Epigenetics of Personality Traits: An Illustrative Study of Identical Twins Discordant for Risk-Taking Behavior

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DNA methylation differences between identical twins could account for phenotypic twin discordance of behavioral traits and diseases. High throughput epigenomic microarray profiling can be a strategy of choice for identification of epigenetic differences in phenotypically different monozygotic (MZ) twins. Epigenomic profiling of a pair of MZ twins with quantified measures of psychometric discordance identified several DNA methylation differences, some of which may have developmental and behavioral implications and are consistent with the contrasting psychometric profiles of the twins. In particular, differential methylation of CpG islands proximal to the homeobox DLX1 gene could modulate stress responses and risk taking behavior, and deserve further attention as a potential marker of aversion to danger. The epigenetic difference detected at DLX1 of ~1.2 fold change was used to evaluate experimental design issues such as the required numbers of technical replicates. It also enabled us to estimate the power this technique would have to detect a functionally relevant epigenetic difference given a range of 1 to 50 twin pairs. We found that use of epigenomic microarray profiling in a relatively small number (15–25) of phenotypically discordant twin pairs has sufficient power to detect 1.2 fold epigenetic changes.

Phenotypic differences between identical, or monozygotic (MZ), twins have been poorly explained by measurable environmental discordance. Various degrees of phenotypic differences have been observed in all traits, ranging from normal behavior and normal traits to manifestation of complex disease (Wong et al., 2005). MZ twins arise from a single fertilization and later split in early embryogenesis to develop into two distinct human beings. Because of their common gametic origin, MZ twins are identical at the DNA sequence level, and yet they often display phenotypic differences later in life (Wong et al., 2005). Traditionally, such twin discordance is attributed to environmental influences acting differently on each twin (Reiss et al., 1991). A number of epidemiological studies in past years questioned this assertion by measuring phenotypic variation in MZ twins raised in the same environment versus those raised apart, and found measurable environmental factors insufficient to explain observed differences, thus bringing into question the underlying mechanisms of MZ twin discordance (Bouchard et al., 1981).

In attempts to address this question, recent attention has returned to molecular studies. Looking beyond the identical DNA sequence in MZ twins, numerous differences in epigenetic patterns between twins have been identified, some of which are believed to result in the observed discordant phenotypes (Fraga et al., 2005; Heijmans et al., 2007; Kuratomi et al., 2007; Petronis et al., 2003; Oates et al., 2006; Zhang et al., 2007). Epigenetic signals are molecular signatures that control various aspects of genome organization, including gene transcription. Epigenetic modifications consist of methylation of cytosines, as well as modifications of histones including methylation, phosphorylation, acetylation, and ubiquitination (Geiman & Robertson, 2002; Li, 2002; Robertson, 2002; Schotta et al., 2004; Wang et al., 2004). In mammals, DNA methylation occurs most commonly where cytosine is directly followed by guanine, forming a CpG dinucleotide. Clusters of CpG dinucleotides are referred to as CpG islands (Takai & Jones, 2002). Many CpG islands across the genome are located

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proximal to gene promoters, and their DNA methylation status can affect levels of gene transcription through modulation of chromatin conformation and sequestration of components of the basal transcription machinery (Holliday et al., 1996; Yeivin & Razin, 1993). DNA methylation has been shown to restrict the access of transcription factors and limit gene expression (Ehrlich & Ehrlich, 1993; Riggs et al., 1998). Conversely, unmethylated DNA is associated with an open chromatin conformation, allowing for access of DNA binding elements and transcriptional activation (Jenuwein & Allis, 2001; Nemeth & Langst, 2004; Rice & Allis, 2001; Strahl & Allis, 2000). In addition to the promoter, the 3' untranslated region (3' UTR) of a gene can also be an important regulator of expression levels and can be directly affected by DNA methylation (Malumbres et al., 1999; Shen et al., 2001). DNA methylation differences between twins may therefore translate into the expression of phenotypic differences through varying levels of gene transcription.

Since epigenetic factors in MZ twins may be the underpinning molecular mechanism to explain phenotypic differences between identical co-twins, it is critical to map epigenetic differences between twins to enable discovery of epigenetic changes, which may be associated with discordance for complex traits. With this in mind, our primary objective was to employ a novel epigenomic microarray profiling strategy on a pair of normal MZ twins. The key question we attempted to address was whether high throughput microarray-based epigenetic profiling can reliably identify DNA methylation differences in normal twins, and if so, what size and how many epigenetic differences can be detected in twins differing for normal psychological traits. The co-twins investigated for this purpose exhibited major differences for various psychometric measures of risk taking behavior. Our secondary objective was to investigate any links between the identified differences and quantified phenotypic differences, and to estimate the power of the technique for identifying possible etiological epigenetic differences in larger populations of twins discordant for normal behavioral traits. In fulfilling these objectives, the study provides an illustrative example of experimental design and power considerations when performing epigenomic microarray profiling in discordant MZ twins.

We undertook a detailed behavioral examination of two 49-year-old female MZ twins, one of whom works as a war journalist and the other as an office manager in a law firm. They spent a close childhood in each other's company. Their parents dressed them the same, ensuring they were essentially indistinguishable. They were bright students, but differed in their favorite subjects. One twin (who will later be referred to as the 'war' twin) enjoyed languages and disliked domestic science, while the reverse applied to her twin (the 'law' twin). At 17 years of age, the war twin left home, setting in place a peripatetic existence. In the process she learned multiple languages. She eventually chose journalism as a career, gravitating to war zones where in a long and distinguished career she covered wars in Africa, the Middle East, and the Balkans. Over the course of 20 years she was exposed to many life threatening situations, saw many people killed and wounded, and lost close colleagues. She married in her forties to a cameraman who also worked in war zones. She never had children and by her own admission never had the maternal urge. She occasionally drinks in excess of nine units of alcohol per week, considered the upper limit of healthy drinking in a woman (Bondy et al., 1999). She does not smoke.

Her co-twin was bereft when her sister left home. She too thought of traveling, but her choice of venue was more cautious, limited as it was to a single English speaking country. She married young, to a lawyer and soon had two children. She works part-time as a manager in a law office. She drinks three to four units of alcohol per week and does not smoke.

Neither twin has a history of psychiatric problems. Despite their geographical separation they remain close emotionally and meet as often as they can.

Methods

The twins underwent psychometric, genetic, and epigenetic testing.

Psychometric assessment

1. The Wechsler Abbreviated Scale of Intelligence (WASI; Wechsler, 1999), a shortened form of the full Wechsler Adult Intelligence Scale (WAIS-III) gave an IQ score.
2. The Minnesota Multiphasic Personality Inventory (MMPI-II; Hathaway, 1989) provided an index of personality attributes. The MMPI-2 is the leading commercially available clinical test of personality and psychopathology. It is composed of 567 true/false items that comprise 10 scales assessing clinical syndromes including anxiety, depression, and psychosis, and personality traits such as coping style and patterns of interpersonal relationships. There are also scales for assessing the validity of the person's overall approach to the test. Numerous supplemental content scales are also available. Interpretation is based on analysis of the profile of scale elevations to provide an analysis of psychopathology and personality style.
3. The Toronto Gambling task (Floden & Stuss, 2004) is a computerized test that assesses the role of risk taking and impulsivity in decision-making processes. On each trial, subjects are presented with a series of five gambles where the probability of 'winning' points systematically increases or, on half the trials, decreases. Subjects are free to select the gamble they prefer in order to win as many points as they can. However, gambles with a higher probability of 'winning' have a lower associated point value, while gambles with a low probability of winning are linked with larger payoffs. Subjects with a risk-taking decision style consistently choose

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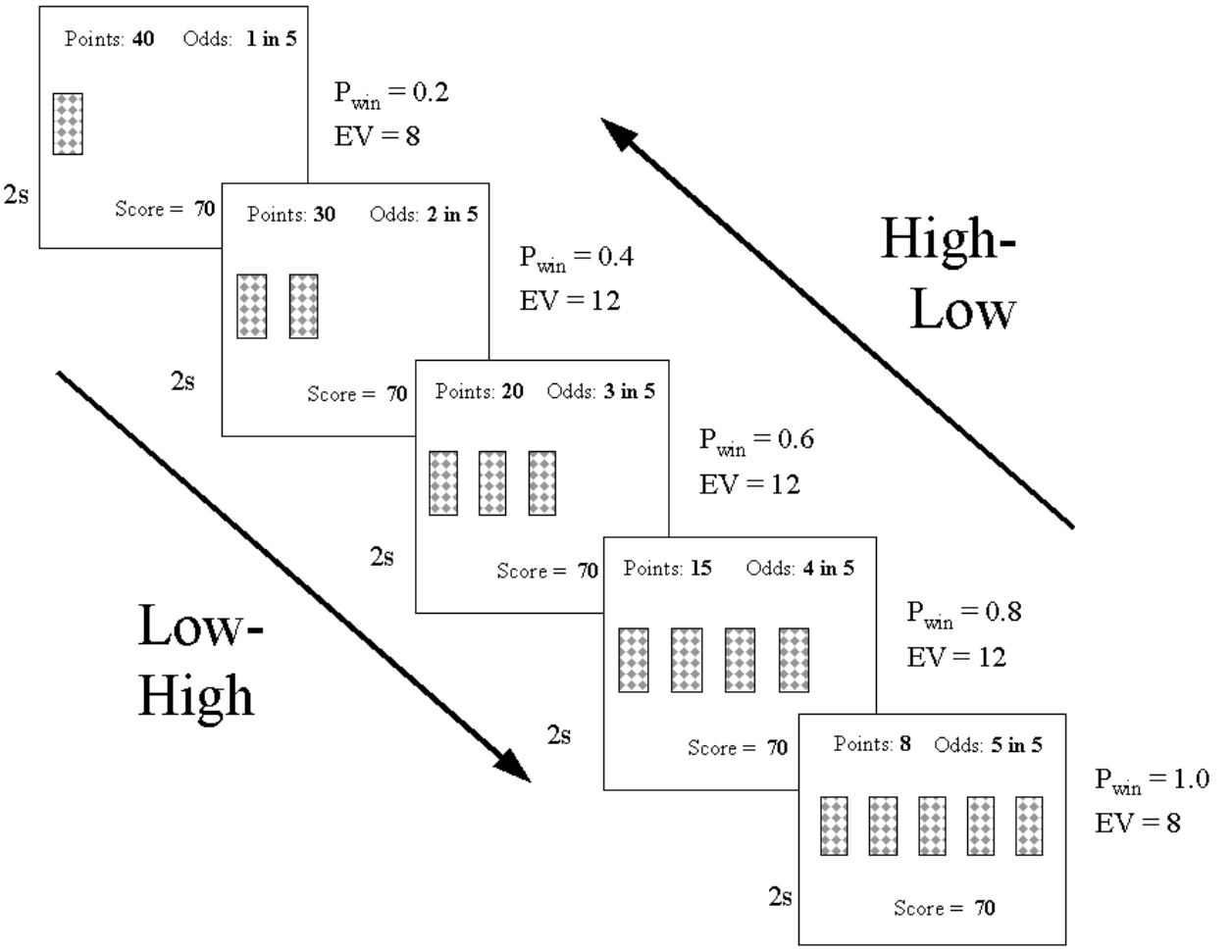

Figure 1

The Toronto Gambling Task displays and contingencies. A schematic diagram of successive displays on one trial of the gambling task.

In the Low–High condition, presentation order moves from bottom to top — from low probabilities/large rewards to high probabilities/small rewards. In the High–Low condition, presentation order moves from top to bottom. Each display is present for 2 seconds or until the subject makes a response and the trial is terminated. Subjects complete 40 trials. Each trial is then followed by a blank screen for 2s (first 20 trials) or 10s (last 20 trials). Subjects initially received five practice trials of each presentation order to ensure comprehension of instructions.

In both conditions, up to five cards are presented face-down in a horizontal array. One of the five cards displays the word 'WIN' on its face whereas the other four cards are blank. Subjects are instructed that they can touch the screen at any time to select the gamble and turn over the cards present. If the WIN card is among the cards present at the response, the subject earns points. If the WIN card is absent at the response, no points are awarded. The position of the WIN card is random on each trial, meaning that the more cards on the screen, the higher the probability that the WIN card will be present. However, the point value of the WIN card is inversely related to the number of cards on the screen (i.e., more cards/higher probability = fewer points). The likelihood of finding the WIN card and its associated value are displayed on the screen at all times during the trial to minimize memory demands. Note that, as the probability of finding the target card increases, the point value decreases. Contingencies are shown adjacent to each display (P_{win} = the probability of finding the target card, EV = expected value of the gamble across multiple trials). Unbeknownst to subjects, the first and last gambling options presented are slightly inferior to the middle options, which do not differ from each other in terms of expected value.

gambles with low probabilities of obtaining a larger reward. This is differentiated from disinhibited responses through comparison of the presentation orders. Subjects with an impulsive or disinhibited decision style choose whichever type of gamble is presented first. Thus, they choose low probability gambles when they are presented in order of increasing probability (i.e., low to high), and choose high probability gambles when they are presented in decreasing order. Details of the full procedure are described in Figure 1.

The performances of the twins were compared to those from a group of 11 healthy controls matched for age and IQ. The control data had been published previously (Floden & Stuss, 2004).

4. The 28-item General Health Questionnaire (Goldberg & Hillier, 1979) is a self-report scale that measures psychological distress. It contains four subscales of 7 questions each that measure somatic symptoms, anxiety, social dysfunction, and depression. The 4-point rating scale is scored 0-0-1-1 for

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each question, giving a range of scores from 0 through 28. By convention, scores ≥5 are considered indicative of psychological distress.

Zygosity Testing

Genomic DNA was sent to a genetic testing company, Proactive Genetics Incorporated (http://www.proactivegenetics.com/), for zygosity testing.

Epigenetic Testing

Epigenetic Testing by microarray-based DNA methylation profiling was performed using the protocol described in by Schumacher et al. (2006). Briefly, differences between twins were investigated by interrogating the enriched unmethylated fractions of total genomic DNA from the co-twins. Genomic DNA was extracted from peripheral blood cells using standard phenol chloroform techniques. Enzymatic digestion was performed with DNA methylation sensitive restriction enzyme HpaII (restriction site: CCGG). After digestion, DNA adaptors were ligated to the restriction fragments, and this was followed by polymerase chain reaction (PCR) amplification, using primers that were complementary to the adaptors. PCR conditions were adjusted in such a way that only fragments that were less than 1kb (i.e., short, digested, and therefore unmethylated) will amplify preferentially. The unmethylated fraction of genomic DNA is then end-labeled with Cy3 and Cy5 (GE Healthcare) dyes, and subjected to hybridization at 42° C for 16 hours. All samples were interrogated on the human CpG island microarray, consisting of 12,192 clones representative of numerous CpG island regulatory elements across the genome (Heisler et al., 2005).

Microarray experiments were performed using a balanced block design. Separate enrichments of four genomic DNA aliquots per twin were produced in order to create a total of eight twin versus co-twin hybridizations (biological variance group) that would stringently control for experimental variability. Hybridization signals in the biological variance group could be compared to seven self–self hybridizations (technical variance group) to determine whether epigenetic differences between the twins were detectable above the technical variance.

Epigenetic microarray profiling was performed on four dye swapped technical replicates on 9 pairs of normal MZ twins obtained from the Queensland Institute of Medical Research, Brisbane, Australia in order to assess levels of DNA methylation variation in a control population of identical twins.

Microarrays were scanned on an Axon 4000b scanner and analyzed using Genepix6.0 Software. Subsequent GPR files were subjected to ratio and print tip loess based normalization. Microarray data were trimmed on the microarray feature annotation, removing mitochondrial genes, translocation hot spots, and repetitive genomic regions. Fold change data, as determined by log transformed loess M ratios, were compared using a paired *t*-test, and subjected to correction for multiple testing by a Benjamini-Hochberg False Discovery Rate (FDR) test, a standard for microarray analysis. Gene IDs within 1 kb proximal to CpG islands were obtained from the microarray annotation data, and cross referenced with the April 2007 build of the Gene Ontology Database (www.geneontology.org) to obtain gene ontology (GO) categories associated with each microarray locus. The average fold change for one identified locus (DLX1) with probable functional significance was compared to the variation exhibited by the separate set of microarray data generated using the 9 sets of control MZ twins. A *z* test was used to compare the absolute value of fold change with the distribution of absolute differences of the normal control MZ twins at this locus.

The spot wise standard deviation of co-twin DNA methylation difference across the 9 normal MZ twin pairs was used to assess the biological DNA methylation variation per locus. The distribution of spot wise standard deviations was calculated from the mean values of 1, 2, and 4 dye swapped technical replicates per twin pair separately, to assess the influence of the number of technical replicates on biological variance. Subsequently, a power analysis was performed in *R* in a spot wise manner for each *SD* distribution to detect the proportion of loci per number of twin pairs (*N*) that would have 80% power to detect a range of fold changes (1.15, 1.2, 1.6) at an α level of 0.001, used to control the family-wise error rate (FWER) resultant from multiple testing. A more conservative Bonferroni corrected α level of 4.1×10^{-6} was also used for power analysis for the 1.2 fold change. A fold change of 1.15 corresponds to the observed fold change threshold for technical variance (Figure 4), while the 1.6 fold change represents the maximum fold change observed of any FDR significant loci. The fold change of 1.2 was of particular interest as it corresponds to the observed fold change of an identified locus proximal to the distaless homeobox gene 1 (DLX1), which we speculate as having functional relevance to phenotypic differences in the war/law twin pair.

Results

Psychometric assessment

Based on the WASI, the twins had similar overall IQs (114 and 115; high average range) and both scored zero on the GHQ. However, differences emerged on the MMPI where the war twin's profile personality profile was within normal limits, while her twin's responses revealed a tendency to overreact to minor problems with anxiety and somatic (physical) symptoms.

The twins' MMPI-2 profiles are presented in Figure 2. Analysis of the validity scales (L and K elevated) indicates that both twins approached the MMPI-2 in a defensive manner, presenting themselves in a favorable light. Although these scores likely underestimate symptoms, this does not affect comparisons between the two twins, as their validity score patterns were similar. If anything, the significant clinical scale elevations noted for the law twin over the war twin may be underestimated, as the law twin was slightly more defensive.

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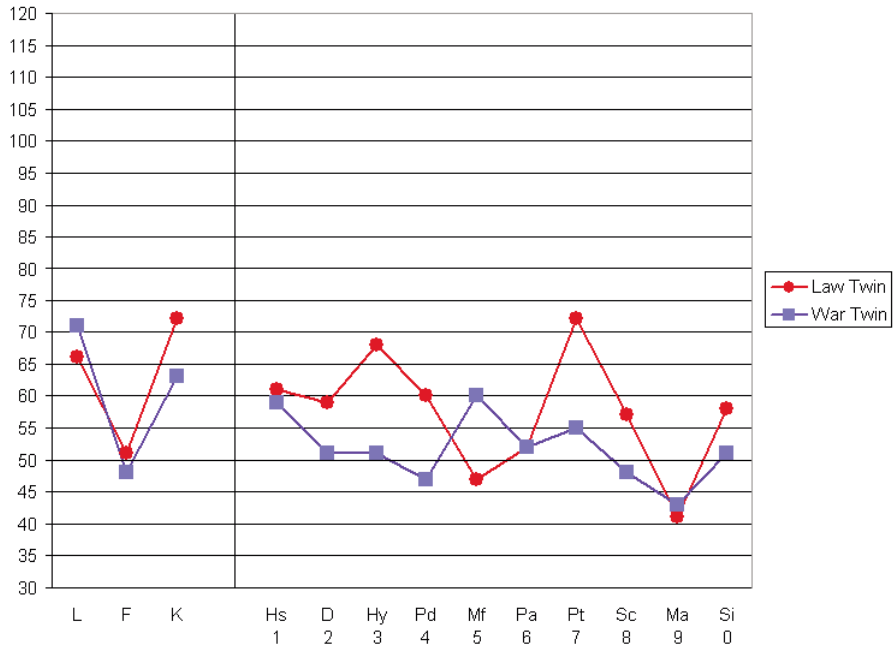

Figure 2

MMPI-2 scores for the law twin (solid line, red circles) and the war twin (dashed line, blue squares). K-Corrected MMPI-2 scores are presented in the standard manner, with the validity scales (L, F, K) followed by the clinical scales.

The law twin's profile of clinical scales included significant elevations on scales 3 (hysteria) and 7 (psychosomatic), a statistically infrequent profile found in less than 1% of the normative sample of women. This profile reflects a high degree of anxiety, tension, and discomfort. Individuals with this pattern tend to be agitated, lacking in self-confidence, perfectionistic, and introverted. They often overreact to problems. The law twin's profile also reflected a lack of insight into the psychological origins of her problems.

The war twin's profile was normal; none of the clinical scales were elevated. It is notable that the war twin produced a relatively high score on scale 5, indicating rejection of traditional female roles, whereas her co-twin produced a relatively low score on this same scale, reflecting more traditionally feminine interests.

Additional sibling differences were present on the Toronto Gambling Task (Figure 3). The war twin preferred high risk gambles where there was a low probability of obtaining a high reward. In contrast, her sister showed risk-averse preferences generally selecting conservative gambles. Both sisters were relatively extreme in comparison to a normal control group of comparable age (mean = 50.6, *SD* = 14.2) and estimated IQ (mean = 115.4, *SD* = 7.0).

Genetics

Monozygosity of the war and law twins was confirmed by Proactive Genetics Incorporated through genotyping of DNA markers D5S818, D13S317, D7S820, D16S539, vWA, TH01, TPOX, and CSF1PO.

Epigenetics

The spot wise fold change exhibited by DNA versus self hybridizations in the technical variance group was compared to that in the twin versus co-twin hybridizations in the biological variance group across 12148

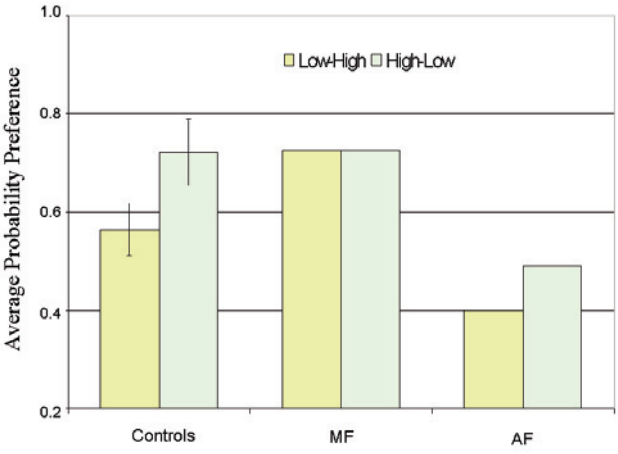

Figure 3

Gambling performance for the twins as well as control subjects (n = 11). Graph depicts the average gamble selection in both the Low–High (increasing probability) and High–Low (decreasing probability) presentation orders. Higher probabilities reflect conservative play whereas lower probabilities reflect riskier play. The law twin performed similar to other controls in the High-Low presentation order but was significantly more conservative in the Low-High presentation order (one-sample $t(10) = 6.1$, $p < .001$). In contrast, the war showed significantly more risk-taking than controls, regardless of presentation order (Low–High order, one-sample $t(10) = 6.2$, $p < .001$; High–Low order, one-sample $t(10) = 6.9$, $p < .001$).

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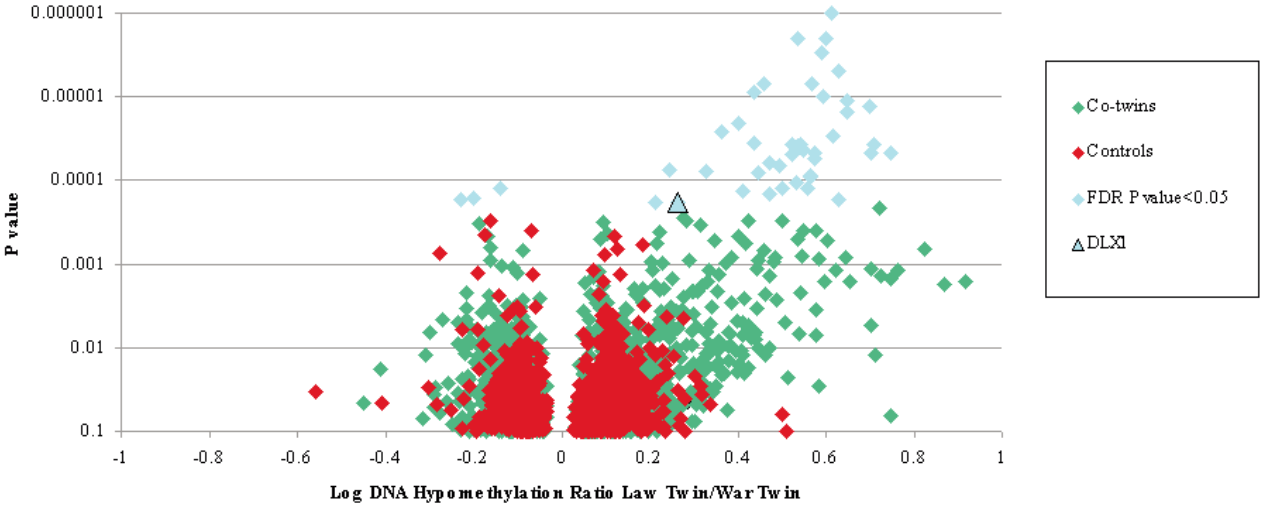

Figure 4

Relative DNA methylation profiles of the war twin vs. law twin

Volcano plot of $\log_2$ transformed ratio information for each microarray element (X axis) vs. the P value of a paired *t*-test (Y axis) comparing DNA methylation differences in the co-twins. Data produced from the biological variance group is in green, while technical variance data is in red. Data values with an FDR corrected *P* value below .05 are in blue, including DLX1, represented by a blue triangle outlined in black.

microarray features, which excludes control and blank spots. The number of loci in the technical variance group with a mean fold change above a threshold of 1.15 was 1496, 591, 309, 258, and 160 for the spot wise average of 3, 4, 5, 6, 7 arrays, respectively. The number of loci in the biological variance group with mean fold change above this threshold was 1020, 644, 418, 486, 499, and 412 loci for 3 to 8 arrays, respectively. Above 4 technical replicates, the biological variance group consistently had 1.5 % more loci above this threshold, on average (Figure 4).

The application of correction for multiple testing using FDR identified 38 loci that consistently exhibit statistically significant differences in DNA methylation levels in the biological variance group. No spots in the technical variance group survived correction for multiple testing, which was the case independent of the number of self-self hybridizations investigated. Only one locus from the technical variance group had a fold change greater than 1.15 ($\log_2(1.15) = 0.2$) beyond a *P* value of < .001 before correction (Figure 4), allowing us to establish an effect size of 1.15 fold change as an experimental variance threshold for significantly different loci between twins.

Gene ontology classification of the most significant genes within 10 kb of the CpG islands revealed 23%

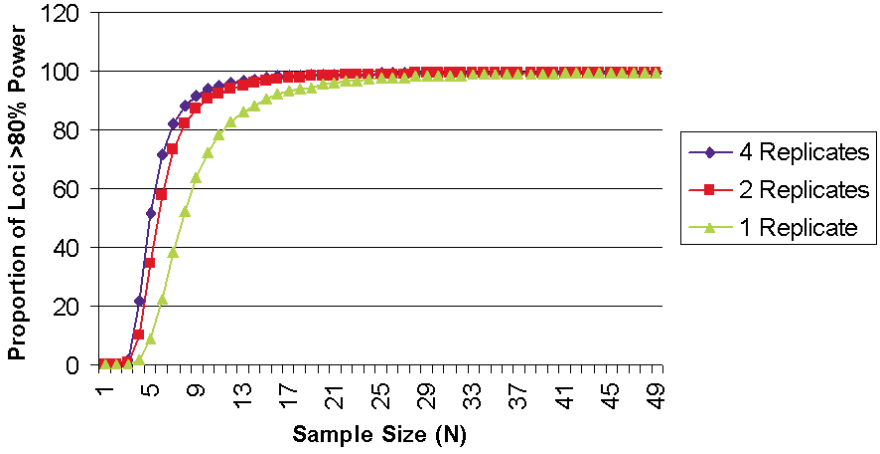

Figure 5

Power vs. technical replicate hybridization number and sample size (*N*) A plot representing the different proportions of loci on the microarray that will achieve > 80% power per sample size when the log fold change across the 9 non-discordant twin pairs used to create a spot wise standard deviation distribution are resultant from an average of 1, 2, and 4 technical replicates. In all cases, the measured effect size was a fold change of 1.2.

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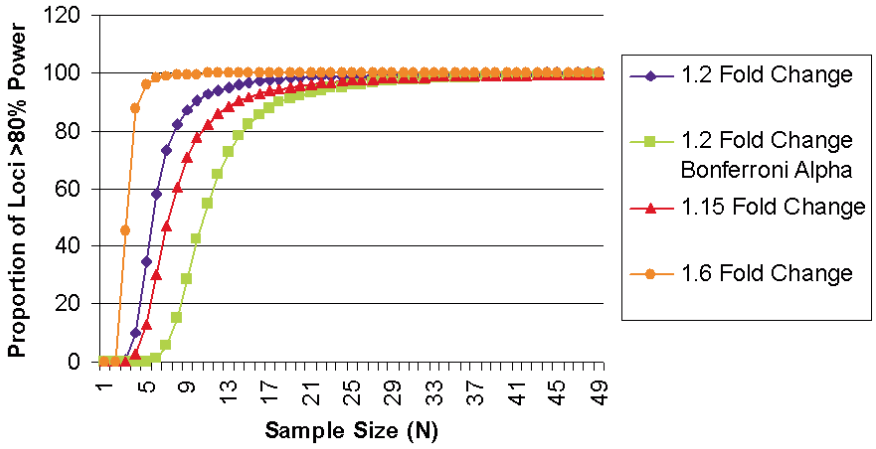

Figure 6

Power vs. effect size and sample size (*N*). A plot of the proportion of loci on the microarray that will achieve > 80% power as a function of the number of twin pairs (*N*) for fold changes of 1.15, 1.2, and 1.6. For the fold change exhibited by the DLX1 locus, power analysis using the most stringent Bonferroni corrected alpha level was also plotted.

involved in transcriptional regulation, including one with potential behavioral implications, namely DLX1, with a *P* value of .047 after correction for multiple testing with FDR. The fold change ratio for this locus was statistically higher than the variation observed in an alternative set of MZ twins ($Z = 3.5$, $P < .0004$).

We also attempted to determine the proportion of the loci on the microarray in total that exhibit > 80% power as a function of sample size. Additionally, we wanted to assess the power of the method as a function of the number of technical replicates per twin pair performed. As expected, we observed that a larger number of technical replicates results in a smaller spot wise variance. Figure 5 depicts the proportion of loci achieving > 80% power per sample size, as a function of the SD distribution produced from the average of 1, 2, and 4 technical replicates per twin pair for an effect size of 1.2 fold change. Similar differences were observed for the other effect sizes (data not shown). In general, dye swapping is useful to eliminate spots resultant from dye bias and cross hybridization, and so subsequent power analysis was performed with the spot wise SD distribution produced from two dye swapped technical replicates, in order to represent the most economical, yet conservative, estimation of sample size requirements. The power analysis prognosticates that with 21, 14, and 6 twin pairs, > 95% of the loci will have an 80% chance of detecting a true DNA methylation difference of 1.15, 1.2, and 1.6 fold between groups, respectively (Figure 6). When applying Bonferroni correction, α is reduced to 4.1 $x10^{-6}$ for a very conservative estimate of the false positive rate. The subsequent sample size in this case for an effect size of 1.2 to achieve > 95% of loci with > 80% power is 25 twin pairs. These power analyses assume that the biological variance in a group of twin pairs with a discordant phenotype will be similar to that of normal pairs.

Discussion

A number of consistent strands run through the twins' phenotypes. The defining characteristics of the war twin's life — dangerous career, married late to a man exposed to similar grave dangers, no children, drinks more alcohol than medically recommended, displays minimal anxiety, and adopts high risk strategies on certain cognitive paradigms — fits well with a demographic and behavioral profile characteristic of war journalists in particular (Feinstein, 2006), and high sensation seekers in general (Zukerman & Kuhlman, 2000). Her twin, by contrast, is the mirror opposite of all these factors and behavioral traits.

Of note was the finding that the war twin's divergent patterns of responses on the Toronto Gambling task were high risk, in keeping with a career choice that included working in zones of conflict. Yet, despite the many life-threatening events that she had confronted, her scores on the GHQ did not reveal emotional distress, a result that matched her healthy personality profile on the MMPI-2. In contrast, her sister who had chosen the more predictable and safer work environment, while also showing no current symptoms of psychological distress on the GHQ, had an MMPI-2 result that revealed a propensity to develop anxiety and somatic complaints when confronting stress. Moreover, her gambling task performance was significantly more risk-averse than controls. Thus, in both twins it is possible to see a connection between career choices and psychometric characteristics.

Biological factors implicated (but not always replicated) in risk taking behaviors have included lower serum levels of monoamine oxidase B (Zukerman, 1994), elevated levels of dopamine (Bardo et al., 1996), and the D4 dopamine receptor (D4DR) exon

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III gene (Ebstein et al., 1997). To date, however, no study has explored potential epigenetic influences.

While MZ twins are genetically identical at the DNA sequence level, a divergence of DNA methylation profiles occurring during development and over time could lead to dissimilar phenotypes. The Human CpG island microarray used to interrogate epigenetic variation between these twins does so at 12,192 loci, representative primarily of CpG island regulatory elements. One of the microarray clones identified as differentially methylated between twins was located on chromosome 2q31.1 within the 3' UTR of the distal-less Homeobox 1 gene (DLX1). When the mean fold change for DLX1 was compared to the general twin versus co-twin *SD* of this locus in an alternative set of 9 MZ twins without known discordant phenotypes, the differences identified between this study's twin pair were significantly higher ($P < .0004$). Epigenetic profiling was carried out in an identical manner in both cases, suggesting that large twin versus co-twin DNA methylation differences at DLX1 in this twin pair have the potential to underlie the psychometric discordance measured.

The DLX1 gene encodes a transcription factor involved in the formation and maintenance of a distinct set of GABAergic interneurons (Cobos et al., 2005; Letinic et al., 2002). DLX1 derived neurons express neuropeptide Y (NPY), a peptide hormone that interacts with the hypothalamic pituitary adrenal axis (HPA), more commonly known as the stress center of the brain (Cobos et al., 2005). DLX1 expression is critical for NPY production, as DLX1 knockout mice show a progressive loss of NPY most likely as a result of interneuronal loss (Cobos et al., 2005). Numerous animal model studies implicate NPY in modulating stress and anxiety, as both NYP receptor antagonists and knockouts produce an anxiolytic effect (Bacchi et al., 2006). This is likely because the stress response produced by the HPA is inhibited by GABA (Kovacs et al., 2004); however, NPY release in turn inhibits the effects of GABA, effectively exciting stress response (Kash & Winder, 2006).

DNA methylation in the DLX1 3'UTR is likely to have important consequences in the regulation of this gene as, like many other homeobox containing genes, the region encoding DLX1 also codes for an overlapping antisense transcript (Coudert et al., 2005; McGuinness et al., 1996), which can modulate gene expression in varying ways (Lavorgna et al., 2004). Like any transcribed region, antisense transcripts can be modulated by DNA methylation status, as exemplified by the epigenetic control of the imprinted KvDMR antisense transcript, KCNQ1OT1. While we did not study the direct mechanisms by which the DLX1 3' UTR methylation modulates gene expression, it is clear that the gene regulatory machinery sensitive to such methylation is in place at the DLX locus, and could have implications for the downstream developmental pathways mediated by this gene. This could result in a reduced overall level of anxiety in the war twin as compared to the law twin, which is consistent with the risk taking behavior differences observed. While peripheral blood should be relatively robust to environmental influence, it remains possible that the observed DNA methylation difference is a downstream effect of the different environments and lifestyles of these two individuals.

To our knowledge, this study represents the first use of microarray-based technology to identify epigenetic differences between identical twins. The technical variance group hybridizations highlight a biological detection threshold of a fold change of 1.15 for differences indicative of true DNA methylation differences. DLX1 represented the most significant identified epigenetic difference that appeared to have functional relevance to the measured phenotypic differences between these twins. While this locus was beyond the threshold of technical variance, and thus represents a true methylation difference between twins, one set of MZ twins is certainly insufficient to make claims that a seemingly relevant epigenetic difference actually accounts for the observed phenotype. Therefore, we used DLX1 as an example of a detectable biological difference, in order to estimate the required sample size of twin pairs with the same discordant phenotype that would be necessary to identify functionally relevant epigenetic changes of this effect size. The results demonstrate that, *pari passo*, were this twin set among a larger sample population of ~15–25 discordant twins with similar behavioral phenotypes, the technique would have 80% power to identify etiologically significant epigenetic differences.

One limitation in the interpretation of the power analysis is that there is a 36-year age difference between the war/law twin pair and the mean age of the cohort of MZ twins used to represent the population DNA methylation variance. The HPLC-based analyses of density of methylated cytosines revealed a consistent age-dependent decrease of global methylation levels in human tissues (Fuke et al., 2004). A more recent study compared MZ co-twin DNA methylation differences in a young and aged cohort of twins, and identified higher levels of DNA methylation variability in the aged cohort (Fraga et al., 2005). The authors hypothesized that DNA methylation patterns may drift over time, causing older MZ twins to be more epigenetically dissimilar than younger ones. Alternatively, a recent study by Heijmans et al. investigated DNA methylation variation at two imprinting control regions in both a young and aged cohort of MZ co-twins, and determined that the observed variation was primarily the result of a heritable influence and that age attributed no effect (Heijmans et al., 2007). Of course, these disparate results could be attributed to differences in the specific genomic regions and twin populations investigated, and to date, there is not a definitive answer regarding DNA methylation variation with

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age. However, it is important to note that such an epigenetic drift, as it was referred to by Fraga et al., could result in a larger population DNA methylation variance, and would, in effect, decrease the power of the microarray technology used in this study in older twin cohorts.

Another important consideration when evaluating the informativeness of epigenetic markers for a given trait is that there could be tissue specific differences between the changes identified in peripheral blood and brain tissue where the gene products of these markers are known to function. That being said, it remains possible that for particular loci, early developmental epigenetic variation occurring prior to major tissue differentiation could be reflected in more peripheral tissue to the area investigated. Some of our laboratory's preliminary epigenetic profiling studies of different tissues between twins find that epigenetic twin differences at particular regions are common between endodermal and mesodermal tissues, suggesting that peripheral blood has the potential to identify epigenetic differences that exist in more distant tissue (unpublished data).

Researchers interested in performing epigenomic microarray profiling of this kind on discordant twin populations should also carefully consider the cost versus power of performing technical replicate microarrays. Our experiments demonstrated that dye swapping was critical to eliminate false positive findings resultant from dye bias (data not shown). Therefore, performing two technical replicates per twin pair comparison is recommended. Beyond this, while averaging the values of more technical replicates reduces the spot wise *SD,* and thus increases power, it appears from Figure 5 that the relative power increase is not sufficient to justify the cost. For example, to detect a fold change of 1.2 with 80% power on > 95% of microarray loci, an *N* of 14 and 12 twin pairs would be required for experiments performed with 2 and 4 dye swapped technical replicates, respectively. The total number of microarray hybridizations required to achieve the same power in each case would therefore be 28 and 48, respectively, and thus it is obvious that performing 2 dye swapped technical replicates is sufficient to detect epigenetic differences without undue cost and effort.

Epigenetic markers cannot by themselves account for why one twin chose war journalism as a career while her co-twin opted for safer environs, for such complex decisions will always transcend genetic determinism. However, what epigenetics may explain is the propensity of one twin to function well and without undue anxiety in highly dangerous situations. In turn, this trait may have influenced a career choice. Whether the same markers are to be found in others pursuing different hazardous occupations is not known, but these results suggest new avenues for research elucidating responses to danger and the ability of some to function well when confronted by risk.

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