Published in final edited form as:
Epigenomics. 2012 June ; 4(3): 261–268. doi:10.2217/epi.12.25.

DNA methylation in schizophrenia subjects: gender and *MTHFR* 677C/T genotype differences

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Abstract

Aim—In schizophrenia, metabolic syndrome incidence is double that of the general population, with women having a higher incidence. Pharmacogenetically regulated folic acid may be related to this risk. DNA methylation and metabolic syndrome within this group has not been previously studied.

Methods—Metabolic syndrome was evaluated with fasting laboratory measurements, and dietary and lifestyle assessments. Methylation analysis used a peripheral sample for the LINE-1 assay. DNA was also genotyped for *MTHFR* 677C/T.

Results—This analysis included 133 subjects. We found a significant relationship between LINE-1 methylation, and an interaction between *MTHFR* and gender, controlling for serum folate ($p = 0.008$). Females with the 677TT genotype had the lowest methylation (56%) compared with the other groups (75%).

Conclusion—TT genotype females had the lowest methylation, which may explain metabolic syndrome gender differences in schizophrenia. Folate supplementation may be a suggested intervention within schizophrenia; however, additional work is required.

Keywords

females; folate; metabolic syndrome; methylation; *MTHFR*; schizophrenia

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

This project was supported by the NIMH (grant number R01 MH082784-01) and the NIH-NCCR, GCRC Program (grant number UL1RR024986), the Chemistry Core of the Michigan Diabetes Research and Training Center (grant number NIH5P60 DK 20572), and the Washtenaw Community Health Organization. None of these funding and support agencies had any further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
No writing assistance was utilized in the production of this manuscript.

Within schizophrenia, folate has begun to garner much attention in relation to negative symptoms, as well as metabolic syndrome risk [1–5]. The metabolism and availability of folate within the Aldo Met cycle is regulated through various pathways, one being pharmacogenetically regulated by *MTHFR* [6], where presence of the 1298A/C or 677C/T variant results in up to a 70% reduction in MTHFR activity. This perturbation to the Aldo Met cycle results in reduced formation of methyltetrahydrofolate from folate, which converts homocysteine to methionine and *S*-adenosyl methionine. In addition, in a separate part of this cycle, *COMT* converts methionine to *S*-adenyl methionine (SAM) and the *COMT* 158Val polymorphism codes for COMT activity that is 30–50% greater than the Met's [7]. The end point function of this Aldo Met cycle, in general, is the production of methyl groups, which are then used to methylate DNA, lipids and proteins [6]. Thus, key to the field of epigenetics and DNA methylation is the regulation of folate and homocysteine metabolism by these pharmacogenetic variants.

Pharmacogenetically, variants of both *MTHFR* and *COMT* have been implicated in the psychopathology seen within schizophrenia [2,8–10], as well as in the development of metabolic syndrome seen with atypical antipsychotic (AAP) use [3–5]. New research has found that for those with a serious mental illness, 30 years of life are lost, primarily due to cardiovascular disease compared with the general population [11,12]. Recent data from animals, as well as historical accounts from notable times of famine, suggest that dietary modulation related to folate may result in DNA hypomethylation, which may result in an increased obesity risk [13,14]. How perturbations within the Aldo Met cycle related to folate and homocysteine regulation affect DNA methylation have been studied previously [15]; however, how it ultimately affects metabolic syndrome risk within schizophrenia is unknown. Thus, the aim of this study was to examine global methylation within living schizophrenia subjects using a peripherally obtained DNA sample to determine differences related to race, folate status, lifestyle factors, AAP use and *MTHFR/COMT* genotype. In addition, we sought to examine the relationship between global DNA methylation and the occurrence of metabolic syndrome within subjects treated with AAPs.

Methods

Subjects

For this investigation, subjects were included from a previous pharmacogenetic study where a peripheral DNA sample had been obtained [3]. Subjects were included in the initial study if they met the inclusion and exclusion criteria.

The inclusion criteria included: males and females ranging in age from 18 to 90 years old; a Diagnostic and Statistical Manual Version 4 (DSM-IV) diagnosis of schizophrenia, schizophreniform disorder, or schizoaffective disorder; and treatment with an antipsychotic for at least the previous 6 months. The exclusion criteria included: inability to give informed consent (assessed using a brief questionnaire about study procedures) or unwillingness to participate; presence of any medical conditions or medications that could significantly affect changes in weight (e.g., cancer, HIV or their treatment); and a diagnosis of active substance abuse. Subjects meeting these criteria were then seen in the Michigan Clinical Research Unit (MI, USA) at the University of Michigan Hospitals and Clinics where they underwent informed consent and the study assessments. This study was approved by the University of Michigan (MI, USA) institutional review board and carried out in accordance with the Declaration of Helsinki (ClinicalTrials.gov identifier: NCT00815854) [101].

Clinical assessments

Following subject consent, physical and physiological function parameters including a physical exam, dietary questionnaire, cigarette smoking status and physical activity assessments were acquired. Prelaboratory assessment measures included a schizophrenia spectrum diagnosis via a structured clinical interview for DSM diagnoses and dietetic assessments of height, weight, BMI and hip and waist circumference. Blood pressure was measured along with fasting blood glucose, folate, B12, homocysteine, insulin, hemoglobin A1c and lipid (i.e., total cholesterol, triglycerides, high- and low-density lipoproteins) levels via blood samples. The blood samples were also used for the genetic and methylation analyses. Upon review of patient data, the status of metabolic syndrome of each patient was assessed using the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP-III) guidelines [16] of having three or more of the following: blood pressure ≥130/85 mmHg (or current antihypertensive treatment); fasting blood glucose ≥100 mg/dl (or current diabetic treatment); large waist circumference (men <40 inches, women <35 inches); high-density lipoprotein cholesterol (men <40 mg/dl, women <50 mg/dl) or triglycerides ≥150 mg/dl (or current hyperlipidemia treatment).

A medication history including over-the-counter and herbal supplement usage was collected via a questionnaire and review of subject records. Subjects receiving clozapine, olanzapine, risperidone, iloperidone, paliperidone or quetiapine were considered to be receiving an AAP. All antipsychotic drugs were standardized by converting dosage regimens to chlorpromazine equivalents based on a mg/kg basis (i.e., dose in chlorpromazine equivalents multiplied by the number of years used, divided by 100) [17]. Each subject was asked to quantify the number of cigarettes smoked per day and the amount of time they have smoked to obtain a pack-year history for each subject. Nonsmokers (defined as having no cigarette use within the past 12 months) were questioned on their past smoking history, including amount and duration of exposure.

A total activity score was computed based on a previously designed questionnaire [18]. Subjects reported their total ‘strenuous activity’ (e.g., jogging, aerobics, swimming and physical labor), ‘moderate activity’ (e.g., housework, light jogging, painting and so on) and ‘mild activity’ (e.g., walking and so on), in minutes per week and the amount of times engaged in such activity per week. A final score (in metabolic equivalent of task [MET]/min) was computed by multiplying the time for each activity by a metabolic equivalent score and summing the scores.

MTHFR & *COMT* genotyping

Genomic DNA was isolated from whole blood with the salt precipitation method [19]. Genotyping was carried out with Pyrosequencing™ Technology (Qiagen, Hilden, Germany). PCR primers were designed using Oligo 6 (MBI, CO, USA). Pyrosequencing primers were designed using Pyrosequencing SNP Primer Design Version 1.01 software [102]. Subjects were genotyped for the *MTHFR* 677C/T (rs1801133) variant, as well as the *COMT* Val158Met (rs4680) variant. For that assay, 45 PCR cycles were performed for reactions in a 20 μl volume with 1.5 mM Mg^{2+}. PCR products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide prior to Pyrosequencing. Briefly, Pyrosequencing is a primer-based extension method of sequencing that utilizes a four-enzyme process performed in a single well. Nucleotides are incorporated into the open 3′-DNA strand in which pyrophosphate is released and used in a sulfurylase reaction emitting ATP. The ATP is then used by luciferace, which is converted to oxyluciferin. Light is discharged as a result of the reaction and collected by a charge-coupled device camera. The light is assembled into a readable format and represented as peaks, commonly called

Pyrogram® (Qiagen) charts. Based on these Pyrograms, each DNA sample can be sequenced and then genotyped [20].

Global methylation assessment

In order to carry out the methylation analysis in living subjects, the peripherally obtained genomic DNA isolated from the study subjects was bisulfate-treated using the EZ DNA Methylation™ kit (Zymo research, CA, USA). Global genomic DNA methylation was analyzed using the LINE-1 sequencing assays for methylation via Pyrosequencing, which have been previously described [21]; briefly, bisulfate-treated DNA is amplified using a specific biotinylated primer pair. The amplified PCR product is then processed and analyzed using the Pyrosequencer methylation software [21].

Statistical analysis

Differences in LINE-1 methylation were examined based on race, gender, smoking status, AAP use and metabolic syndrome criteria using simple Student's t-tests, and analysis of variance for differences related to genotype. Linear regression was used to determine the relationship between global methylation and the dependent variable of metabolic syndrome, controlling for various factors such as age, race, AAP use, folate level and *MTHFR*/*COMT* genotypes and the interaction of gender and *MTHFR/COMT* genotypes. A p-value < 0.05 was predetermined as significant with the sample size used in this study. Statistical analysis was performed with JMP 9® (SAS, NC, USA) and values are reported as mean ± standard deviation.

Results

A total of 133 patients were included in the analyses within the study. Table 1 is a summary of the sample population demographics. While the mean age of our group was 46 years, the majority of subjects were male and racially categorized themselves as predominantly white/Caucasian, followed by black/African–American. A total of 46% of subjects meet criteria for metabolic syndrome, 71% of subjects were receiving an AAP and 58% currently smoked cigarettes. The mean serum folate concentration was 16 ng/ml, which is above the threshold of 3.0 ng/ml set for folate deficiency.

The *MTHFR* 677C/T and *COMT* 158Val/Met genotypes both were in Hardy–Weinberg equilibrium ($p > 0.3$) and there were no differences in either genotype based on gender ($\chi^2 = 0.17$; $p = 0.92$). Although in general, the *MTHFR* 677 T allele was more common in the Caucasian subjects (T-allele frequency = 0.45) compared with the African–American and other subjects (T-allele frequency = 0.23 and 0.37, respectively), there were no statistical differences ($\chi^2 = 3.9$; $p = 0.14$).

No differences were seen in global methylation percentage measured by the LINE-1 assay in relation to race, gender, smoking, AAP or metabolic syndrome status ($p > 0.1$ for all). For the *MTHFR* 677TT genotype group, a lower mean methylation was found (67 vs 74% for C-allele carriers); however, this was not statistically significant ($p = 0.1$). No differences in methylation were noted based on the *COMT* 158Val/Met genotype. Additional data are shown in Table 2.

Looking at our regression model for global methylation using the LINE-1 assay, both gender ($t = 2.88$; $p = 0.004$) and *MTHFR* 677C/T genotype ($t = 1.6$; $p = 0.11$) contributed to the significance of the whole model ($F[5,110] = 3.02$; $p = 0.01$). Furthermore, gender and the *MTHFR* genotype interacted to significantly affect LINE-1 methylation ($F[2,2] = 4.63$; $p = 0.01$). The group with the lowest global methylation was females who possessed the *MTHFR* 677TT genotype (standard error: 56.1% ± 5.5). Table 3 is a breakdown of global

methylation percentage based on gender and genotype. Given that dietary folate intake is an important cofactor in the Aldo Met cycle, as it relates to the *MTHFR* genotype and DNA methylation, we also examined LINE-1 global methylation based on gender and *MTHFR* genotype after controlling for serum folate levels, the interaction between gender and genotype still remained significant ($F[2,2] = 7.7$; $p = 0.008$). We did not find any relationship between LINE-1 methylation and any of the specific components of the metabolic syndrome (cholesterol, glucose, blood pressure or waist circumference); however, methylation was significantly associated with homocysteine ($F[1,3] = 4.1$; $p = 0.04$, correlation coefficient [r] = 0.10).

Discussion

Overall, the results of this investigation show few differences in global DNA methylation, as measured using a LINE-1 assay, related to our study population. Most interestingly, we did not find a significant relationship between the *MTHFR* genotype and global methylation; however, lower levels of global methylation were seen in those with the TT genotype. Given the role *MTHFR* plays in the Aldo Met cycle with regard to folic acid metabolism and methionine production, we expected to see greater differences in methylation in relation to this genotype.

When our methylation results were stratified by gender, overall, our female subjects showed a trend towards lower levels of global methylation, but this was not statistically significant. In looking at gender in relation to other variables associated with global methylation, our regression analysis showed that both the gender and the *MTHFR* genotype interacted, where females with the *MTHFR* 677TT genotype had the lowest overall levels of global methylation (Table 3). This relationship remained significant after controlling for serum folate levels. In looking at this relationship, we did postulate that some of the gender differences seen may be due to differences in *MTHFR* genotype distribution between the two groups. However, when this was more closely examined, no differences in the *MTHFR* genotype distribution were seen between males and females ($p = 0.91$).

Examining the literature, there is some work, also using the LINE-1 Pyrosequencing assay, which reports that in the general population, women have lower global methylation compared with men [22]. Overall, the authors found that in men, mean global methylation ± standard error was 75.0 ± 2.5% and in women, the mean methylation ± standard error was 73.2 ± 3%. In comparing these values with our group, similar yet greater gender differences were found. As outlined in Table 3, we observed significant differences in methylation based on *MTHFR* genotype and gender, some of which significantly depart from reports from the general population. In addition, as part of this investigation we found a positive relationship between homocysteine and LINE-1 methylation, which was not surprising, knowing homocysteine's role within the Aldo Met cycle.

While the cross-sectional design of this study prevents us from making definite causality statements regarding this relationship, the results are interesting given the previously reported relationship between metabolic syndrome and lower global methylation [14,22]. It should be noted that in general, women with schizophrenia are at the greatest risk for the development of metabolic syndrome. This was first reported by investigations examining data from the CATIE trial. According to a baseline investigation for CATIE, McEvoy and colleagues found that after controlling for age, race, ethnicity and body mass, men were 87%, and women were 137% more likely to have metabolic syndrome relative to the National Health and Nutrition Examination Survey matched controls [23]. The reason for this gender inequality has not been fully elucidated within the literature, and the differences in DNA methylation, which we report here, as well as the *MTHFR* genotype which we have

previously reported [4], may be contributing factors for this increased metabolic syndrome risk. Thus, targeted folate interventions in females with the *MTHFR* 677TT genotype may be a viable option for attenuation of metabolic syndrome; however, further investigation is needed.

The field of epigenetics is still in its infancy; however, research regarding how diet affects obesity, and *in utero* influences of diet on offspring has been longstanding [14]. Specifics regarding epigenetic risks for metabolic syndrome development are still unknown, and there is a paucity of data regarding the epigenetic influences at work within mental health, and especially schizophrenia.

The Dutch famine of the 1940s has provided a lot of insight into the effects of extreme starvation on long-term cardiovascular effects [24]. While many have closely examined this well-documented historical tragedy, it is the first report of how epigenetic influences may affect the worldwide occurrence of obesity. Complementary to much of the data related to this famine, animal studies have shown that administration of a methyl-supplemented diet that induces DNA hypermethylation during development, results in the prevention of body weight accumulation compared with animals at risk for obesity who were given a standard diet [13]. Given folate's role in DNA methylation and some of our pharmacogenetic work being done regarding risk of metabolic syndrome in those who have a reduced ability to effectively utilize dietary folate, folate supplementation within this high-risk population may be a viable intervention [3,4].

While folate is a primary focus for our research group, others have also examined the epigenetics of mental illness. The first group to report global methylation differences within mental illness was published by Dempster and colleagues [25]. By investigating epigenetic changes between monozygotic and dizygotic twins at risk for schizophrenia and bipolar disorder, this group was able to conclude that peripheral DNA methylation differences mediate the phenotype and etiology of these two mental illnesses, specifically citing hypomethylation within the promoter region of the gene encoding ST6GALNAC1 as being associated with schizophrenia. Unfortunately, this investigation did not examine gender-related methylation differences. However, as previously discussed, others within the epigenetics field have reported lower overall methylation within the female population, which would support the gender differences we saw within our schizophrenia population [22]. However, these investigators, did not find any differences in methylation related to body composition. For our data, we did not see any differences in folate status based on gender ($p = 0.56$); however, recent reports have shown that folate intake within women of child bearing age is still not within targeted ranges despite the mandatory dietary folate fortification [26]. Therefore, as suggested previously, folate supplementation within this population may provide a unique opportunity to address this cause-and-effect relationship with regard to global DNA methylation and metabolic syndrome within women with schizophrenia.

Limitations of this study

Perhaps the greatest limitation of this study is the relatively small sample size of 133 subjects, of which only 53 were female. In addition, our small number of females with the *MTHFR* 677TT genotype needs to be identified as a limitation; however, this group is within the estimated frequency of 10–15% for the *MTHFR* genotype. As stated in our discussion, the cross-sectional design without a control group of healthy individuals does not allow us to assume causality associated with the methylation differences we are reporting. In addition, we did not have the ability to determine the dietary habits of our study participants' mothers while pregnant, which is a standard assessment often seen in epigenetic studies

related to obesity development. Along the same lines, folate status is a reflection of short-term folate balance, and our data may not be representative of a subject's true average folate level. In our study, global DNA methylation was measured using one assay. Although several methods currently exist that allow for measures of global DNA methylation, consensus has not been reached as to the most appropriate method. Our study also utilized a peripherally obtained DNA sample for methylation analysis, which is different to most mental illness investigations examining methylation difference and disease risk. However, other investigations within the schizophrenia and healthy populations have shown that peripherally obtained DNA samples closely mimic a brain-originated sample, and as such, our samples should closely represent subject brain-tissue methylation [27,28]. However, there are limitations to these correlations and further validation is needed. For this investigation, we only examined one *MTHFR* genotype; however, previous work carried out by our group has examined other *MTHFR* variants (1298A/C) and has found strong linkage between these two [3,4]. Last, owing to the exploratory nature of this investigation we did not strictly control our level of significance to control for multiple testing. Regardless of these limitations, the results of this work are intriguing and deserve further targeted investigation with a larger sample.

Conclusion

In conclusion, while very few differences were found related to global DNA methylation in subjects with schizophrenia being treated with antipsychotics, we report an interesting interaction between gender, *MTHFR* 677C/T genotype and overall global methylation. The hypomethylation seen within the female *MTHFR* 677TT group may help to explain gender related differences seen within the schizophrenia population related to the occurrence of metabolic syndrome; however, additional research is needed to confirm these results. Folate supplementation, especially targeted in females with the *MTHFR* 677TT genotype, potentially before metabolic syndrome occurrence, may be suggested as a preventative intervention within schizophrenia; however, much more work within this area is required to support this conclusion.

Future perspective

The field of epigenetics is still in its infancy but is rapidly expanding. Within the next 5–10 years clinical practice may change to encompass a more global view of mental health to include not only the biology of the illnesses, but also the impact of the environment on the occurrence and treatment of psychiatric illness. Understanding the epigenetic relationship related to the occurrence of metabolic adverse events from medications is a first step within this direction.

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Executive summary

Background

- Currently, folic acid, its pharmacogenetically regulated metabolism and its potential relationship to DNA methylation, is of great interest to the field of psychiatry.
- For those with schizophrenia, up to 30 years of life are lost due to cardiovascular disease, and the risk of sudden cardiac death is three-times that compared with the general population.
- Having a baseline understanding of potential mechanisms behind these risks will allow for intervention development related to the prevention of this cardiovascular morbidity and mortality for those with mental illness.

Methods

- Global methylation was assessed by the LINE-1 assay using peripheral DNA.
- The *MTHFR* 677CT and *COMT* 158ValMet genotypes were determined by pyrosequencing.
- Each subject underwent medication, dietetic and metabolic syndrome assessments.

Results

- For this study, differences in global DNA methylation were found in relation to the *MTHFR* genotype and gender.
- Females with the *MTHFR* 677TT genotype had the lowest LINE-1 methylation (56%) compared with the other groups (72–79%).
- Levels of methylation seen within our female *MTHFR* 677TT group were lower than what has been reported in the general population and may be related to metabolic syndrome occurrence.

Conclusion

- Replication will be needed in other cohorts in order to confirm our findings.
- Use of folate as a targeted supplement may help to prevent or ameliorate some of the cardiovascular risk factors seen in schizophrenia patients, but more specifically for female subjects with the *MTHFR* 677TT genotype.
- Future work is needed to determine the appropriate dose, duration and efficacy of this proposed intervention.

Table 1

Summary of patient demographics (n = 133).

Demographics	Mean ± standard deviation
Mean age (years)	46.1 ± 11.1
Caucasian/African–American/other (%)	64/27/9
Males/females (%)	64/36
Mean plasma folate (ng/ml)	15.6 ± 6.56
Meeting metabolic syndrome criteria (%)	46
Currently receiving an atypical antipsychotic (%)	71
Antipsychotic chlorpromazine equivalents (mg)	381.29 ± 432.49
Current cigarette smokers (%)	58
Total activity level (METs/min)	2340 ± 2347
MTHFR 677C/T genotype, CC/CT/TT (%)	61/30/9
COMT 158Val/Met genotype, ValVal/ValMet/MetMet (%)	30/50/20

MET: Metabolic equivalent of task.

Table 2

Percentage LINE-1 methylation in relation to demographic variables.

Demographic	LINE-1 % (standard error)	p-value
Race		
Caucasian	71.0 (1.9)	0.24
African–American	76.1 (3.0)	
Other	77.7 (5.1)	
Gender		
Males	75.1 (1.4)	0.21
Females	72.1 (1.8)	
Smoking status		
Yes	74.1 (1.7)	0.97
No	74.0 (1.5)	
Atypical antipsychotic use		
Yes	74.4 (1.4)	0.56
No	73.0 (2.1)	
Metabolic syndrome		
Yes	74.5 (1.5)	0.60
No	73.3 (1.8)	
MTHFR *677C/T genotype*		
CC	73.7 (1.4)	0.11
CT	76.6 (2.0)	
TT	67.6 (3.9)	
COMT *158Val/Met gentoype*		
Val/Val	74.3 (2.5)	0.44
Val/Met	72.7 (1.6)	
Met/Met	76.1 (2.0)	

Table 3

LINE-1 methylation by gender and *MTHFR* 677C/T genotype.

***MTHFR* genotype (gender)**	**Males (% ± standard error)**	**Females (% ± standard error)**
CC (n = 49 males/32 females)	74.7 ± 1.8	71.8 ± 2.3
CT (n = 25 males/15 females)	75.2 ± 2.6	78.7 ± 3.3
TT (n = 6 males/6 females)	79.0 ± 5.4	56.1 ± 5.5

s.e.: Standard error.