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The Study of X Chromosome Inactivation Mechanism in Klinefelter's Syndrome by cDNA Microarray Experiment

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Abstract

To investigate the XIST gene expression and its effect in a Klinefelter's patient, we used Klinefelter's syndrome (XXY) patient with azoospermia and also used a normal male (XY) and a normal female (XX) as the control. We were performed cytogenetic analysis, Y chromosomal microdeletion assay (Yq), semi-quantitative RT-PCR, and the Northern blot for Klinefelter's syndrome (KS) patient, a female and a male control. We extracted total RNA from the KS patient, and from the normal cells of the female and male control subjects using the RNA prep kit (Qiagen). cDNA microarray contained 218 human X chromosome-specific genes was fabricated. Each total RNA was reverse transcribed to the first strand cDNA and was labeled with Cy-3 and Cy-5 fluorescein. The microarray was scanned by ScanArray 4000XL system. XIST transcripts were detected from the Klinefelters patient and the female by RT-PCR and Northern blot analysis, but not from the normal male. In the cDNA microarray experiment, we found 24 genes and 14 genes are highly expressed in KS more than the normal male and females, respectively. We concluded that highly expressed genes in KS may be a resulted of the abnormal X inactivation mechanism.

Keywords: X Inactivation, XIST, Klinefelter's syndrome, cDNA chip

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Introduction

X-chromosome inactivation (XCI) is initiated from the X-inactivation center (Xic) in female. X inactivation normally occurs randomly, with an equal probability of either the paternally (Xp) or maternally (Xm) inherited X being inactivated (Eichhorn *et al.*, 1990). Theoretically, the observation of a single active X chromosome in cases of X-chromosome aneuploidy suggests that one X chromosome is always chosen to be active rather than different numbers of X chromosomes being chosen to be inactive. For example, X-chromosome anueploidy like nXY (n>2) consists of X+ X- Y and X+ X- X- Y (X+, active X chromosome; X-, inactive X chromosome; Y, Y chromosome) to be active.

Klinefelter's syndrome (KS) is defined by male with one Y chromosome and at least two X chromosome. KS is found in approximately 1 of 500 men. Clinical features include small testes (<10ml), disproportionately long arms, legs, gynecomastia (breast development), elevated gonadotrophin levels, and azoospermia.

To obtain the X chromosome expression profile in subjects with KS, experiments for X chromosome inactivation were performed. The human X chromosome genes were used as a model for the cDNA microarray, which mainly consisted of 218 genes on X chromosome.

For detection of X-chromosome inactivation in a KS patient, the expression of XIST in a normal male (XY), a normal female (XX), and KS patient (XXY) were checked. The XIST transcript in humans is a large non-protein-coding transcript that coats the inactive X chromosome (Brockdorff *et al.*, 1992; Brown *et al.*, 1992). A previous study reported the isolation and characterization of the novel gene, XIST, which was expressed from Xi but not from Xa chromosomes. This discovery represents a candidate for a gene that is either involved in or strongly influenced by X inactivation (Brown *et al.*, 1991). The XIST expression can indicate a possible role of the X inactivation for normal spermatogenesis (Salido *et al.*, 1992). We estimated the state of the X chromosome inactivation to determine whether or not the XIST gene can be expressed in a normal male, a normal female, and a KS patient.

Materials and Methods

Patients and Controls

A normal male and female, and a Klinefelters syndrome with azoospermia and 47 XXY, at the CHA General Hospital, College of Medicine, Pochon CHA University (Seoul, Korea), gave informed consent for the use of their blood.

Cytogenetic and Y Chromosome Microdeletion Assay

Cytogenetic analysis was performed on metaphase spreads of cultured lymphocytes. For Yq deletion studies, DNA was extracted from the peripheral blood and amplified by a multi-PCR method with primers to 13 loci on the Y chromosome, including one SRY and 12 sequence-tagged sites (STSs) (AZF-a region: sY84, sY86; AZF-b region: sY 134, sY 138, MK5; AZF-c region: sY 152, sY 147, sY 254, sY 255, SPGY1, sY 269, sY 158) (Simoni, 2001; Simoni *et al.*, 1999).

Cell and Its culture condition

We obtained EBV-lymphoblstoid cell lines from a normal male, female and Klinefelters patient. Cells were grown in a CO_2 incubator (37℃, 5% CO_2) in RPMI 1640 medium (Gibco BRL, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Cergy Pontoise, France), 1% L-Glutamine and 1% Antibiotic-antimicotic.

RNA Extraction and DNAse treatment

Total RNA lymphoblastoid cell from normal (male and female) and KS patient isolated by using the Qiagen RNeasy kit according to the recommendation of the manufacturer protocol (Qiagen, Valencia, CA). Gel electrophoresis and spectrophotometric reading assessed RNA quality. RNA preparation was treated for 10 min at 37℃ with RNAse-free DNAse I (Roche Boehringer Mannhein, Germany) and stored at −80℃.

Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR for *XIST* expression performed using QIAGEN OneStep RT-PCR Kit (Qiagen, Valencia, CA) with PCR DNA Thermal Cycler (Perkin Elmer, Norwalk, CT). First-strand cDNA was synthesized from total RNA at 50℃ for 30 min, activated HotStarTaq DNA Polymerase by heating at 95℃ for 15 min. PCRs of first-strand cDNA were performed under conditions of 40s at 94℃, 40s at 56℃ and 1 min at 72℃ each cycle. 40 cycles were done, followed by extension for 10 min at 72℃. The XIST expression detected using the following primer pairs: XIST 1, 5'-GAA GTC TCA AGG CTT GAG TTA GAA G-3'; XIST 3a, 5'-ACA TTT TTC TCT AGA GAG CCT GGC-3'; XIST 3b, 5'-GCC AGG CTC TCT AGA GAA AAA TGT-3'; XIST 5, 5'-TGT CTG CAT AAA AGC AGA TT-3'; Pyruvate dehydrogenase (PDH) 1, 5'-GGA ATG GAT GAG GAC CTG GA-3'; Pyruvate dehydrogenase (PDH) 2, 5'-CTT CCA CAG CCC TCG ACT AA-3' (Brown *et al.*, 1991). These primers can generate 705bp (XIST 1 and 3a), 380bp and 240bp (XIST 3b and 5) and 105bp (PDH1 and PDH2) PCR fragment, respectively. The RT-PCR products were subjected to electrophoresis on a 1% agarose gel.

Northern Blot Analysis

Total RNA (20 μg) was electrophoresed on 1.25 % agarose gel containing 37% formaldehyde and 20X MOPS for 5hr at 150V, and then transferred to positively charged nylon. Then, blots were hybridized with ^{32}P-labeled DNA fragment from 705 bp PCR product using XIST primer 1 and 3a as a probe for 19.3 kb XIST RNA. The probe of Human GAPDH (1.28 kb) was used for northern blot analysis as a control.

Array Fabrication

218 cDNA clones of human cDNA collection were kindly provided from the Center for Functional analysis of Human Genome, Korea. Clone inserts were PCR-amplified from culture and purified by PCR Cleanup Kit (Millipore, Billerica, Mass., USA). PCR from plasmid miniprep DNA was carried out for 96 well microtiter plates. PCR products were dissolved for spotting on slide by 50% DMSO, and then spotted onto Corning GAPS II slides (Corning), coated with an amino-silane surface chemistry.

Probe Preparation and Hybridization to cDNA microarray

Ribonucleic acid (40 μg) was reversed transcribed using Superscript II RT kit (Gibco, Grand Island, USA) with 1 mM Cy3 and Cy5 labeled dUTP (NEN). To purify labeled cDNAs from unincorporated nucleotides, probes were filtered through Ethanol preparation. The human cDNA microarray (spotted by Digital Genomics) that contained 218 human genes were prehybridized in 10 mg/ml BSA, 5× SSC, 0.1% SDS and 25% formamide for 45 min at 58℃, washed by dipping five times in Milli_Q_water, and dried the slide by centrifuge at 650 rpm at room temperature for 5 min. Fluorescent cDNA probes were dried after Ethanol preparation and resuspended in 12 ml hybridization buffer (25% formamide, 5x SSC, 0.1% SDS). After combination of Cy3 and Cy5 labeled, we added 0.025-0.1 mg/ml Cot-1 DNA and 0.5 mg/ml poly

A to samples, all was denatured at 95℃ for 5 min. The probes were applied to a prehybridized array, covered with a 22 X 22 mm glass coverslip (Corning), and placed in a humidified hybridization chamber. After hybridization, the slide was washed for 5 min with 2X SSC/ 0.1% SDS at room temperature, and washed again for 10 min with 0.1X SSC/0.1% SDS at room temperature, and finally washed four times for 1 min with 0.1X SSC with continuous agitation.

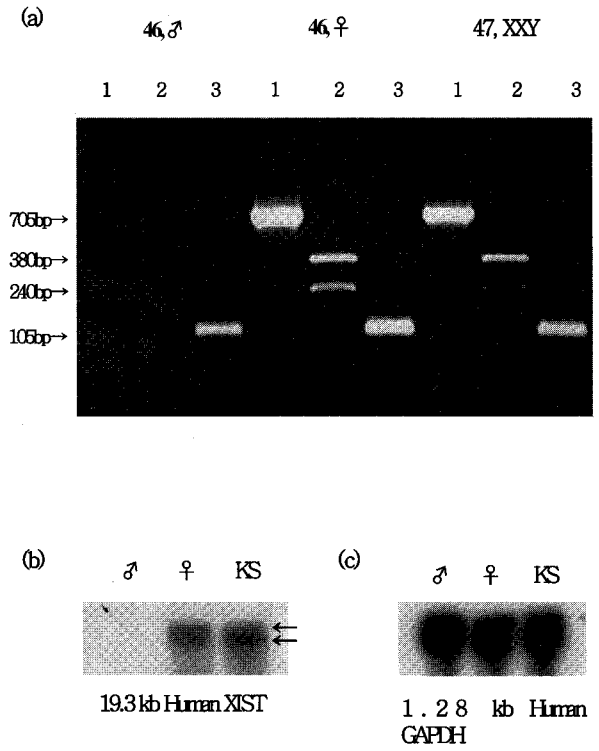


Fig. 1. Detection of the XIST transcripts by semi-quantitative RT-PCR (a) and Northern blot analysis (b) and (c)in a KS patient, a normal male, and a normal female.
RT-PCR analysis was performed to detect the expression of XIST transcript in the samples. (a) Semi-quantitative RT-PCR for the XIST and PDH genes in all samples (Lane 1, RT- PCR with primer pair XIST 1 and XIST 3a; Lane 2, RT-PCR with primer pair XIST 3b and XIST 5; Lane 3, RT-PCR with primer pair PDH 1 and PDH 2). (b) Northern blot analysis for the XIST (19.3 kb) transcript was carried out to confirm RT-PCR results. Male (♂), female (♀), and the KS patient's RNA was fractionated on a formaldehyde-agarose gel and was transferred to a positively charged nylon yielding three lanes. The figure shows bands in a lane of the female and the KS patient using a XIST probe. The lane containing the male (♂) is not shown, because there was no hybridization signal. Interestingly, XIST transcripts, the two bands, are indicated by arrow symbols. (c) Northern blot for human GAPDH transcript illustrates the same expression in the male, the female, and the KS patient.

Analysis of Gene Expression

The slide was dried by centrifugation, scanned by ScanArray 4000XL system and analyzed with GeneSight 3.2 (BioDiscovery, USA). The intensity of each spots were expressed numerically with autoalign method in program, the data were normalized with global normalization method on GeneSight (BioDiscovery, USA). The data generated by image processing were imported into GeneSpring (version 6 Silicon Genetics, Redwood, CA) to classify by category. The ratio threshold was set at 2.0 in our study. Only those genes, which showed as increase or decrease of 2.0 fold or greater, were considered as differentially expressed.

Results

The identification of X inactivation in a normal male and female, and KS patient by semi-quantitative RT-PCR and northern blot analysis

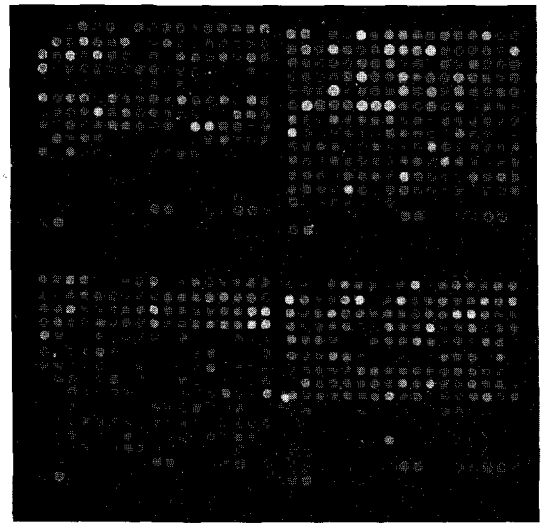
To identify whether KS has an inactive X chromosome or not, the expression of XIST (X-inactivation specific transcript) was analyzed from a normal male (46, ♂), a normal female (46, ♀), and a KS patient (47, XXY) by semi-quantitative RT-PCR (Fig. 1a). The analysis showed that XIST transcripts can be detected in all blood samples from a normal female (46, ♀) and a KS patient (47, XXY) with two X chromosomes. On the other hand, the expression of XIST transcripts in a normal male (46, ♂) was negative. All samples were positive for pyruvate dehydrogenase (PDH) transcripts according to the semi-quantitative RT-PCR. After semi-quantitative RT-PCR, we confirmed this result by northern blot analysis (Fig. 1b and c). However, the results of the northern blot by using XIST probe were different from the semi-quantitative RT-PCR results of the female(♀) and KS subject in terms of the band size of the XIST transcript (Fig. 1b). There was the same positive expression for GAPDH in the normal male, the normal female, and the KS patient, respectively (Fig. 1c).

Differential expression of X chromosomal genes in a normal male vs KS patient and a normal female vs KS patient

The cDNA's were generated through reverse transcription from three samples, and cDNA's of a normal female and male were labeled Cy3, and cDNA of a KS patient was Cy5 (Fig. 2a and b). In the DNA microarray, 218 genes were X chromosome specific genes. When we compared the KS patient with a normal female, we found that 14 genes were deferentially expressed (Table 1). When

comparing a normal male with the KS patient in the same way, their 24 genes were differentially expressed (Table 1). These genes showed higher expression by two-folds in the KS patient than that of the male or female cDNA. According to a previous study, Sudbrak *et al.* (2001) found through the cDNA microarray experiment of normal females, normal males, and females with supernumerary X chromosome that many genes showed elevated expression level in females that have excess X chromosome.

(a)



(b)

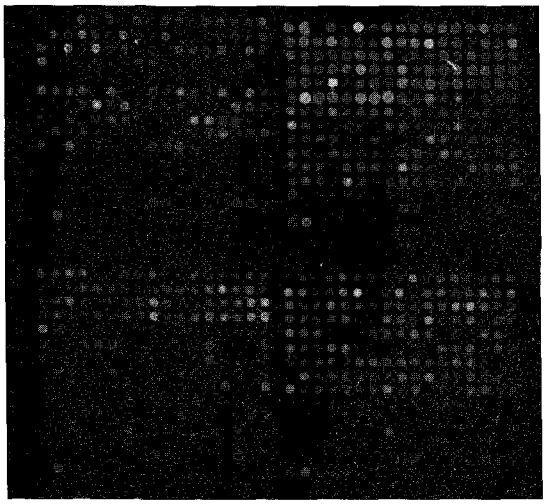


Fig. 2. The X chromosome-specific cDNA microarray hybridized to Cy3- and Cy5- labeled cDNA from lymphoblastoid cell line. (a) Hybridization from cDNA of a normal female (Cy3-labeled cDNA) and a KS patient (Cy5-labeled cDNA). (b) Hybridization of a normal male (Cy3-labeled cDNA) and a KS patient (Cy5-labeled cDNA).

Discussion

In this study, we demonstrated the expression pattern of XIST transcripts in the normal male (46, ♂), the normal female (46, ♀) and the KS patient (47, XXY) by semi-quantitative RT-PCR (Fig. 1a). The XIST expression in the KS patient can be an identifying factor for the existence of X inactivation. Interestingly, there was a different expression between the female and the KS patient for the 240 bp band in Fig. 1a; there was more expression in the female than the KS patient. Further research is necessary to narrow the possible reasons this data. Then, we verified the XIST expression by semi-quantitative RT-PCR and through northern blot analysis (Fig. 1b and c). Interestingly, XIST transcript showed the double band for the female (46, ♀) and the KS patient (47, XXY) in Fig. 1b. Hong *et al.* (1999) and Memili *et al.* (2001) presented the structural data for the murine Xist gene. Two major isoforms of Xist can be detected by northern analysis, which are consistent with differential polyadenylation. According to the recent structure of human XIST, there is a high sequence similarity between the 3' regions of human XIST and mouse Xist. However, human XIST has an intron in this region, and this intron spans the majority of the sequence of high similarity between the mouse and human (Hong *et al.*, 2000). Our data may indicate that the human XIST double band may be, in fact, alternatively spliced forms of 19.3kb and 16.5kb transcripts.

From observing the results of the semi-quantitative RT-PCR, the process of how X inactivation occurs in the KS patient was a mystery. A comparative experiment of X chromosome gene expression in a normal male, a normal female, and a KS patient was performed by cDNA microarray. We have identified the significantly elevated expression of 14 genes and 24 genes on the X chromosome of the KS patient than in female and male controls (F/M), respectively. We suggest that X inactivations of KS through genes are highly expressed tend to have an abnormal X inactivation mechanism.

According to Sudbrak *et al.* (2001), females with a supernumerary X chromosome have many genes that were found to escape X-inactivation. In cells with four or five X chromosomes for such as XIST, intracellular mRNA concentrations should therefore be from three to four times higher than in normal female cells. Although both the normal male and the KS patient have a same man with Y chromosome, the expression difference is not due to the numbers of X chromosomes. The data

Table 1. Genes that showed elevated expression levels over twice signal intensity in cell of KS patient than cell of female and male

	Gene ID	Gene symbol	Cytogenic band	
1	NM_002637	PHKA1	Xq12-q13	F
2	NM_004135	IDH3G	Xq28	F
3	NM_015698	T54	Xp11.23	F
4	NM_014500	HTATSF1	Xq26.1-q27.2	F/M
5	NM_024539	RNF128	Xq22.3	F/M
6	NM_014138	PRO0659	Xp11.23	F/M
7	NM_000169	GLA	Xq22	F
8	NM_022052	NXF3	Xq22-q23	F/M
9	NM_004726	REPS2	Xp22.22	F
10	AB051488	DXS7007E	Xq23	F
11	AF207881	APK1	Xq25-26.2	F
12	AK024601	FAM3A	Xq28	F
13	AL832829	MGC39350	Xp11.4	F
14	AL833629	GDI1	Xq28	F
15	NM_019848	SLC10A3	Xq28	M
16	NM_024859	FLJ21687	Xp11.23	M
17	NM_001000	RPL39	Xq22-q24	M
18	NM_000276	OCRL	Xq25-q26.1	M
19	NM_006044	HDAC6	Xp11.23	M
20	NM_004208	PDCD8	Xq25-q26	M
21	NM_005229	ELK1	Xp11.2	M
22	NM_001007	RPS4X	Xq13.1	M
23	NM_004344	CETN2	Xq28	M
24	NM_003254	TIMP1	Xp11.3-p11.23	M
25	NM_014060	MCT-1	Xq22-q24	M
26	NM_001666	ARHGAP4	Xq28	M
27	NM_012280	FTSJ1	Xp11.23	M
28	AK024746	stSG42464	Xq13.1	M
29	AK074087	FLJ12525	Xq12-13	M
30	AK074449	PGK1	Xq13	M
31	AK095887	FLJ38568	Xq21.1	M
32	AL161985	TFE3	Xp11.22	M
33	AY007119	DSIPI	Xq22.3	M
34	BC028142	CA5B	Xp21.1	M

F: Normal female
M: Normal male
F/M: Normal female and male

comparing the KS patient and the male possibly show similar results to the data between the KS patient and the female because the genes that are supposed to be inactivated have been activated.

Among the genes that are expressed higher in the KS patient, muscle isoform of the phosphorylase kinase alpha subunit (PHKA1) (Table 1) and ribosomal protein S4, X-linked (RPS4X) genes (Table 1) are known to be physically linked with XIST near the X inactivation center (XIC) (Fisher *et al.*, 1990; Lafreniere *et al.*, 1993). Further investigation of the specific gene expression near Xic may help clarify the mechanism of X chromosome inactivation.

Moreover, members of the ETS oncogene family (ELK1) (Table 1) belong to the ETS oncogene superfamily, and the expression of this gene is enhanced in a testicular germ cell tumor with an excess of active X chromosomes (Kawakami *et al.*, 2003). Previous studies (Hasle *et al.*, 1995; Rapley *et al.*, 2000) reported that KS can be a risk factor to the germ cell tumor. Consequently, we cannot exclude the relationship between a highly expressed gene in the KS patient and testicular germ cell tumor of a normal male.

Therefore, through cDNA microarray we gained the general gene expression profile on the X chromosome of a KS patient. However, for a systemic study on X inactivation mechanism a repetition of the experiment using more samples is necessary, and further research

may be especially needed on the highly expressed genes of a KS patient.

Acknowledgement

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