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Changes in methylation within the STK32B promoter are associated with an increased risk for generalized anxiety disorder in adolescents

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ABSTRACT

Generalized anxiety disorder (GAD) is highly prevalent among adolescents. An early detection of individuals at risk may prevent later psychiatric condition. Genome-wide studies investigating single nucleotide polymorphisms (SNPs) concluded that a focus on epigenetic mechanisms, which mediate the impact of environmental factors, could more efficiently help the understanding of GAD pathogenesis. We investigated the relationship between epigenetic shifts in blood and the risk to develop GAD, evaluated by the Development and Well-Being Assessment (DAWBA) score, in 221 otherwise healthy adolescents. Our analysis focused specifically on methylation sites showing high inter-individual variation but low tissue-specific variation, in order to infer a potential correlation between results obtained in blood and brain. Two statistical methods were applied, 1) a linear model with limma and 2) a likelihood test followed by Bonferroni correction. Methylation findings were validated in a cohort of 160 adults applying logistic models against the outcome variable "anxiety treatment obtained in the past" and studied in a third cohort with regards to associated expression changes measured in monocytes. One CpG site showed 1% increased methylation in adolescents at high risk of GAD (cg16333992, $p_{adj.}$ = 0.028, estimate = 3.22), as confirmed in the second cohort (p = 0.031, estimate = 1.32). The identified and validated CpG site is located within the *STK32B* promoter region and its methylation level was positively associated with gene expression. Gene ontology analysis revealed that *STK32B* is involved in stress response and defense response. Our results provide evidence that shifts in DNA methylation are associated with a modulated risk profile for GAD in adolescence.

1. Introduction

There is a high prevalence of anxiety and mood disorders among adolescents (Polanczyk et al., 2015). Compared to their healthy counterparts, adolescents at risk for anxiety disorders are two to three fold more likely to develop a diagnosable mental health condition (Naylor et al., 2012) or to show health-compromising behaviors such as substance abuse and suicide (Fergusson and Woodward, 2002). One of the most common psychiatric illnesses shown in younger individuals is generalized anxiety disorder (GAD), a condition associated with many comorbidities and heavy costs to society. Extrinsic experiences are assumed to provoke changes at the epigenetic level, especially during adolescence, which consequently have the ability to modify the psychiatric phenotype (Mitchell et al., 2016). However, the epigenetic regulation of GAD development is not yet understood. Epigenetic and transcriptional mechanisms that play a role in GAD pathogenesis are yet to be defined, which could allow for early and effective prevention strategies against GAD.

In adolescence, the difficulty to cope with stressful factors, e.g. social pressure, academic performance, family stability, together with a reprograming of distinct signaling mechanisms, leads to excessive worry and inability to control it (McLaughlin and Hatzenbuehler, 2009). Genome-wide studies have shown that common single nucleotide polymorphisms (SNPs) explained only 7.2% of the variance in GAD symptoms, suggesting that demographic and lifestyle factors (e.g. age, diet, exercise) contribute to disease susceptibility and outcome to a significant extent (Davies et al., 2015; Dunn et al., 2017). Different gene regulatory mechanisms, such as DNA methylation (DNAm) or histone variants as well as post-translational modifications orchestrate human brain development and may underpin the psychopathology of neuropsychiatric disorders. DNAm, the most studied epigenetic mechanism, has been shown to be highly modulated by environmental

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factors (Kofink et al., 2013; Rutten and Mill, 2009) and is furthermore cell type- and tissue-specific (Hardy et al., 2017). Indeed, a large portion of the methylome varies greatly between tissues as a result of cell specialization and shows only limited differences between individuals (Davies et al., 2012). Methylated cytosines in the CpG context can recruit proteins with repressive domains or block the binding of transcription factors at gene promoters, causing a decrease in gene expression.

Few studies have investigated the relationship between methylation patterns and GAD. Wang et al. performed a targeted analysis in peripheral blood, reveling higher methylation levels at the glucocorticoid receptor gene (*NR3C1*) in patients with GAD (Wang et al., 2017). Recently, altered methylation levels at the *ASB1* gene promoter were detected in an epigenome-wide association study (EWAS) in whole blood comparing severely anxious and healthy participants (Emeny et al., 2018).

Given that blood is an easy accessible tissue, the majority of studies analyzing epigenetic changes in psychiatric disorders used blood methylation levels as a proxy for brain DNAm profiles (Cordova-Palomera et al., 2015; Ciuculete et al., 2017; Bostrom et al., 2017; Rukova et al., 2014). Supporting this approach, tissue-specific epigenetic profiles have been recently characterized in the Encyclopedia of DNA Elements (ENCODE) and the NIH Epigenomics Roadmap projects to gain more knowledge about chromatin states and gene-gene interactions in different tissues (Ernst et al., 2011; Roadmap Epigenomics et al., 2015). Additionally, previous studies analyzed the correlation between DNAm variation in blood and postmortem brain tissue (Walton et al., 2016; Davies et al., 2012; Hannon et al., 2015). In the study performed by Walton et al. (2016), 7.9% of the CpG sites showed a strong and significant correlation between blood and brain methylation patterns in 12 individuals. Moreover, using a larger sample (n = 122) and four brain regions (prefrontal cortex, entorhinal cortex, superior temporal gyrus and cerebellum), Hannon et al. (2015) concluded that some methylation sites show a greater inter-individual variation than the variation between tissues.

Our aim was to identify methylation patterns linked to the risk to develop GAD in adolescents. Importantly, we intended to reliably extrapolate our results obtained in blood to the brain. Therefore, we considered tissue-specific methylation signatures by focusing only on methylation sites for which inter-individual variation outweighs differences in brain-blood methylation. The identified and validated CpG sites and the associated genes were examined for their functional relevance in biological pathways using several different bioinformatics tools.

2. Methods

2.1. Discovery dataset

Our study comprised 221 non-related adolescents aged 14 to 16 years who were recruited between 2012 and 2014 in Uppsala, Sweden (see Supplementary File). DNAm analyses of the subjects were performed at two different time points, meaning that some adolescents were analyzed at time point 1, and some other adolescents at time point 2 (Ciuculete et al., 2017). Measurements of both time points were considered in our discovery analyses in order to increase the analysis power. The psychiatric phenotype was assessed by the standardized DAWBA questionnaire (Goodman et al., 2000) and validated in a Brazilian sample (Fleitlich-Bilyk and Goodman, 2004). The questionnaire allows generating *in silico* scores ranging between less than 0.1% to over 70% probability that an adolescent (aged 5–17 years) is characterized by anxiety disorders, depression, post-traumatic stress disorder, autism, separation anxiety disorder and obsessive compulsive disorder, based on DSM-IV and ICD-10 (Goodman et al., 2011). The GAD DAWBA band consists of questions concerning specifically general anxiety, such as e.g. the level of worry about schoolwork, dying or own appearance and to what extent these worries are associated with physical symptoms. The study was approved by the Regional Ethics Committee in Uppsala and all participants gave their written informed consent.

2.2. Validation dataset

For validation analyses, an independent, published dataset stored in the Gene Expression Omnibus database (GEOD) was used (E-GEOD-72680). More details about this cohort can be found in Supplementary File. This dataset was used to confirm the epigenetic findings associated with elevated GAD risk in an adult cohort suffering from anxiety. Briefly, this cohort consisted mostly of females aged between 18 and 77 years, who were characterized by the use of drugs against anxiety in the medical history (Zannas et al., 2015). Out of the 392 individuals originally present in the dataset, we excluded subjects with reported body mass index (BMI) higher than 30, in order to standardize the dataset for our study. A total of 160 individuals were thus included in the validation analysis.

2.3. Expression dataset

We used an additional independent open-access dataset (E-GEOD-56047) containing both transcriptomic and methylome data from $CD14^+$ samples of 1202 participants (44-83 years-old) (Reynolds et al., 2014) (more details in Supplementary File). For our analyses, both methylation and expression data was checked for outliers using graphical visualization and were removed from further calculations. The dataset was used to investigate the effect of methylation shifts on gene expression.

2.4. Probe selection

Hannon et al. investigated the correlation between epigenetic patterns in whole blood and four different brain regions and identified 22,459 CpG loci for which tissue-specificity of methylation was minimal. Across these probes, individual differences explain more than 90% of the variance in DNA methylation at 16,285 (73%) sites. For the remaining ones (27%), inter-individual variation explained more than 50% of the total variance (see Supplementary File). These CpG sites fulfilled the following equation:

$$M_{tot} = M_{indiv} + M_{tissue}, \text{ where } M_{indiv} > 50\% \text{ of total variance}$$

Where M_{tot} is the measured methylation level, M_{indiv} is the methylation level predicted by the individual and M_{tissue} is the effect of tissue (blood or brain).

We considered only probes lying within ± 2000 base pairs (bp) from the transcriptional start site (TSS), as Wagner et al. demonstrated that DNA methylation and gene expression are closely related within this region (Wagner et al., 2014). After the removal of probes with low detection p-value, a total of 13,156 loci were included in further analyses.

2.5. Data analysis and statistical tests

Data analysis was performed as summarized in the flow chart to answer three main questions:

1) Are there any differentially methylated probes between adolescents at high and low risk of GAD in blood?

Adolescents were grouped in individuals with low and high risk of GAD according to their scores defined by the DAWBA band ("gen-band"). A score below 15% was defined as "Low-risk" (category 0) (74.8%) and included the levels 0 ($< 0.1\%$), 1 ($\approx 0.5\%$) and 2 ($\approx 3\%$) of the DAWBA generalized anxiety band. The individuals with levels 3