Epigenetic Study of XIST Gene from Female and Male Cells by Pyrosequencing

Hwan-Hee Kim, Yeo-Jin Yun, Min-Ae Song, Suman Lee*

Functional Genomics Lab, CHA Stem Cell Research Institute, School of Medicine, CHA University, Bundang-Gu, Sungnam-Si, Kyunggi-Do, 463-836, Korea

남성과 여성에서 XIST 유전자의 후성학적 비교 연구

김환희 · 윤여진 · 송민애 · 이수만*

차의과학대학교

목 적: X 염색체 불활성화는 여성과 남성 사이에 X 염색체의 유전자 발현 유지를 위해 여성의 X 염색체 중 하나가 불활성화 되는 현상이다. 이러한 X 염색체 불활성화는 해독되지 않는 XIST 유전자에 의해 조절된다. XIST 유전자는 오직 불활성화된 X 염색체 에서만 발현되고, 활성화된 X 염색체 에서는 발현되지 않는다. 따라서 체세포에서 활성화된 X 염색체의 XIST 유전자는 promoter 부분이 메틸화 되어있고, 불활성화된 X 염색체에서는 메틸화가 거의 되어 있지 않다.

연구방법: 본 연구에서는 정상 여성과 정상 남성의 XIST 유전자의 promoter와 5'-end 지역의 메틸화 차이를 측정하기 위해 정상여성과 남성의 혈액에서 DNA를 추출하여 파이로시퀀싱 (Pyrosequencing) 방법을 통해 XIST 유전자의 총 8부분의 CpG 영역 (-1696, -1679, -1475, -1473, -1469, +947, +956, +971)을 분석하였다.

결 과: 총 8부분의 CpG 영역을 분석한 결과, promoter 부분인 CpG 1-5 영역 (-1696, -1679, -1475, -1473, -1469)에서는 여성과 남성의 메틸화 정도에 차이가 없었다. 그러나 5'-end 부분인 CpG6-8 영역 (+947, +956, +971)에서는 여성이 45.2% 49.9% 44.2%, 남성이 90.6%, 96.7%, 87.8%으로 메틸화 정도가 차이를 나타냈다.

결 론: 따라서 본 연구에 사용한 방법은 XIST 유전자의 메틸화 패턴의 차이를 기존의 방법보다 신속하고 정확하게 분석할 수 있다는 장점이 있기 때문에 유용하게 사용될 수 있을 것이다.

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중심단어: XIST, CpG, DNA 메틸화, 파이로시퀀싱

X inactivation is the silencing one of the two X chromosomes in XX female mammals for dosage compensation which ensures that an equal number of X-linked genes is expressed in both sexes.[1] X inactivation occurs at random, and the inactive X forms a discrete body within the nucleus called a Barr body.[2] The human X chromosome carries approximately 1,500 genes, some of which represent potential targets for the common genetic alterations that are observed in human diseases.[3]

X inactivation in mammals is controlled by the X-

주관책임자: Suman Lee Ph.D., Functional Genomics Lab, CHA Stem Cell Research Institute, School of Medicine, Bundang Campus, School of Medicine, CHA University, 222 Yatap-Dong, Bundang-Gu, Seongnam-Si, Kyunggi-Do, 463-836, Korea.
Tel: 82-31-725-8372, Fax: 82-31-725-8398
e-mail: suman@cha.ac.kr
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Table 1. Primer sets and PCR condition for methylation analysis of CpG site (1-8) in XIST

Primer	Sequence	Size (bp)	Sequencing primer	PCR condition
CpG1-2	F; 5'-GGTTGGAGTGTAGTGGTATTATTT-3' R; 5'-(biotin) TAACAAACACCTATAATCCCAACT-3'	109	5'-GGAGTGTAGTGGTATTATTT-3'	10X PCR buffer, 0.2 mM each of dNTPs, 0.2 μM each of Primer, 5X Q solution, 1U HotStarTaq DNA polymerase (QIAGEN)
CpG3-5	F ; 5'-GTGTTGGGATTATAAGTATGAGTT-3' R; 5'-(biotin) CCAAACCTCTAATACAACTTTATC-3'	115	5'-GGGATTATAAGTATGAGTTA-3'	
CpG6-8	F; 5'-GTAGAGAATGATTTTGTAGTTAAGTTAAGG-3' R; 5'(biotin)-ATCTTACCTCCCTAATTTAACTTAACAC-3'	133	5'-GTTATTTTAGATTTGT-3'	2.5 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.2 μM each of primers, 2U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA)

CpG1; -1696, CpG2; -1679, CpG3; -1475, CpG4; -1473, CpG5; -1469, CpG6; +947, CpG7; +954, CpG8; +971

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inactivation center (XIC), and triggered by a non-coding RNA called X (inactive)-specific transcript (XIST) that spreads along the entire length of the X chromosome.[4] XIST RNA is expressed from the inactive X (Xi) but not from the active X chromosome (Xa). In human, the candidate region for the XIC is located within band Xq13 on the proximal long arm of the X chromosome, and the longest human XIST transcript may be 19.3 kb in length.[5]

The CpG sites of promoter and 5' end in XIST is fully methylated on the active X chromosome and completely demethylated on the inactive X chromosome by methylation-specific PCR (MSP) and direct bisulfite sequencing, suggesting that DNA methylation may be involved in controlling allele-specific transcription of XIST.[6,7]

In this study, we investigated a simple method to discriminate between XX and XY based on the CpG methylation patterns within the promoter and 5'-end of XIST gene by pyrosequencing.

MATERIALS AND METHODS

1. Sample

Female (XX, N=1) and male (XY, N=1) whole blood samples were obtained from the CHA General Hospital, College of Medicine, CHA University (Seoul, Korea).

2. DNA isolation and bisulfite modification

Genomic DNA (gDNA) was isolated from whole blood samples by a DNeasy Tissue Kit (Qiagen, Valencia, CA). Sodium bisulfite treatment of gDNA was performed with the EZ methylation-Gold KIT™ (Zymo Reserch, Orange, CA). The bisulfite- treated DNA after isolation was stored at -20℃ until further use.

3. Pyrosequencing

PCR amplification for the pyrosequencing of XIST was performed in a 50 μL reaction mixture; the thermal cycling was done as follows: CpG1-2 and CpG3-5 sites PCR of XIST were done for 40 cycles with an annealing

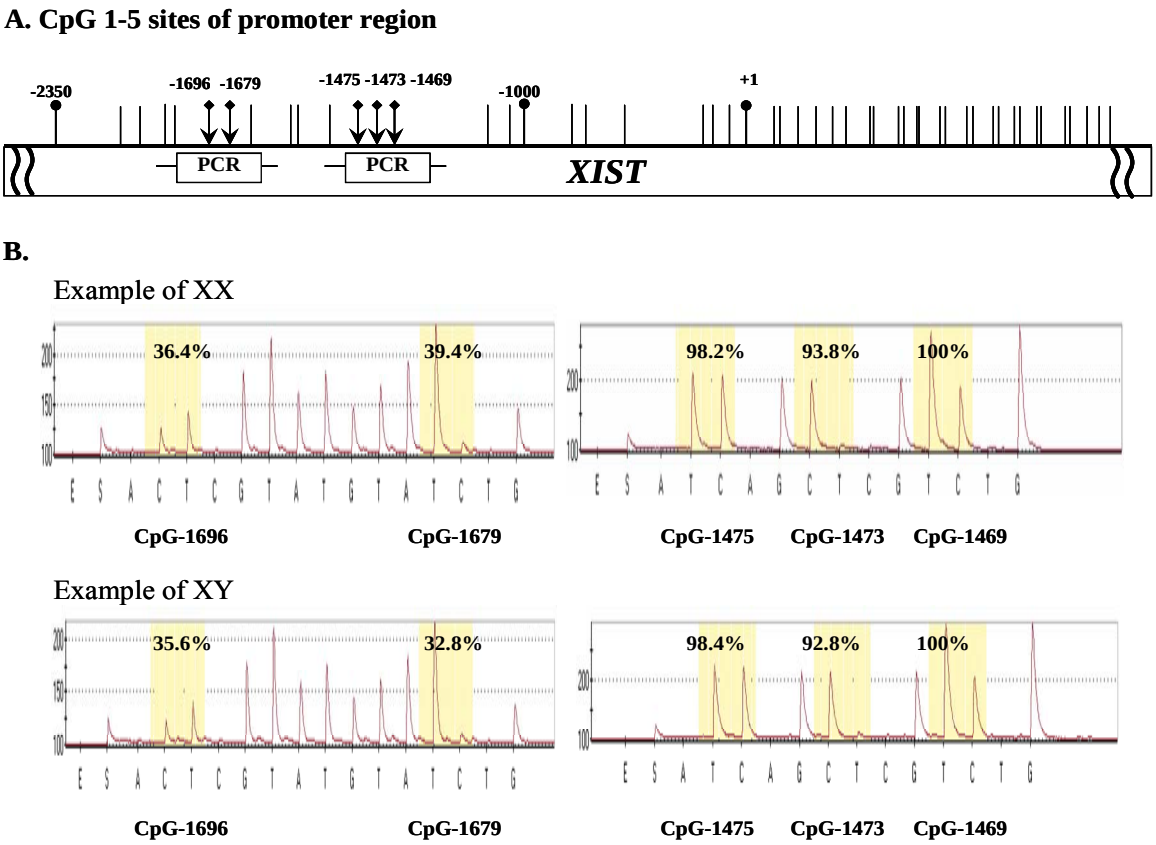

Figure 1. The design for the methylation analysis of promoter of XIST and its quantification by pyrosequencing. **(A)** Each CpG site within promoter of XIST is indicated by vertical lines. The five CpG sites for analysis are indicated by arrows. **(B)** Nucleotide dispensation orders (nDO) of the CpG methylation were designed using PSQ 96MA 2.1 software. The shaded regions represent 5 CpG sites quantified at the promoter of XIST from the example of XX and the example of XY.

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temperature of 60℃ and 55℃, respectively. CpG6-8 sites PCR of XIST was performed with an annealing temperature of 55℃ for 50 cycles. The sequences of primer sets and PCR conditions were indicated in Table 1. All the reactions were constructed as recommended by the manufacturer's instructions (Biotage AB, Uppsala, Sweden). The pyrosequencing reaction was performed automatically using a PSQ 96MA system along with an SNP Reagent Kit.

RESULTS

We quantified CpG methylation percentages of 5 CpG sites (CpG1-5) within the promoter, and 3 CpG sites (CpG6-8) within 5'-end of XIST by pyrosequencing from normal XY and XX (Figure 1 and 2). Figure 1B and 2B showed the examples of pyrograms resulting from pyrosequencing. Methylation of CpG1-5 sites at the promoter region did show the similar methylation patterns from both XX and XY by three independent assays. At the promoter region of XIST gene, the average methylation percentages were 37.1 (±1.0) at CpG1, 33.9 (±4.7) at CpG2, 98.9 (±0.6) at CpG3, 93.2 (±0.4) at CpG4, 100 (±0.00) at CpG5 site from normal XX. From normal XY, the average methylation percentage was; 35.4 (±1.7) at CpG1, 33.1 (±1.4) at CpG2, 98.8

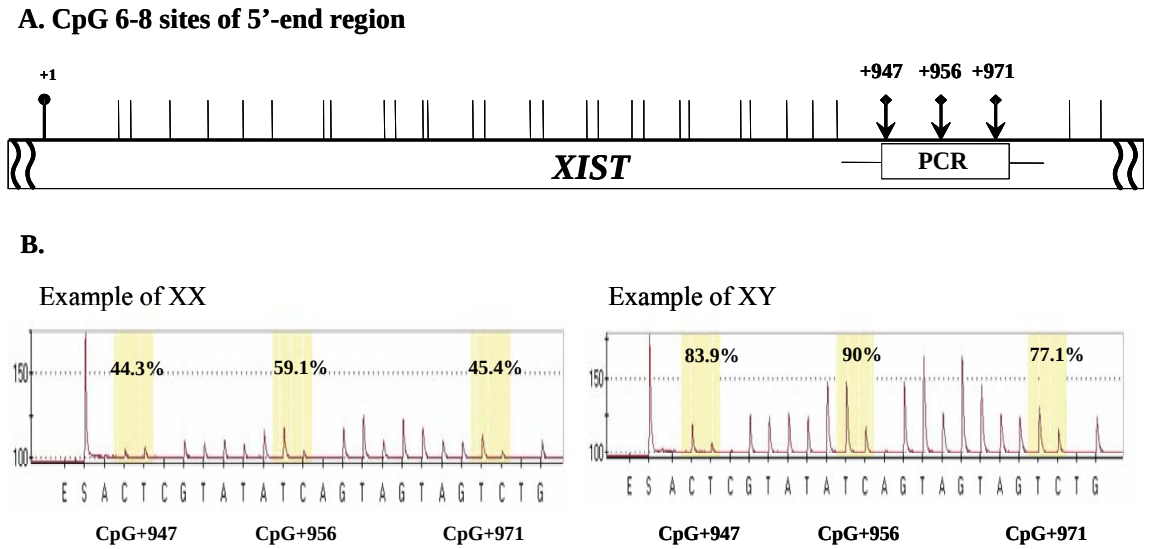

Figure 2. The design for the methylation analysis of 5'-end region of XIST and its quantification by pyrosequencing. **(A)** Each CpG site within XIST is indicated by vertical lines. The three CpG sites for analysis are indicated by arrows. **(B)** Nucleotide dispensation orders (nDO) of the CpG methylation were designed using PSQ 96MA 2.1 software. The shaded regions represent three CpG sites quantified at the 5'-end region of XIST from the example of XX and the example of XY.

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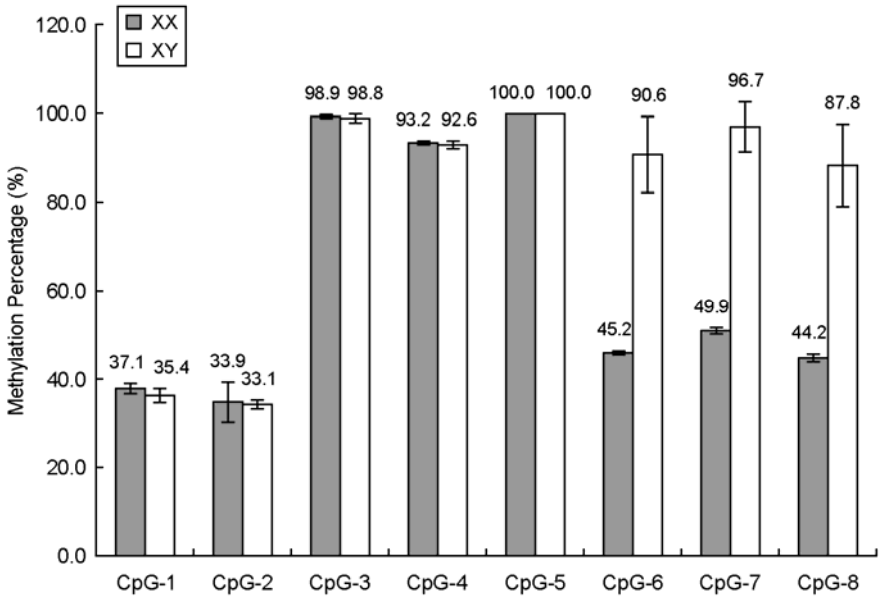

Figure 3. Pyrosequencing data of XIST methylation percentages. Representative graph of methylation percentage of eight CpG sites from blood DNA. The SDs of the mean of three independent experiments were indicated above the bar.

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(±1.1) at CpG3, 92.6 (±0.7) at CpG4, 100 (±0.00) at CpG5 site.

Interestingly, the different CpG methylation pattern was found in the 5' end of XIST. The methylation percentages of CpG sites (CpG 6-8) in normal XX were 45.2 (±0.4) at CpG6, 49.9 (±1.0) at CpG7, and 44.2 (±0.5) at CpG8. However, there were hyper CpG methylation pattern at the 5' end of XIST in normal XY.

The methylation percentages were 90.6 (±8.4) at CpG6, 96.7 (±5.8) at CpG7 and 87.8 (±9.3) at CpG8. Our results showed the different methylation of 5'end in XIST were found from XX and XY (Figure 3).

DISCUSSION

Gene dosage on the X-chromosome between male and female mammals can be compensated by the whole chromosomal transcriptional silencing of extra X chromosome characteristic of female cells.[8] The X inactivation in female cells occurs early in development, and the initiation of X inactivation is generally random. The XIST gene produces a large noncoding RNA that localizes to the inactive X chromosome and XIST RNA expression was correlated with epigenetic regulation.[9,10]

There were various methods to analyze DNA methylation, including Bisulfite sequencing,[11,12] MSP (methyl specific PCR),[7] DNA microarray,[13] and pyrosequencing.[14,15] Pyrosequencing is a direct sequencing by synthesis method originally developed to overcome artifacts of secondary structure and avoid gel electrophoresis.[16] Pyrosequencing is not restricted to restriction enzyme sites, and avoided cloning of bisulfite-treated, amplified DNA in bacterial expression vectors and subsequent isolation of plasmids form numerous bacterial clones. Also, this method was allowed accurate qualitative and quantitative analysis of multiple CpG methylation sites in the same reaction.[17]

Alterations of the X inactivation which can lead to the activation of oncogenes or loss of function of tumour suppressors may be the common genetic events that cause cancer include female[18~20] and male breast cancer.[21,22] Previously, the abberent methylation change of XIST gene has been reported for male reproductive cancers, such as prostate cancer and testicular germ-cell tumors.[7,23] And the XIST methylation status that the same as in females and Klinefelter's syndrome patients.[24] Therefore, CpG methylation analysis of XIST may be important for X-linked gene regulation. We investigated the methylation pattern of XIST gene form male and female cells by pyrosequencing in this study.

Our pyrosequencing method is sensitive for quantifying the small percentage change in the methylation status of XIST, and may be used for early diagnosis and monitoring of various diseases including X chromosome abnormality.

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= **Abstract** =

Objective: X inactivation is the silencing one of the two X chromosomes in female mammals for gene dosage on the X-chromosome between female and male. X inactivation is controlled by X inactive-specific transcript (XIST) gene, untranslated RNA. XIST is expressed only from the inactive X (Xi), not expressed from the active X (Xa). The Xist promoter is methylated on the silent Xist allele on the Xa in somatic cells, and less methylated on the Xist-expressing Xi. We investigated the difference of XIST methylation pattern of the promoter and 5'-region of XIST from male (XY) and female (XX) subjects.

Methods: The direct quantification of XIST methylation is required for clinical application of normal XX and XY blood. Methylation percentage of eight CpG sites (-1696, -1679, -1475, -1473, -1469, +947, +956, +971) of XIST gene were diagnosed by pyrosequencing.

Results: We directly quantitated the methylation percentage of the promoter and 5'-end of XIST by pyrosequencing. The average methylation percentages at CpG6-8 sites (+947, +956, +971) were 45.2% at CpG6, 49.9% at CpG7, and 44.2% at CpG8 from normal female and normal male were 90.6%, 96.7%, 87.8%, respectively. Nether CpG 1-5sites (-1696, -1679, -1475, -1473, -1469) had any effect on XX and XY.

Conclusion: This method is sensitive for quantifying the small percentage change in the methylation status of XIST, and may be used for diagnosis.

Key Words: XIST, CpG, DNA methylation, Pyrosequencing