Clin. Lab. 2025;71:XXX-XXX

ORIGINAL ARTICLE

Homozygosity of the Xq13.2q21.1 Region and Specific SNPs Correlates with Nonrandom X Chromosome Inactivation

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SUMMARY

Background: **Nonrandom X chromosome inactivation (XCI) is thought to contribute to symptom expression in female carriers of X-linked diseases, yet the mechanisms remain unclear. This study investigated the relationship between genetic factors on the X chromosome and XCI status.**
Methods: **We used chromosomal microarray analysis (CMA) and whole-exome sequencing (WES) to analyze five stem cell lines with nonrandom XCI and four with random XCI. We compared copy number variation (CNV), regions of homozygosity (ROH), single nucleotide variants (SNVs), and XCI status in these cell lines.**
Results: **The total number of CNVs and their distribution did not differ significantly between groups. No CNVs larger than 400 kilobase pairs (Kb) on the X chromosome were detected, and no pathogenic CNVs were identified in any of the cell lines. ROH in the Xq13.2q21.1 region was present in four out of five nonrandom XCI cells but was absent in all random XCI cells. Sequencing identified an average of 27.2 and 25 nonsynonymous variants in nonrandom XCI cells and random XCI cells, respectively. Nine SNPs were specific to the X chromosome in nonrandom XCI cells, whereas one unique SNP was detected in random but not in nonrandom XCI cells.**
Conclusions: **Homozygosity in the Xq13.2q21.1 region and specific SNPs may be associated with nonrandom XCI status, suggesting a potential genetic basis for XCI patterns.**
(Clin. Lab. 2025;71:xx-xx. DOI: 10.7754/Clin.Lab.2024.241050)

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Manuscript accepted October 11, 2024

KEYWORDS

X-chromosome inactivation, region of homozygosity, Xq13.2q21.1, genetic variation, SNPs

LIST OF ABBREVIATIONS

XCI - X chromosome inactivation
hESC - human embryonic stem cell
iPSC - induced pluripotent stem cell
CMA - chromosomal microarray analysis
WES - whole exome sequencing
Kb - kilobase pairs
Mb - megabase pairs
CNV - copy number variation
ROH - region of homozygosity
SNV - single nucleotide variant

SNP - single nucleotide polymorphism
snoRNA - small nucleolar RNA
lncRNA - long non-coding RNA

INTRODUCTION

X-chromosome inactivation (XCI) is the silencing of the overall expression level of one of the two X chromosomes in female cells [1]. Typically, XCI occurs randomly, with some cells silencing the paternal X and others the maternal X, resulting in an approximate 50:50 distribution [2]. However, in nonrandom XCI, one chromosome is preferentially inactivated in most cells (more than 80%) [2,3].

Nonrandom XCI has been implicated in the varied symptoms seen in female carriers of X-linked diseases [3-7]. For instance, while conditions like Dent disease, Menkes disease, and retinal dystrophy are primarily X-linked, some female carriers exhibit symptoms due to nonrandom XCI [8-10]. Conditions like Duchenne/Becker muscular dystrophy (DMD/BMD) show variability in carrier symptoms, ranging from mild to severe, with 2.5% to 18% of female carriers having muscular or cardiac involvement [3-7].

XCI generally occurs early in development, and previous research has relied on mouse embryos, human embryonic stem cells (hESCs), or induced pluripotent stem cell (iPSC) models to study its mechanisms [11-13]. Our previous studies suggested differences in CNVs, ROH, and point mutations between cell lines with random and nonrandom XCI [14]. This study aimed to further investigate whether specific genetic factors on the X chromosome correlate with XCI status.

MATERIALS AND METHODS

Sample preparation

Five human embryonic stem cells (hESCs) with nonrandom XCI status (FY-hESC-5, -8, -11, -27, and -39) and four hESCs with random XCI status (FY-hES-10, -22, -25, and -35) previously established in our laboratory were used in this study [14,15]. All of the abovementioned cells were cultured in vitro and harvested for analysis. DNA was extracted using the QIAamp DNA Blood Mini Kit (no. 51106, Qiagen, Germany), and this experiment was approved by the Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University (no. 2020017).

Chromosome microarray analysis (CMA)

An Affymetrix CytoScan 750K from Thermo Fisher (Thermo Fisher, USA) was used. The extracted DNA was digested, ligated, PCR amplified, purified, fragmented, and labeled for hybridization according to the instructions. The washed and stained chip was scanned, and the data were collected and analyzed using Chromosome Analysis Suite (ChAS 4.3) software. Copy number variation (CNV) and region of homozygosity (ROH) were examined. CNV analysis was performed using a threshold of 400 kilobase pairs (Kb) and 50 probes, and ROH analysis was performed using a threshold of 5 megabase pairs (Mb) and 50 probes. CNV scoring criteria were based on the American College of Medical Genetics and Genomics (ACMG)/ClinGen interpretation guidelines.

Whole-exome sequencing

One microgram of genomic DNA was cleaved into 200 - 250 base pair (bp) fragments using the Covaris S2 system, and after end repair, the library was constructed using second-generation sequencing kits (Agilent, USA). The library was constructed and sequenced on the HiSeq 2500 platform.

After decontamination and trimming, the raw sequencing reads were subjected to BWA-GATK (https://software.broadinstitute.org/gatk/) with ClinSV (https://bio.tools/clinsv) for bioinformatics analysis. Then, all single nucleotide variants (SNVs) and small insertion/deletion variants were annotated using ANNOVAR software (http://ANNOVAR.openbioinformatics.org/), and all structural variants were annotated using AnnotSV software (https://lbgi.fr/AnnotSV/). The SNVs, deletions, insertions, frameshifts, nonsense mutations, and missense variants on the X chromosome were statistically analyzed to compare the differences in each mutation between the two different inactivation statuses of hESCs.

RESULTS

CNV analysis

Seven duplications and four deletion CNVs larger than 400 kb were detected in five nonrandom XCI cells, and two duplications and three deletion CNVs larger than 400 kb were detected in four cells with random XCI status. A deletion in the 16p11.2 region was common in both groups, but no CNVs larger than 400 kb appeared on the X chromosome (Table 1, Figure 1). There was no significant difference in the total number or distribution of CNVs between the random and nonrandom XCI cells. None of the CNVs detected were pathogenic.

ROH analysis

With a threshold of 5 Mb and 50 probes, we identified 12 ROHs in nonrandom and 10 in random XCI cells, out of which 9 and 7 were located on the X chromosome, respectively. Four out of five nonrandom XCI cells had ROH in the Xq13.2q21.1 region, while it was absent in random XCI cells (Figure 2, Supplementary Table S1). This region includes several long noncoding RNAs (lncRNAs) associated with XCI regulation, such as *TSIX*, *XIST*, *JPX*, *FTX*, and *ATRX*, that were located in this region (Supplementary Table S1). Further analysis pinpointed two specific ROH regions - chrX:72473982-75010148 and chrX:75572224-77231335 - which

Table 1. Analysis of CNVs between cells with random and nonrandom XCIs.

Cell lines	XCI Status	Copy number	Chromosome position	Size (Kb)	OMIM genes (MIM number)
FY-hESC-5	nonrandom	4	15q11.1q11.2(20016316_22673387)	2,657	NBEAP1 (601889), POTEB (608912)
		3	3p12.3(75404163_75894520)	490	ZNF717 (618405)
		4	8p23.1(7186620_7798839)	612	FAM90A7P (613044), FAM90A10P (613047), DEFB4A (602215)
FY-hESC-8	nonrandom	1	16p11.2(32208734_32642700)	433	/
FY-hESC-11	nonrandom	1	8p23.1(7250368_7744052)	412	DEFB103B (606611), SPAG11B (606560), FAM90A7P (613044), FAM90A10P (613047)
		4	2p11.2(89133112_89545331)	412	/
		3	16p11.2(32791257_33952889)	1,161	/
		4	15q11.1q11.2(20585976_22588019)	493	NBEAP1 (601889), POTEB (608912)
FY-hESC-27	nonrandom	1	16p11.2(32524765_33742056)	1,217	TP53TG3 (617482)
FY-hESC-39	nonrandom	1	11p11.12(50068987_50501403)	432	/
		3	19p13.3(713294_1438839)	725	PALM (608134), MISP (615289), PTBP1 (600693), PLPPR3 (610391), AZU1 (162815), PRTN3 (177020), ELANE (130130), CFD (134350), MED16 (604062), RNU6-1 (180692), KISS1R (604161), ARID3A (603265), GRIN3B (606651), TMEM259 (611011), CNN2 (602373), ABCA7 (605414), ARHGAP45 (601155), POLR2E (180664), GPX4 (138322), SBNO2 (615729), STK11 (602216), ATP5F1D (603150), MIDN (606700), CIRBP (602649), EFNA2 (602756), NDUFS7 (601825), GAMT (601240), DAZAP1 (607430), RPS15 (180535)
FY-hESC-10	random	3	8p23.1(11881847_12358169)	476	USP17L2 (610186), FAM86B1 (616122), FAM86B2 (616123)
FY-hESC-22	random	1	16p11.2(32014955_33815401)	1,800	TP53TG3 (617482)
FY-hESC-25	random	3	3p26.3(1043530_1467343)	423	CNTN6 (607220)
FY-hESC-35	random	1	7q11.21(64665478_65148399)	483	ZNF92 (603974)
		1	16p11.2(32937323_33815554)	878	/

encompass the *XIST*, *TSIX*, *ATRX*, and *ATP7A* genes (Figure 3).

Sequencing analysis of the X chromosome

On average, nonrandom XCI cells had 27.2 SNVs or small indels, compared to 25 in random XCI cells (data not shown). We applied a filter to identify variants specific to XCI type: those present in at least 80% of nonrandom XCI cells but absent in random cells, and vice versa. This revealed 9 SNPs unique to nonrandom XCI cells and one SNP unique to random XCI cells (Table 2).

Table 2. Specific SNPs between cells with random and nonrandom XCI.

Chr.	Gene	Pos.	SNP ID	Ref.	Random XCI				Nonrandom XCI				
					H10	H22	H25	H35	H11	H27	H39	H5	H8
X	LOC389906	3824080	rs62576922	G	./.	./.	./.	./.	G/A	./.	G/A	G/A	G/A
X	FAM47A	34674970	rs5928617	C	./.	./.	./.	./.	C/G	G/G	C/G	C/G	G/G
X	MED14	40571413	rs4827226	T	./.	./.	./.	./.	T/C	T/C	T/C	T/C	T/C
X	MED14	40573157	rs6520683	G	./.	./.	./.	./.	./.	G/A	G/A	G/A	G/A
X	CXorf36	45050973	rs4239958	C	./.	./.	./.	./.	T/T	T/T	T/T	T/T	T/T
X	RPS4X	71495582	rs3747302	G	./.	./.	./.	./.	G/A	G/A	G/A	G/A	A/A
X	TCEAL6	101395783	rs34325441	G	./.	./.	./.	./.	G/GG	./.	GG/GG	G/GG	GG/GG
X	DOCK11	117700141	rs2286977	A	./.	./.	./.	./.	A/G	G/G	A/G	A/G	A/G
X	UTP14A	129058882	rs2281278	T	./.	./.	./.	./.	./.	T/C	T/C	T/C	T/C
X	ADGRG2	19017997	rs3924227	T	./.	T/C	T/C	C/C	./.	./.	./.	./.	./.

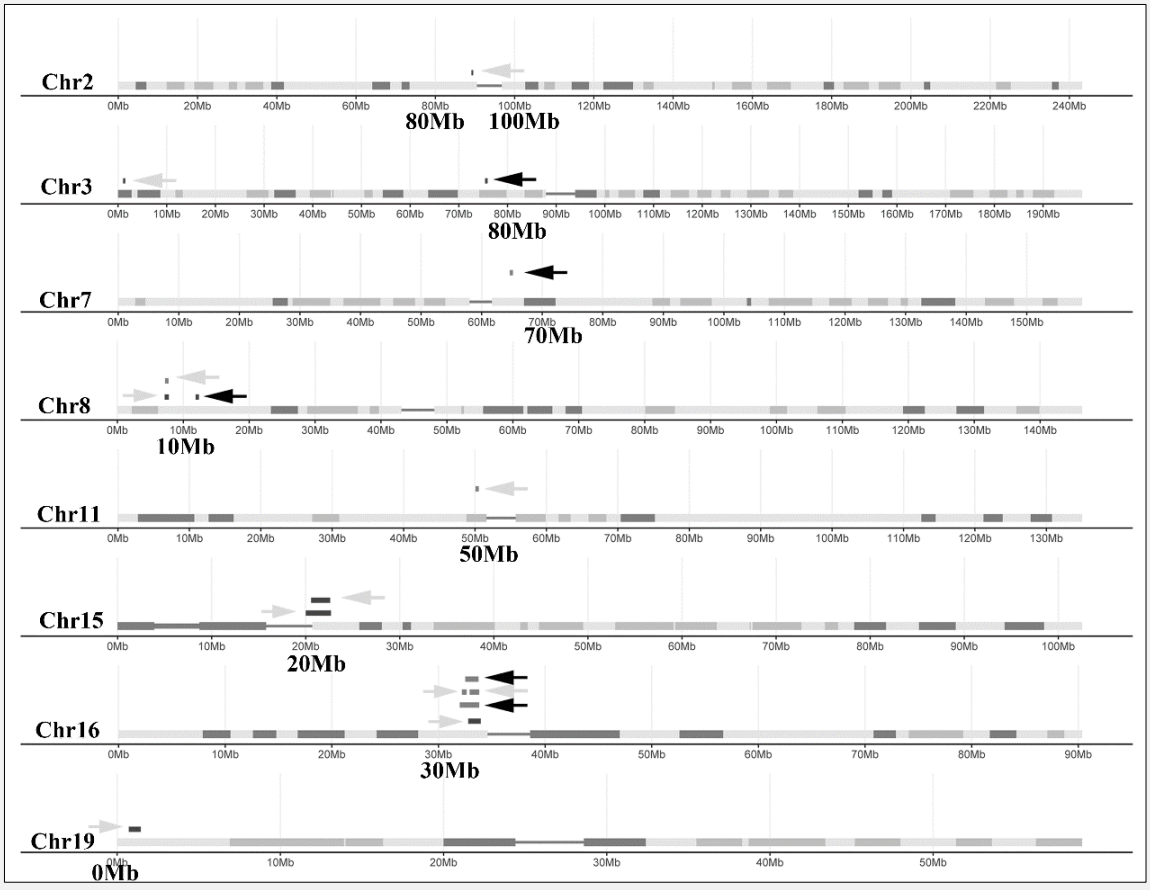

Figure 1. Analysis of CNV distribution.

A total of seven duplications and four deletion CNVs larger than 400 kb were detected in nonrandom XCI cells (gray arrows), and two duplications and three deletion CNVs larger than 400 kb were detected in random XCI cells (black arrows). The CNVs were distributed on chromosomes 2, 3, 7, 8, 11, 15, 16, and 19. 16p11.2 was identified as a hot spot in both groups. No CNV larger than 400 kb was detected on the X chromosome.

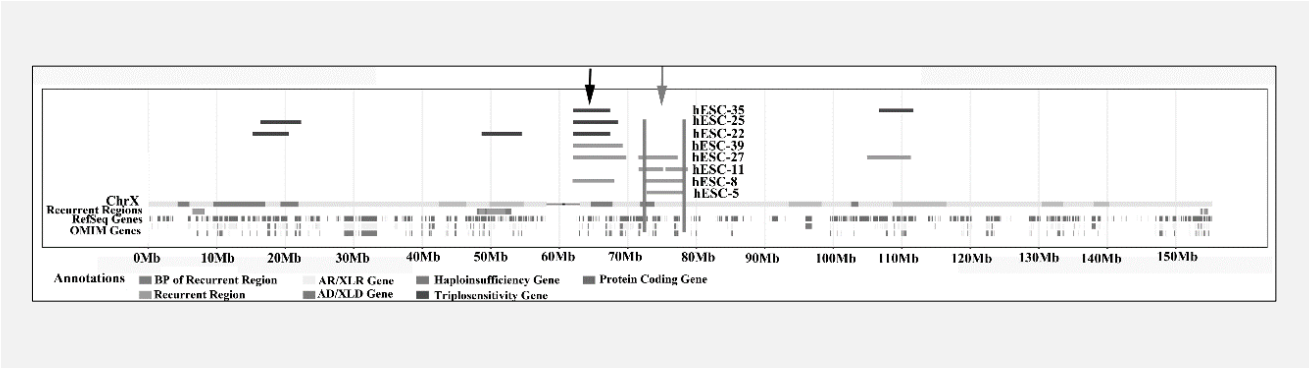

Figure 2. Identified ROHs on chromosome X.

Nine ROHs on the X chromosome were observed in the nonrandom XCI cells (gray rectangle, cell lines 5, 8, 11, 39, and 27), whereas 7 ROHs on the X chromosome were observed in the random XCI cells (black rectangle, cell lines 35, 25, and 22). Four out of five nonrandom XCI cells had ROH in the Xq13.2q21.1 region (gray line and gray arrow), whereas no ROH was detected in the random XCI cells (gray arrow). The ROH at the Xq11.1q13.1 region showed no difference between random and nonrandom XCI cells (black arrow). The black rectangle indicates the ROH of cells with random XCI, and the gray rectangle indicates the ROH of cells with nonrandom XCI.

DISCUSSION

The X chromosome contains over 1,000 functionally important genes and plays a critical role in several complex diseases [16,17]. The random pattern of inactivation protects female carriers of X-linked diseases from developing the disease. Skewed XCI is defined as "preferential" (80:20%) or "extreme" (90:10%) XCI status and may indicate the presence of an X-linked pathogenic variant, even in affected females [18]. As nonrandom XCI has been reported in carriers of various X-linked genetic diseases [3,19-21], it is essential to explain the differences in pathogenesis and phenotype in carriers of X-linked diseases with nonrandom XCI.

The mechanisms behind XCI are intricate and incompletely understood [22-25], with key regulatory lncRNAs (such as *XIST*, *JPX*, and *TSIX*) located on the Xq13.2 region [26-28]. Among them, the *XIST* gene is the core element of XCI, and one of the critical pathways for its regulation of XCI is through the binding of polycomb repressive complex 2 (PRC2) through the RepA repeat region on the first exon of *XIST* [29]. Our previous research indicated that ROH in the Xq13.2q21.1 region may correlate with nonrandom XCI [14]. By using more nonrandom or random XCI cells, this study further supports these findings, showing that ROH in Xq13.2q21.1 was present in four of five nonrandom XCI cells but was absent in random XCI cells. ROH, a state where specific alleles are homozygous over a region, is also common in tumors and has been associated with skewed XCI [30]. Studies in mice have shown that deletion-type loss of heterozygosity (LOH) in XCI regulatory genes such as *XIST* and *TSIX* can lead to skewed XCI [31]. When copy number neutral ROH mutations occur in the two alleles that regulate the critical factors of XCI, they can lead to the inactivation of all or none of the two X alleles in the cells of female mice [32]. Thus, ROH in Xq13.2q21.1 might affect XCI through epigenetic regulation, providing a potential marker for identifying carriers of X-linked diseases.

In the present study, we further analyzed the observed ROH regions and found that all four cell lines overlapped in the chrX:72473982-75010148 and chrX:75572224-77231335 regions. Genetic analysis of these two regions revealed that the *XIST* and *ATRX* are located in these regions. *XIST* and *ATRX* play critical roles in XCI [25,33], and the associations between the ROH of these genes and nonrandom XCI are currently unknown.

In addition to ROH, the influence of genetic factors on XCI is also a hot topic of research [34,35]. In this study, we also analyzed CNVs and SNVs on the X chromosome and did not find any pathogenic CNVs on the X chromosome, and we did not find any pathogenic SNVs in the Xq13.2q21.1 region by whole exome sequencing; therefore, we did not find any effect of CNVs or XCI regulatory region variants on XCI in this study.

Our sequencing results also highlighted nine SNPs unique to nonrandom XCI cells. Notably, we found SNP rs3747302 in the *RPS4X* gene near the Xq13.2q21.1 region in random XCI cells but found it absent in nonrandom cells. The function analysis of *RPS4X* revealed it is a component of the small ribosomal subunit [36,37]; however, the role of *RPS4X* in XCI remains uncertain. Two SNPs (rs4827226 and rs4239958) in the *MED14* and *CXorf36* genes, respectively, were found in all five nonrandom XCI strains but not in random XCI cells. The effect of these SNPs on XCI may suggest its potential functional relevance.

A limitation of this study is the small sample size, which warrants further investigation. While this study provides evidence linking specific ROH and SNPs to nonrandom XCI, future research should explore the functional roles of these genetic markers.

In conclusion, this study identifies ROH in the Xq13.

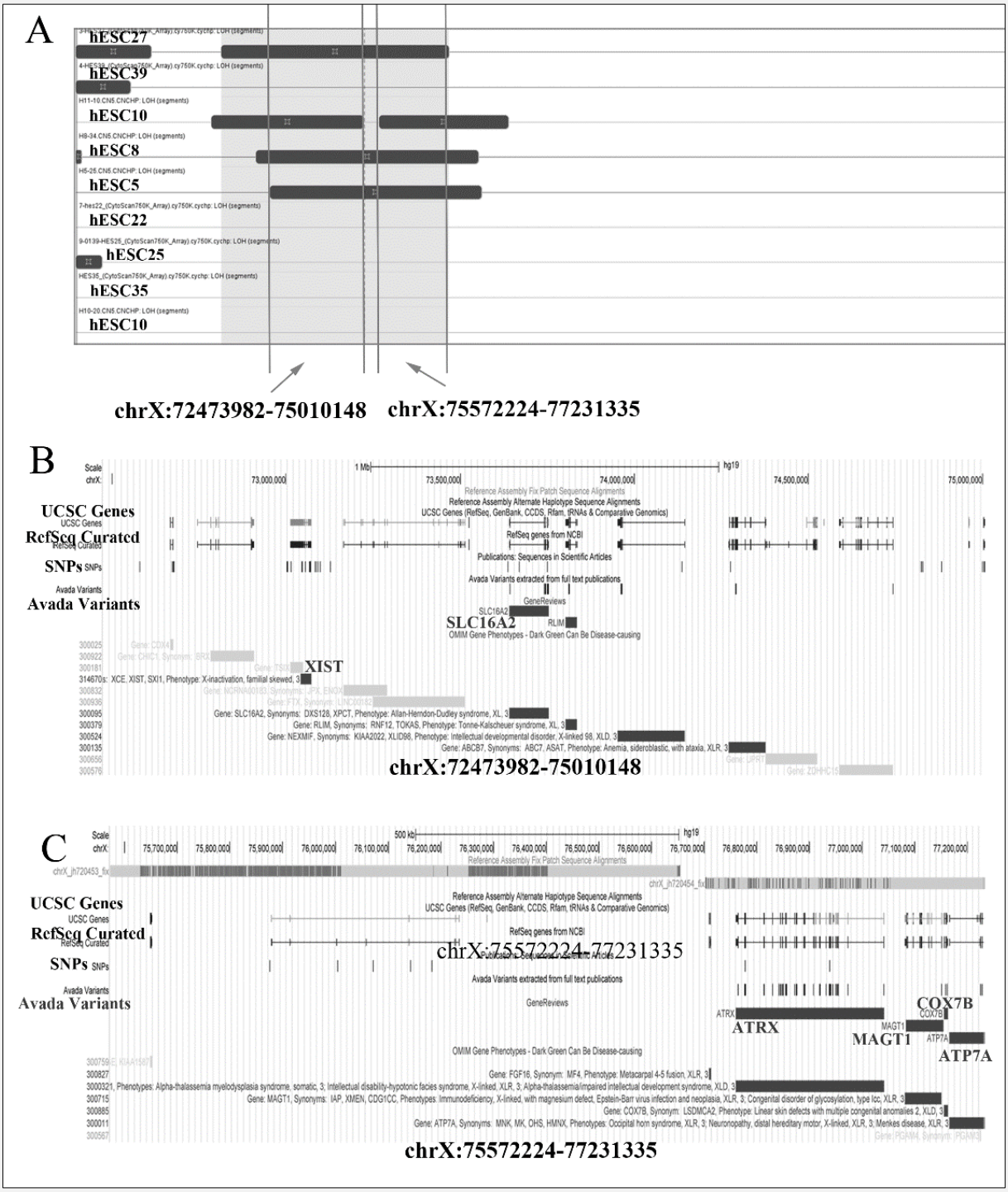

Figure 3. Key region of ROH and genes associated with XCI.

(A) Two key ROH regions at chrX:72473982-75010148 and chrX:75572224-77231335 were identified in the nonrandom XCI cells, showing that the ROH at the Xq13.2q21.1 region is critical for XCI. (B) *XIST, TSIX*, and other key genes are located in the region chrX:72473982-75010148. (C) The *ATRX, ATP7A, MAGT1*, and *COX7B* genes are within the region chrX:775572224-77231335. The black rectangle indicates disease-causing genes.

2q21.1 region and nine specific SNPs in the X chromosome regulatory center as potential markers associated with nonrandom XCI. These findings provide insights into the mechanisms of XCI and suggest broader implications for the understanding of X-linked diseases.

Acknowledgment:
The authors thank Bing Song and Yinghong Yang from the Third Affiliated Hospital of Guangzhou Medical University for the sample preparation in this work.

Source of Funds:
This research was supported by the Natural Science Foundation of Guangdong Province (2019A1515011302).

Declaration of Interest:
The authors declare no competing financial interests.

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