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DNA hypermethylation and X chromosome inactivation are major determinants of phenotypic variation in women heterozygous for *G6PD* mutations

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ABSTRACT

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X-linked incompletely dominant enzyme deficiency that results from *G6PD* gene mutations. Women heterozygous for *G6PD* mutations exhibit variation in the loss of enzyme activity but the cause of this phenotypic variation is unclear. We determined DNA methylation and X-inactivation patterns in 71 G6PD-deficient female heterozygotes and 68 G6PD non-deficient controls with the same missense mutations (*G6PD Canton* c.1376G>T or *Kaiping* c.1388G>A) to correlate determinants with variable phenotypes. Specific CpG methylations within the *G6PD* promoter were significantly higher in G6PD-deficient heterozygotes than in controls. Preferential X-inactivation of the *G6PD* wild-type allele was determined in heterozygotes. The incidence of preferential X-inactivation was 86.2% in the deficient heterozygote group and 31.7% in the non-deficient heterozygote group. A significant negative correlation was observed between X-inactivation ratios of the wild-type allele and G6PD/6-phosphogluconate dehydrogenase (6PGD) ratios in heterozygous *G6PD Canton* ($r = -0.657$, $p < 0.001$) or *Kaiping* ($r = -0.668$, $p < 0.001$). Multivariate logistic regression indicated that heterozygotes with hypermethylation of specific CpG sites in the *G6PD* promoter and preferential X-inactivation of the wild-type allele were at risk of enzyme deficiency.

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most common inherited enzymopathies, affecting approximately 400 million people worldwide, predominantly in Africa, the Middle East and Southeast Asia [1]. G6PD deficiency is caused by mutations in the *G6PD* gene and more than 160 different mutations associated with *G6PD* have been described [2]. The inheritance of G6PD deficiency shows a typical X-linked incomplete dominant pattern and most G6PD deficient patients are men. The majority of heterozygous women have been reported with normal G6PD activity, and variable loss of enzyme activity has been observed in some heterozygous females [1,3], suggesting that stratification according to *G6PD* mutation does not fully explain the clinical heterogeneity of heterozygotes for G6PD deficiency.

G6PD expression is regulated by many factors such as mutations in *G6PD*, exonic splicing silencer, and transcription factors as well as epigenetic states [1,4–7]. For female heterozygotes, DNA methylation and X chromosome inactivation (XCI) might be major factors in the variation in loss of enzyme activity [1,6,7]. DNA methylation is a type of epigenetic silencing and hypermethylation of CpG islands near transcription start sites (TSSs) is crucial to gene silencing [8]. Toniolo et al. reported that in mice, *G6PD* expression is associated with the methylation status of CpG islands in the 5′-promoter region of *G6PD* [6]. However, little is known about the impact of specific gene methylations on G6PD enzyme activity in humans. Women heterozygous for *G6PD* mutations are genetic mosaics because of XCI. XCI is regarded as an essentially stochastic process. However, in certain situations, nonrandom XCI might be under the control of genetic determinants [9]. In a heterozygote, skewed inactivation of the X chromosome with normal allele results in expression of the mutant allele [10]. Consequently, these women can be as susceptible

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to a G6PD deficiency as a G6PD-deficient male. Prior reports indicated that skewed XCI influences G6PD activity in elderly women heterozygous for *G6PD* mutations [7,11]. However, they didn't exclude the variable effect of different *G6PD* mutations and the X chromosome with the *G6PD* mutant allele was not determined, so the relationship between XCI and phenotype might partly be explained by lack of persuasive power. Further research is necessary to investigate the causes of phenotypic variation in women heterozygous for *G6PD* mutations.

In this study, we investigated the impact of DNA methylation and XCI on G6PD activity using 71 G6PD-deficient heterozygous women and 68 non-deficient controls with either *G6PD Canton* (c.1376G>T) or *Kaiping* (c.1388G>A) mutation. The results showed that *G6PD* promoter hypermethylation and preferential XCI of the wild-type allele reduced G6PD activity, suggesting that these factors contributed to phenotypic variation in women heterozygous for *G6PD* mutations.

Materials and methods

Subjects

Recruited subjects were 21- to 43-year-old women and their sons: 71 women heterozygous with G6PD-deficiency and 68 non-deficient controls, all with either *G6PD Canton* or *Kaiping* mutation. Characteristics of the individuals in this study are in Supplemental Table S1. The clinical diagnosis of G6PD deficiency in the Chinese population was carried out through the G6PD/6-phosphogluconate dehydrogenase (6PGD) ratio method [12]. G6PD/6PGD positively correlated with the G6PD-enzyme activity: G6PD-deficiency, G6PD/6PGD <1.3; G6PD non-deficiency, G6PD/6PGD ≥ 1.3. Genomic DNA was extracted from leukocytes in peripheral blood samples by standard phenol/chloroform extraction method. Genotypes for *G6PD* were determined by multiplex primer extension/denaturing high performance liquid chromatography (PE/DHPLC) [13]. The local medical research ethics committee reviewed the study, and informed consent was obtained from all participants.

Examination of CpG methylation within the G6PD promoter

Bisulfite modification of DNA was performed as described [14] and treated DNA was amplified using PCR primers G6PD-F and G6PD-R (Supplemental Table S2). Amplification efficiency was verified by gel electrophoresis and sequencing (Invitrogen Life Technologies) using primer G6PD-F. Unmethylated C was converted to U by modification with sodium bisulfite and subsequently replaced by T after PCR. The presence of C at CpG sites indicated a methylated C allele in bisulfite-converted DNA. The methylation status of CpG sites was calculated from C and T peaks using the BioEdit Sequence alignment Editor v7090 (Carlsbad, CA, USA) [14].

Determination of the X chromosome inactivation pattern

The XCI patterns (XCIPs) in heterozygous women were evaluated by androgen receptor assay [15]. The CAG polymorphism in the *androgen receptor* (*AR*) gene enables the maternal and paternal alleles to be discriminated. The *Hpa*II site is methylated on the inactivated X chromosome, which blocks the restriction site, while the site is cut by *Hpa*II on the active X chromosome. The *Hpa*II tiny fragment island, which is associated with the promoter region of the *MIC2* gene, is unmethylated on the active and inactive X and Y chromosomes [16], and was used as a reference gene as it can also be amplified and digested by *Hpa*II. A Beckman GeXP Genome Lab Genetic Analyzer was used to study the CAG repeat on the *AR* gene. In brief, 1 μg of heterozygous female genomic DNA was treated overnight with and without *Hpa*II. Reactions were stopped by incubation at 70 °C for 20 min, and processed DNA (200 ng) was used in duplex PCR. Untreated genomic DNA samples from the sons were amplified with primers HUMARA-F and HUMARA-R. Primers are in Supplemental Table S2. All amplification products were analyzed with Beckman GeXP Genome Lab Genetic Analyzer and Genome-Lab software (Beckman Coulter, USA). For families, the allele genotypes of the heterozygous mother were determined from the fragment length and *G6PD* genotype of the son's gene. Assuming that the wild-type allele is I, the mutant allele is II. The degree of XCI was calculated as area I / (area I + area II × E), where areas I and II were the peak areas in an electropherogram with *Hpa*II digestion, and E was the ratio of amplification of allele I and allele II without digestion [11].

Statistical analysis

Multivariate logistic regression was performed for specific CpG methylations in the *G6PD* promoter and XCIPs of the wild-type allele as independent variables and G6PD/6PGD ratios as the dependent variable. Statistical analysis was done using the Mann–Whitney test, Spearman's correlation test, Fisher's exact test, chi-square test and multivariate logistic regression. $p < 0.05$ was regarded as statistically significant. All statistical analyses were conducted using SPSS 13.0 for Windows (SPSS Version 13, SPSS Inc., 2003).

Results

Hypermethylation of specific CpG sites in the G6PD promoter diminishes G6PD activity

To ascertain whether specific CpG methylations in the *G6PD* promoter were involved in reducing G6PD activity similar to genome-wide methylation, we evaluated the DNA methylation patterns of the *G6PD* promoter. The region in *G6PD* promoter containing 15 CpG sites located −15 to −200 bp upstream of the transcription start site was mapped (Fig. 1A). The methylation levels of the CpG sites were investigated for women heterozygous for *G6PD* with enzyme deficiency and non-deficient controls, and data were analyzed by the Mann–Whitney U-test. In women with *G6PD Canton* mutation (Fig. 1B), deficient heterozygous women had a higher degree of methylation than controls at CpG sites 6, 12, 13 and 14 ($p = 0.046$, 0.022, 0.042 and 0.040, respectively). Similar results were obtained in women with *Kaiping* mutation (Fig. 1C), with deficient heterozygous women showing greater methylation than controls at CpG sites 1, 8, 10, 11, 12, 13, 14 and 15 ($p = 0.049$, 0.028, 0.038, 0.005, 0.041, 0.018, 0.013 and 0.002, respectively). Significant CpG methylations ($p < 0.05$) were included in subsequent statistical analyses.

Correlation between the selected CpG methylations and G6PD activity was analyzed by Spearman's rank correlation test. Women with *G6PD Canton* mutation had significantly negative correlations between G6PD activity and methylation levels of CpG 12 and CpG 14 ($r = -0.298$ and -0.266) (Table 1). Women with *G6PD Kaiping* mutation had significantly negative correlations between G6PD activity and methylation levels of 7 CpG sites (1, 10, 11, 12, 13, 14 and 15) ($r = -0.347$, -0.311, -0.291, -0.307, -0.322, -0.266 and -0.373, respectively). The results indicated that hypermethylation of specific CpG sites within the *G6PD* promoter reduced enzyme activity in female heterozygotes with the same *G6PD* mutations.

Preferential X-inactivation of the wild-type allele reduces G6PD activity

To verify the impact of X-inactivation on enzyme activity in the *G6PD* heterozygotes, 71 G6PD-deficient heterozygous families and 68 G6PD non-deficient heterozygous families were examined for X-inactivation status of the *G6PD* wild-type allele using the polymorphic trinucleotide repeat sequence CAG in the *androgen receptor* (*AR*) gene. Effective tests were conducted on 65 deficient heterozygous families and 63 non-deficient heterozygous families; 11 were excluded because of homozygous polymorphic alleles (Supplemental Table S3A). Varying levels of XCIPs were observed for the cohort of heterozygous women (Fig. 2) ranging from 4.01% to 97.28% (mean ± SD = 54.66 ± 21.95%). Skewed

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