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X-chromosome inactivation shapes clinical heterogeneity among affected mother-daughter carriers of X-linked disorders

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Research Article

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Abstract

Background

X-chromosome inactivation (XCI) generates cellular mosaicism in females by silencing one X chromosome while the other remains transcriptionally active. This process may modify phenotypic expression in carriers of X-linked disorders. We evaluated the relationship between XCI-derived variant-allele activity and clinical heterogeneity in female carriers, and explored its potential utility for phenotype stratification and genetic counseling.

Methods

We retrospectively studied mother–daughter or mother–female fetus carrier pairs from unrelated families carrying pathogenic or likely pathogenic variants in X-linked genes (*DMD*, *RPGR*, *IKBKG*, *RPS6KA3*, *OFD1*, *COL4A5*, *CACNA1F*, and *NDP*). XCI ratios were measured by methylation-sensitive multiplex quantitative polymerase chain reaction (PCR) after HpaII digestion, and the variant-bearing X chromosome was phased by linkage analysis. The variant-allele activity ratio was calculated by integrating the degree and direction of XCI. We assessed intergenerational XCI concordance, compared variant-allele activity across phenotype strata, performed subgroup analyses according to XCI escape status, and followed female fetal carriers identified by prenatal diagnosis.

Results

All carriers had informative XCI and phasing results, enabling calculation of variant-allele activity. Mother–daughter analyses showed no significant correlation or concordance of variant-allele activity, indicating limited intergenerational predictability of XCI patterns. In the overall cohort, variant-allele activity showed a trend toward higher values in more severe phenotypes but did not reach statistical significance. In contrast, among carriers of variants in non-escape genes, higher variant-allele activity was significantly associated with greater phenotype severity and was increased in severe compared with non-severe carriers. Within clinically discordant paired families, the more severely affected carrier consistently showed the higher variant-allele activity ratio. In non-escape genes, a variant-allele activity ratio ≥ 80% was associated with severe phenotypes in this cohort, whereas > 20% activity showed high sensitivity but limited specificity. Families involving escape or variably escaping genes and prenatal cases showed gene-specific patterns that were not fully explained by simple activity thresholds.

Conclusions

XCI is an important modifier of clinical heterogeneity in female carriers of X-linked disorders, particularly in non-escape genes. Within-family comparisons demonstrate a consistent allele-dosage effect, where higher variant-allele activity is associated with more severe phenotypes. Variant-allele activity may enable preliminary risk stratification in non-escape genes, although its clinical utility requires validation in larger cohorts. Prenatal XCI assessment may provide complementary information for fetal risk evaluation when interpreted with gene-specific and longitudinal clinical data.

Introduction

X-linked disorders are caused by pathogenic variants in genes located on the X chromosome. They have traditionally been classified as X-linked recessive or X-linked dominant according to whether heterozygous females are expected to manifest disease. Although useful for describing inheritance, this framework is often insufficient in clinical practice. Female carriers of classically recessive disorders, such as Duchenne muscular dystrophy (DMD) and Fabry disease, may be asymptomatic, have only mild clinical abnormalities, or develop clinically significant disease[1, 2]. Conversely, females carrying variants in X-linked dominant or male-lethal disorders may show wide phenotypic variability[3]. These observations indicate that conventional dominant–recessive terminology does not fully explain clinical heterogeneity in female carriers and that more refined approaches are needed for phenotype assessment and genetic counseling.

X-chromosome inactivation (XCI) is a major epigenetic mechanism of dosage compensation in female mammals. During early embryonic development, one of the two X chromosomes is transcriptionally silenced as the inactive X chromosome (Xi), whereas the other remains transcriptionally active as the active X chromosome (Xa), resulting in cellular mosaicism for X-linked gene expression[4]. According to the Lyon hypothesis, XCI is generally initiated as a random process, so that either the maternal or paternal X chromosome may be selected for inactivation[4]. In female carriers of X-linked disorders, this mosaic state can partly explain why manifestations are often milder or more variable than those in hemizygous males. However, whether and to what extent XCI contributes to phenotypic heterogeneity among female carriers remains incompletely defined.

In normal circumstances, XCI is expected to approximate a balanced 50:50 pattern. Marked deviation from this balance is described as skewed XCI. Ratios of at least 80:20 are commonly used to define skewed XCI, whereas ratios of at least 90:10 are often considered extremely skewed [5]. Skewing can occur in healthy females due to stochastic variation during early embryogenesis or genetic and epigenetic influences on the XCI choice process [6, 7]. Studies in healthy female populations have shown that skewed XCI is not rare and that mother–daughter concordance of XCI patterns is generally limited [5, 8], although the heritability of XCI remains debated [9, 10].

X-linked disorders may also be associated with non-random XCI through secondary selection. After XCI is established, survival-related selective pressures may confer a competitive advantage on cells in which the reference allele remains on Xa [7]. This mechanism is particularly relevant for severe X-linked dominant or male-lethal disorders. For example, in Rett syndrome [11], ATR-X syndrome [12], and incontinentia pigmenti [13], affected males are often severely affected or nonviable, whereas some heterozygous females show relatively mild manifestations accompanied by extremely skewed XCI. These

extreme cases suggest that secondary selection may shape XCI patterns and contribute to phenotypic variability in female carriers. Similar associations between XCI skewing and clinical severity have been reported in a range of X-linked disorders, including Fabry disease [14], dystrophinopathy [15], and hemophilia [16], although not all studies have found a consistent relationship between XCI skewing and clinical severity [17, 18, 19].

Another important source of complexity is XCI escape. Approximately 15% of X-linked genes consistently escape XCI, and additional genes show variable escape across tissues or individuals [20, 21]. For such genes, Xi may still contribute residual expression, weakening the direct correspondence between XCI ratio and functional allele dosage. Therefore, studies that evaluate XCI without considering escape status may underestimate or obscure gene-specific effects.

In this study, we refined a family-based analysis of female carriers of X-linked disorders using a multi-locus XCI assay and linkage-based phasing. The cohort included 10 mother–daughter or mother–female fetus carrier pairs, including four pairs identified through prenatal diagnosis. Rather than interpreting the XCI ratio alone, we calculated the variant-allele activity ratio by integrating the degree and direction of XCI. We aimed to evaluate the relationship between XCI and clinical heterogeneity, assess intergenerational concordance of XCI patterns, explore the potential threshold value of variant-allele activity in non-escape genes, and examine the role of prenatal XCI testing in genetic counseling.

Methods

Study design and participants

This study retrospectively screened whole-exome sequencing (WES) cases evaluated at the Affiliated Women and Children's Hospital of Ningbo University between July 2021 and July 2024. During this period, WES was performed in 4,741 cases, of which 151 were found to harbor pathogenic or likely pathogenic variants in X-linked disease genes. At the family level, a total of 3,265 families underwent WES, among which 108 families were identified with pathogenic or likely pathogenic variants in X-linked disease genes. These 108 families were subsequently assessed for study eligibility. Eligible families contained one mother–daughter or mother–female fetus carrier pair sharing the same heterozygous pathogenic or likely pathogenic X-linked variant, with sufficient clinical information and available DNA for X-chromosome inactivation analysis. Families were excluded when additional pathogenic variants could confound phenotype interpretation, when the target variant was homozygous or somatic mosaicism was suspected, or when biological samples were unavailable. Ultimately, 10 unrelated families, each containing a confirmed mother–female offspring carrier pair, were included in the study (Fig. 1). The study was approved by the institutional ethics committee (approval number: EC2020-048), and written informed consent was obtained.

Clinical and genetic data

Clinical data were obtained from medical records and follow-up, including age, symptoms, relevant laboratory and imaging findings, family history, and postnatal outcomes for prenatal cases. Known gene–disease relationships and expected phenotypic spectra were reviewed using Online Mendelian Inheritance in Man (OMIM), GeneReviews, and relevant literature, with particular attention to major manifestations reported in hemizygous male patients. Variants were classified according to criteria based on the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) framework [22].

Before interpreting phenotypes in relation to XCI, female carriers were classified according to predefined severity criteria. Carriers were considered phenotype-negative when no clinical, laboratory, or imaging abnormality attributable to the familial variant was identified. Mild phenotypes were defined as subclinical, biochemical, imaging, or incomplete clinical manifestations without major functional impairment, major structural anomalies, or severe organ involvement. Severe phenotypes were defined as major organ involvement, marked functional impairment, or major fetal structural anomalies, with manifestations generally approaching those expected in hemizygous male patients with the corresponding disorder.

XCI assay and phasing

Genomic DNA was extracted from peripheral blood and amniotic fluid samples using a DNA extraction kit (Qiagen, China). XCI ratios were measured using a methylation-sensitive multiplex polymerase chain reaction (PCR) assay targeting four highly polymorphic loci distributed across the X chromosome: Xp11, Xp35, Xp47, and Xq67. The numeric suffixes indicate the approximate physical distances, in megabases, from the Xp telomere, and Xq67 corresponds to the androgen receptor (*AR*) locus.

For each sample, paired undigested and HpaII-digested DNA aliquots were prepared. In the HpaII-digested aliquot, unmethylated alleles on Xa were cleaved, whereas methylated alleles on Xi remained intact and amplifiable. Both aliquots were subsequently amplified using an XCI assay kit (Jingzhun Biomedical, China). The amplification products were separated by capillary electrophoresis on an ABI 3500 DX Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and allele sizes and peak heights were analyzed using GeneMapper software v5.0 (Applied Biosystems, Foster City, CA, USA).

For each informative locus, the higher and lower post-digestion peaks were designated d1 and d2, respectively, and their corresponding pre-digestion peaks were designated u1 and u2. The XCI ratio was calculated as $\frac{d1/u1}{d1/u1+\ d2/u2}$× 100%. For samples informative at multiple loci, the mean value across the informative loci was used as the final sample-level result. XCI was categorized as random (50% to < 80%), skewed (80% to < 90%), or extremely skewed (≥ 90%).

To determine whether the familial variant was located on Xa or Xi, linkage analysis was performed using the four XCI loci and five additional X-chromosomal short tandem repeat (STR) markers (DXS7132, DXS9902, GATA165B12, IDXp, and IDXq). The five STR markers were amplified by multiplex PCR using the

AneuFiler kit (Jingzhun Biomedical, China). Amplification products were separated on an ABI 3500 DX Genetic Analyzer and analyzed using GeneMapper software v5.0 (Applied Biosystems, Foster City, CA, USA). This nine-marker approach was used to improve genomic coverage and reduce phasing errors caused by recombination. The variant-allele activity ratio was then calculated as the estimated proportion of cells in which the X chromosome carrying the familial variant was active.

Statistical analysis

Continuous data were summarized as medians and interquartile ranges when appropriate. Mother–daughter paired differences in variant-allele activity ratio were evaluated using paired tests according to the distribution of differences, with nonparametric sensitivity analysis. Pearson and Spearman analyses were used to assess intergenerational correlation. Concordance of XCI categories was assessed using kappa statistics. For within-pedigree analyses, each paired mother–daughter or mother–female fetus comparison was treated as the basic unit, and XCI patterns and clinical phenotypes were compared within each family. Differences across phenotype strata were evaluated using Kruskal–Wallis tests, and severe versus non-severe comparisons were assessed using Mann–Whitney U tests. Because of the small sample size and gene heterogeneity, threshold analyses were considered exploratory. All statistical analyses were performed using SPSS 27.0.

Results

Cohort characteristics and variant spectrum

The final cohort comprised 10 unrelated families with X-linked disorders, each contributing one paired mother–daughter or mother–female fetus comparison, yielding a total of 20 female carriers. Among these pairs, four were mother–female fetus pairs identified through prenatal diagnosis, and six were mother–daughter pairs. Eight X-linked disease genes were represented: *DMD* in three families, and *RPGR*, *IKBKG*, *RPS6KA3*, *OFD1*, *COL4A5*, *CACNA1F*, and *NDP* in one family each. All familial variants were classified as pathogenic or likely pathogenic according to ACMG/AMP-based criteria. The variant spectrum included nonsense, missense, frameshift, and multi-exon deletion variants. Seven families involved non-escape genes (*DMD*, *RPGR*, *RPS6KA3*, *COL4A5*, and *CACNA1F*), whereas three involved escape or variably escaping genes (*IKBKG*, *OFD1*, and *NDP*). These cohort characteristics are summarized in Table 1.

Table 1
Familial variants and XCI status of the enrolled pedigrees

Family	Gene	Variant	Protein effect	Variant type	ACMG classification	Pathogenicity classification
1	*DMD*	NM_004006.3: c.4843A > T	p.Lys1615*	Nonsense	PVS1+PM2_Supporting+PP4	P
2	*RPGR*	NM_001034853.2: c.178G > A	p.Gly60Ser	Missense	PS4_Supporting+PM2_Supporting+PM5 + PP3+PP4	LP
3	*IKBKG*	NM_001099857.5: exons 4–10 del	p.(?)	Copy number loss	PVS1_Strong+PS4+PP1_Strong+PS3+PM2_Supporting	P
4	*RPS6KA3*	NM_004586.3: c.2162T > G	p.Val721Gly	Missense	PM2_Supporting+PP3_Moderate+PP1 + PP2+PP4	LP
5	*OFD1*	NM_003611.3: c.1597_1598del	p.Lys533GlufsTer23	Frameshift	PM2_Supporting+PVS1	LP
6	*COL4A5*	NM_033380.3: c.395G > A	p.Gly132Glu	Missense	PM2_Supporting+PP3_Strong+PS4_Supporting+PP4	LP
7	*DMD*	NM_004006.3: exons 49–50 del	p.(?)	Copy number loss	PVS1+PM2_Supporting+ PS4_Supporting	P
8	*CACNA1F*	NM_001256789.3: c.1423del	p.Asp475MetfsTer14	Frameshift	PVS1+PM2_Supporting	LP
9	*NDP*	NM_000266.4: c.279del	p.His94ThrfsTer10	Frameshift	PM2_Supporting+PVS1_Strong+PS4_Supporting+PP4	LP
10	*DMD*	NM_004006.3: exons 4–7 del	p.(?)	Copy number loss	PVS1+PM2_Supporting+ PS4_Supporting	P

del deletion, *ACMG* American College of Medical Genetics and Genomics, *P* pathogenicity, *LP* likely pathogenicity

Clinical manifestations in female carriers

Among the 20 female carriers, 17 (85.0%) had clinical, laboratory, or imaging abnormalities considered attributable to the familial variant, whereas three (15.0%) were phenotype-negative at the last available assessment. Six of the 10 paired families (60.0%) showed evident intrafamilial phenotypic differences

despite carrying the same familial variant. In Family 1 (*DMD* c.4843A > T), the mother had mild hyperCKemia, whereas the daughter showed mild muscle weakness, reduced exercise tolerance, and marked hyperCKemia. HyperCKemia was defined as a persistent serum creatine kinase (CK) elevation exceeding 1.5 times the upper limit of normal according to the European Federation of Neurological Societies criteria [23]. In Family 3 (*IKBKG* exons 4–10 deletion), the mother had limited pigmentary changes, whereas the daughter showed typical skin and dental involvement. In Family 4 (*RPS6KA3* c.2162T > G), the mother had severe intellectual disability and characteristic facial features, whereas the daughter showed milder intellectual involvement. In Family 5 (*OFD1* c.1597_1598del), the mother had oral abnormalities, whereas the female fetus showed severe central nervous system malformations. In Family 6 (*COL4A5* c.395G > A), the mother had microscopic hematuria and moderate proteinuria, whereas the daughter had gross hematuria and massive proteinuria. In Family 10 (*DMD* exons 4–7 deletion), the mother had no definite manifestations, whereas the daughter had mild hyperCKemia after birth (Table 2).

XCI patterns and mother–daughter concordance

All 20 carriers yielded informative XCI results and successful X-chromosome phasing (Table 3). Fourteen carriers (70.0%) showed random XCI, three (15.0%) skewed XCI, and three (15.0%) extremely skewed XCI. Among carriers with skewed but not extremely skewed XCI, the variant allele was located on Xi in all three cases. Among carriers with extremely skewed XCI, the Family 1 daughter and Family 4 mother showed preferential inactivation of the X chromosome carrying the reference allele, leaving the variant allele predominantly on Xa; the Family 3 mother showed preferential inactivation of the variant-bearing X chromosome. These findings emphasize that XCI direction is clinically more informative than the numerical XCI ratio alone.

Table 3
XCI patterns of mother-daughter carrier pairs in 10 pedigrees

Family	Gene	Subject	Assay age	Sample	XCI(%)	Skewing type	Preferential inactive X	Variant activity (%)
1	*DMD*	Mother	36 years old	Blood	60.1	Random	Variant	39.9
		Daughter	3 years old	Blood	93.7	Extremely skewed	Wild-type	93.7
2	*RPGR*	Mother	33 years old	Blood	70.8	Random	Wild-type	70.8
		Daughter	5 years old	Blood	66.8	Random	Wild-type	66.8
3	*IKBKG*	Mother	30 years old	Blood	96.2	Extremely skewed	Variant	3.8
		Daughter	1 day old	Blood	84.9	Skewed	Variant	15.1
4	*RPS6KA3*	Mother	31 years old	Blood	93.4	Extremely skewed	Wild-type	93.4
		Daughter	10 years old	Blood	67.1	Random	Variant	32.9
5	*OFD1*	Mother	34 years old	Blood	74.9	Random	Variant	25.1
		Daughter	31 weeks' GA	Amniotic fluid	54.0	Random	Variant	46.0
6	*COL4A5*	Mother	43 years old	Blood	82.2	Skewed	Variant	17.8
		Daughter	21 years old	Blood	56.3	Random	Wild-type	56.3
7	*DMD*	Mother	65 years old	Blood	67.5	Random	Variant	32.5
		Daughter	28 years old	Blood	60.6	Random	Variant	39.4
8	*CACNA1F*	Mother	24 years old	Blood	58.3	Random	Variant	41.7
		Daughter	24^{+6} weeks' GA	Amniotic fluid	54.6	Random	Variant	45.4
9	*NDP*	Mother	37 years old	Blood	63.1	Random	Variant	36.9
		Daughter	19^{+1} weeks' GA	Amniotic fluid	51.8	Random	Wild-type	51.8
10	*DMD*	Mother	34 years old	Blood	84.4	skewed	Variant	15.6
		Daughter	19 weeks' GA	Amniotic fluid	70.7	Random	Wild-type	70.7

XCI X-chromosome inactivation, *GA* gestation age

Intergenerational analysis did not support stable transmission of XCI characteristics. Five of the 10 pairs differed in whether the variant-bearing X chromosome was predominantly active or inactive. Five pairs also differed in XCI category, including four with opposite directions of preferential inactivation. The median variant-allele activity ratio was 34.7% (interquartile range, 19.6%–41.2%) in mothers and 48.9% (40.9%–64.2%) in daughters or fetuses. The paired differences did not significantly deviate from normality according to the Shapiro–Wilk test (W = 0.900, P = 0.217); therefore, a paired t test was used as the primary analysis. Although daughters or fetuses had a higher variant-allele activity ratio than mothers on average, with a mean paired difference of 14.06 percentage points, the difference was not statistically significant (t = 1.337, P = 0.214; 95% confidence interval, − 9.74 to 37.86 percentage points). A Wilcoxon signed-rank sensitivity analysis yielded the same conclusion (V = 12, exact two-sided P = 0.131). Neither Pearson nor Spearman analysis showed a significant correlation between maternal and daughter/fetal variant-allele activity ratios (Pearson r = 0.073, P = 0.841; Spearman r_s = 0.030, P = 0.934).

Agreement between maternal and daughter/fetal XCI categories was poor and did not differ from random expectation (Cohen's κ = −0.042, 95% CI, − 0.308 to 0.225; P = 0.846).

Association between variant-allele activity and phenotype in the overall cohort

In the overall cohort, higher variant-allele activity tended to be associated with greater phenotype severity, although the association was not statistically significant. The median activity ratios were 45.4% (30.5%–48.6%) in phenotype-negative carriers, 38.2% (27.0%–52.6%) in mildly affected carriers, and 93.4% (69.7%–93.6%) in severely affected carriers. The between-group difference was not significant (Kruskal–Wallis H = 4.723, P = 0.094). When phenotype severity was coded as 0, 1, and 2, its correlation with variant-allele activity was positive but not significant (Spearman r_s = 0.364, P = 0.115).

Within-family comparison of variant-allele activity and phenotype

Because comparisons across unrelated families may be influenced by gene function, variant type, disease mechanism, and XCI escape status, variant-allele activity and clinical severity were further compared within each paired family carrying the same variant. Across the six clinically discordant families, the more severely affected individual consistently had the higher variant-allele activity ratio.

Among the 10 families, Families 4 and 5 are highlighted as representative examples of the association between variant-allele activity and phenotypic severity (Fig. 2). In Family 4, the mother carrying the *RPS6KA3* variant had 93.4% variant-allele activity and severe intellectual disability, with a phenotype resembling that of her affected hemizygous son, whereas her daughter had 32.9% variant-allele activity and milder intellectual disability. In Family 5, the female fetus had 46.0% variant-allele activity and presented with multiple central nervous system (CNS) abnormalities, whereas her mother, who carried the same *OFD1* variant, had 25.1% variant-allele activity and only mild oral manifestations.

Subgroup analysis of non-escape genes

Because XCI escape may weaken the relationship between XCI ratio and effective variant-allele dosage [21], carriers from non-escape gene families were analyzed separately (n = 14). In the two phenotype-negative carriers, the variant-allele activity ratios were 15.6% and 45.4%. In the 10 mildly affected carriers, the median ratio was 40.8% (34.5%–64.2%), whereas the two severely affected carriers had ratios of 93.4% and 93.7%. The overall difference among the three phenotype strata approached statistical significance (Kruskal–Wallis H = 5.349, P = 0.069). Variant-allele activity was significantly correlated with phenotype severity (Spearman r_s = 0.597, P = 0.024), and severe carriers had significantly higher activity ratios than non-severe carriers (Mann–Whitney U = 24, exact two-sided P = 0.022).

Families carrying *DMD* variants provided an additional gene-specific example. Among six female carriers of *DMD* variants, four had random XCI, one had skewed XCI, and one had extremely skewed XCI. The four carriers with random XCI showed no definite muscle weakness and had only mild hyperCKemia. The Family 10 mother showed protective skewing, with preferential inactivation of the variant-bearing X chromosome, and was clinically and biochemically unaffected. In contrast, the Family 1 daughter showed extremely skewed XCI in a risk-enhancing direction, leaving the variant allele predominantly on Xa, and had marked hyperCKemia and mild muscle weakness in childhood (Table 4).

Table 4
Phenotype and XCI patterns in *DMD* variant carriers

Pedigree	Variant	Subject	Last follow-up age	Symptoms	CK level	XCI(%)	Preferential inactive X	Variant activity (%)
1	c.4843A > T	Mother	39y	No	Mild hyperCKemia	60.1	Variant	39.9
		Daughter	5y	Mild muscle weakness, exercise intolerance	Markedly elevated CK	93.7	Wild-type	93.7
7	exons 49-50del	Mother	69y	No	Mild hyperCKemia	67.5	Variant	32.5
		Daughter	31y	No	Mild hyperCKemia	60.6	Variant	39.4
10	exons 4-7del	Mother	35y	No	Normal	84.4	Variant	15.6
		Daughter	0.5y	No	Mild hyperCKemia	70.7	Wild-type	70.7

del deletion, *CK* creatine kinase, *XCI* X-chromosome inactivation

Exploratory threshold analysis in non-escape genes

Exploratory threshold analysis was performed in the non-escape gene subgroup to evaluate whether variant-allele activity could stratify phenotypic risk. Based on conventional definitions of skewed XCI, we evaluated ≥ 80% variant-allele activity as a threshold for severe phenotypes and > 20% activity as a threshold for phenotype-positive status (Fig. 3).

In this small subgroup, the ≥ 80% threshold separated severe from non-severe carriers: both severe carriers had ratios ≥ 80%, whereas all 12 non-severe carriers had ratios below 80%. This finding should be interpreted cautiously because only two severe cases were present. A cutoff of > 20% for phenotype-positive status had 91.7% sensitivity (11/12), 50.0% specificity (1/2), 91.7% positive predictive value, 50.0% negative predictive value, and 85.7% accuracy. Thus, ≤ 20%, > 20% to < 80%, and ≥ 80% variant-allele activity may represent provisional risk bands for non-escape genes rather than validated diagnostic cutoffs.

Escape or variably escaping genes

The three families involving *IKBKG*, *OFD1*, and *NDP* were analyzed descriptively because their escape or variable escape status may limit interpretation of variant-allele activity ratios. In the family carrying the *IKBKG* variant, both carriers showed skewed or extremely skewed XCI toward preferential inactivation of the variant-bearing X chromosome (Fig. 4). The mother had a lower variant-allele activity ratio of 3.8% and relatively mild residual pigmentary changes, whereas the daughter had a higher ratio of 15.1% and presented with more typical neonatal cutaneous involvement and dental abnormalities. In the orofaciodigital syndrome type 1 (OFD1) family, the mother showed a near-skewed but conventionally random XCI pattern (74.9%), with the variant-bearing X chromosome predominantly inactive, and had oral abnormalities. In contrast, the female fetus showed random XCI (54.0%) and severe central nervous system malformations. In the prenatal family carrying the *NDP* variant, both the mother and female fetus showed random XCI patterns (63.1% and 51.8%, respectively); the mother had mild myopia, whereas the child had no definite ophthalmologic abnormality at 1 year of age. These observations indicate that escape or variably escaping genes did not conform to the simple XCI threshold pattern observed in non-escape genes.

Prenatal XCI findings and postnatal follow-up

Four families underwent prenatal XCI assessment (Families 5, 8, 9, and 10). In Family 10, the female fetus carried a *DMD* exons 4–7 deletion and showed a random XCI pattern according to conventional classification, whereas phasing indicated a variant-allele activity ratio of 70.7%. At six months, the infant had mild hyperCKemia without overt motor delay. In Families 8 and 9, the female fetuses carrying *CACNA1F* and *NDP* variants, respectively, both showed random XCI, and no definite ophthalmologic abnormalities were observed at the postnatal 1-year follow-up. In Family 5, the female fetus carrying the *OFD1* variant also showed random XCI but presented with severe fetal central nervous system malformations, leading to pregnancy termination.

Discussion

This study refines the interpretation of XCI in female carriers of X-linked disorders by integrating multi-locus methylation analysis, linkage-based phasing, family-matched variant comparisons, and statistical evaluation of variant-allele activity. By incorporating highly polymorphic X-linked markers beyond the classical *AR* locus used in the human androgen receptor assay (HUMARA) [24], the assay achieved informative results in all carriers, reducing inconclusive findings caused by homozygosity. Moreover, XCI ratios derived from the HUMARA assay are influenced by the methylation status of CpG sites at a single locus and may not fully reflect the overall X-chromosome inactivation pattern [25, 26]. The family-based design enabled determination of whether the variant allele was preferentially located on Xa or Xi and allowed direct intrafamilial comparison between XCI patterns and phenotypic outcomes in carriers with the same variant.

Our findings support XCI as an important modifier of phenotypic variability in female carriers, particularly for variants in non-escape genes. Paired comparisons of female carriers carrying identical pathogenic variants provided family-level evidence that XCI contributes to clinical heterogeneity. Across the cohort, the variant-allele activity ratio showed a positive trend with phenotype severity. This association became stronger and statistically significant when restricted to families with non-escape genes, supporting the biological relevance of XCI in modulating disease expression when the affected gene is subject to X-linked dosage regulation. These results indicate that XCI should not be regarded merely as a background epigenetic phenomenon, but as a clinically meaningful modifier influencing the penetrance and expressivity of X-linked variants in female carriers.

A major strength of this study is that XCI was not interpreted as a ratio alone. By combining methylation-based multi-locus analysis with linkage-based phasing, we converted the conventional XCI ratio into a variant-allele activity ratio. This distinction is clinically important because the same degree of skewing can have opposite implications depending on which X chromosome is preferentially inactivated. For example, a 90:10 XCI pattern may increase disease risk if the variant-bearing X chromosome is predominantly Xa, but may reduce risk if the variant-bearing X chromosome is predominantly Xi. In this cohort, extremely skewed XCI provided bidirectional evidence supporting this interpretation. The daughter in Family 1 and the mother in Family 4 showed preferential inactivation of the X chromosome carrying the reference allele and more severe manifestations, whereas the mother in Family 3 showed preferential inactivation of the variant-bearing X chromosome and had a milder phenotype. These observations show that the pathogenic relevance of skewed XCI can be interpreted properly only after determining whether the predominantly active allele is the variant or reference allele.

Within-family comparisons further supported a functional allele dosage effect. In most informative families, the more severely affected individual had a higher variant-allele activity ratio. The *RPS6KA3* family was representative: the mother, whose variant-allele activity ratio reached 93.4%, had a severe

phenotype comparable to that of her hemizygous son, whereas her daughter, with a ratio of 32.9%, showed milder developmental involvement [27]. Similarly, in families carrying *DMD* variants, random XCI or protective skewing was associated with minimal clinical manifestations, whereas extreme skewing favoring the variant allele was associated with early biochemical abnormalities and mild muscular involvement. Previous studies have also indicated that XCI is an important modifier underlying phenotypic variability among female carriers of *DMD* variants [28, 29]. These observations suggest that variant-allele activity provides biologically meaningful information beyond genotype alone.

The lack of stable mother–daughter XCI concordance highlights the need for individualized assessment in genetic counseling. In this cohort, maternal variant-allele activity ratio, XCI category, and clinical phenotype did not reliably predict the daughter's XCI status or disease severity. This finding is consistent with the view that XCI patterns are largely shaped by stochastic events during early embryogenesis and may be further modified by cell selection during development, rather than being inherited in a stable and predictable manner [7]. Therefore, maternal XCI status alone should not be used as a surrogate for risk estimation in daughters or female fetuses carrying the same familial X-linked variant. Direct assessment of the carrier's own XCI pattern and variant-allele activity ratio may provide a more precise basis for evaluating potential phenotypic consequences.

The exploratory thresholds observed in non-escape genes are biologically plausible and clinically informative, but should be interpreted cautiously. The ≥ 80% threshold was selected according to the conventional definition of skewed XCI. When a pathogenic variant is located predominantly on Xa, a variant-allele activity ratio ≥ 80% may approximate a functional hemizygous state and increase the risk of a severe or male-like phenotype. Conversely, a variant-allele activity ratio ≤ 20% was used as a reference boundary for phenotype-negative status, whereas > 20% activity was explored as a threshold for phenotype-positive status. In this cohort, the 80% threshold identified severe phenotypes in all non-escape gene carriers, whereas the > 20% threshold for any phenotype was less specific. Two discordant observations illustrate the limitations of a simple threshold model. One mildly affected female carrier of a *COL4A5* variant had a low variant-allele activity ratio, which may reflect the age-dependent evolution of renal manifestations in female carriers of *COL4A5* variants [30]. Conversely, one young female child with a *CACNA1F* variant had a moderate variant-allele activity ratio but no definite phenotype at the current follow-up, possibly reflecting early disease stage and limited follow-up [31]. Therefore, the proposed ≤ 20%, > 20% to < 80%, and ≥ 80% bands should be viewed as a preliminary framework for risk discussion rather than as a validated prediction model.

Escape or variably escaping genes require a gene-specific interpretive approach, particularly when associated with severe or male-lethal X-linked disorders. In our cohort, two families carried variants in male-lethal X-linked disease genes: Family 3 with an *IKBKG* variant and Family 5 with an *OFD1* variant [32, 33]. Among the four female carriers from these families, three showed skewed or near-skewed XCI that preferentially inactivated the variant allele and survived with clinical manifestations. These included the mother in Family 3 with an XCI ratio of 96.2%, the mother in Family 5 with a ratio of 74.9%, and the daughter in Family 3 with a ratio of 84.9%. In contrast, the female fetus carrying the *OFD1* variant, with a random XCI pattern of 54.0% presented with severe intrauterine central nervous system malformations, resulting in pregnancy termination. In intrafamilial comparisons, more severe phenotypes were associated with higher variant-allele activity ratios, whereas milder individuals showed lower ratios. However, deviations from a simple threshold model indicate that additional gene-specific mechanisms should be considered.

One possible explanation is that highly pathogenic variants in *IKBKG* and *OFD1* may exert strong cellular selection during development. Cells in which the variant-bearing X chromosome remains active may be selectively disadvantaged, whereas cells with preferential activation of the X chromosome carrying the reference allele may gain a survival advantage, leading to secondary XCI skewing. Consistent with this possibility, Thauvin-Robinet et al. reported skewed XCI in 30% of 25 patients with OFD1, higher than the approximately 8% reported in the general female population [5, 34]. Increased frequencies of skewed XCI have also been observed in other male-lethal X-linked disorders, including Aicardi syndrome, Rett syndrome, and microphthalmia with linear skin defects syndrome [35, 36, 37]. In these conditions, skewed XCI may reflect selection against cells with an active variant allele and may partly mitigate disease severity by reducing overall variant-allele activity. This interpretation may explain why the Family 5 fetus, who failed to establish favorable skewing, developed severe intrauterine abnormalities.

Another important consideration is that both *IKBKG* and *OFD1* are known to escape XCI [21]. Therefore, even when XCI appears favorable, a variant allele located on Xi may retain residual expression. Depending on the variant mechanism and gene function, this residual expression may contribute to disease through toxic variant-derived products or dosage imbalance [38]. This may explain why the mother in Family 3 still exhibited clinical manifestations despite highly skewed XCI favoring activation of the X chromosome carrying the reference allele. However, the phenotypic impact of XCI escape remains uncertain. One study has suggested that biallelic expression of escaping genes may, in certain contexts, have a buffering effect, whereby limited additional gene product from Xi partially mitigates the consequences of the variant [3]. It should also be emphasized that escape from XCI is usually incomplete [21]. Thus, XCI remains an important modifier of phenotypic heterogeneity in these families, but its effect should be interpreted together with residual escape expression, variant mechanism, and gene function.

Prenatal XCI analysis may offer additional information for assessing clinical risk in female fetal carriers. In Family 10, the female fetus carried a *DMD* exons 4–7 deletion predicted to disrupt the reading frame, a variant type typically associated with classic DMD in hemizygous males [39]. Prenatal analysis showed random XCI, with a variant-allele activity ratio of 70.7%. At six-month follow-up, the infant had no overt motor delay but had mild hyperCKemia. Although this does not establish future clinical progression, prenatal XCI information may help identify female fetal carriers who warrant closer postnatal biochemical and neuromuscular surveillance. In Families 8 and 9, the female fetuses carrying *CACNA1F* and *NDP* variants, respectively, both showed random XCI, and neither child had definite ophthalmologic abnormalities at current follow-up. These early outcomes are reassuring but not definitive, because visual phenotypes may emerge or become more recognizable with age. In contrast, Family 5 demonstrates an important limitation: the female fetus carrying the *OFD1* variant showed random XCI but had severe fetal central nervous system malformations. Random XCI therefore does not necessarily imply a benign outcome, particularly for highly pathogenic variants and genes with escape or variable escape. Taken together, prenatal XCI testing may offer potentially informative guidance, consistent with recent studies of *DMD* variants [40] and large X-linked deletions [41], but it should always be interpreted in the context of gene function, disease natural history, variant mechanism, and other clinical data.

In this study, XCI analysis was performed on genomic DNA from peripheral blood or amniotic fluid. Although these surrogate tissues differ from disease-relevant target tissues, several systematic studies support their practical value for XCI evaluation. Bittel et al. analyzed 26 female individuals across 34 tissues from all three germ layers and reported generally high concordance of XCI patterns across tissues within individuals [42]. Tukiainen et al. quantified allele-specific expression across human tissues using RNA-sequencing data and showed that, despite variability related to tissue-specific escape genes, XCI patterns are largely conserved across tissues [21]. These findings support peripheral blood and amniotic-fluid DNA as clinically useful sources for XCI assessment, particularly in systemic diseases or disorders involving tissues derived from multiple germ layers.

Several limitations warrant consideration. First, the sample size was small, particularly for severe and phenotype-negative carriers, limiting statistical power and the stability of threshold estimates. Second, the cohort included multiple diseases with different natural histories, mechanisms, and age-dependent manifestations, restricting comparability across pooled phenotypic categories. Third, follow-up was limited for some infants and prenatal cases; therefore, absence of a current phenotype does not exclude future clinical expression. Fourth, the escape-gene subgroup was too small for formal threshold analysis or robust stratified evaluation. Larger, gene-stratified, multicenter studies with longitudinal follow-up are required to validate these findings, refine the proposed interpretive thresholds, and clarify the clinical application of XCI and variant-allele activity analysis in female carriers of X-linked disorders.

In conclusion, XCI is an important modifier of clinical heterogeneity in female carriers of X-linked disorders, with the strongest effect observed in non-escape genes. Within-family comparisons support a functional allele-dosage effect, where higher variant-allele activity is associated with more severe phenotypes. In non-escape genes, variant-allele activity may provide preliminary risk stratification, although proposed thresholds require validation in larger gene-specific cohorts. Gene escape status and variant mechanism may modify this relationship, and intergenerational XCI patterns are not reliably concordant. Therefore, maternal phenotype or XCI status alone is insufficient for outcome prediction. Prenatal XCI assessment may offer adjunctive information for fetal risk evaluation when interpreted in a gene-specific and longitudinal clinical context.

Declarations

Ethics approval and consent to participate

This work was approved by the Medical Ethical Committee of the Affiliated Women and Children's Hospital of Ningbo University (Approval number: EC2020-048). Written informed consent was obtained from each enrolled patient.

Conflict of interests

No financial or non-financial benefits have been received or will be received from any party related directly or indirectly to the subject of this article.

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Author Contribution

MWJ, XJYand LHB contributed to the conception and design of the study, acquisition of data, statistical analysis and interpretation of the data, and drafting of the article. XJY, XM and YLL contributed to acquisition of data, analysis and interpretation of the data, and critically revised the article for important intellectual content. AY, WSS and CHH contributed to data acquisition and critically revised the article for important intellectual content. ZT and LHB contributed to the conception and design of the study, critically revised the article for important intellectual content, and supervised the study. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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Data availability:

All data generated or analyzed during this study are included in this published article. The datasets are also available from the corresponding author on reasonable request.

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Table 2

Table 2 is not available with this version.

Figures

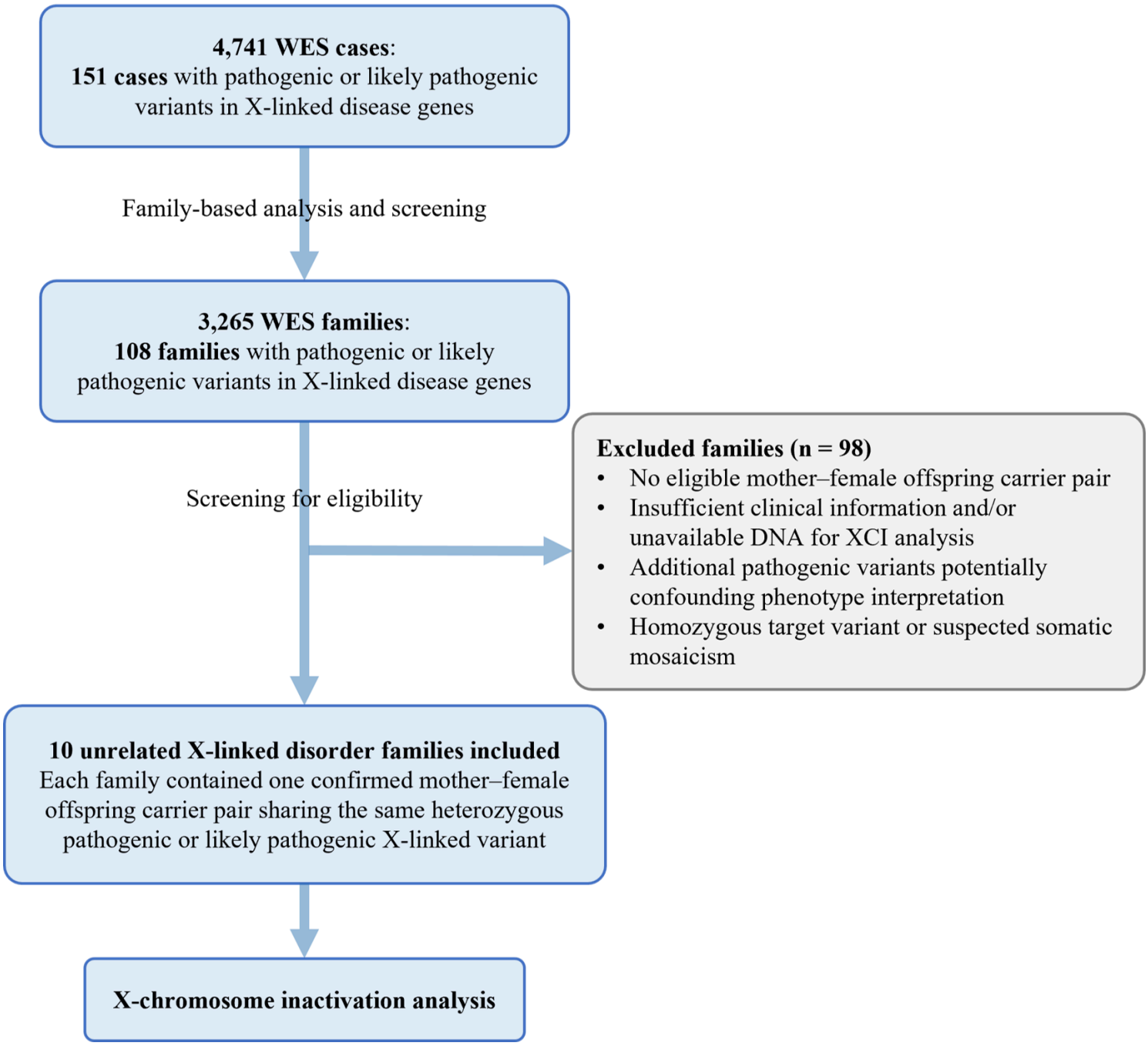

Figure 1

Flowchart of family-based cohort selection for XCI analysis.

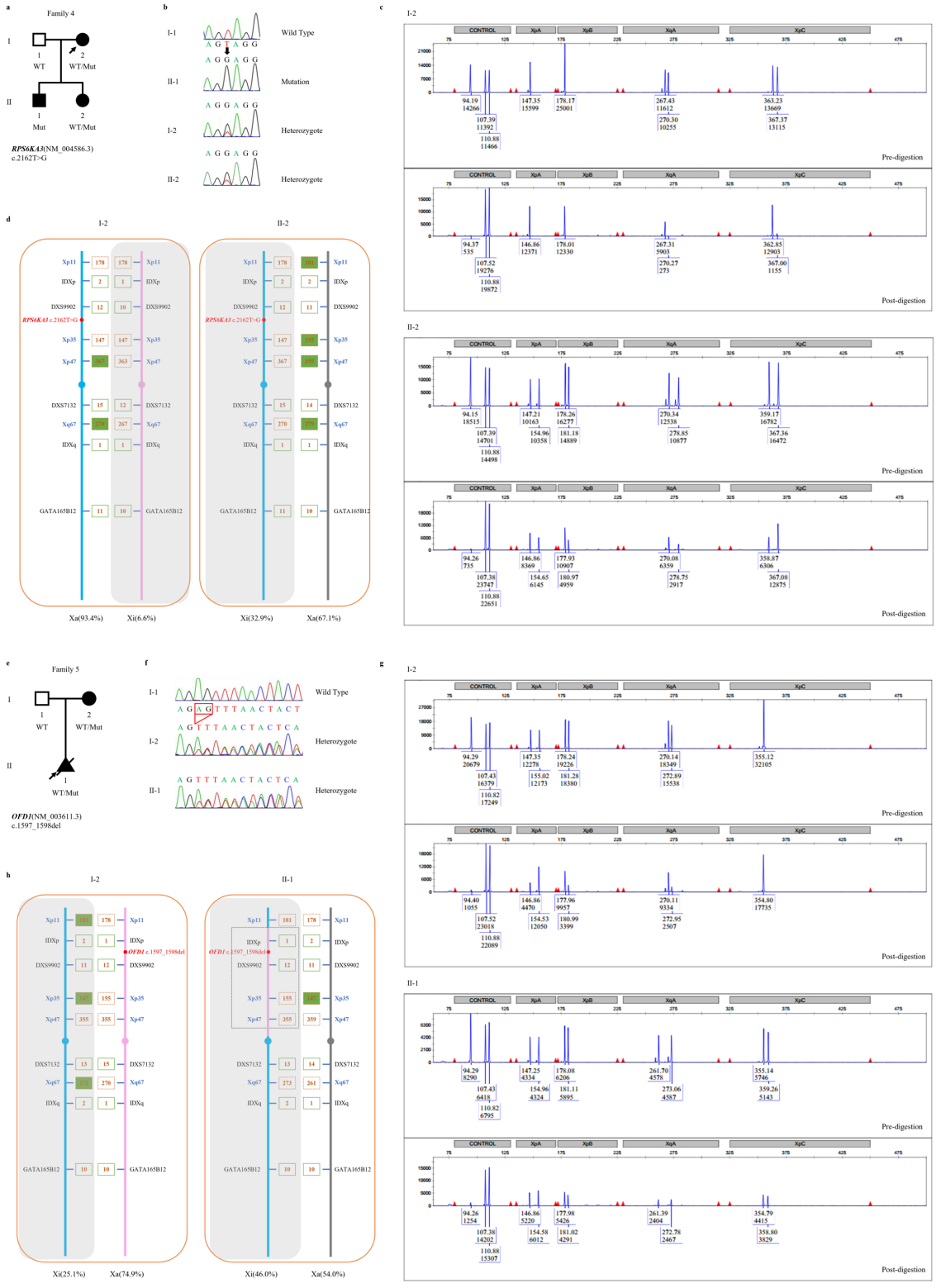

Figure 2

XCI analysis and allele phasing in two representative families carrying *RPS6KA3* and *OFD1*variants. a, e Pedigrees of Family 4, carrying the *RPS6KA3* variant, and Family 5, carrying the *OFD1*variant, respectively. **b, f** Confirmation of the *RPS6KA3* and *OFD1*variants by Sanger sequencing, respectively. **c, g** Capillary electropherograms of the four polymorphic loci used for XCI analysis. XpA, XpB, XpC, and XqA denote the amplified fragments from loci Xp35, Xp11, Xp47, and Xq67, respectively; CONTROL denotes the quality-control fragment. **d, h**XCI allele phasing in the mother–female offspring pairs. The two maternal X chromosomes are shown in pink and blue, and the paternal X chromosome inherited by each female offspring is shown in gray. Allele sizes (bp) at the four XCI loci are indicated in yellow boxes, and repeat numbers at the five linked STR markers (IDXp, DXS9902, DXS7132, IDXq, and GATA165B12) are indicated in green boxes. *Ref* reference allele, *Var* variant allele, *Xa* active X chromosome, *Xi* inactive X chromosome.

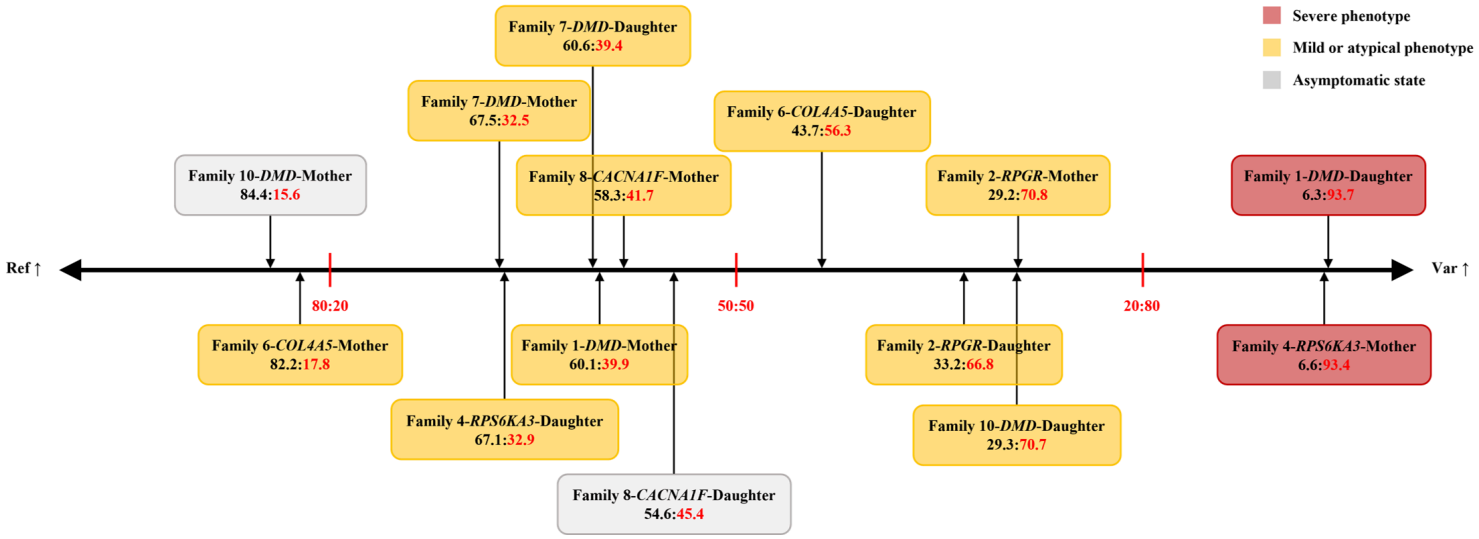

Figure 3

Distribution of XCI ratios and corresponding clinical phenotypes among carriers of variants in non-escape genes. The horizontal scale represents a continuum of XCI ratios, expressed as the ratio of reference-allele activity to variant-allele activity (Ref:Var). For each carrier, the variant-allele activity value is highlighted in red. *Ref*reference allele, *Var* variant allele.

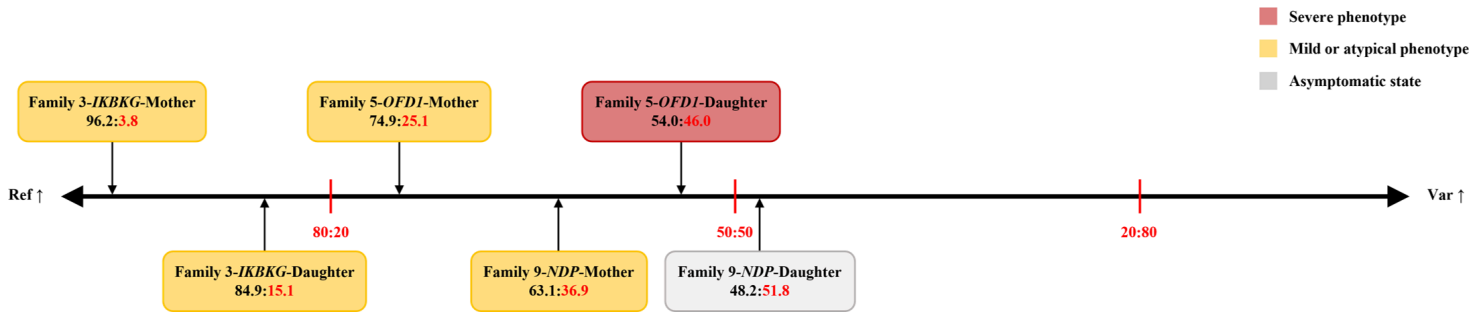

Figure 4

Distribution of XCI ratios and corresponding clinical phenotypes among carriers of variants in escape genes. The horizontal scale represents a continuum of XCI ratios, expressed as the ratio of reference-allele activity to variant-allele activity (Ref:Var). For each carrier, the variant-allele activity value is highlighted in red. *Ref*reference allele, *Var* variant allele.

Supplementary Files

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