Discordant MZ Twins With Cleft Lip and Palate: A Model for Identifying Genes in Complex Traits

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Monozygotic (MZ) twins may be discordant for complex traits due to differential environmental exposure in utero, epigenetic variability in imprinting, X chromosome inactivation, or stochastic effects. Occasionally MZ twins may be discordant for chromosomal and single gene disorders due to somatic mosaicism. For complex traits, which are due to the interactive effects of multiple genes and environmental factors, the affected twin of a discordant MZ pair offers the possibility for identifying somatic mutations in candidate genes. DNA sequencing of candidate genes in discordant MZ twins can identify those rare etiologic mutational events responsible for the different phenotypes since the confounding effects of common single nucleotide polymorphisms are eliminated, as DNA sequences should be identical in MZ pairs. In this report we describe the extensive DNA sequencing of 18 candidate genes in a sample of MZ and dizygotic (DZ) twins with nonsyndromic cleft lip with or without cleft palate. We were unable to identify any somatic differences in approximately 34 Kb of DNA sequenced in 13 MZ pairs, for a total of approximately 900 Kb of sequence comparisons, supporting the hypothesis that nonetiologic posttwinning mutations are rare. While no etiologic variants were identified in this study, sequence comparisons of discordant MZ twins can serve as a tool for identifying etiologic mutations in clefting and other complex traits.

The search for factors contributing to the etiology of complex traits is challenging due to population, locus, and allele heterogeneity, the unequal contribution of the environment in different settings, and the likely large number of genes and environmental triggers involved (Thomas et al., 2003; Wyszynski & Beaty, 2002). Genetic investigations of such traits often require large case collections, as well as substantial molecular and analytic resources in order to be successful. The identification of genetic factors is further complicated when there are multiple interacting loci and when the condition is either rare (i.e., birth defects) or associated with older age of onset (i.e., Alzheimer's disease), making phenotyping and family collection more difficult. Since hundreds or even thousands of affected family members may be necessary for genome-based linkage approaches, the problem can challenge the investigative capabilities of even the largest laboratories (Botstein & Risch, 2003).

An alternative to these traditional gene identification strategies that recently resulted in the successful identification of the gene for autosomal dominant Van der Woude Syndrome (VWS; Kondo et al., 2002) is to search for a posttwinning mutational event in a pair of monozygotic (MZ) twins discordant for the phenotype. In this report, two unaffected parents had MZ twins with one child born with a cleft lip and pathognomonic lip pits as the cardinal manifestations of the VWS, while the other twin was free of any clinical abnormality. Screening of candidate genes identified a stop codon mutation in the IRF6 gene in the affected twin, with normal sequence in the unaffected twin. This was the first case in which a

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hypothesized postzygotic mutational event in a single gene has been clearly documented between MZ twins in humans.

This finding suggested that a similar approach could be used for complex traits with multiple interacting genes and where high-quality candidate genes can be identified through studies of gene expression or metabolism. MZ twins are particularly advantageous in that the background somatic mutation rate between them is expected to be extremely low (Nachman & Crowell, 2000). In addition, the confounding effect of single nucleotide polymorphisms (SNPs), each of which could be etiologic when in or near a gene of interest and which occur at rates of about 1 SNP per 1000 bp of DNA, will be eliminated (Kruglyak & Nickerson, 2001). Since some SNPs are found in less than 1 in 1000 individuals in the general population, the challenge is that screening a very large number of controls might be needed for confirmatory evidence that any particular sequence change found is a polymorphism rather than an etiologic variant. This is particularly important for those variants that will involve regions of DNA involved in gene regulation or expression whose recognition is more difficult than for nonsense or frameshift mutations. In addition, taking advantage of the comparative genome sequence available between the human and animal models such as the chimpanzee, mouse, rat, dog and fish (Thomas et al., 2003), one can now target regulatory regions to look for mutations in discordant MZ twins. Mutations found only in the affected MZ twin are likely to be causal (Mitchell, 2002). We describe here our preliminary efforts to undertake a search for etiologic mutations that may contribute to causation of nonsyndromic cleft lip with or without cleft palate (NSCL/P). As explained below, this strategy can also be applied to other complex traits.

Methods

Study Samples

For this study, 56 pairs of twins were collected. Twenty-two pairs are from the Philippines (8 concordant and 14 discordant for nonsyndromic cleft lip with or without cleft palate [NSCL/P]). They were identified through *Operation Smile* (Murray et al., 1997; Schultz et al., 2003).

Seventeen pairs were recruited from the Netherlands Twin Registry (NTR; Boomsma et al., 2002), 2 concordant and 15 discordant for NSCL/P. The NTR has recruited around 50% of new-born twins in the Netherlands since 1986. Parents of twins complete a questionnaire after registration when the twins are a few months old. Twin pairs were identified from this questionnaire. A few additional pairs applied for inclusion in the study after reading a short description of the study in the yearly NTR newsletter.

Ten pairs were obtained from Denmark collected by the Danish Twin Registry (Skytthe et al., 2003), 1 concordant and 9 discordant for NSCL/P. Four pairs were obtained from the disease checklists in several surveys of the Australian Twin Registry, 1 concordant and 3 discordant for NSCL/P (Bucholz et al., 1998; Heath et al., 2001). The remaining pairs included a discordant pair with Say Syndrome (Ashton-Prolla & Felix, 1997) and two more MZ pairs discordant for nonsyndromic cleft lip with or without palate, one from Colombia and the other reported previously (Wyszynski & Beaty, 2002). Whole blood was collected for DNA extraction after signed informed consent following Institutional Review Board (IRB) approval in all cases.

Zygosity Testing

Zygosity was confirmed by analyzing 15 high heterozygosity microsatellite markers: D12S1042, D4S2639, D10S1230, D9S1118, D5S2498, D5S2496, D6S1027, D6S1034, D6S1270, D5S1470, D2S2972, D1S1656, D2S1360, D8S1132, and D8S1130. Zygosity is also continuously reconfirmed by the identification of normal SNPs in the sequencing of candidate genes.

DNA Sequencing

DNA sequence analysis was carried out on a collection of genes (and some or all of the exons in those genes) based on their status as candidates for NSCL/P. Candidate status was based on previous reports of that gene in a syndromic form of CL/P that might mimic isolated CL/P: MSX1 (Jezewski et al., 2003; van den Boogaard et al., 2000;), FGFR1 (Dode et al., 2003), IRF6 (Kondo et al., 2002), P63 (Barrow et al., 2002), and other genes that cause syndromes that include clefting or have expression patterns suggestive of contributing to CL/P. These include JAG2, (Shawber et al., 1996), LHX8 (Zhao et al., 1999), PTCH (Kimonis et al., 1997; Mansilla et al., 2004), PVRL1 (Sozen et al., 2001; Suzuki et al., 2000), SKI1 (Colmenares et al., 2002), and TBX10 (Bush et al., 2004), FGFR2 (Rice et al., 2004), FGF10 (Rice et al., 2004), SATB2 (FitzPatrick et al., 2003), FOXE1 (Castanet et al., 2002; Clifton-Bligh et al., 1998), and TGFB3 (Lidral et al., 1998; Vieira et al., 2003). PVR, PVRL2 and MSX2 were analyzed as homologs of candidate genes.

Templates for sequencing were generated by PCR in an Applied Biosystems Gene Amp PCR System 9700. The 10 µl reactions contained 1.5 mM Mg^{2+}, 200 µM dNTPs, 0.3 mM each primer, Bioexact reaction buffer (Bioline), 0.25 units of Bioexact and 20 ng DNA, and were performed in corresponding cycle for each gene. Sequencing then was performed with the DNA Sequencing Kit, Big Dye™ Terminator Cycle Sequencing. (Applied Biosystems). The 10 µl sequencing chemistry reactions contained Big Dye Terminator Mix, Big Dye Terminator Buffer reaction, 0.075 mM of the corresponding primer, 1.25 ng/100 bp of the PCR product, and 5% of DMSO. The standard cycle

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suggested by the manufacturer was carried out using at least 35 cycles.

Sequencing reactions were run on an ABI Prism 3700 analyzer.

Sequences were analyzed by the Polyphred 4.0 program and Consed and manually inspected to verify differences and detect possible mutations missed by the algorithm.

Deletion Assays

We also examined this set of samples for the 22q11.2 deletion or DiGeorge Critical Region (DGCR) using a real time PCR assay that was suited to the available DNA samples (Kariyazono et al., 2001). Since some cases of 22q- have clefts, this approach could identify an alternate mechanism to point mutations by searching for *de novo* deletions resulting from genomic rearrangements.

PCR was carried out as proposed by Kariyazono et al. (2001) using primers and fluorescent probes for two genes: ubiquitin fusion degradation gene (UFD1L), reported to play a role in the DGCR, and the S100B gene (mapped to chromosome 21) used as a control target and triplicated in chromosome 21 aneuploidy. Deletion 22q and Trisomy 21 controls were included.

Results

Zygosity Confirmation

Table 1 summarizes the zygosity status of the 56 pairs of twins tested. No difference in either marker was found among the MZ twin pairs, whereas the dizygotic (DZ) twin pairs showed genotypic differences in at least two markers.

Thirteen MZ discordant, 4 MZ concordant and 4 DZ discordant twin pairs were selected for sequencing and deletion assays (Table 2).

Variants

The exons and regulatory regions shown in Table 3 were sequenced. All the samples were analyzed in forward and reverse directions. Table 4 shows the collected data from the sequencing results.

Table 1

Zygosity Status Based on the Genotypes of the 15 Microsatellites Markers

	P	A	N	D	C	W	F	Total
MZ Disc	6	2	1	1	1	1	1	13
MZ Conc	3	1	1					5
DZ Disc	8	1	14	8				31
DZ Con	5		1	1				7
Total								56

Note: MZ = Monozygotic; DZ = Dizygotic; Conc = Concordant; Disc = Discordant; P = Phillipines; A = Australia; N = Netherlands; D = Denmark; C = Colombia; W = Wyszynski et al., 1996 and 2002; F = Ashton-Prolla & Felix, 1997.

Table 2

Twins Pairs Used for Sequencing in this Study

	P	A	N	D	C	W	F
MZ Disc	6 (2/4)	2 (1/1)	1(1/0)	1(0/1)	1 (1/0)	1 (0/1)	1 (0/1)
MZ Conc	2 (1/1)	1 (0/1)	1(1/0)				
DZ Disc	2 (1/1)			2 (1/1)			

Note: Male/female ratio in parenthesis.
MZ = Monozygotic; DZ = Dizygotic; CONC = Concordant; DISC = Discordant; P = Phillipines; A = Australia; N = Netherlands; D = Denmark; C = Colombia; W = Wyszynski et al., 1996 and 2002; F = Ashton-Prolla & Felix, 1997.

Nineteen new polymorphisms not previously found in disease or normal populations were found (Table 5). A synonymous SNP in MSX1 exon 2, which was inherited from the unaffected father, was found in a pair of MZ twins with a concordant cleft phenotype. In the same pair of twins, a SNP was identified in a putative regulatory region 3' of the MSX1 gene inherited from the unaffected mother and a G to A transition 13 base pairs upstream of exon 15 in FGFR1, inherited from the unaffected father. The MSX1 regulatory region SNP was not detected in 300 unaffected controls but the FGFR1 SNP was found in 3 out of 300 unaffected controls.

Two new single nucleotide polymorphisms were also identified that result in missense mutations: G676A (Glu226Lys) in PVR was present in a DZ affected twin but absent in the unaffected co-twin, while C533T (Thr178Met) was identified in PVRL2 in a pair of MZ discordant twins. DNA from the

Table 3

Genes, Exons and Regulatory Regions Analyzed

Gene	Exons	Regulatory regions
FGF10	1–3	
FGFR1	2–18	
FGFR2	2–18	
FOXE1	1	
IRF6	1–9	81 & 100 kb
JAG2	13, 14 & 15	
LHX8	5	
MSX1	1 & 2	1–8, 10–13
MSX2	1	
P63	1, 3–14	
PTCH	6, 9 & 15	
PVR	1–8	
PVRL1	1–6	
PVRL2	2–6	
SATB2	3–11	
SKI1	2 & 3	
TBX10	1–5 & 8	
TGFB3	2	

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Table 4

Registry of the Sequencing Data

Gene		MSX1	MSX1	IRF6	TBX10	PVRL1	PVRL2
Region		Exon 2	R5	Exon 7	Exon 3	Exon 3	Exon 2
Nucleot			C-3170T				C-207T
Prot		G263G		V274I	K101T		
mz disc	Phil-2-A						
	Phil-2-U						
	Phil-4-A						
	Phil-4-U						
	Aus-2-A						
	Aus-2-U						
	Phil-5-A						
	Phil-5-U						
	Say-1-A						
	Say-1U						
mz con	Phil-7-A						
	Phil-7-U						
	Aus-6-A						
	Aus-6-U						
dz disc	Phil-10-A						
	Phil-10-U						
	Aus-8-A						
	Aus-8-U						

Note: The variants found in the sequence procedure are registered in a table, identifying gene and region sequenced, nucleotide (Nucleot) or amino acid (Prot) change found. Zygocity (mz: monozygotic; dz: dyzigotic) and discordancy (con: concordant; dis: discordant) for the twins pairs is also indicated. The genotypes are registered following the pattern code.

parents for both twin sets is not available to confirm origin of the mutations, but we are currently sequencing unaffected controls for comparison frequencies.

No variant differences were found in the Say Syndrome (Ashton-Prolla & Felix, 1997) discordant MZ pair, nor in the interesting family previously reported by Wyszynski et al., (1996), and Wyszynski and Beaty (2002). This family had MZ twin sisters discordant for clefting, but each had an affected child with cleft (Wyszynski & Beaty, 2002).

In this study, approximately 900 Kb of DNA in 14 pairs of discordant MZ twins were compared and no sequence differences found. For the DZ pairs ~200 Kb were compared, and in total 25 differences were confirmed between the two probands of a pair. This rate of 1 SNP/8 Kb is lower than the reported background DNA differences (Kruglyak & Nickerson, 2001). This is due to the small number of samples included in this study and the genetic relatedness of DZ twins which diminishes the nucleotide diversity between them.

Deletion Assays

No deletions were found in any members of the twin population (Figure 1).

Discussion

In this report, an initial effort to collect and study a pilot set of samples of MZ twins discordant for isolated cleft lip and palate is described. DNA sequencing and microdeletion searches were carried out for a panel of 18 highly plausible candidate genes selected for their previously identified role in isolated cleft lip and palate or on the basis of their gene expression or mouse knockout phenotypes.

We determined the zygosity for the 56 pairs of twins (18 MZ and 38 DZ) for comparative purposes. The female pairs of all these twins will be useful for studies of X chromosome inactivation and, similarly, all the twin pairs could be used for studies of epigenetic effects involved in the etiology of oral clefts, such as imprinting.

Our sequencing results show no differences in approximately 900 Kb of comparative sequence between MZ twins. This suggests that somatic mutation events are indeed rare, as has been suggested by Crow (2000). Although confirmed mutations have not yet been identified in any of the twin pairs, the approach used here appears valid and efficient, as high throughput DNA sequencing can be carried out reliably when DNA samples are of similar quality and established protocols are in place. We used automated reads of DNA sequence to provide an initial view of possible sequence differences and then verify suspected variants by inspection and subsequently by resequencing, reverse sequencing, and sequencing of parents to identify those which are *de novo*. This general strategy can now be extended to additional twin pairs and genes to determine the frequency with which such mutations might arise.

Table 5

List of New Polymorphisms (HMH = Human Mouse Homology)

Gene	Region	Base	Amino acid
MSX1	Exon 2	C3553A	G263G
	3′ HMH	C+1055T	
	3′ HMH	C+2588T	
FGFR1	Intron 14	G+137A	
	Exon 18 (3′ UTR)	G2477A	
FGFR2	Exon 2 (5′ UTR)	G–46A	
	Exon 16	A2112G	P704P
FOXE1	Exon 1	C210G	P70P
IRF6	3′ HMH	G+79247A	
P63	Intron 8	C+125G	
	Intron 8	G+144A	
	Exon 9	G1131T	P377P
	Intron 10	G+65A	
	Intron 10	G+86A	
PVR	Exon 2	T207C	H69H
	Exon 3	C609T	T203T
	Exon 3	G676A	E226K
PVRL2	Exon 3	C533T	T178M
	Exon 3	G684A	S228S

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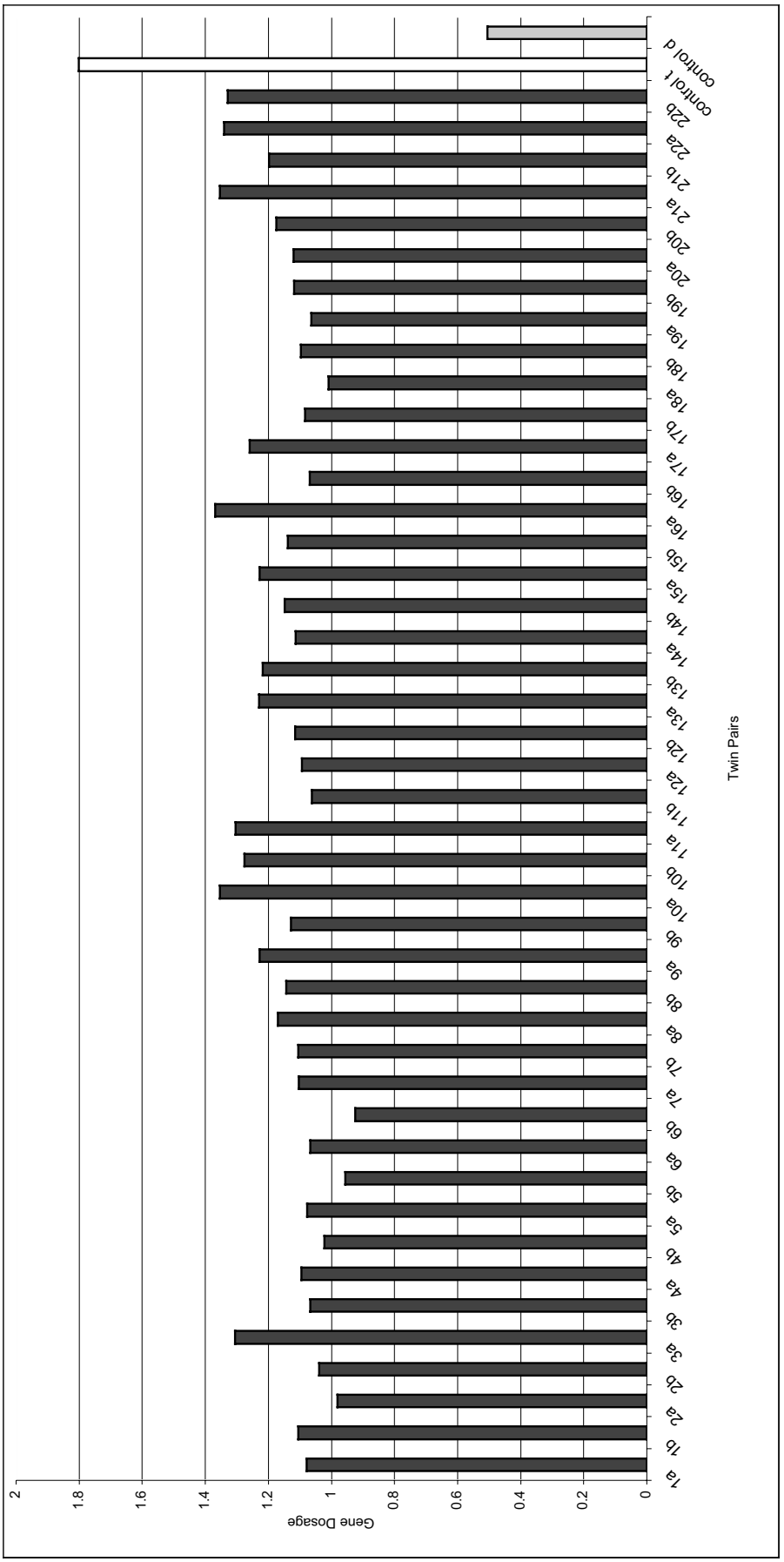

Figure 1
22q deletion assay. The gene dosage is obtained using S100B as an internal control for the 22q11.2 deletion (R22 = UFD1L/100B) and UFD1L as an internal control for the trisomy 21 (R21 = S100B/UFD1L). Samples heterozygous for the UFD1L are expected to yield R22 = 0.5, homozygous normal samples r21,22 = 1.0 and trisomy 21 R21 = 1.5. Twin pairs are indicated as 1a–1b to 22a–22b (black bars), trisomy control (control t, white bar), and deletion control (control d, gray bar).

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There are likely to be multiple causes of the discordances found in MZ twins of which postzygotic twinning mutations will only be one. Other factors related to penetrance and/or stochastic factors could certainly play a role as could environmental differences such as twin-to-twin transfusions and other vascular, anatomic or positional differences found in MZ twins. Similarly, X chromosome inactivation (in female pairs), imprinting and other phenomena of gene expression could also play a role.

There are several limitations to our approach. Mosaicism in the affected twin might make mutations difficult to detect. Similarly, vascular anastomoses between the twin pairs could also result in both twins having evidence of mutation; however, the unaffected twin would have had this mutation identified only in DNA derived from cellular components of blood that could be exchanged through the anastomoses. Routinely collecting buccal swabs or other sources of tissue from such twin pairs would give one the opportunity to determine whether the mosaicism was localized to a single tissue. There are many strengths to this approach as well. First, as noted above, it almost eliminates the concern that variants found are rare single nucleotide polymorphisms. Except in unusual cases where these are located in mutation hot spots (Goriely et al., 2003), the background rate of mutations appears low enough that investigating them in more detail should be well within the capacity of a screening protocol. If complex traits are caused by digenic or triallelic disorders (Kajiwara et al., 1994; Katsanis et al., 2001), the technique can identify a mutation arising in one member of that path. Mutations can be found in autosomal recessive as well as dominant disorders where a second hit within the candidate gene will present as the mutation in recessive conditions.

MZ twins discordant for complex traits such as hypertension or diabetes are common (~50% of twin pairs; Martin et al., 1997). Because of the high prior probability that the study genes would have been selected for strong candidate status and because there is a low background rate of somatic mutations, the identification of one likely etiologic mutation (such as a nonsense or frame shift variant in a discordant pair) would be, almost by itself, confirmatory of etiology.

Finally, this approach can also continue to be used for rare syndromic disorders in which such discordant pairs are occasionally reported. This may be particularly relevant for those birth defect disorders that are of uncertain etiology and may be secondary to chromosomal microdeletions, multiple gene mutations, imprinting or other difficult-to-assay causes. In these cases, expensive or high-risk protocols such as comparative genomic hybridization might be successfully applied to a small number of cases to provide a cost-efficient search. Unusual multiple malformation disorders such as OEIS or VATER (Bohring, 2002) might particularly benefit from this approach.

Overall, we believe that the use of discordant MZ twin pairs and high-throughput sequencing, as well as genetic searches based on genes selected from gene expression data hold great promise for identifying mutations contributing to the genetic component of complex traits. As sequencing costs continue to drop, this approach should be considered as an adjunct to other gene/mutation mapping strategies.

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