. Our study is the first attempt on evaluating global m6A level in SCZ patients, however the low detection limit led to inconclusive results; further studies with better detection methods and larger samples could shed light on its impact in psychosis.

Conclusion

This study examined the DNA methylation, hydroxymethylation, and formylation, as well as m6A RNA methylation in patients diagnosed with SCZ and studied their association with their response to treatment with atypical antipsychotics. The methylation pattern of 5mC and 5hmC showed a significant increase in patients compared to control, and when patients were stratified as responders and non-responders to antipsychotics, there was a clear increase in the levels of 5mC and 5hmC in the non-responder group. This result along with the literature on the pharmaco-epigenetics and drug-induced methylation changes made us hypothesize the possible reversal/demethylation of global methylation in response to treatment in the responders. However, there is lack of reversal/demethylation of 5mC and 5hmC in TNR SCZ subjects to antipsychotics. The limitation of this study is the small sample size and also lack of baseline methylation of the patients before the treatment, which could have highlighted the significance of our results. Evaluation of baseline global methylation before treatment and after treatment will further enhance the possibility of using global methylation marks as candidate biomarkers to distinguish the treatment response in schizophrenia clinical cases.

Data Sharing Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

This study complies with the Declaration of Helsinki and was performed according to ethics committee approval. Ethical approval for the study was obtained from the Institutional Ethics Committee of Schizophrenia Research Foundation (Ref. No. SRF-CR/09/MAR-2020).

Consent for Publication

No personal details of patients (including individual details, images or videos) were used anywhere in this manuscript. Written informed consent to participate in the research study is obtained after explaining the study.

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

Disclosure

Ravi Sudesh was a DHR Young Scientist at Dr ALM PG Institute of Basic Medical Sciences, University of Madras, at the time the study was conducted. The authors declare that they have no competing interests in this work.

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