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Epigenetic dysregulation of *HTR2A* in the brain of patients with schizophrenia and bipolar disorder

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ABSTRACT

Introduction: *HTR2A* gene has been the subject of numerous studies in psychiatric genetics because LSD, which resembles serotonin causes psychosis and atypical antipsychotic drugs target the *HTR2A* receptor. However, evidence for the role of *HTR2A* polymorphism(s) in schizophrenia (SCZ) and bipolar disorder (BD) has been elusive. We hypothesized that epigenetic dysregulation of *HTR2A* may be involved in psycho-pathogenesis and analyzed promoter DNA methylome and expression of *HTR2A* in SCZ, BD and control subjects.
Method: DNA derived from post-mortem brains of patients with SCZ and BD and matched control subjects (each 35) were obtained from the Stanley Medical Research Institute. While bisulfite DNA sequencing was used to screen and quantify cytosine methylation in the *HTR2A* promoter, corresponding gene expression was analyzed by qRT-PCR.
Results: We found strong evidence for epigenetic fine-tuning of *HTR2A* expression. In general, the expression of *HTR2A* in individuals carrying the C allele of T102C (or G allele of −1438A/G polymorphism) was higher than TT genotype. Interestingly, promoter DNA of *HTR2A* was hypermethylated at and around the −1438A/G polymorphic site, but was hypomethylated at and around T102C polymorphic site in SCZ and BD compared to the controls. Furthermore, epigenetic down-regulation of *HTR2A* was associated with early age of disease onset in SCZ and BD.
Conclusion: Epigenetic dysregulation of *HTR2A* may contribute to SCZ, BD and earlier age of disease onset. Further research is required to delineate the dysregulation of other components of serotoninergic pathway to design new therapeutics based on the downstream effects of serotonin.

1. Introduction

Serotonin is required for proper synapse formation and neuronal functions (Broman and Fletcher, 1999). Environmental factors stimulate serotonin release acting on seven types of serotonin receptors which through cAMP and CREB orchestrate transcription of many genes to produce proteins required for neuronal growth and long-lasting structural changes (Kandel, 2001). Serotonin also regulates many human drives such as appetite, sexual desire as well as cognitive processes and mood state (Sodhi and Sanders-Bush, 2004).

The serotonin receptor type-2 (*HTR2A*) is the main target of atypical antipsychotic drugs and harbors several polymorphisms which some of them have been interrogated for functional consequences (Williams et al., 1997; Abdolmaleky et al., 2004a; Ghadirivasfi et al., in press). While the promoter −1438A/G single nucleotide polymorphism (Enoch et al., 1998; Walitza et al., 2002) has been shown to affect the promoter activity (Parsons et al., 2004), it is in perfect linkage disequilibrium with T102C polymorphism in exon-1 that does not cause an amino-acid change. In a meta-analysis on the association between T102C polymorphism of *HTR2A* and schizophrenia (SCZ), we found a significant allelic heterogeneity between East Asian and European population (Abdolmaleky et al., 2004a). In the East Asian population (including Chinese, Japanese,

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Singaporeans and Koreans) the C allele was not associated with SCZ, and instead we found evidence for the involvement of the T allele in SCZ pathogenesis. Some studies reported that the abundance of the *HTR2A* receptors expressed by the C allele is less than the T allele in normal individuals and SCZ patients (Turecki et al., 1999; Polesskaya and Sokolov, 2002; Khait et al., 2005). However other studies found no difference in the expression of either C or T allele (Hernandez and Sokolov, 2000;.Bray et al., 2004).

Several lines of evidence suggest that in addition to genetic polymorphisms epigenetic factors may also play a role in the regulation of *HTR2A* expression. For instance, Kato et al. (1996) presented evidence for genomic imprinting of *HTR2A*. Bunzel et al. (1998) showed that, approximately 22% of individuals have monoallelic expression of the *HTR2A* due to polymorphic imprinting in the brain. DNA methylation (DNAM) is the main mechanism of genomic imprinting and is influenced by many metabolic and environmental factors (Singal and Ginder, 1999; Bird, 2002). Gene promoter DNAM pattern in response to environmental impacts can also have deleterious effects similar to those derived from mal-functional polymorphisms (Petronis et al., 2003; Grayson et al., 2005; Abdolmaleky et al., 2004b, 2005, 2006; Huang and Akbarian, 2006; Akbarian, 2010). This may also be the cause of inconsistency in *HTR2A* mutation and expression studies in different ethnic groups (Abdolmaleky et al., 2004a, 2004b). As the G at position −1438 follows C, and C at position 102 is followed by G, these polymorphisms provide additional candidate cytosines (CpG) for methylation that may influence the level of gene expression (Petronis, 2000). In support of this hypothesis, it was reported that C allele-specific methylation of these polymorphisms correlates with the reduced expression of *HTR2A* in the temporal cortex of normal individuals, and the degree of methylation correlates with expression of DNA methylase-1 *DNMT1* (Polesskaya et al., 2006). It should be noted that, although there is no conventionally defined CpG island proximal to the *HTR2A* start site, there are two CpG islands in intron-1 and exon-2 within the 3850 bp downstream of the coding start site. Furthermore, the CpG enriched sequences of the *HTR2A* promoter (2 kb upstream of the coding region) harbor 30 CpGs, among which 20 are within DNA sequences surrounded by the −1438A/G and T102C polymorphic sites. Here, we report the analysis of human post mortem brains for DNAM in the entire *HTR2A* promoter region, including the polymorphic sites and cytosines flanking the CpG enriched sequences and "CpG island shores" that my play major roles in gene expression (Irizarry et al., 2009) to define SCZ and BD pathogenesis.

2. Materials and methods

2.1. Brain samples and statistical analysis

We obtained 105 DNA/RNA samples prepared from the frontal lobe of patients with SCZ, BD and matched controls from the Stanley Medical Research Institute. Univariate, bivariate and multivariate statistical tests were used for data analysis. In general, two-tailed *t* test was used for the quantitative data analysis and the exceptions are indicated in the Results section.

Parametric tests and large sample approximations (*e.g.*, Chi-square) were used wherever possible; otherwise, for small samples Fisher's Exact Test was applied.

2.2. Polymorphisms or mutations in the HTR2A

The genotype of −1438 A/G and T102C polymorphisms were determined as previously described using enzymatic restriction of PCR products (Arranz et al., 1995).

2.3. Analysis of gene expression by quantitative-real-time PCR

Total RNA was reverse transcribed and the cDNA was amplified using gene specific primers which introduced elsewhere (Abdolmaleky et al., 2008a; Ghadirivasfi et al., in press) and shown in Table 1 and Platinum Taq DNA polymerase (Invitrogen). Target genes were normalized to β-actin. Quantitative real-time PCR was performed using the SYBR green methodology as previously described (Abdolmaleky et al., 2006). The ΔΔCT method was used for quantification (ABI) and the fold changes are reported as $2^{-\Delta\Delta CT}$.

2.4. Mapping differentially methylated CpGs and determination of methylation status

Bisulfite DNA sequencing was followed by quantitative methylation specific PCR (qMSP) to assess the *HTR2A* promoter DNAM as described in detail elsewhere (Abdolmaleky et al., 2008a). Briefly, genomic DNA was chemically modified with sodium bisulfite (Qiagen, EpiTec Bisulfite Kit, Cat#59104) to convert the unmethylated cytosines to uracils (Frommer et al., 1992). Five sets of primers were used to amplify the entire *HTR2A* promoter region and nested primers were used for direct bisulfite DNA sequencing (Table 1). Whenever there was low level of DNAM (*e.g.* −1177 and −1149 cytosine residues), the PCR product was cloned using the pGEM-T kit and transformed into the JM109 cells (Promega cat#L1001). Ten colonies per PCR product were sequenced to verify the result of direct bisulfite sequencing. The degree of DNAM at the polymorphic sites and neighboring cytosines was calculated based on direct bisulfite sequencing (M/M + U, where M and U are the height of the traces of C or T, representing methylated or unmethylated cytosines, respectively).

DNAM of the predominantly unmethylated sites (<20%, based on bisulfite sequencing) was evaluated by qMSP analysis. In order to obtain accurate results, optimal conditions were established to generate reliable standard curves and a single PCR product using unmethylated and methylated templates (placental DNA and *in vitro* methylated DNA, respectively) during the trial experiments (Abdolmaleky et al., 2008a). PCR products corresponding to methylated and unmethylated DNA templates were normalized to PCR product of the β-actin promoter amplified in separate reactions using primers corresponding to a CpG free region (Table 1).

3. Results

3.1. Genotypes of −1438A/G (and/or T102C) polymorphisms of HTR2A and expression analysis

The allele frequency of the T102C polymorphism established no significant differences between the patients and controls as shown in Table 2. Quantitative real-time PCR analysis revealed that overall, the expression of *HTR2A* in individuals with the TT genotype was the same in patients and the control subjects. However, it was variable and significantly higher (Fig. 1) in individuals heterozygous for the TC and the CC genotypes ($p = 0.008$ and $p = 0.0034$, respectively) compared to the TT genotype.

Additionally, *HTR2A* expression was lower by 24% in SCZ compared to the controls ($p = 0.04$). There was a similar trend for decreased *HTR2A* expression in BD (~18%, $p = 0.1$). However, the difference was significant in BD patients who were antipsychotic free (at least for a month) at the time of death (~33%, $p = 0.027$, one-tailed *t* test) compared to the controls. When we considered the effect of antipsychotic use on *HTR2A* expression, while the difference was more prominent in individuals with CC *versus* the TT genotype (~40%, $p = 0.012$), TC heterozygotes were in the mid-range. The SCZ patients heterozygous for TC had lower expression of *HTR2A* compared to the controls ($p = 0.02$) corresponding mostly to the left brain. On the contrary, there was decreased *HTR2A* expression in the right brain in the BD patients ($p = 0.03$, one-tailed *t* test). In general, *HTR2A* expression increased with age in individuals carrying the C allele, particularly in the controls with CC genotype ($p = 0.04$, <44 *versus* >44), but this was less pronounced in patients with SCZ and BD and did not reach statistical significance. There