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Association of Protein Kinase B (AKT) DNA Hypermethylation with Maintenance Atypical Antipsychotic Treatment in Patients with Bipolar Disorder

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Abstract

Study Objective—Atypical antipsychotics cause insulin resistance that leads to an increased risk of diabetes mellitus and cardiovascular disease. Skeletal muscle is the primary tissue for uptake of glucose, and its dysfunction is considered one of the primary defects in the development of insulin resistance. Protein kinase B (AKT) plays an important role in overall skeletal muscle health and glucose uptake into the muscle. The objective of this study was to measure *AKT* isoform–specific gene methylation differences in the skeletal muscle of patients with bipolar disorder treated with atypical antipsychotic or mood stabilizer maintenance therapy.

Design—Cross-sectional, observational study.

Setting—Clinical research services center at an academic center.

Patients—Thirty patients with a confirmed diagnosis of bipolar disorder who were treated with either an atypical antipsychotic (16 patients) or mood stabilizer (14 patients) at a consistent dose for at least 3 months.

Interventions—A fasting skeletal muscle biopsy was performed in the vastus lateralis in each patient. Patients also underwent fasting blood sample collection and a standard, 75-g oral glucose tolerance test.

Measurements and Main Results—Skeletal muscle DNA methylation near the promoter region for three genes—*AKT1, AKT2,* and *AKT3*—was measured by methylation-sensitive high-resolution melting. Gene methylation was analyzed based on atypical antipsychotic versus mood stabilizer maintenance therapy. Associations between gene methylation, insulin resistance, and

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glucose tolerance were also analyzed. In patients treated with atypical antipsychotics, *AKT1* and *AKT2* methylation was increased compared with patients treated with mood stabilizers (p=0.03 and p=0. 0.02, respectively). In addition, for patients receiving atypical antipsychotics, a positive trend for *AKT2* hypermethylation with increasing insulin resistance was observed, whereas for patients receiving mood stabilizers, a trend for decreased *AKT2* methylation with increasing insulin resistance was observed.

Conclusion—Overall, our findings suggest that the *AKT* gene is differentially methylated in the skeletal muscle of patients taking atypical antipsychotics or mood stabilizer maintenance therapy. These results may direct future approaches to reduce the harmful adverse effects of atypical antipsychotic treatment.

Keywords

antipsychotic; mood stabilizer; muscle; protein kinase B; AKT; epigenetics; bipolar

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Atypical antipsychotics (AAPs) are an effective treatment used for the management of symptoms in patients with psychiatric diseases such as schizophrenia and bipolar disorder. Their use is associated with an increased risk of cardiometabolic adverse effects including weight gain and insulin resistance (1). The insulin resistance observed in patients taking AAPs contributes to an increased risk of diabetes mellitus and metabolic syndrome (2, 3). It has been demonstrated that patients with severe mental illness (schizophrenia and bipolar disorder) have higher mortality rates compared to the general population due to cardiovascular disease that is partly related to AAP-induced insulin resistance and metabolic adverse effects (4–7). However, other factors may contribute to this increased risk such as lifestyle factors and dietary and hereditary factors.

Despite the epidemiologic evidence linking AAP-induced metabolic adverse effects such as insulin resistance to increased mortality, the mechanisms by which it occurs remain unknown. Previous work using blood from clinical populations has pointed to dysregulations in various molecular systems including genetic, epigenetic, and lipidomic (8–12). However, deriving mechanistic understandings of pharmacologic effects can be difficult when analyzing blood given its heterogeneous collection of cell types. Additionally, insulin resistance may be driven by dysfunction in tissues responsible for insulin-stimulated glucose uptake (13). Skeletal muscle is considered the primary insulin-responsive tissue, accounting for up to 80% of insulin-stimulated glucose uptake (14). Dysfunction in skeletal muscle glucose uptake is considered the primary defect in insulin resistance, thus serving as a primary candidate tissue for investigating the mechanisms of AAP-induced insulin resistance (15, 16).

Protein kinase B (AKT) is a serine/threonine kinase with a critical role in several biological processes throughout the body including cell migration, apoptosis, and glucose metabolism and transport (17). This wide range of function is dictated by three isoforms—protein kinase B α, β, and γ—that are encoded by three separate genes—*AKT1, AKT2*, and *AKT3*, respectively. Dysfunction in AKT and its pathways has been implicated in several disease states such as cancer and insulin resistance (18). Additionally, AKT has a hypothesized role in the pathogenesis of mental illness including both schizophrenia and bipolar disorder (19–

22). It is hypothesized that AAPs and other antipsychotics may exert some of their treatment effects by influencing the levels and/or function of AKT. Studies in PC12 cell lines, rodent central nervous system, human blood, and postmortem brain have demonstrated an effect of AAPs on AKT levels and/or activity (23–28). Other studies have suggested that AKT effects in the central nervous system may be due to hyperinsulinemia caused by AAPs peripherally (29). We hypothesized that AAPs may influence AKT in a tissue-dependent and isoform-specific manner. It may be that AAPs influence AKT, such that therapeutic effects are observed in one tissue (i.e., brain), but adverse effects occur in others (e.g., metabolic tissues). In support of this hypothesis, a study by England colleagues showed that AKT activity was disrupted with AAP treatment of L6 rat skeletal muscle cells, a commonly used cell model for human skeletal muscle (30). Despite this work implicating AAP effects on AKT in the skeletal muscle, to our knowledge, this has not been translated to humans.

The purpose of this study was to measure gene-specific methylation differences of *AKT1*, *AKT2*, and *AKT3* in an insulin target tissue (skeletal muscle) based on maintenance drug treatment in a sample of patients with bipolar disorder. This work may provide evidence for the effects of AAPs on AKT specifically in skeletal muscle, which can be used to direct future investigations into reducing AAP-induced adverse effects, morbidity, and mortality.

Methods

Clinical Population

Potential study patients were recruited from the greater Detroit, Michigan, area through public postings and invited to the Wayne State University Clinical Research Services Center (CRSC; Detroit, MI) to undergo full informed consent, screening, and associated study procedures. Patients were included if they had a diagnosis of bipolar disorder (I, II, or not otherwise specified [NOS]), verified by the Mini International Neuropsychiatric Interview (MINI) and performed by a trained research assistant (31). Patients also must have been treated with either an atypical antipsychotic or mood stabilizer at a consistent dose for at least 3 months. Patients were excluded if they had a known metabolic disturbance prior to starting their drug therapy, had diabetes, had an active substance abuse or dependence diagnosis (by history and urine dipstick), had an allergy to lidocaine, had a history of bleeding, or had a primary relative with diabetes, or if peripheral intravenous access could not be obtained. We defined maintenance therapy as the main psychiatric medication (i.e., AAP or mood stabilizer) used to manage psychiatric symptoms; therefore, patients were excluded if they were currently being treated with more than one antipsychotic and/or mood stabilizer in combination. All protocols and procedures were approved by the Wayne State University Institutional Review Board and were in accordance with the Declaration of Helsinki.

Clinical Assessments

Patients meeting inclusion criteria underwent several assessments including a medication history questionnaire. Medications were verified by pharmacy records, and patients were asked about their psychiatric history and current symptoms. Anthropometrics were measured including vital signs, height, weight, hip and waist circumferences, and percentage of body

fat by bioelectric impedance. Patients underwent fasting blood sample collection and a standard, 75-g oral glucose tolerance test (OGTT) for analysis of insulin sensitivity. Glucose level was measured by a bedside YSI 2300 Stat Plus glucose analyzer (YSI Inc., Yellow Springs, OH), and fasting insulin level was measured through the Detroit Medical Center Hospital Laboratory. An index of insulin resistance was calculated by the homeostatic model assessment of insulin resistance (HOMA-IR) (32). Glucose tolerance was defined as normal (2-hour OGTT glucose level <140 mg/dL) or impaired (2-hour OGTT glucose level 140-199 mg/dL) (33). A fasting skeletal muscle biopsy was performed in the vastus lateralis using the modified Bergstrom needle technique under local anesthesia (34, 35). Biopsies were immediately cleaned of blood, fat, and connective tissue using 1× phosphate-buffered saline and were snap frozen with liquid nitrogen within 30 seconds until further processing.

DNA Methylation Analysis

Frozen muscle (10 mg) was homogenized in lysis buffer on a Minilys Bead Homogenizer (Bertin Corp., Rockville, MD). Genomic DNA was extracted from the homogenate using the Qiagen AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Germantown, MD) on a Qiagen Qiacube automated preparation machine. Extracted DNA was measured on a Qubit fluorometer (Thermo Fisher Scientific, Carlsbad, CA), and 500 ng was bisulfite converted using the Qiagen EpiTect Bisulfite Kit. Bisulfite-converted DNA was used for analysis of DNA methylation by polymerase chain reaction (PCR) followed methylation-sensitive high-resolution melting (MS-HRM) analysis on a LightCycler 480 Instrument II Real-Time PCR machine (Roche, Indianapolis, IN). Primers were designed to analyze the transcription start area (within 500 base pairs) of each gene according to the methods of Wojdacz and colleagues (36, 37). Primer sets, along with genomic location coordinates, are detailed in Table 1. Particularly of note, *AKT1* and *AKT2* transcription start regions contained CpG islands whereas *AKT3* did not. PCR reactions were performed with Roche LightCycler 480 High Resolution Melting Master, 2.5 mM of magnesium chloride and 4-10 ng of bisulfite-converted DNA, according to the manufacturer's recommendations. PCR and high-resolution melting conditions are available upon request. All reactions were carried out in triplicate, and each plate of samples contained positive controls (0-100% methylated controls in 10% intervals), negative controls and genomic template (non–bisulfite-converted) controls. Reactions with a coefficient of variation greater than 5% were discarded. Percent methylation of each gene was estimated by first obtaining the difference curves relative to the 0% methylation standard using the Roche LightCycler 480 Gene Scanning Software. Area under the curve (AUC) was estimated for each sample by using the trapezoidal rule in Microsoft Excel (Microsoft Corp., Redmond, WA). A standard curve was then plotted using the calculated AUCs for the standards. Percent methylation for each sample was extrapolated from the standard curve. This methodology has been used for other MS-HRM analyses to obtain quantitative methylation values comparable to that of pyrosequencing (38, 39).

Statistical Analysis

All data are presented as means with standard deviations. Demographic and clinical variables between maintenance treatment groups were analyzed with Student *t* tests, χ^2 tests, or Fisher exact tests where appropriate (e.g., continuous variables such as age utilized

independent *t* tests, categorical variables such as glucose tolerance utilized χ^2 tests). The outcome of interest was mean difference in skeletal muscle *AKT1, AKT2*, or *AKT3* gene methylation between the AAP and mood stabilizer groups by independent Student *t* test. A follow-up linear regression was performed to adjust for age, sex, and body mass index (BMI), which can influence DNA methylation independently. Additional analyses were performed to understand the relationship between *AKT* gene methylation and insulin resistance or glucose tolerance. A p value less than 0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of the Clinical Population

A total of 30 patients consented to the study and underwent muscle biopsies. The average age of the sample group was 43.5 ± 14.3 years, 57% were Caucasian, 63% were female, 53% were taking an AAP, and 47% were taking mood stabilizers. Of those taking an AAP, 31% were taking quetiapine, 25% risperidone, 19% olanzapine, 13% asenapine, and 13% aripiprazole. For the mood stabilizer group, 43% were taking lamotrigine, 43% lithium, and 14% valproic acid. Demographic and clinical characteristics by group are presented in Table 2.

Skeletal Muscle *AKT* Gene Methylation and Maintenance Therapy

AKT methylation was successfully measured in the majority of samples. One sample was removed for *AKT1* and one for *AKT3* due to a high coefficient of variation among the sample triplicates (11% and 8%, respectively). Overall, *AKT1* had a mean methylation of 1.71 ± 0.573%, *AKT2* had a mean methylation of 29.0 ± 9.62%, and *AKT3* had a mean methylation of 55.2 ± 12.0%. Skeletal muscle gene methylation of *AKT1* and *AKT2* was associated with treatment status. For *AKT1*, patients taking AAPs had higher methylation (or hypermethylation) at 1.91 ± 0.66% compared to patients taking mood stabilizers at 1.45 ± 0.29% (t=−2.29, p=0.0301). Similarly, for *AKT2*, patients taking an AAP had hypermethylation (33.0 ± 10.8%) when compared to patients taking mood stabilizers (24.3 ± 5.33%, t=−2.51, p=0.0192). Methylation at *AKT3* was not significantly different between patients taking AAPs (56.9 ± 13.2%) compared to patients taking mood stabilizers (53.1 ± 10.4%, t=−0.800, p=0.431). The associations for *AKT1* and *AKT2* remained statistically significant when performing a linear regression with *AKT* methylation as the dependent variable and maintenance therapy at the independent variable of interest while adjusting for age, sex, and BMI (F(4,29)=3.18, p=0.0322 and F(4,30)=4.42, p=0.0095, respectively). The adjusted model for *AKT3* was nonsignificant (F(4,29)=1.29, p=0.3048).

AKT Gene Methylation, Glucose Tolerance, and Insulin Resistance

Given the importance of AKT in glucose transport and insulin sensitivity, we investigated associations between methylation of the *AKT* gene, measures of glucose tolerance, and insulin resistance (HOMA-IR) based on maintenance treatment. When analyzing the association of *AKT* methylation with HOMA-IR or glucose tolerance in the overall population, no significant relationships were observed for any gene isoform (all p>0.2). When comparing the same relationships between *AKT* methylation, HOMA-IR, and glucose

tolerance based on maintenance treatment with an AAP or mood stabilizer, there were nonsignificant but opposing trends observed for *AKT2*. In patients taking an AAP, *AKT2* methylation and HOMA-IR were positively correlated (p=0.3), whereas in patients taking a mood stabilizer, the relationship between *AKT2* methylation and HOMA-IR was negatively correlated (p=0.1). In the analysis of glucose tolerance, for patients taking an AAP, *AKT2* methylation was higher for those with impaired glucose tolerance compared to those with normal glucose tolerance (38.5 ± 14.4% vs 29.2 ± 7.56%, t=−1.54, p=0.1510). In contrast, patients taking a mood stabilizer with impaired glucose tolerance had lower *AKT2* methylation compared to patients taking a mood stabilizer with normal glucose tolerance (22.1 ± 4.08% vs. 27.6 ± 5.78%, t=1.89, p=0.0915).

Discussion

This study identified isoform-specific associations of the *AKT* gene with AAPs and mood stabilizer treatment in the skeletal muscle of patients with bipolar disorder. Additionally, opposing trends were observed between skeletal muscle *AKT2* gene methylation and markers of insulin resistance and glucose tolerance. These relationships between *AKT2* methylation and insulin resistance and glucose tolerance did not reach statistical significance and thus should be considered preliminary. To our knowledge, this is the first study to obtain muscle biopsies from a population with a psychiatric illness and to analyze DNA methylation of *AKT* to identify potential mechanisms of AAP-induced adverse effects.

Association of *AKT* Gene Methylation with Atypical Antipsychotic Treatment

Our findings suggest that both *AKT1* and *AKT2* gene methylation in the skeletal muscle may be increased in patients taking AAPs compared to patients taking mood stabilizers. The *AKT1* gene, coding for the corresponding AKT1 (protein kinase B α) protein, has high expression in the liver, muscle, nervous system, and endothelial cells and may have a more specific role for cellular growth and angiogenesis (40). Studies suggest that it has a potentially important role in skeletal muscle health and in neuroprotection (41–43). AKT2 (protein kinase B β) is considered to have a pivotal role in the phosphoinositide 3-kinase (PI3K) pathway involved in the activation and recruitment of glucose transporter type 4 (GLUT-4) to the surface of skeletal muscle cells for the uptake of glucose in response to insulin (i.e., insulin-stimulated glucose uptake) (44). Furthermore, increased methylation (hypermethylation) in the promoter area of a gene, which was observed for *AKT1* and *AKT2* in patients taking AAPs, is generally associated with lower levels of gene expression (45). It can be hypothesized that treatment with AAPs may cause lower levels of AKT1 and AKT2 protein available in the skeletal muscle to facilitate important functions in skeletal muscle health and glucose uptake. This may influence the ability of these AKT isoforms to perform their functions in the skeletal muscle of patients treated with AAPs. These findings require further follow-up, assessing both protein abundance and regulation in the skeletal muscle of patients treated with AAPs or mood stabilizers.

Our investigation did not identify correlations between skeletal muscle *AKT3* promoter methylation and AAP treatment compared to mood stabilizer treatment. AKT3 (protein kinase B γ) is thought to have an important role in postnatal neuronal development (46). Our

finding is consistent with another study that did not identify an effect of antipsychotic treatment on AKT3 in brain tissue. However, within this same study, they identified an effect only on AKT1 and not AKT2 (47). The findings from our work, coupled with literature, may point to tissue-specific effects by AAPs on AKT isoforms.

Atypical Antipsychotics and Skeletal Muscle AKT DNA Methylation in Glucose Dysregulation

Since the skeletal muscle is considered the primary tissue involved in peripheral glucose uptake, and AKT is a major protein involved in the molecular pathway of glucose uptake, we analyzed the association between *AKT* gene methylation and measures of insulin resistance in our patients. Although we did not identify any significant associations, which may have been due to the limited sample size, we observed opposing trends where reduced insulin resistance and glucose tolerance was associated with increased *AKT2* DNA methylation in the AAP group and decreased *AKT2* methylation in the mood stabilizer group. In the presence of insulin resistance or glucose intolerance, a state of hyperinsulinemia is generally observed to increase insulin-stimulated uptake of glucose into sites such as the skeletal muscle. AKT facilitates glucose uptake (48), and increased expression and activity of AKT is generally expected in response to insulin resistance or glucose intolerance. Therefore, a decrease in methylation, as observed in the mood stabilizer group, may be expected as a compensatory response. Our observation of an opposite trend in AAP-treated patients suggests that this compensatory response in the skeletal muscle may be disrupted by AAP treatment. Our work does not explain how this disruption may be occurring; however, overlap in the dopamine, serotonin, and AKT pathways has been demonstrated (20, 49, 50). Further work is needed to verify this potential finding and elucidate the exact mechanism with prospective studies.

Limitations

Some limitations should be considered in this study. First, our samples size was limited for the analyses of *AKT* methylation and insulin resistance or glucose tolerance. The analyses identifying trends may have been underpowered to detect a true effect. Therefore, follow-up studies using larger samples are required. However, to our knowledge, this study is the first to obtain muscle biopsies from a psychiatric population taking AAPs or mood stabilizers and serves as proof-of-concept for future work in this field. Additionally, we were still able to identify significant associations between maintenance drug treatment and *AKT* gene methylation. Another limitation to consider is that the type of AAP or mood stabilizer was heterogeneous in each group. Although our findings give evidence for a class-wide AAP effect on *AKT* gene methylation in skeletal muscle, future work will need to validate these findings for individual AAPs. This work was cross-sectional in nature, and therefore only associations can be drawn. Prospective studies that assess changes in DNA methylation before and after AAP use will give further insight into acute versus chronic effects of AAPs in the AKT pathway.

Conclusion

To our knowledge, this is the first study to investigate molecular-level changes in the skeletal muscle of patients taking AAPs or mood stabilizers for 3 months or longer. We identified gene methylation changes in a tissue (skeletal muscle) and a key insulin-signaling protein (AKT) that plays a critical role in the development of insulin resistance. Our work also suggests a potentially dysfunctional response of AKT in the setting of insulin resistance for patients receiving AAP treatment. With future work, a better understanding of the effect of AAPs on skeletal muscle health and function may enable future precision medicine approaches designed to reduce AAP adverse effects and the mortality observed in patients with psychotic illness.

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Table 1

Description of Genomic Area Analyzed for Each Gene

Gene	Genomic Area Analyzed	No. of CpG Sites	Forward Primer
			Reverse Primer
AKT1	Chr14: 105262025 – 105262130 (105bp)	9	5′ – TAGACGGGGAAATTGAGGTT – 3′
			5′ – ACAACTCCCGACGACCTAA – 3′
AKT2	Chr19: 40791288-40791462 (174bp)	22	5′ – TTCGTTTAGAGGGTGGGAGG – 3′
			5′ – CACGCTACGCTAATTCCCTTTC – 3′
AKT3	Chr1: 244006164- 244006346 (182bp)	3	5′ – TTTGGCGATAGAGTGAGATTT – 3′
			5′ – ACCAATCACGCCTACCCAAATAATAA – 3′

Genomic coordinates are given in Genome Reference Consortium GRCh37/hg19. Numbers of CpG sites were analyzed in each amplicon by using methylation-sensitive high-resolution melting.

Table 2

Demographic and Clinical Characteristics of the Study Patients

Characteristic	Atypical Antipsychotic Group (n=16)	Mood Stabilizer Group (n=14)
Age (yrs)	41.9 ± 15.3	42.8 ± 12.6
Female sex[a]	12 (75)	7 (50)
Race		
Caucasian	8 (50)	9 (64)
African-American	7 (44)	4 (29)
Asian	1 (6)	1 (7)
Body mass index (kg/m^2)	32.2 ± 10.1	32.4 ± 6.3
Waist-to-hip ratio	1.01 ± 0.110	1.00 ± 0.041
Body fat (%)	36.3 ± 9.05	34.9 ± 6.42
Glucose tolerance		55/45
Normal	10 (63)	8 (57)
Impaired	6 (38)	6 (43)
HOMA-IR	1.78 ± 3.65	2.31 ± 2.66
Atypical Antipsychotic	Quetiapine: 5 (31) Risperidone: 4 (25) Olanzapine: 3 (19) Asenapine: 2 (13) Aripiprazole: 2 (13)	NA
Mood Stabilizer	NA	Lamotrigine: 6 (43) Lithium: 6 (43) Valproic acid: 2 (14)
***AKT1* Methylation (%)**[c]	1.91 ± 0.66	1.45 ± 0.29
***AKT2* Methylation (%)**[c]	33.0 ± 10.8	24.3 ± 5.33
***AKT3* Methylation (%)**	56.9 ± 13.2	53.1 ± 10.4

Data are mean ± SD values or no. (%) of patients.

NA = not applicable.

[a] p=0.1 for the difference between the atypical antipsychotic group and the mood stabilizer group.

[b] Glucose tolerance (glucose level measured after 2-hour oral glucose tolerance test) was defined as normal if glucose level was <140 mg/dL or impaired if glucose level was 140-199 mg/dL.

[c] p<0.05 for the difference between the atypical antipsychotic group and the mood stabilizer group.