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Brief Research Communication

Evidence for Monozygotic Twin (MZ) Discordance in Methylation Level at Two CpG Sites in the Promoter Region of the Catechol-O-Methyltransferase (*COMT*) Gene

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Monozygotic (MZ) twin concordance for a range of psychiatric conditions is rarely 100%. It has been suggested that epigenetic factors, such as DNA methylation, may account for a proportion of the variation in behavioral traits observed between these genetically identical individuals. In this study we have quantitatively assessed the methylation status of two CpG sites in the promoter region of the *COMT* gene in 12 MZ twins-pairs discordant for birth weight, but otherwise clinically unaffected. DNA was obtained at age 5-years using buccal swabs, and modified using sodium-bisulfite treatment. Methylation profiles were assessed using Pyrosequencing™, a technology enabling the precise degree of methylation to be assessed at any CpG site. We found that the degree of methylation at the two CpG sites was highly correlated, but there was considerable variation in the concordance of methylation levels between MZ twin-pairs. Some MZ twin-pairs showed a high degree of methylation concordance, whereas others differed markedly in their methylation profiles. Such epigenetic variation between genetically identical individuals may play a key role in the etiology of psychopathology, and explain the incomplete phenotypic concordance observed in MZ twins. **© 2006 Wiley-Liss, Inc.**

KEY WORDS: ***COMT*; low birth-weight; monozygotic (MZ) twins; Pyrosequencing; Methylation; CpG**

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Studies of twins have been widely used to investigate the genetic contribution to disease etiology in psychiatric disorders. It is widely observed that, in many psychiatric conditions, monozygotic (MZ) twins are more concordant that dizygotic (DZ) twins, strongly implicating inherited genetic factors in pathogenesis. However, another common observation is that there are few, if any, non-Mendelian psychiatric conditions in which the concordance rate for MZ twins is actually 100%. In the classical twin-study approach, in which MZ twins are assumed to be genetically identical, any discordance between MZ twins is attributed to 'non-shared' environmental factors. A recent alternative explanation, however, is that some of the observed phenotypic differences between MZ twins may be the result of epigenetic factors [Kato et al., 2005; Wong et al., 2005].

Epigenetics is the study of heritable (mitotic or meiotic) variation in gene function not resulting from an actual change in DNA sequence. Of particular interest is the phenomenon of cytosine methylation at CpG sites, a molecular process that is intrinsically linked to the regulation of gene expression, although other epigenetic mechanisms including the post-translational modification of histones, are also important. Methylation at CpG sites, principally located in CpG-islands in the promoter regulatory regions of many genes, disrupts the binding of transcription factors and attracts methyl-binding proteins that are associated with gene silencing and chromatin compaction. Petronis [2001] have argued that these epigenetic mechanisms can explain a number of the non-Mendelian features observed in a range of complex psychiatric disorders such as schizophrenia. Unlike DNA sequence variation, which is generally highly stable, epigenetic processes are highly dynamic even within an individual: they can be tissue specific, developmentally regulated, and induced by exposure to a range of environmental stressors. The dynamic nature of epigenetic processes is illustrated in a recent report by Fraga et al. [2005] that suggests epigenetic differences between MZ-twins accumulate over time, resulting in significant differences in elderly twins.

Little actual empirical work has been performed to investigate MZ-twin methylation discordance in relation to specific candidate genes that are strongly postulated to play a role in psychopathology. The first such study was undertaken by Petronis et al. [2003], who investigated the methylation status of CpG sites in the regulatory region of the dopamine D2 receptor (*DRD2*) gene in two MZ twin-pairs—one discordant and the other concordant for a diagnosis of schizophrenia. They found variation in the *DRD2* methylation patterns within both sets of twins, but also observed that this epigenetic difference was larger in the twin-pair discordant for schizophrenia. The aim of our study was to investigate whether MZ-twin methylation discordance occurs in the promoter-region of another widely-studied psychiatric candidate gene, Catechol-O-Methyltransferase (*COMT*). Given that the immediate post-partum environment has been postulated to influence promoter methylation at CpG sites [Weaver et al., 2004], and low-birth weight has been shown to interact with risk variants at the

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COMT locus to increase the risk of psychopathology [Thapar et al., 2005], we selected MZ-twins who were highly discordant for birth weight to investigate whether such early-life factors were associated with any methylation differences observed.

The *COMT* gene is located on chromosome 22q11, a region strongly implicated in the etiology of several psychiatric disorders, in particular schizophrenia [O'Donovan et al., 2003]. While several studies have found associations between polymorphic markers in this gene and schizophrenia, such findings are far from universally replicated and a recent meta-analysis of the common val108met polymorphism failed to find overall evidence for a significant association, suggesting that other etiological processes involving this gene may be important in pathogenesis [Munafo et al., 2005]. Interestingly, a recent paper by Thapar et al. [2005] has shown that *COMT* is associated with early-onset antisocial behavior in children with attention-deficit hyperactivity disorder, but only among individuals exposed to low-birth weight.

Murphy et al. [2005] have recently examined the methylation status of six CpG sites in the promoter region of *COMT*. Using bisulfite-genomic sequencing they found that four of the sites were totally methylated in all individuals assessed, but that two adjacent CpG sites (incorporating cytosines 23 and 27 in their analysis) showed evidence for only partial methylation and some degree of between individual variation. In the present study we have accurately quantified the degree of methylation at these two CpG sites in a series of MZ twins. For our analyses we used the Pyrosequencing^{TM} system (Biotage, Uppsala, Sweden), a sequencing-by-synthesis method that relies on the luminometric detection of pyrophosphate release upon nucleotide incorporation via an enzymatic cascade. Because there is a direct correlation between sequence data and the amount of nucleotides incorporated during the reaction, it provides an accurate method for detecting the precise methylation level at any specific CpG site giving truly quantitative data that are not achievable using standard bisulfite-sequencing protocols [Tost et al., 2003].

We selected 12 Caucasian MZ twin-pairs (6 male, 6 female) on the basis of a discordant environmental risk history of birth weight from a register of twins based in the UK [see Trouton et al., 2002]. The average twin discordance for birth weight in this sample was 970.66 g (see Table I). Buccal cell DNA was obtained at age 5, as described by Freeman et al. [2003], and treated with sodium bisulfite using a modified version of the protocol outlined by Olek et al. [1996]. Briefly, ~800 ng samples of genomic DNA (in a volume of 21 μl) were denatured at 95°C for 10 min, followed by incubation with 4 μl 2 M NaOH solution at 50°C for 15 min. DNA was mixed with 50 μl of 2% low-melting agarose, and 8 μl beads were formed in prechilled mineral oil. Bisulfite conversion was performed with a 5 M sodium bisulfite solution at 50°C for 4 hr, under exclusion of light. The beads were washed twice for 15 min in TE buffer (pH = 8.0). Desulfonation was done in 0.2 M NaOH, twice for 15 min each, followed by two additional washing steps, again, with TE buffer. Single beads were washed with water and used for subsequent PCR reactions. Two primers, specific to bisulfite-treated DNA, were designed on the reverse-complement strand to flank the two *COMT* CpG sites (F: 5′-GAG TAG GTT GTG GAT GGG TTG TA-3′ and R: 5′-*biotin*-ACA TTT CTA AAC CTT ACC CCT CTA-3′). Purification with streptavidin beads and Pyrosequencing^{TM} reactions were performed according to the manufacturer's standard protocol using the sequencing primer 5′-GTA ATA TAG TTG TTA ATA GTA GA-3′. Example output traces, showing the percentage methylation at the two CpG sites, can be seen in Figure 1.

Table I gives the methylation profile across the two *COMT* CpG sites for the 12 MZ twin-pairs. All individuals assayed were partially methylated at the two CpG positions, and we found a strong correlation between the degrees of methylation at both CpG sites ($r = 0.77$, $P < 0.01$). Site one in our assay (cytosine 27 in the study of Murphy et al. [2005]) had an average methylation level of 53.1%, whereas site two (cytosine 23) had an average methylation level of 48.7%. These data differ from the pattern of methylation observed in lymphocyte and brain DNA by Murphy et al. [2005], in which site 1 (cytosine 27) was generally 100% methylated, with a couple of exceptions, and only site 2 (cytosine 23) was consistently partially methylated, thus suggesting some tissue-specificity in methylation profiles. The sample analyzed by Murphy et al. [2005] also included three MZ twin-pairs, and they found no twin differences. However, the methodology used in this previous report (the direct-sequencing of bisulfite-PCR products) is not able to give truly quantitative estimates of CpG methylation in the same way that Pyrosequencing can [Paul and Clark, 1996]. Thus, while their data can inform us whether MZ twins are fully methylated, fully unmethylated, or semi-methylated they do not provide information about whether twins are concordant or discordant for the actual *level* of methylation.

The main aim of this study was to examine the relationship of methylation status at the two *COMT* CpG sites within MZ twin-pairs. The within MZ twin-pair differences in methylation for both CpG sites are illustrated in Table I and Figure 2. Overall, MZ twins appear to be more concordant for methylation at CpG site 1 than at CpG site 2: the average within twin-pair difference for methylation level at CpG site 1 was found to be 10.3% and at site 2 was 16.1%. The average within MZ twin-

TABLE I. Birth-Weight and Methylation Profiles at the 2 *COMT* Promoter CpG Sites for the 12 MZ Twin-Pairs

MZ twin-pair		Birth weight (g)			CpG 1 methylation (%)			CpG 2 methylation (%)			Average difference (%)
ID	Sex	Twin 1	Twin 2	Diff	Twin 1	Twin 2	Diff	Twin 1	Twin 2	Diff	
1	M	1787.63	2780.75	993.12	53.3	53.4	0.1	51.8	50.0	1.8	0.95
2	F	3362.44	2355.13	1007.31	58.5	52.0	6.5	46.8	47.4	0.6	3.55
3	F	2809.13	1844.38	964.75	59.4	53.9	5.5	54.5	49.7	4.8	5.15
4	F	3376.63	2468.63	908.00	36.7	34.0	2.7	28.0	37.3	9.3	6.00
5	M	1986.25	2837.50	851.25	46.3	48.5	2.2	43.6	55.6	12.0	7.10
6	M	2043.00	3121.25	1078.25	56.4	59.9	3.5	39.3	53.3	14.0	8.75
7	M	1702.50	2553.75	851.25	53.1	63.5	10.4	45.1	33.9	11.2	10.80
8	M	1844.38	2837.50	993.12	41.1	54.0	12.9	33.8	48.0	14.2	13.55
9	F	2780.75	1674.13	1106.62	62.8	49.0	13.8	62.6	41.3	21.3	17.55
10	M	2979.38	2099.75	879.63	62.6	46.6	16.0	64.6	43.8	20.8	18.40
11	F	2951.00	1844.38	1106.62	49.2	68.6	19.4	43.9	74.4	30.5	24.95
12	F	2724.00	1816.00	908.00	71.4	40.7	30.7	86.7	34.4	52.3	41.50
Average		2440.84		970.66	53.1		10.3	48.7		16.1	13.19

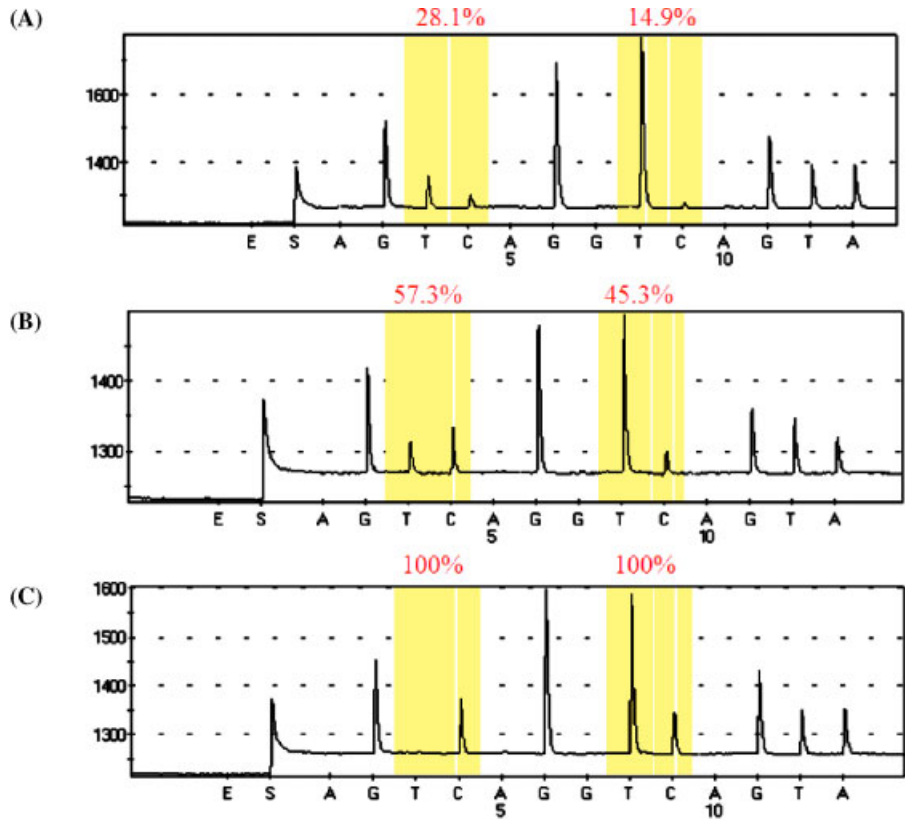

Fig. 1. Example pyrosequencing output traces for the two *COMT* CpG sites assayed in this study. Samples A and B demonstrate between-individual methylation variation for the two CpG sites. Sample C is a positive methylated control sample showing 100% methylation in DNA from plasmids treated with SssI methyltransferase.

pair difference in methylation for the two sites combined was 13.19%. As expected, given the strong correlation between the level of methylation observed at CpG site 1 and CpG site 2, there is a strong correlation between MZ-twin differences in methylation at the two sites ($r = 0.87$, $P < 0.001$).

The results are interesting in that several of the MZ pairs are highly concordant for methylation status, whereas other twin-pairs are highly discordant. Twin-pairs 1–6, for example, show a less than 4% difference in methylation level for the first CpG site and less than a 9% overall methylation difference. Conversely, there are two MZ twin-pairs that show quite dramatic differences in cytosine methylation at both CpG sites, with twin-pair 12 showing an average methylation difference of almost 42%. These data are interesting given that DNA was obtained from the MZ-twins at age 5. We thus find evidence for MZ-twin discordance in methylation at an early age unlike

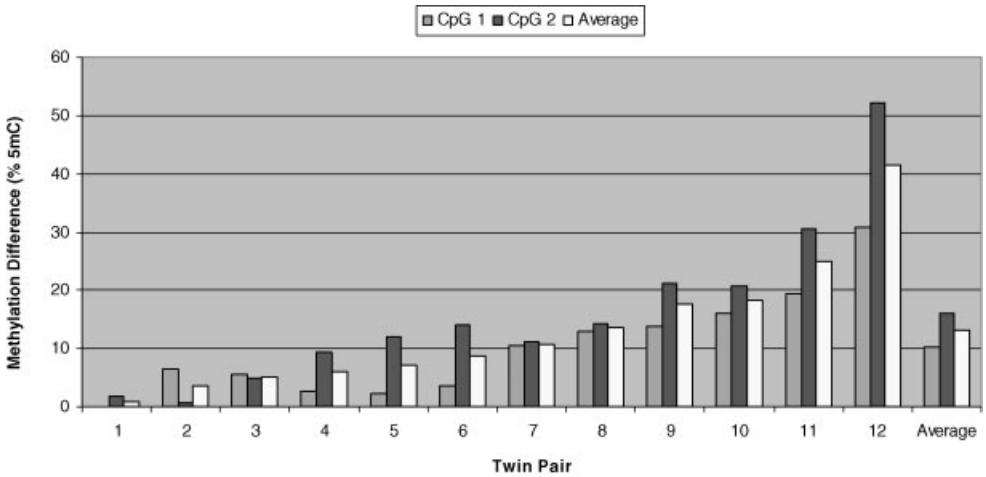

Fig. 2. Within MZ twin-pair differences in cytosine methylation for the two CpG sites in the promoter-region of *COMT*. The difference (expressed in percent methylation difference) for CpG site 1 (blue), CpG site 2 (red), and the average (yellow) is shown for twin pairs 1–12 arranged in ascending order.

Fraga et al. [2005] who report that epigenetic differences are not apparent until later in life. This is an important observation and suggests that potentially functional epigenetic differences are present across the life-course and do not arise only in elderly individuals. It should be noted, however, that our study only investigated epigenetic differences at the *COMT* locus, whereas Fraga et al. [2005] have assessed genome-wide patterns of methylation.

Overall, our data suggest that the MZ concordance rate for CpG methylation in the promoter region of the *COMT* gene is not 100%, and that there is considerable between twin-pair variation in the level of discordance. While all twin-pairs were concordant for having partial methylation at both CpG sites, several MZ twins were highly discordant for the amount of methylation observed at either site. These data are similar to those reported by Petronis et al. [2003] who found MZ twin discordance for methylation in the promoter region of another candidate psychopathology gene, *DRD2*. Tsujita et al. [1998] performed restriction landmark genome scanning analysis to identify differences between MZ twins discordant for schizophrenia. They identified differences between MZ twins at several loci, probably attributable to differences in the methylation status at *Not*I restriction enzyme sites between the twins. Given the role of DNA methylation in regulating gene expression, such findings provide an insight into how phenotypic differences may occur between genetically identical MZ twins. Future work will focus on examining how methylation status in the promoter region of the *COMT* gene influences expression, and whether or not such MZ twin differences also occur in tissues of the nervous system, where their influence on psychopathology may be more direct.

MZ twin differences in methylation could occur for a number of reasons. Two likely causes of such epigenetic variation are random stochastic events and differential exposure to environmental factors. Several studies have indicated that there is some infidelity in the maintenance of methylation patterns in mammalian cells, and that de novo methylation events could occur at a rate of up to 3%–5% per mitosis [Riggs et al., 1998]. Petronis [2004] have proposed that epigenetic dysregulation is likely to play a key role in the etiology of diseases such as schizophrenia and that such stochastic methylation events may contribute to the high level of MZ-twin discordance in these disorders. Given the undoubtedly similar rearing environments experienced by MZ twin-pairs, it has been proposed that such stochastic epigenetic events may account for much of the differences currently attributed to the non-shared environment [Wong et al., 2005]. However, there is also considerable evidence to suggest that epigenetic processes are in fact strongly affected by environmental factors [Sutherland and Costa, 2003], and thus non-shared environmental experiences could still mediate MZ twin discordance via mechanisms such as differential DNA methylation. In relation to the *COMT* gene and its association with schizophrenia, it is interesting that a recent study has found an association between the gene and psychotic symptoms, but only in individuals exposed to an environmental pathogen (cannabis) at a specific developmental time point [Caspi et al., 2005]. It is plausible that the molecular mechanism behind this and other similar gene–environment interactions involves epigenetic processes that act to alter levels of gene expression [Kramer, 2005]. To investigate whether early-life stress influences methylation at the *COMT* locus, we selected MZ-twins who were highly discordant for birth weight. We found no overall association between birth weight and the degree of *COMT* methylation, nor any evidence to suggest that MZ-twin methylation discordance can be explained by birth-weight differences. These data do not, however, discount the role of other postpartum environmental factors in mediating epigenetic differences between MZ twins. It will be interesting to follow the development of these twins to assess whether there are any future behavioral consequences associated with these methylation differences observed in the *COMT* gene, and to examine whether the discordance in methylation increases or decreases with age.

To conclude, we have quantitatively examined the methylation level of two CpG sites in the promoter region of the human *COMT* gene in buccal DNA taken from 12 MZ twin-pairs. We found that while some MZ twins are highly concordant for the degree of methylation at both sites, other twin-pairs showed large differences with one twin showing a higher degree of methylation than the other. It is possible that such MZ epigenetic differences account for some of the discordance observed in MZ twin-pairs for a range of complex psychiatric conditions and behavioral traits.

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