

# Dysregulation of X-Linked Gene Expression in Klinefelter's Syndrome and Association With Verbal Cognition

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Klinefelter's Syndrome (KS) is a chromosomal karyotype with one or more extra X chromosomes. KS individuals often show language impairment and the phenotype might be due to overexpression of genes on the extra X chromosome(s). We profiled mRNA derived from lymphoblastoid cell lines from males with documented KS and control males using the Affymetrix U133P microarray platform. There were 129 differentially expressed genes (DEGs) in KS group compared with controls after Benjamini–Hochberg false discovery adjustment. The DEGs included 14 X chromosome genes which were significantly over-represented. The Y chromosome had zero DEGs. In exploratory analysis of gene expression–cognition relationships, 12 DEGs showed significant correlation of expression with measures of verbal cognition in KS. Overexpression of one pseudoautosomal gene, *GTPBP6* (GTP binding protein 6, putative) was inversely correlated with verbal IQ ( $r = -0.86$ ,  $P < 0.001$ ) and four other measures of verbal ability. Overexpression of *XIST* was found in KS compared to XY controls suggesting that silencing of many genes on the X chromosome might occur in KS similar to XX females. The microarray findings for eight DEGs were validated by quantitative PCR. The 14 X chromosome DEGs were not differentially expressed in prior studies comparing female and male brains suggesting a dysregulation profile unique to KS. Examination of X-linked DEGs, such as *GTPBP6*, *TAF9L*, and *CXORF21*, that show verbal cognition–gene expression correlations may establish a causal link between these genes, neurodevelopment, and language function. A screen of candidate genes may serve as biomarkers of KS for early diagnosis. © 2007 Wiley-Liss, Inc.

**KEY WORDS:** XXY; XY; brain; lymphocytes; microarray; *XIST*; *GTPBP6*; *TAF9L*; *CXORF21*

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## INTRODUCTION

Men with Klinefelter Syndrome (KS) have one extra X chromosome (XXY) and vary considerably in their cognitive and behavioral phenotype [Jacobs and Strong, 1959]. The cells with at least one extra X chromosome are believed to create deficiencies in male sexual characteristics [Smyth and Bremner, 1998]. It is for this reason that XXY individuals frequently are undetected until adolescence until they present to a physician for lack of normal male sexual growth [Smyth and Bremner, 1998]. It is at this time that they are often prescribed testosterone. Many XXY males then obtain heterosexual mates, but are rarely fertile.

Individuals with XXY karyotype generally have normal to low-normal intelligence, but many display subtle verbal disabilities, while their non-verbal abilities are spared [Nielsen, 1969; Netley and Rovet, 1982; Theilgaard, 1986; Bender et al., 2001; Boone et al., 2001; Fales et al., 2003]. Dyslexia and attention deficit disorder are diagnosed more frequently in children with the XXY syndrome than in their XY peers [Bender et al., 1986; Temple and Sanfilippo, 2003]. Behavioral abnormalities reported range from depression to bipolar disorder to schizophrenia, although the majority of XXY men are psychiatrically normal. KS is significantly more frequent among hospitalized patients with schizophrenia than the general population [reviewed in DeLisi et al., 1994]. We began a series of investigations to determine whether this naturally occurring human chromosome anomaly could serve as a neurogenetic research model for determining specific sex chromosome and/or autosomal genes whose abnormal expression may put individuals at risk for developing schizophrenia or other serious psychotic disorder. We previously reported that XXY males had gray and white matter structural brain differences compared with normal XY males and that they had an excess of psychotic symptoms, as well as verbal deficits [DeLisi et al., 2005].

## MATERIALS AND METHODS

### Subjects

Adult males with a previously diagnosed XXY karyotype were recruited primarily from advertisements on websites for regional US support groups designed specifically to aid people with KS and other chromosomal anomalies, and by contacting

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the leadership of these organizations directly [see DeLisi et al., 2005 for recruitment details]. Eleven males with previous written documentation of an XXY karyotype [DeLisi et al., 2005] agreed to participate in this study. For the current study, laboratory controls consisted of six consecutively obtained control males who were recruited for other genetic studies without psychiatric conditions.

A structured psychiatric interview was performed on all XXY subjects using the Diagnostic Interview for Genetic Studies [DIGS; Nurnberger et al., 1994] and a psychiatric diagnosis made according to DSM-IV Criteria for both Axes I and II psychiatric disorders. These results have been reported in DeLisi et al. [2005]. In addition, the symptom categories obtained that were relevant to a diagnosis of schizophrenia or affective disorder were scored as present or absent (multiple delusions, auditory hallucinations, major depression, mania).

A battery of cognitive tests was performed and examination of group differences between XXY men and XY controls was reported previously [DeLisi et al., 2005]. Since differences between XXY men and controls were primarily within the domains of verbal functioning and attention, those were specifically chosen for correlation to the gene expression results in the current study. The scores used came from the following domains and specific tests:

- (1) *General Verbal Ability*—Full-scale IQ and Verbal IQ obtained from the full WAIS-III;
- (2) *Receptive Language*—The Token Test is a series of oral commands about colored circles and squares given in increasing complexity, and the Peabody Picture Vocabulary Test (PPVT). The critical dependent variables were the total scores;
- (3) *Expressive Language and Academic Skills*—The Wide Range Achievement Test, 3rd Edition (WRAT), The Boston Naming Test, and Controlled Oral Word Association (COWA) examined both category fluency and phonological fluency;
- (4) *Verbal Memory*—Wechsler Memory Scale-III, Wechsler Logical Memory I and II; Haylings sentence completion test, immediate and delayed, sentence repetition, and the word attack test;
- (5) *Concentration/Attention*—Trail-making Tests Parts A and B.
- (6) *Academic Skills*—The Wide Range Achievement Test, 3rd Edition (WRAT).

In all tests, but trail making, which is a measure of psychomotor speed, the higher the score, the better the functioning. Exploratory cognition—gene expression correlations were conducted using Pearson's correlations with unadjusted  $\alpha = 0.05$ . The New York University School of Medicine, The Nathan S. Kline Institute for Psychiatric Research, and The University of California human subjects' review boards approved this study. All participants gave written informed consent prior to their participation.

## LABORATORY

A blood sample was drawn from all KS subjects for confirmation of XXY or XY karyotypes and high-resolution karyotyping was performed by JL at Coriell Institute in Camden, New Jersey directly on cultured T-lymphocytes using standard techniques [DeLisi et al., 2005]. Transformed lymphocytes from each subject were obtained from the Coriell Institute and grown until confluent. The procedure for generating cell lines and extraction of total RNA was as previously described [Vawter et al., 2006]. RNA was extracted from  $\sim 5 \times 10^7$  lymphoblastic cells using the standard TRIzol

isolation protocol (Invitrogen, Carlsbad, CA). The total RNA was cleaned by passing over silica-based mini-spin columns (Qiagen RNeasy Mini Kit, Valencia, CA) and analyzed on a 2100 Bioanalyzer (Agilent, Palo Alto, CA) for quantification of 28S and 18S ribosomal RNA peaks.

## Oligonucleotide Microarrays

The Affymetrix oligonucleotide microarray chip (Human GeneChip U133P 2.0) expression profiling experiments were carried out following the manufacturer's technical protocol (Affymetrix, Santa Clara, CA). Briefly, total RNA from human lymphoblastoid cells was reverse-transcribed to cDNA using a T7-(dT)<sub>24</sub> (HPLC purified) primer (Superscript cDNA Synthesis Kit, Invitrogen). The cDNA was cleaned (Qiagen Gene Chip Sample Cleanup Module) and synthesized into cRNA (MegaScript T7, Ambion, Austin, Texas), incorporating biotinylated-UTP and -CTP (PE Lifesciences, MA). Biotinylated cRNA was cleaned (Qiagen Gene Chip Sample Cleanup Module), quality-checked (Bioanalyzer 2100, Agilent), fragmented, and hybridized to the Affymetrix Human Genome U133P Arrays. Arrays were washed, stained, and scanned on the Affymetrix Fluidics Station and G7 Affymetrix high-resolution scanner using GCOS 1.3. The cell files derived from the U133P chips were analyzed with a robust multiarray condensation algorithm (RMA) (Irizarry et al., 2003). The RMA condensation of Affymetrix probes was accomplished using a custom Affymetrix chip definition file for ENSEMBL genes (Dai et al., 2005) was available at: [http://brainarray.mhri.med.umich.edu/Brainarray/Database/CustomCDF/genomic\\_curatedCDF.asp](http://brainarray.mhri.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curatedCDF.asp). There has been a significant increase in EST, cDNA, and genomic sequence information since the design of the Affymetrix chip on the Unigene 133 build [Dai et al., 2005].

The RMA expression values were subjected to a within array (subject) z-score transformation [Cheadle et al., 2003]. The z-score transformation sets the mean for each individual array at 0 and the standard deviation at 1. The z-score data is analyzed for KS and normal karyotype males for differential gene expression with a two-tailed *t*-test ( $P < 0.05$ ). The *t*-tests for each gene were subjected to Benjamini-Hochberg correction for false discovery rate (Partek Genomics Solution, v 6.2, St. Louis, MO). All genes were utilized in the Benjamini Hochberg FDR calculation; there was no prescreening of genes from the entire list.

## Quantitative Real-Time PCR

Primers were designed within a 3' exon using the Primer Express program (Applied Biosystems, Foster City, CA). A nucleotide BLAST (NCBI) was performed to check the specificity of the primers. The same purified cDNA preparation was used for microarray analysis and quantitative real-time PCR. A 1:100 dilution of cDNA (5  $\mu$ l) was added to each Q-PCR reaction containing SYBR Green PCR Master Mix (Applied Biosystems), and amplification was carried out on the 7000 Sequence Detection System (Applied Biosystems). A standard curve used to quantitate the products was generated from dilutions of known concentrations of genomic DNA. All samples and standards were run in triplicate. The average Ct values were used for *t*-tests to compare KS and control group. The Ct for a housekeeping gene (*SLC9A1*) previously shown in lymphocytes to be a stable reference gene [Vawter et al., 2006] was subtracted from each target gene for normalization.

## Bioinformatic Analysis

Differentially expressed genes (DEGs) that passed Benjamini-Hochberg correction from microarray analysis were

compared to the actual number of genes in particular KEGG (Kyoto Encyclopedia of Genes and Genomes), GO, and GenMapp pathways. Fisher exact tests were calculated for gene classification enrichment (Hosack et al., 2003). The EASE software application (Hosack et al., 2003) was obtained from the NIH website (<http://apps1.niaid.nih.gov/David/upload.asp>). Functions of genes were annotated using Database for Annotation, Visualization, and Integrated Discovery (DAVID) [Dennis et al., 2003]. The over-representation analysis was subjected to Bonferroni correction for multiple testing of pathways.

The Novartis SymAtlas website was consulted for tissue profiling of each significant gene to determine brain and blood expression profiles <http://symatlas.gnf.org/SymAtlas/> [Su et al., 2004]. The number of brain regions in the Novartis SymAtlas database that showed above the median tissue expression for each DEG was used as a surrogate index of brain expression. There are 19 brain tissues assayed in duplicate, including fetal brain, and other tissues and cell lines [Su et al., 2004], for which the median expression levels are calculated. Inspection of the results compared to Unigene library representation showed good agreement.

Dr. DeLisi at New York University School of Medicine directed the identification, evaluation and collection of blood samples from all XXY men for this project. The microarray analysis and quantitative PCR studies of lymphoblastoid cell lines were conducted under the direction of Dr. Vawter at UC Irvine. Neuropsychological test scores were analyzed in collaboration with Dr. Harvey at Mt. Sinai School of Medicine.

## RESULTS

After Benjamini–Hochberg false discovery correction of the  $P$ -values, there were 129 DEGs found in the KS males compared with the control males (Table I). All genes were utilized for calculation of the Benjamini Hochberg FDR. The  $P$ -values, fold changes, and number of brain regions that show expression for each significant DEG are shown (Table I and Supplementary Table I). There were 14 DEGs on the X chromosome: XIST, MBTPS2, CXorf21, ZNF275, DSYS155E, SMCX, ZFX, MECP2, RPS4X, TAF9L, GTPBP6, SLC35A2, YY2, PGK1. There were 0 DEGs located on the Y chromosome,

and the remaining 115 DEGs were located on autosomes. All 129 DEGs were analyzed for pathway over-representation analysis with EASE. There were no Gene Ontology or KEGG pathway classifications that exhibited significant over-representation for 129 DEGs following Bonferroni correction. Each chromosome was tested for over-representation by Fisher's Exact Test ( $2 \times 2$  table) for the number of genes on the chip/number of genes on the chromosome and compared to the number of DEGs on the chip/number of DEGs on the chromosome. The Fishers' Exact Test used the hypergeometric distribution. The X chromosome DEGs were significantly over-represented after Bonferroni correction (adjusted  $P = 0.005$ ) and there were no other chromosomes that showed significant over-representation. The criterion for significant DEGs was then relaxed (uncorrected  $P < 0.0001$ ), and upregulated genes and downregulated genes were run as separate lists in EASE. The regulation of transcription GO biological process category and similar categories hierarchical GO categories (transcription, DNA binding) were over-represented ( $P < 0.0003$  following Bonferroni correction). For downregulated genes using the relaxed criterion, the mitochondrion GO cellular localization category was also significantly over-represented ( $P < 0.03$  following Bonferroni correction). An example of three significant X chromosome gene differential expression for KS compared to XY males is shown (Fig. 1A and Supplemental Fig. 1B,C).

### DEG Correlations With Cognitive Variables

A total of 12 of the 129 DEGs showed significant correlations (nominal  $P < 0.05$ ) with 1 or more of the verbal cognitive test scores in KS subjects (Table II). Of these 12 DEGs, 7 showed increased gene expression in KS and an inverse correlation to verbal cognition so that higher expression of the gene correlated with worse functioning. The other five DEGs were decreased in KS and showed positive correlations with verbal cognition, that is lower activity correlated with worse functioning.

Expression of one pseudoautosomal gene, *GTPBP6* (GTP binding protein 6, putative; alias *PGPL*) was inversely correlated with verbal IQ ( $r = -0.85$ ,  $P < 0.001$ ) and four other measures of verbal cognition (Table II). *GTPBP6* is located on

TABLE I. X-linked Gene Expression Loci

Cytoband	Symbol	$P$ -value	Fold change (Klinefelter's/control)	Number of brain regions above median expression (Novartis SymAtlas)	ENSEMBL gene
Xq13.2	XIST	6.28E-15	136.53	3	OTTHUMG00000021839
Xq13.2	XIST	4.88E-15	79.06	3	OTTHUMG00000021839
Xp21.3	<b>CXorf21</b>	3.08E-07	2.62	17	ENSG00000120280
Xq28	ZNF275	4.54E-08	2.02	13	OTTHUMG00000024196
Xp22.32	DXYS155E	2.49E-06	1.95	8	OTTHUMG00000021063
Xp11.22-p11.21	SMCX	1.85E-06	1.81	5	ENSG00000126012
Xp21.3	ZFX	3.57E-09	1.81	5	OTTHUMG00000021264
Xp22.33/Yp11.32	<b>GTPBP6</b>	2.59E-06	1.65	8	ENSG00000178605
Xq28	MECP2	3.07E-07	1.36	1	OTTHUMG00000024229
Xq13.1	RPS4X	6.83E-07	1.18	10	ENSG00000198034
Xp11.23-p11.22	SLC35A2	2.66E-07	0.8	16	OTTHUMG00000024129
Xp11.23-p11.22	SLC35A2	1.50E-07	0.78	16	ENSG00000102100
Xp22.2-p22.1	YY2	1.25E-07	0.72	6	OTTHUMG00000021237
Xp22.1-p22.2	MBTPS2	1.93E-08	0.54	7	ENSG00000012174
Xq13	PGK1	2.11E-06	0.29	2	ENSG00000187325
Xq13.1-q21.1	<i>TAF9L</i>	2.45 E-06	0.56	5	OTTHUMG00000021887

The fold change,  $P$ -values are from microarray U133P 2.0 Affymetrix chips, are shown for genes that passed Benjamini–Hochberg false discovery. The gene symbols in bold correspond to gene expression markers that significantly correlated with cognitive measures in Table II. The data for the number of brain regions above median expression is from Novartis SymAtlas website, and the italicized number of brain regions above median expression indicate fetal brain expression of the gene. Some genes are shown twice, due to different probesets that BLAST to different ENSEMBL transcripts. Data for blood expression from Novartis SymAtlas website is shown in a supplementary table and the expression loci on autosomal chromosomes are also listed in a supplementary table.

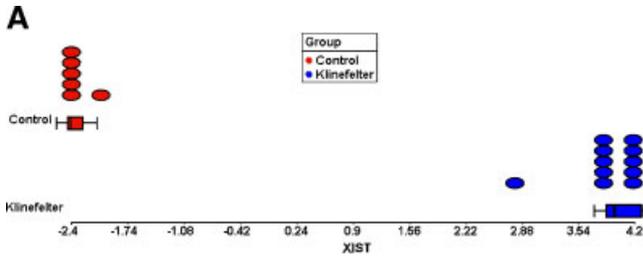


Fig. 1. Dotplot of Klinefelter's Syndrome and control males showing differential gene expression. Sample plots are from DEGs that passed Benjamini Hochberg false discovery and were verified with quantitative real-time PCR were selected: (A) XIST, (B) CXORF21, and (C) ZNF275. B, C: These are shown in Supplemental Figures. The units on the x-axis represents the standard z-score transformed expression value [Cheadle et al., 2003] for each subject on each microarray (z-score mean for all genes = 0, standard deviation = 1). Thus, for XIST gene, the males with XXY karyotype showed 2.8–4 standard deviations above mean signal intensity compared to all genes on the array, while XY males showed a signal intensity 1.74–2.4 standard deviation units below mean expression. There is a standard deviation (z-score unit) spread between XY and XXY expression of XIST.

the pseudoautosomal region of chromosomes X and Y. Since 3 of the 12 genes that displayed correlations with cognitive measures were X-linked, this suggested that the X-linked genes may be over-represented in relation to the remaining genes and the enrichment was at a trend (Chi-square = 3.61,  $P = 0.057$ ,  $df = 1$ ).

#### Validation of Microarray Gene Expression Profile

Ten candidate genes that passed the Benjamini–Hochberg false discovery rate correction were analyzed with SybrGreen quantitative real-time PCR (Q-PCR) assays. Eight of these genes showed significant differences ( $P < 0.05$ ) between KS and control male groups by Q-PCR (Table III). These eight X-linked candidate genes that were validated by Q-PCR (XIST, SMCX, SNF275, CXORF21, GTBP6, SLC35A2, TAF9L, and MBTPS2), showed an average  $\pm 2.2$ -fold dysregulation by real-time Q-PCR in KS compared to control subjects. As expected from prior studies of XIST mRNA expression [Kleinheinz and Schulze, 1994; Vawter et al., 2004], this gene showed an absence of expression in XY males and upregulation in XXY subjects, so the fold changes are biased and not included in the average fold change calculations.

#### Brain Expression of 129 DEGs at Novartis SymAtlas

The brain expression for each DEG (Table I) was queried at the Novartis SymAtlas website which contains data from Affymetrix microarrays for human tissues and cell lines [Su et al., 2004]. In the Novartis gene expression human tissue profile database, there were 19 brain regions assayed in duplicate samples. The total number of brain regions that exceeded median expression microarray values for each of the 129 DEGs are shown (Table I) and also separately for fetal brain expression. For all 14 X-linked DEGs, they were expressed in multiple brain regions above median expression (Table I). Three X-linked DEGs that show significant exploratory correlations with cognitive performance were expressed in 17 (CXORF21), 8 (GTPBP6), and 5 (TAF9L) brain regions, and might play roles in brain function since they are expressed in multiple brain regions. CXORF21, GTPBP6, and TAF9L were expressed in fetal brain as shown in Novartis SymAtlas (Table I).

## DISCUSSION

We initiated studies of XXY males because of reports of the increased prevalence of this karyotype among men with schizophrenia [reviewed in DeLisi et al., 1994]. The original hypothesis was that XXY males would serve as a neurogenetic model for schizophrenia. Thus dysregulated genes expressed among XXY men could be candidates for abnormal expression of a risk-variant in the gene (other than having an extra X chromosome) in some patients with schizophrenia.

The major findings of the present study are that there were significantly more X chromosome genes dysregulated between KS and controls than expected by chance in a genome wide microarray gene expression scan. The results further replicated prior work that showed X gene inactivation most likely occurs in XXY individuals due to the significant XIST gene mRNA expression first reported in XXY lymphocytes [Kleinheinz and Schulze, 1994]. Second, in exploratory analysis of cognition–gene expression correlations, we find further support for the KS candidate genes. Third, a majority of the KS candidate genes (both autosomal and X-linked) are also expressed in brain tissue including fetal brain, suggesting these candidate genes might influence brain development, language functioning, and perhaps later vulnerability to psychosis. Fourth, the broad categories of transcription regulation and mitochondrion were over-represented in upregulated and downregulated gene transcripts in KS.

The present results suggesting candidate genes for KS and verbal cognition may not be simply a reflection of male and female gender differences in gene expression. Although we do not have conclusive data to support this assertion, several prior gender studies did not report gene expression differences that overlap with candidate KS genes [Carrel et al., 1999; Galfalvy et al., 2003; Craig et al., 2004; Vawter et al., 2004]. These studies examined different brain and rodent/human somatic cell hybrids, thus creating some differences due to tissue specificity.

Expression of XIST in XXY subjects creates X-inactivation and was first reported in XXY lymphocytes [Kleinheinz and Schulze, 1994]. KS subjects have a Y chromosome in addition to two X chromosomes that could lead to overexpression of many genes that escape normal X inactivation in the pseudoautosomal regions which have gene copies on both X and Y chromosomes [Carrel et al., 1999]. One such gene, protocadherin 11X (PCDH11X), which was differentially expressed in brain [Galfalvy et al., 2003] is known to be unmethylated and thus likely to escape inactivation in females [Ross et al., 2006]. However, the PCDH11X and PCDH11Y genes were not differentially expressed in KS in our study, but PCDH11X was differentially expressed perhaps due to methylation differences in KS subjects [Ross et al., 2006]. The levels of PCDH11X or PCDH11Y in the present study found in lymphocytes were extremely low (at  $-3$  z-score units), that is, in the lowest 5% expression. Studies of brain reported higher expression of PCDH11X in female compared to male brain [Galfalvy et al., 2003; Lopes et al., 2006], suggesting in tissues that express these genes at higher levels there might be differences in KS subjects. Sudbrak et al. [2001] also found that PCDH11X was overexpressed in 10 of 20 lymphocyte samples from individuals with 4–5 X chromosomes.

PGK1 gene expression was downregulated in KS and the gene is located in the region of the X chromosome that is known to be inactivated (Pfeifer et al., 1990). Since PGK1 is known to be X-inactivated, if we compared XX females to XY males, it might be expected that there would also be an expression difference, that is, perhaps lower in XX females. Further, since our findings strongly show the expression of XIST is increased in KS (which silences some X chromosome gene expression) then it logically follows that PGK1 would be downregulated

TABLE II. Pearson's Correlations Were Run for Differentially Expressed Genes Between XXY (Klinefelter) and XY Controls Shown in Table I

Gene symbol	CXORF21	CHSY1	DKFZP56 40243	KIAA0460	GTPBP6	PKMYT1	ADPGK	RECQL	TAF9L	DNAJA2	AMD1	TCF8
<i>ENSEMBL</i> gene	ENSG00000120280	ENSG00000131873	ENSG00000042022	ENSG00000163125	ENSG00000178605	ENSG00000127564	ENSG00000159322	ENSG0000004700	OTTHUMG00000021887	ENSG0000009345	ENSG0000000123505	ENSG0000000148516
Klinefelter/ control FC <sup>#</sup>	2.62	1.84	1.77	1.72	1.65	1.31	1.27	0.46	0.56	0.64	0.66	0.79
Verbal IQ	-0.41	0.14	0.38	-0.09	-0.85	-0.64	-0.60	-0.52	0.02	-0.03	-0.26	0.04
Receptive language	-0.67	0.52	0.46	0.33	-0.29	-0.04	-0.02	-0.70	-0.45	-0.33	-0.51	-0.30
Token test	-0.53	0.53	<b>0.63</b>	0.24	-0.76	-0.24	-0.28	-0.73	-0.44	-0.34	-0.55	-0.26
Peabody PVT												
Expressive language	-0.30	0.53	<b>0.63</b>	0.57	-0.56	-0.08	0.08	-0.80	-0.71	-0.62	-0.32	-0.23
Boston naming test												
COWA												
Animal	-0.51	0.03	0.05	<b>0.61</b>	-0.50	-0.08	0.14	-0.39	-0.36	-0.53	-0.16	-0.10
Vegetable	-0.51	<b>0.63</b>	0.45	0.26	-0.60	-0.16	-0.17	-0.48	-0.34	-0.11	-0.39	-0.12
Fruit	-0.38	0.45	0.45	0.14	-0.43	-0.23	-0.09	-0.63	-0.49	-0.18	-0.44	-0.07
Similarities	-0.26	-0.19	0.25	-0.32	-0.62	-0.64	-0.71	-0.22	0.36	0.17	-0.05	0.10
Phonological Verbal	-0.21	0.24	0.47	0.08	-0.53	-0.48	-0.51	-0.61	-0.07	-0.07	0.09	0.17
Memory Logical												
Memory												
LOGMEM1	-0.01	0.07	<b>0.63</b>	0.26	-0.14	0.04	0.16	-0.63	-0.35	-0.42	-0.37	-0.63
LOGMEM2	-0.26	0.07	0.50	0.51	-0.12	0.22	0.43	-0.59	-0.47	-0.57	-0.53	-0.78
Sentence	-0.41	<b>0.68</b>	0.48	0.24	-0.27	0.09	0.11	-0.79	-0.59	-0.40	-0.68	-0.54
Repetition												
Haylings A	-0.25	0.33	0.19	-0.31	0.06	0.27	-0.15	-0.17	-0.11	-0.16	-0.57	-0.46
Haylings B	0.34	-0.24	0.19	-0.16	-0.37	-0.35	0.01	-0.21	-0.07	-0.11	-0.27	-0.26
Word Attack	-0.56	0.52	0.04	0.01	-0.47	-0.07	-0.37	-0.25	-0.15	-0.00	-0.27	0.14
Concentration/ attention												
Trails A	0.29	-0.20	-0.32	-0.24	<b>0.58</b>	0.04	0.22	0.42	0.36	0.43	0.26	0.09
Trails B	0.19	-0.11	0.04	-0.29	0.27	-0.12	-0.38	0.44	0.52	0.45	<b>0.64</b>	0.47
Academic skills												
WRAT	-0.57	0.51	0.08	0.34	-0.60	-0.13	-0.19	-0.57	-0.36	-0.32	-0.23	0.02

The expression markers for which correlations were significant ( $P < 0.05$ ) on a cognitive test battery emphasizing verbal functioning are indicated in bold.  
<sup>#</sup>Fold Change for each gene is shown for Klinefelter/control.

TABLE III. Real-Time Quantitative PCR Was Run for 10 Differentially Expressed Genes Between XXY (Klinefelter Syndrome) and XY Controls Focusing on X Chromosome Genes Except RECQL

Gene	QPCR				Gene	Microarray			
	XXY (ave delta Ct)	XY (ave delta Ct)	Fold change	<i>t</i> -test ( <i>P</i> -value)		XXY ave	XY ave	FC	<i>t</i> -test ( <i>P</i> -value)
<i>XIST</i>	-2.29	11.03	10231.05	0.00006	<i>XIST</i>	4.77	-2.33	136.53	6.28E-15
<i>ZNF275</i>	-1.65	-0.23	2.68	0.00031	<i>ZNF275</i>	2.90	1.89	2.02	4.53E-08
<i>CXorf21</i>	-0.90	0.14	2.06	0.05957	<i>CXorf21</i>	1.07	-0.32	2.62	3.08E-07
<i>SMCX</i>	0.24	1.27	2.05	0.04849	<i>SMCX</i>	1.47	0.61	1.81	1.85E-06
<i>GTBP6</i>	2.06	3.03	1.96	0.00716	<i>GTBP6</i>	0.00	-0.38	1.65	2.59E-06
<i>MECP2</i>	2.05	2.06	1.00	0.99409	<i>MECP2</i>	0.70	0.26	1.36	3.07E-07
<i>RECQL</i>	-0.62	-0.86	0.84	0.46164	<i>RECQL</i>	-0.50	0.01	0.46	7.18E-07
<i>SLC35A2</i>	3.17	2.29	0.55	0.03687	<i>SLC35A2</i>	-1.18	-0.83	0.78	1.50E-07
<i>TAF9L</i>	3.53	2.66	0.55	0.09789	<i>TAF9L</i>	-0.83	0.01	0.56	2.45E-06
<i>MBTPS2</i>	4.81	3.19	0.32	0.00040	<i>MBTPS2</i>	-0.89	0.01	0.54	1.93E-08

The QPCR results are sorted by descending fold change and show strong similarity to microarray analysis. Eight of the 10 QPCR assays validated the microarray results.

also in Klinefelters. This explanation requires experimental verification of differences in XX and XXY compared with XXY subjects for the *PGK1* gene. Both KS and XX females have one active and one inactive *PGK1* allele as reflected in methylation study of KS [Ross et al., 2006] but such studies might not be concordant with expression. Thus the present results show a decreased *PGK1* expression in KS compared to XY subjects which could result from increased methylation of the *PGK1* promoter region.

Several X-linked genes are overexpressed by 2.2-fold by Q-PCR. There were X-linked genes under expressed by 2.2-fold in XXY males compared to XY males. The reasons for these downregulated X genes in XXY males compared to XY control males is not clear. A recent report that about 15% of X-linked genes escape inactivation in normal females [Carrel and Willard, 2005], further leaves open the question of how decreased expression of X-linked genes occurs in KS compared to XY males. One overly simplistic speculation is that overexpression of some X-linked genes might regulate transcription of other X-linked genes. It would seem probable that XXY males are not completely similar in X chromosome expression to normal females; otherwise there would be no behavioral or cognitive variables to distinguish them from normal females. The X-linked dysregulated genes reported in this study are plausible candidates for further study. The present data suggests completely normal Y chromosome gene expression, and that the majority of DEGs in KS show changes in both X-linked and autosomal genes compared to XY males. It is tempting to speculate that dysregulation of autosomal gene expression is initiated by one of the extra X chromosomes.

A survey of brain tissues from a prior publication [Su et al., 2004] shows that all of the 14 X-linked DEGs are in fact present in brain tissue. This does not necessarily imply that these genes directly cause KS or verbal cognition impairments. There were five genes that were differentially expressed in KS, expressed in fetal and adult brain, and correlated with verbal cognitive functioning (*CHSY1*, *CXORF21*, *GTPBP6*, *PKMYT1*, *TAF9L*) and these five genes are further described from NCBI data repositories (PubMed, *ENTREZ* gene, OMIM, BLAST). *CHSY1* is involved in synthesis of chondroitin sulfate, a glycosaminoglycan polymer expressed on the surface of most cells and in extracellular matrices [Kitagawa et al., 2001]. This gene could be important in modulating cellular adhesion. *CXORF21* is hypothetical protein FLJ11577 and no information from conserved domains was available. This gene although predicted to have three exons has unknown functions. *GTPBP6* (alias *PGPL*) is a pseudoautosomal gene that escapes X inactivation [Gianfrancesco et al., 1998]. The present

microarray and QPCR data are consistent with this report, even though XXY males express high level of *XIST*, *GTPBP6* was overexpressed in XXY males consistent with the escape from inactivation and the presence of a pseudoautosomal gene. The *GTPBP6* gene is expressed in fetal and adult brain and was negatively correlated with language functioning. Overexpression of this gene might be deleterious to functions associated with language development. The *PKMYT1* gene appears to regulate the progression of cell cycle through *cdc2* kinase and is a target of Akt. Overexpression of *PKMYT1* was shown to inhibit progression of cell cycle, was a negative regulator of the G(2)/M transition, and thus overexpression in KS males might also inhibit cell-cycle progression [Booher et al., 1997; Wells et al., 1999; Okumura et al., 2002; Passer et al., 2003]. Finally, *TAF9L* (human *TAF9L*Like, encoded by a *TAF9* paralog gene) was shown to be a subunit of TFIID [Frontini et al., 2005]. TFIID is comprised of the TATA box binding protein (TBP) and 14 TBP-associated factors (TAFs). This gene appears to be expressed in brain and blood [Su et al., 2004] and regulates transcription. These five genes described above along with the broad category of genes that were significantly overexpressed in KS compared to XY males belong to transcription regulation category. The increased expression of genes that regulate transcription might be particular to lymphoblastic cell lines, so that further research in specific brain regions associated with language will be critical to begin to establish causality. However, these genes suggest that if similar function in brain cells are involved, that critical cell cycle and transcription regulation gene functions might be related to the pathophysiology in language impairments.

Taken together, overexpression of *XIST* may create a mosaic of cells in tissues that are inactivated [Vawter et al., 2004]. Since X-linked gene expression abnormalities in KS persist with *XIST* expression, it is planned to compare XXY subjects to XX subjects to further narrow the candidate list of genes found in the present study. The genes that do escape X inactivation in aneuploidies may have influence on the expression of autosomal genes as suggested by the results of the current study, and the autosomal genes that are brain expressed, in turn may be crucial to the behavioral syndromes observed in these individuals. Further examinations of these candidate genes and proteins in postmortem brain from KS subjects are needed to strengthen links for neurobehavioral effects. Many of the 129 candidate KS genes presented in this article show no overlap in expression between KS and XY controls, thus presenting the opportunity for a simple screening of a panel of these genes for early detection of KS. The probing of brain function with lymphocyte biomarkers and cognitive measures

represents a useful method for screening genes associated with behavioral functions.

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