Original Article

Loss of X Chromosome Inactivation in Androgenetic Complete Hydatidiform Moles With 46, XX Karyotype

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Summary: Most of complete hydatidiform moles (CHMs) showcase an androgenetic nature of the nuclear genome. In the normal female embryo, one of the 2 X chromosomes is inactive. However, the status of X chromosome inactivation (XCI) in androgenetic CHMs remains unknown. Seventy-one androgenetic CHM tissues with the 46, XX karyotype were collected. Seventy-four normal female villi and 74 normal male villi were collected as controls. The expression of XCI markers (XIST, TSIX, and XACT) and an X-linked gene (CDX4) was detected by real-time polymerase chain reaction. Other XCI-associated genes were also examined, including the methylation status of the human androgen receptor gene (HUMARA) by methylation-specific polymerase chain reaction, and the expression of H3K27me3, USP21, and Nanog by Western blot and immunofluorescence, respectively. In addition, 126 CHMs and 63 normal female villous samples were collected for CDX4 immunohistochemical staining. The expression of XIST RNA was significantly lower, and TSIX RNA expression was significantly higher in androgenetic CHMs than that in normal female villi (both \( P < 0.01 \)). The expression of CDX4 mRNA in androgenetic CHMs was elevated compared with that in normal male and normal female villous samples (both \( P < 0.01 \)), and CDX4 protein expression was also higher than that in normal female villous samples (both \( P < 0.01 \)). The expression of H3K27me3 was lower in androgenetic CHMs compared with that in normal female villi (both \( P < 0.01 \)). The methylation pattern of HUMARA was found lacking in androgenetic CHMs. The expression of Nanog and USP21 protein in androgenetic CHMs was higher than that in normal villi (both \( P < 0.01 \)). Both X chromosomes are active in androgenetic CHMs with the 46, XX karyotype, and the USP21-Nanog pathway may be involved in the disruption of XCI during this process.

Key Words: Complete hydatidiform moles—X chromosome inactivation—USP21—Nanog.

Complete hydatidiform mole (CHM) is characterized by hydropic swelling, trophoblastic proliferation, and vascular immaturity in the stromal cells of chorionic villi. CHM is an uncommon disease in western countries, but the incidence has increased in Asian women, accounting for \( \sim 5.0 \) in 1000 pregnancies.
in China (1). CHM has been classified into 2 types according to the genetic origin: the androgenetic CHM and biparental CHM (2). Androgenetic CHM, which arises from the fertilization of an enucleate egg and 1 or 2 haploid sperms, accounts for 90% to 95% of all CHMs. There are 2 kinds of androgenetic CHMs, the monospermic CHM, arising from the fertilization of an enucleate egg and a single haploid sperm, accounts for 70% of CHM, while the dispermic CHM, resulting from fertilization of an enucleate egg by 2 sperms, makes up 20% to 25% of CHM. Biparental CHM is quite rare with a diploid, but of biparental origin. The development of CHM has remained mysterious. Disordered genetic imprinting (3), defective angiogenesis (4), oxidative stress (5), abnormal apoptosis (6), and the dysfunction of mitochondria (7) may be involved in the development of CHM. However, all these findings failed to offer conclusive proof, and the detailed mechanisms of CHM development remain unknown.

X chromosome inactivation (XCI), is considered a dosage compensation strategy to balance sex chromosome contents between female individuals and male individuals, and is believed to be connected with the regulation of embryonic development, as well as with some diseases. The effect of XCI on recurrent miscarriage is contentious because of different inclusion criteria in the studies involved. Some studies suggested that the abnormal XCI may be associated with recurrent miscarriage, while Kaare et al. (8) found that abnormal XCI was not the cause of recurrent miscarriage. In the context of gestational trophoblastic disease, Djuric et al. (9) showed a normal XCI pattern in biparental CHM, but the status of XCI in androgenetic CHM with the 46, XX karyotype has still not been reported, although Siu et al. (10) claimed that Nanog, was an important factor in XCI (11), was overexpressed in gestational disease. Androgenetic CHM is the most common type among CHMs, and understanding its status of X chromosome inactivation may assist in better understanding of the mechanisms of the development of this disease.

In the study, we detected the expression of XCI markers (XIST, TSIX, and XACT) and an X-linked gene (CDX4), and examined other XCI-associated factors, including the methylation status of human androgen receptor gene (HUMARA), and the expressions of H3K27me3, USP21, and Nanog in androgenetic CHMs with the 46, XX karyotype. The aim of our study was to confirm the status of XCI and explain the possible association with the development of CHM.

MATERIALS AND METHODS

Sample Collection
Molar tissue samples were collected from patients treated at Women’s Hospital, School of Medicine, Zhejiang University, China. Villous tissue samples from normal early pregnancy patients were selected as controls. All normal villous tissues were donated by women who underwent surgical abortion due to unexpected pregnancy. All samples were immediately snap-frozen in liquid nitrogen and stored at −80°C for PCR and Western blot assays. Every sample was pathologically diagnosed by a senior pathologist in our hospital, and the immunohistochemical staining of p57 was performed according to a previous report (12) to confirm the diagnosis of CHM. Thereafter, gender identification was performed on the basis of the detection of sex-related gene (SRY) by PCR (13). CHM cases with the 46, XY karyotype were excluded. After gender identification, a polymorphic marker, HUMARA, was used to exclude the biparental CHMs. The HUMARA assay was performed, as previously described (14). A total of 219 fresh tissues were identified as being eligible for this study. They included 71 androgenetic CHMs with the 46, XX karyotype and 74 normal villi with 46, XX karyotype and 74 normal villi with the 46, XY karyotype. Among these, 27 CHMs with 46, XX karyotype, 30 normal villi with 46, XX karyotype, and 30 normal villi with the 46, XY karyotype, collected during April 2012 to June 2014, were used to detect the expression of XIST, TSIX, and CDX4. The remaining samples that were collected from May 2015 to December 2017 were used to detect USP21, Nanog, H3K27me3, and HUMARA-MSP. In addition, paraffin-embedded tissues, including 126 CHMs and 63 normal villi with 46, XX karyotype, collected from January 2010 to March 2012, were used for immunohistochemistry analysis of CDX4.

Sample collection was approved with the informed consent of each woman and by the institutional ethical review board of the hospital.

Real-Time Polymerase Chain Reaction
Trophoblastic tissues were washed twice with PBS to remove the blood and other impurities. Total RNA was extracted using Trizol reagent in accordance with the manufacturer’s instructions (Invitrogen, Paisley, UK). Real-time polymerase chain reaction was performed using the SYBR Premix Ex Taq PCR Kit (Takara Bio, Shiga, Japan), as previously reported (15). The relative expression of RNA was calculated using the 2−(△△Ct) method. The primers used are shown in Table 1.
Western Blotting Analysis

Trophoblastic tissues were lysed in RIPA buffer. The protein concentration was measured using the Bradford Protein Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Western blot was performed as previously described (16). The membranes were incubated with primary antibodies (all from Abcam, Cambridge, UK) against H3K27me3 (ab192985, 1:1000), USP21 (ab38864, 1:200), Nanog (Abcam, ab109250, 1:1000), and GAPDH (Abcam, ab8245, 1:10000). The bands were detected with FDbio-Dura ECL (FUDE, Zhejiang, China) using the Imagequant LAS4000mini (GE Healthcare, Buckinghamshire, UK). Image J software (NIH, Bethesda, MD) was used to measure the gray value of each band. The signal of each band was normalized to that of GAPDH in the same membrane before comparison.

Immunohistochemistry

Immunohistochemical staining was performed according to a previous report (12). Negative controls (substitution of primary antibody for rabbit IgG) were run simultaneously. Each sample was assigned to one of the following categories: 0 (0%–4%), 1 (5%–24%), 2 (25%–49%), 3 (50%–74%), or 4 (75%–100%). The intensity of immunostaining was graded as follows: 0, negative; 1 +, weak; 2 +, moderate or 3 +, strong. A final score was obtained by multiplying 2 scores. Scores of 0 to 4 were defined as “low expression,” scores of 5 to 8 were defined as “moderate expression,” and scores of 9 to 12 were defined as “high expression.”

Immunofluorescence Analysis

Normal villi tissues and CHM tissues were voluntarily donated by women who underwent surgical abortion. After gender identification, the trophoblastic tissue sections were immunostained with the primary antibodies (H3K27me3, Abcam, ab192985, 1:1000; USP21, Abcam, ab38864, 1:200; Nanog, Abcam, ab109250, 1:1000), as discussed before (17) and examined using confocal microscopy (Olympus, Tokyo, Japan).

HUMARA-MSP

The methylation-specific polymerase chain reaction (HUMARA-MSP) assay was used to analyze XCI in androgenetic CHM. Genomic DNA was isolated from trophoblastic tissues using the TIANamp Genomic DNA Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. Genomic DNA was used for bisulfite conversion procedure using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The bisulfite-treated DNA was then amplified via polymerase chain reaction with 2 sets of primers listed in Table 1, as previously described (18). The products were examined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Statistical Analysis

Statistical analysis was performed with the SPSS 19.0 software package (IBM, Armonk, NY). All experiments were repeated 3 times. Differences among groups were first analyzed by 1-way analysis of variance. The LSD test was then used to further analyze the differences between each of the 2 groups. Wilcoxon rank sum test was used to analyze the difference in expression of CDX4 between CHMs and normal villi. P<0.05 was considered as statistically significant.

RESULTS

The XCI Pattern in CHM

Lower Expression of XIST and Higher Expression of TSIX in Androgenetic CHMs With the 46, XX Karyotype

Real-time polymerase chain reaction revealed that the expression of XIST RNA was significantly decreased and the expression of TSIX RNA was significantly increased in

| TABLE 1. The sequence of the primers |
|-------------------------------------|
| Gene       | Forward primer | Reverse primer |
|------------|----------------|----------------|
| SRY        | 5′AGAGAATCCCAGAATGCGAAAC3′ | 5′CTTCCCGAGGTTGATACTT3′ |
| XIST       | 5′CCATGAGATACCCAGTGC3′ | 5′GTCCTCAGTCTTACATGCTC3′ |
| TSIX       | 5′GTGATCTCTACGGACTGCAAC3′ | 5′AGCTGGTCAGGAGGTCAA3′ |
| XACT       | 5′TGCTGATGATGTCTCTAACCCTG3′ | 5′AGAGGTGTTTGGAGATGAGCA3′ |
| USP21      | 5′GAGCTCACTGAGGCGCTTG3′ | 5′CCAGGAGGGAGTCTGGAAC3′ |
| Nanog      | 5′AAGGGCCGGTTCAAGAAGACAG3′ | 5′CTGATGTCGTTGCGACAGAA3′ |
| CDX4       | 5′CGAGAAGACTTGAGCGGTGA3′ | 5′TCCAGAATCTGTCAGACGTGC3′ |
| GAPDH      | 5′GCACCGTGACGGTGAAC3′ | 5′AACCAATAAACCTATAAAACCTACG3′ |
| HUMARA     | 5′GCCTGTAAGAGTTGTCGTCTTCTCAT3′ | 5′CAAATACCATTAAACCTACG3′ |
| HUMARA-M   | 5′CGAACGATGATATTTCGCG3′ | 5′CAAATACCATTAAACCTACG3′ |
| HUMARA-U   | 5′GTTGTAGTGTAGTATTTCGTTG3′ | 5′CAAATACCATTAAACCTACG3′ |
androgenetic CHMs with the 46, XX karyotype, compared with that in normal female villi (both \( P < 0.01 \)), while the expression of XIST RNA and TSIX RNA expression in androgenetic CHMs was similar to that in normal male villi (Fig. 1). We also detected the expression of XACT, another noncoding RNA, which can compete with XIST in the control of XCI during human early development. Although the expression of XACT was higher in androgenetic CHMs compared with that in normal villi, it was not significantly different from the levels found in the other 2 groups \( (P_{1} (\text{CHMs vs. normal female villi}) = 0.488; P_{2} (\text{CHMs vs. normal male villi}) = 0.381) \).

![Image](image_url)

**FIG. 1.** The expression of XIST, TSIX, XACT, and CDX4. (A) XIST RNA was significantly decreased in androgenetic complete hydatidiform moles (CHMs) compared with that in normal female villi \( (P < 0.01) \), but was similar to that in normal male villi \( (P = 0.573) \). (B) The level of TSIX RNA was significantly increased in androgenetic CHMs \( (P < 0.01) \), but was similar to that in normal male villi \( (P = 0.641) \). (C) XACT RNA was overexpressed in androgenetic CHMs compared with that in normal villi, but no significant difference was observed \( (F = 0.431, P = 0.651, P_{1} = 0.488, P_{2} = 0.381, P_{3} = 0.854) \). (D) The expression of CDX4 mRNA in androgenetic CHMs was significantly higher than that in normal female and male villi (both \( P < 0.01 \)). (E) CDX4 immunostaining intensity in CHMs and normal villi. (F) The immunohistochemistry of CDX4 protein in CHM (200×). (G) CDX4 immunostaining intensity in normal female villous (200×). (H) The immunohistochemistry of p57 protein in CHM (200×). Data are expressed as mean ± SEM. **\( P < 0.01 \). P1: androgenetic CHMs versus normal female villi, P2: androgenetic CHMs versus normal male villi, P3: normal female villi versus normal male villi.
Higher Expression of CDX4 in Androgenetic CHMs With the 46, XX Karyotype

We detected the expression of CDX4 mRNA in androgenetic CHMs with the 46, XX karyotype (n = 27) and normal villi with the 46, XX (n = 30) and 46, XY karyotype (n = 30) by qPCR, and found that the expression of CDX4 mRNA in androgenetic CHMs was significantly higher than that in normal female and male villi (both P < 0.01), as seen in Figure 1D. We further detected CDX4 protein expression in paraffin-embedded samples, including 126 CHMs and 63 normal female villi, by immunohistochemistry, and found that the expression of CDX4 protein in CHMs was significantly higher than that in normal female villi (z = −7.75, P < 0.01), as shown in Figures 1E–G and Table 2.

Decreased Expression of H3K27me3 in Androgenetic CHMs With the 46, XX Karyotype

We measured the levels of H3K27me3 in androgenetic CHMs with the 46, XX karyotype (n = 44), in male villi (n = 44), and in female villi (n = 44) by Western blot. The levels of H3K27me3 in androgenetic CHMs were significantly lower than those in normal female villi (P < 0.01), but was not significantly different from those in normal male villi (P > 0.01). We further detected the expression and localization of H3K27me3 using immunofluorescence analysis. H3K27me3 was mainly localized in the cell nucleus (Fig. 2B), and normal female villi displayed increases in fluorescence intensity of H3K27me3 compared with androgenetic CHMs and normal male villi, consistent with the results of Western blot.

Lack of HUMARA Methylation in Androgenetic CHMs With the 46, XX Karyotype

We detected the methylation status of XCI CpG sites in exon1 of the HUMARA X chromosome gene in androgenetic CHMs using HUMARA-MSP. After electrophoresis, the number of bands in each lane indicated the genetic origins of samples. Specifically, 1 band meant that the sample only contained androgenetic genetic material, while 2 bands indicated that the samples had biparental genetic origin. If the products amplified with HUMARA-M primers existed, there was an inactive X chromosome in the sample, and vice versa. The lengths of the PCR products from the CAG repeat region in the HUMARA was ~200 bp. The products amplified with HUMARA-U primers showed 2 bands in normal female villi, while there was only 1 band in normal male villi and androgenetic CHMs. In addition, the products amplified with HUMAR-M primers showed 2 bands in normal female villi, but no bands were evident in normal male villi and androgenetic CHMs (Fig. 2C). These results indicated that there was no inactive X chromosome in androgenetic CHM. In other words, both X chromosomes were active in androgenetic CHM with the 46, XX karyotype.

The Expression of USP21 and Nanog in Androgenetic CHMs With 46, XX Karyotype

Measurement of the expression of Nanog and USP21 protein in androgenetic CHMs with the 46, XX karyotype (n = 44), in male villi (n = 44), and in female villi (n = 44) by Western blot revealed the higher expression of Nanog and USP21 protein in androgenetic CHMs than that in normal villi (both P < 0.01) (Figs. 3A, B). Furthermore, we examined the localization of USP21 and Nanog by immunofluorescence analysis. We found that USP21 was colocalized with Nanog in villous tissues (Fig. 3C). Meanwhile, the fluorescence intensity of USP21 and Nanog was higher in androgenetic CHMs than in normal villi.

DISCUSSION

Androgenetic CHM is the main kind of CHM, and prone to undergoing malignant transformation. However, the mechanisms involved in the development of this kind of CHMs remain unclear. X chromosome inactivation is a very important process during the development of normal female mammals. Failure of X chromosome inactivation blocks normal embryogenesis shortly after implantation. Although it was confirmed that biparental CHM possessed a normal XCI pattern, the status of XCI in androgenetic CHM with the 46, XX karyotype has been unknown. Here, for the first time, we find that loss of X chromosome inactivation may have something to do with the development of androgenetic CHM.

Many methods have been established to evaluate the XCI pattern according to the characteristics of the inactive X chromosome and the mechanisms of XCI. Compared with the active X chromosome, the inactive X chromosome showed many characteristics of constitutive heterochromatin (19), including maintenance of the condensed...
state throughout interphase, late DNA replication, decreased abundance of histone acetylation, lower methylation level of histone H3 lysine-4, and higher methylation level of histone H3 lysine-9 and H3 lysine-27. These characteristics have been used to evaluate the XCI pattern (20,21). It has also been confirmed that XIST is important in the process of XCI. Thus, the detection of XIST RNA expression and XIST RNA coating in cis using...
Fluorescence in situ hybridization has been widely used to examine the XCI pattern (22). Here, we monitored the expression of XCI markers, including XIST, TSIX, and H3K27me3, and using HUMARA-MSP to elucidate the status of XCI in androgenetic CHMs. The levels of XIST RNA were significantly decreased, while those of TSIX RNA were significantly increased in androgenetic CHMs compared with those in normal female villi, but the levels of both XIST and TSIX RNA were similar to those in normal male villi. The levels of H3K27me3 in androgenetic CHMs were significantly lower than those in normal female villi, but were similar to those in normal male villi. Moreover, there was a lack of HUMARA methylation in CHMs. The collective results suggested that both the X chromosomes were activated in androgenetic CHMs with the 46, XX karyotype. Previous studies demonstrated that the X-linked CDX4 gene is essential for the development of the villi in a variety of ways, including interaction with the Wnt pathway and enhancing the function of CDX2 (23–26). The latter can cooperate with SOX2 and GATA3 and prompt the proliferation of trophoblastic cells during trophoderm formation. In our study, CDX4 was overexpressed in androgenetic CHMs compared with normal villi. Thus, our results suggest that androgenetic CHM lacks the normal mechanics of XCI, and the loss of XCI may contribute to the development of androgenetic CHM by inducing the overexpression of CDX4.

Ubiquitination played a role in the process of XCI by stabilizing many factors (27,28). USP21, a wildly explored deubiquitinase, has been confirmed to have the ability to deubiquitinate and stabilize Nanog (29), a star molecule involved in the process of XCI. Nanog can regulate XCI in many ways, which include regulating the expression of XIST by binding to its first intron (11), occupying approximately one-third of the PRC2-occupied genes, which are closely related with embryonic development (30),
and interacting with many chromatin-remodeling factors, such as SWI-SNF (31) and NuRD (32) complexes. In this study, the expressions of Nanog and UPS21 protein were both higher in androgenetic CHMs than those in normal villi, which was consistent with the previous description of Nanog overexpression in gestational trophoblastic disease (10). Meanwhile, we found UPS21 colocalized with Nanog in villous tissues. Thus, our results hinted that UPS21 may cause the disruption of XCI in androgenetic CHM via stabilization of Nanog.

However, there are some shortages in our study. First, early complete moles often present with minimal amount of choriocarcin villi and frequently admixed with large quantity of gestational endometrium in a curettage specimen; hence, no matter how careful we are, it is very difficult to totally avoid the maternal tissue contamination, which to some extent, may lower the possibility of our results, which show that 2 X chromosomes are both active in CHMs. Second, androgenetic CHMs with 46, XX karyotype can be further divided into 2 categories, monospermic CHM and dispermic CHM, and, although they are all of androgenetic origin, they still have some difference, which to some extent has some effect on our results. Third, due to the inaccessibility of the CHM cell lines, all our research studies have been carried out on tissues. As a result, although we have found that 2 X chromosomes may all be active in androgenetic CHMs, and the expressions of UPS21 and Nanog are higher in androgenetic CHMs, we still do not know whether they are the cause or the result of androgenetic CHMs. Further research studies are needed in order to better understand the mechanisms involved in the initiation and development of CHMs.

CONCLUSIONS

To our knowledge, these findings, for the first time, claimed that both X chromosomes are active in androgenetic CHMs with the 46, XX karyotype, and that the UPS21-Nanog pathway may be involved in the disruption of XCI during this process. These findings may be relevant as one of the mechanisms involved in the development of androgenetic CHM.

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