J Hum Genet (2003) 48:374–379
DOI 10.1007/s10038-003-0040-4

ORIGINAL ARTICLE

Kazuyo Sato · Masaki Hashiyada · Shigeki Uehara
Masayuki Nata · Kunihiro Okamura

CpG dinucleotide methylation patterns in the human androgen receptor gene and X-chromosome inactivation in peripheral blood leukocytes of phenotypically normal women

Received: 4 April 2003 / Accepted: 6 May 2003 / Published online: 1 July 2003

Abstract To evaluate methylation patterns in CpG dinucleotides (CpGs) of the human androgen receptor gene (*HUMARA*) and X-chromosome inactivation (XCI) status in phenotypically normal women in a general population, bisulfite genomic sequencing and methylation-specific PCR of genomic DNA extracted from peripheral blood samples of 124 phenotypically normal women were examined. CpGs methylation patterns were based on bisulfite genomic sequencing of the region containing nine CpGs in the *HUMARA* exon 1. The results of methylation status in CpGs from 43 independent colonies of 14 women revealed that not all CpGs were methylated even in highly methylated *HUMARA* alleles, and that the methylation status in CpGs varied between clones, by the position of CpGs methylation and in each subject. Evaluation of XCI was based on the method of an *HUMARA* (CAG)n polymorphism assay after bisulfite modification of DNA samples. The *HUMARA* allele size ratios of the women (82 heterozygotes) varied over a wide range and the distribution patterns of the ratios approached a 'normal distribution'. Since excessive skewing of XCI was observed in 11–12% of women, female carriers of an X-linked hereditary disease manifest its clinical symptoms or signs possibly in maximum 5–6%.

K. Sato (✉) · K. Okamura
Department of Obstetrics and Gynecology,
Tohoku University Graduate School of Medicine,
1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
E-mail: kazusato@ob-gy.med.tohoku.ac.jp
Tel.: +81-22-7177254
Fax: +81-22-7177258

M. Hashiyada · M. Nata
Department of Forensic Medicine,
Tohoku University Graduate School of Medicine,
Sendai, Japan

S. Uehara
Department of Obstetrics and Gynecology,
Tohoku Kosai Hospital, Sendai, Japan

Keywords CpG dinucleotide methylation · Human androgen receptor gene · Methylation-specific PCR · Polymorphism · Skewing of X-chromosome inactivation

Introduction

X-chromosome inactivation (XCI) was first identified by Lyon (1961); in mammalian females, one of the two X chromosomes is thought to be randomly inactivated to compensate for the difference in X-linked gene dosage between males and females. However, of the normal female population without known X-linked diseases, 5–20% appear to be subject to nonrandom (skewing) XCI in analyses of peripheral blood leukocytes (Fey et al. 1994; Belmont 1996; Gale et al. 1997; Plenge et al. 1997; Lanasa et al. 1999a). Busque and co-workers (1996) also suggested that the incidence of nonrandom XCI increased with age.

In higher order eukaryotes, DNA is methylated only at 5′-CpG-3′ dinucleotides (CpGs), and this DNA modification has important regulatory effects on gene expression, especially in CpG-rich areas (known as CpG islands) located in the promoter regions of many genes (Razin and Riggs 1980; Bird 1986, 1992). As extensive methylation of CpG islands is associated with transcriptional inactivation of several genes on one of the two X-chromosomes in females (Pfeifer et al. 1989; Riggs and Pfeifer 1992), such X chromosomes result in an inactive condition. Therefore, patterns of XCI can be evaluated by assessing the differential methylation between active and inactive X chromosomes.

Several methods have been utilized to evaluate XCI patterns in X-linked genes with known nucleotide polymorphisms, such as intron 1 of the phosphoglycerate kinase gene which includes a *Bst*XI site polymorphism, and exon 1 of the human androgen receptor gene (*HUMARA*) which includes a CAG triplet repeat [(CAG)n] polymorphism (Vogelstein et al. 1987; Lanasa et al. 1999a, 1999b; Sangha et al. 1999; Uehara et al.

2000). To assess differential methylation, samples are first treated with a methylation-sensitive endonuclease or sodium bisulfite, and then PCR-amplified. After PCR of bisulfite-reacted DNA, almost all (99.7%) unmethylated cytosines were converted to thymine, while methylcytosines were not (Clark et al. 1995). In contrast, methylation-specific endonuclease methods can result in incomplete digestion of DNA (Uehara et al. 2001). Therefore, the bisulfite treatment is far more sensitive for analyzing patterns of DNA methylation than endonuclease predigestion. Bisulfite DNA modification has been utilized frequently in previous studies.

Because, in XCI evaluation using bisulfite modified DNA, numbers of CpGs in sequences of primer pairs effect on sharpness of the results, we chose the region of *HUMARA* exon 1 which contains four or five CpGs in adequate primer sequences and a (CAG)n polymorphism.

As nonrandom XCI may play a role in induction of X-linked hereditary disease in female carriers, it is clinically important to quantify XCI skewing in women in the general population. Thus, in the present study, methylation patterns in CpGs of the *HUMARA* were initially analyzed in bisulfite-reacted DNA obtained from phenotypically normal women. Once overall CpGs methylation patterns were obtained, we selected primers and analyzed XCI patterns using the *HUMARA* (CAG)n polymorphism, and revealed a quantitative degree of XCI skewing among normal women.

Subjects and methods

Subjects

Peripheral blood obtained from 124 unrelated healthy female volunteers (age, range 21–48 years; average age 28.6 ± 5.5 years; 59 women were nulliparous and 65 were mothers of one or more children) was screened for heterozygosity at the *HUMARA* locus. This protocol was approved by the human research board of the ethical committee of Tohoku University School of Medicine.

DNA extraction

Peripheral blood samples were obtained in tubes containing EDTA-2K. Genomic DNA was extracted from the blood samples with Sepagene kit (Sanko Junyaku, Tokyo, Japan), according to the manufacturer's instructions. After ethanol precipitation, DNA was dissolved in distilled water and stored at −20 °C.

Bisulfite modification of DNA

DNA samples were treated with sodium bisulfite by the method of Herman et al. (1996). The bisulfite modification alters cytosines to uracils, while methylcytosines in the inactive X chromosome were not affected. Briefly, 3 μg of DNA were dissolved in a total of 40 μl distilled water and denatured by the addition of 4.5 μl of 3 M sodium hydroxide and incubated at 37 °C for 15 min. Twenty-five microliters of 10 mM hydroquinone (Sigma-Aldrich Japan, Tokyo) and 431 μl of 3.6 M sodium bisulfite (Sigma-Aldrich, Japan) were added to the DNA, incubated at 55 °C for 16 h, and then purified using the Wizard DNA clean-up system (Promega, Madison, Wis., USA). The DNA samples were flushed from columns by adding 50 μl of distilled water, and the chemical reaction was completed by the addition of 5 μl of 3 M sodium hydroxide. Following ethanol precipitation, the modified DNA was resuspended in 25 μl of distilled water. The extent of recovery of DNA during the bisulfite modification was 80–90%.

Nucleotide sequencing

Bisulfite genomic sequencing is a sensitive method, which gives a positive signal for methylated cytosines. The sequencing method is based on reaction of genomic DNA with bisulfite under conditions such that cytosine is deaminated to uracil but methylcytosine remains unreacted. Strand-specific primers to bisulfite-deaminated DNA are used to amplify the target sequence. The region containing nine CpGs in *HUMARA* was amplified in bisulfite-reacted DNA by PCR. The primer sites are indicated in Fig. 1 and the sequences are shown in Table 1. Amplification reaction was carried out on bisulfite treated DNA (100 ng) from 14 randomly selected women subjects for 35 cycles with denaturation at 94 °C, annealing at 58 °C, and extension at 72 °C. The PCR products were gel purified, inserted into the pDrive Cloning Vector, and then recombinant plasmids were transformed into the QIAGEN EZ

Fig. 1 Primer design for bisulfite genomic sequencing and methylation-specific PCR for XCI evaluation. A part of the original genomic sequence of *HUMARA* exon 1 is shown. *Highlighted capitalized* CpGs indicate potential methylation sites. Primers for bisulfite sequencing (*open arrows*) were placed in regions completely lacking CpGs to amplify both methylated and unmethylated alleles with equal efficiency. The PCR product for bisulfite sequencing contained nine CpGs between the primers. Primers for XCI evaluation (*plain arrows*: M-PCR, *broken arrows*: U-PCR) were placed in regions containing four or five CpGs to amplify either methylated or unmethylated alleles selectively

Table 1 PCR primer pairs. The primer pairs were designed to amplify the same region of the human androgen receptor gene (*HUMARA*) exon 1 (Kubota et al. 1999)

Primer pair	Sequence (5′ → 3′)
For sequencing	
forward	GAGTTTTTTAGAATTTGTTTTAGAG
reverse	AACCAAATAACCTATAAAACCTCTAC
For XCI evaluation	
M-PCR	
forward	GCGAGCGTAGTATTTTTCGGC[a]
reverse	AACCAAATAACCTATAAAACCTCTACG
U-PCR	
forward	GTTGTGAGTGTAGTATTTTTTGGT[a]
reverse	CAAATAACCTATAAAACCTCTACA

[a] Fluorescently labeled with 6-FAM (carboxy-fluorescein)

Competent Cells with QIAGEN PCR Cloning^{plus} kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. After isolation the colonies containing the correct insert (90 independent colonies from 14 women) were examined with BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif., USA).

Evaluation of XCI

Evaluation of XCI is based on the method of an *HUMARA* (CAG)n polymorphism assay after bisulfite modification of DNA samples. As cytosines are altered to uracils by sodium bisulfite, while methylcytosines are not, PCR products can be amplified using primers specific for methylated versus unmethylated alleles (Kubota et al. 1999). Briefly, two primer sets were prepared; M-PCR forward and reverse primers for PCR amplification of methylated *HUMARA* alleles on the inactive X-chromosome and U-PCR forward and reverse primers for PCR of unmethylated alleles on the active X-chromosome (Kubota et al. 1999). The primer sites are indicated in Fig. 1 and the sequences are shown in Table 1. The M-PCR and U-PCR forward primers were fluorescently labeled at the 5′ end with a 6-FAM (6-carboxyfluorescein) single isomer (Molecular Probes, Eugene, Ore., USA) during the oligonucleotide synthesis. We designated the PCR amplification of methylated alleles as M-PCR, and the PCR of unmethylated alleles as U-PCR. Subsequent to bisulfite modification, DNA samples (200 ng) were subjected to both M-PCR and U-PCR, each in a total volume of 50 μl, containing 5 μl of 10×PCR buffer, 1 μl of 25 mM $MgCl_2$, 5 μl of a 2.0 mM solution mix of each dNTPs, 1 μl of each of the 100 mM forward and reverse primers, and 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems). Amplification was performed in a GeneAmp PCR System 9600 Thermalcycler (Perkin-Elmer, Foster City, Calif., USA) with use of a hot start (10 min at 95 °C). Reactions were cycled for 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C for 35 cycles, followed by a final extension for 10 min at 72 °C. Aliquots of the PCR products (0.5 μl) were mixed with 0.3 μl of the TAMURA 500 size standard (Applied Biosystems) and 0.8 μl of STR 2×loading solution (Promega), and subjected to denaturing polyacrylamide gel electrophoresis.

DNA bands, the molecular weights of which were approximately 200 bp, were detected using an ABI PRISM 377 DNA Sequencer (Perkin-Elmer), and peak patterns were visualized using a GeneScan Analysis 2.0.2 software (Perkin-Elmer). When two discernible peaks (molecular size variants were definitively detectable on peak patterns when products differed by 6 bp) were observed in a pattern, the woman was considered to be a heterozygote. The areas of the heterozygous peaks were measured, and then the ratios of the two peaks (area of the peak with smaller molecular size/total areas of two peaks), which we designated as *HUMARA* allele size ratios, were calculated.

In random XCI, there is an equal chance that either the paternal or maternal X chromosome is inactivated by allelic methylation in a given cell. Thus, band peaks from cells heterozygous for an allelic polymorphism show similar areas even after bisulfite modification. On the other hand, differences in the size ratio of the heterozygous peak patterns indicate unequal methylation of the bi-parental X chromosomes, suggesting nonrandom or skewed XCI.

Criteria for excessive skewing

When the *HUMARA* allele size ratio was less than 0.2 or more than 0.8 both in the M-PCR and U-PCR products, and the peak size difference was opposite between M-PCR and U-PCR (refer to Fig. 4 for details), the XCI pattern was considered to be excessive in skewing. This ratio is widely accepted in the literature to delineate clonal derivation of cells.

Statistics

Pearson correlation coefficient (r) was calculated to test for significant association between M-PCR and U-PCR. Statistical analysis was performed by using StatView 4.5 software.

Results

CpG methylation patterns

Out of 90 independent clones from14 women, 47 were ones in which all cytosines in the sequence were changed to thymines and the other 43 were ones in which cytosines except for CpGs were completely changed to thymines. The representative DNA sequence data of methylation status are shown in Fig. 2.

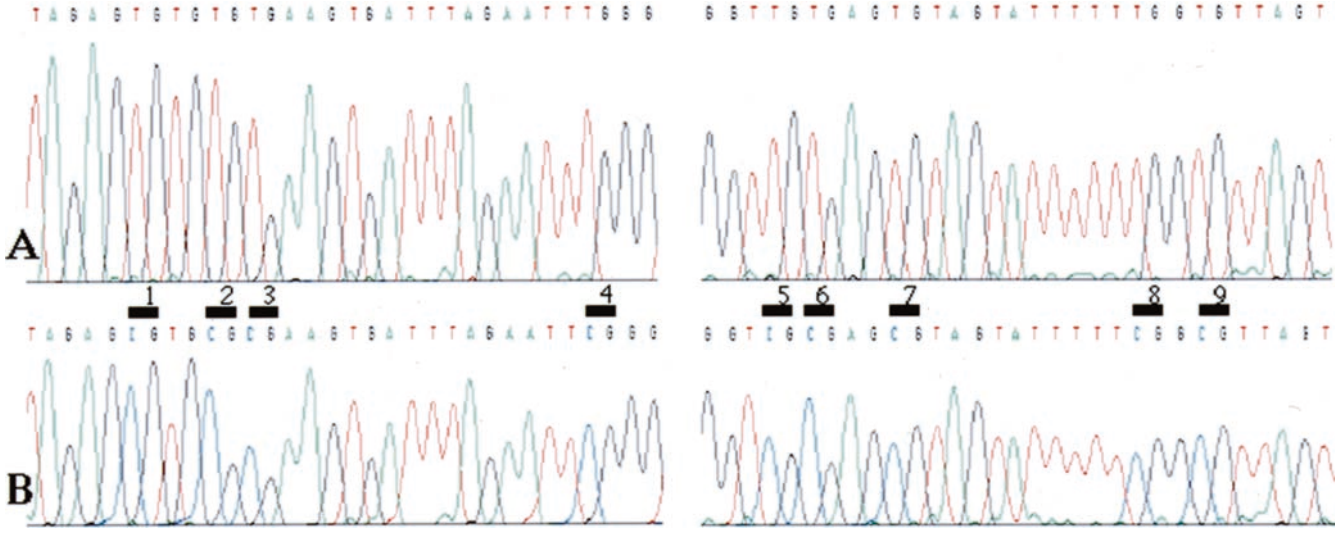

Fig. 2A, B Partial sequences of *HUMARA* exon 1. **A** In the *upper panels*, all cytosines in the sequence are changed to thymines. **B** In the *lower panels*, cytosines except for CpGs are completely changed to thymines, but cytosines in CpGs remain unchanged. Panel **A** represents the clones derived from unmethylated alleles, whereas the panel **B** represents the clones from methylated alleles. *Black bars* represent CpGs

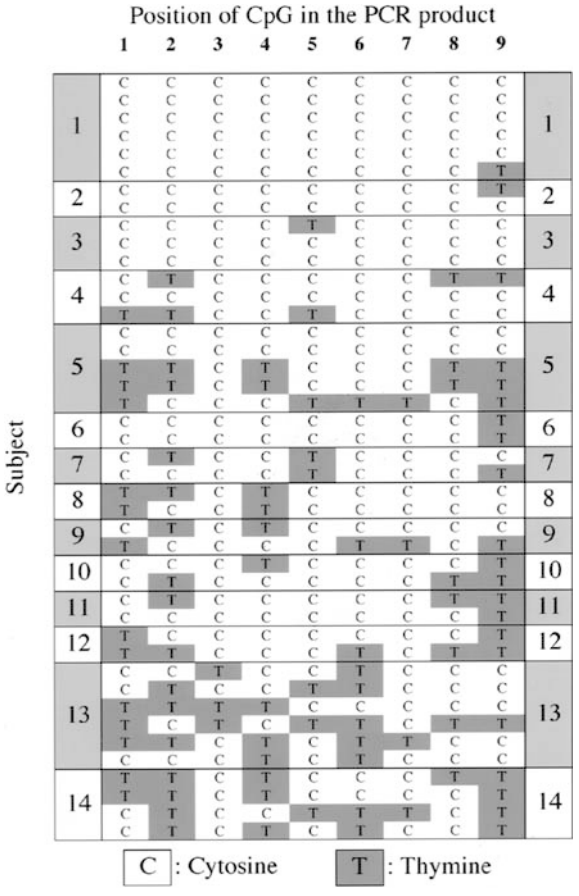

Fig. 3 The sequencing results of the PCR product. The sequencing results of 43 clones containing methylated alleles are summarized. While there are some clones in which almost all cytosines in CpGs were changed to thymines, there are no clones in which all cytosines in CpGs remained as cytosine. Although there are some exceptional cases, 3 and 7 commonly remained as cytosine in the clones in which cytosines remained unchanged in CpGs

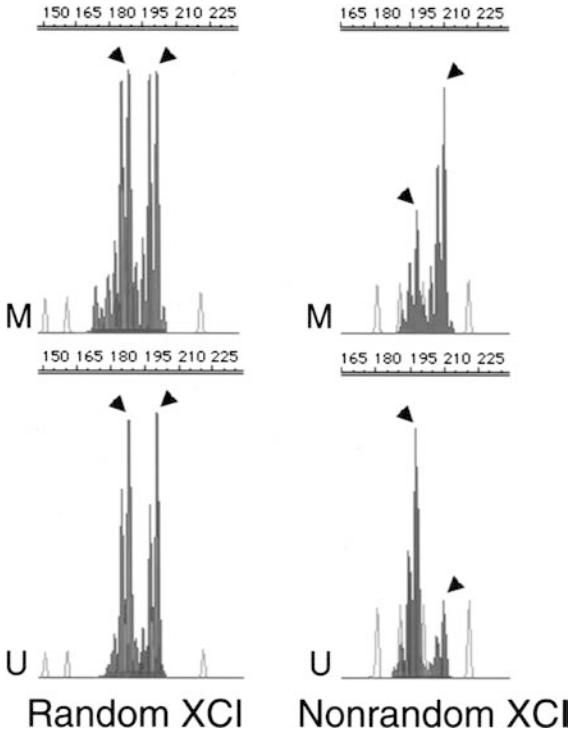

Fig. 4 Representative peak patterns demonstrating random XCI and nonrandom XCI. In each panel the X-axis represents molecular mass, and the Y-axis represents the fluorescent intensity of electrophoretic bands. The *upper panels* (*M*) show peak patterns derived using primers specific for methylated alleles (M-PCR), and the *lower panels* (*U*) show patterns derived using primers specific for unmethylated alleles (U-PCR). The *left panel pair* (*Random XCI*) demonstrates the heterozygous peaks (arrowheads) in which the sizes are similar. The *right panel pair* (*Nonrandom XCI*) demonstrates the heterozygous peaks (*arrowheads*) whose size ratio is opposite in the (*M*) and (*U*) panels. Peaks not shadowed represent size standards confirming that PCR products show adequate molecular sizes (approximately 200 bp)

The former clones were derived from unmethylated alleles of active X-chromosomes, whereas the latter clones were from methylated alleles of inactive X chromosomes. To reveal the methylation status in CpGs, the dinucleotides located in the PCR amplified region were numbered from 1 to 9, and the sequencing results of methylated alleles are summarized in Fig. 3. While there were some clones in which almost all cytosines in CpGs were changed to thymines, there were some clones in which all cytosines in the dinucleotides remained unchanged. Although there were some exceptional cases, nucleotides 3 and 7 in the PCR product commonly remained unchanged as cytosine in the clones containing CpGs.

Selection of heterozygotes

Eighty-two out of 124 subject women were heterozygous for the *HUMARA*, because two discernible peaks were observed in GeneScan analyses.

Variation of HUMARA allele size ratios

Figure 4 demonstrates representative peak patterns of random and nonrandom (skewed) XCI. As shown in Fig. 5, the *HUMARA* allele size ratios of 82 heterozygotes) are scattered throughout a wide range. While some women showed similar peak size differences in M-PCR and U-PCR products, most women had much larger peak size differences between M-PCR and U-PCR products. The distribution patterns of the ratios approached a 'normal distribution' in M-PCR and in U-PCR: the ratios of most women ranged from 0.3 to 0.7, and the mean ratio in M-PCR was 0.56 ± 0.16, and U-PCR was 0.51 ± 0.17. Pearson correlation coefficient (r) was -0.67.

Excessive skewing was observed in 10/82 (12.2%) women with M-PCR and 9/82 (11.0%) women with U-PCR. There were only three women who showed severely excessive skewing both in M-PCR and U-PCR, but moreover there were five women whose allele ratio was from 0.2 to 0.25 or from 0.75 to 0.8 both in the

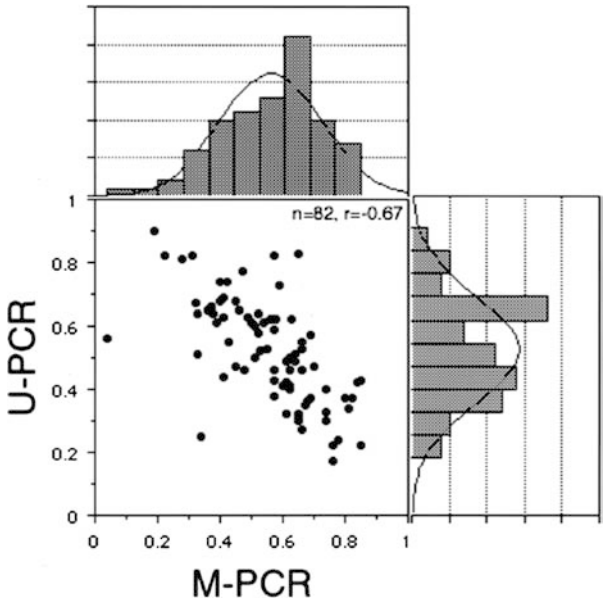

Fig. 5 The distribution patterns of the *HUMARA* allele size ratios between M-PCR and U-PCR. The X-axis represents the size ratio in M-PCR, and the Y-axis represents the size ratio in U-PCR. The ratios are scattered throughout a wide range, and the distribution patterns approach a 'normal distribution'. Excessive skewing, which is defined by the ratio (less than 0.20 or more than 0.80), was observed in ten women in M-PCR and nine women in U-PCR

M-PCR and U-PCR; thus, in sum, 8/82 (9.8%) showed almost excessive skewing.

Discussion

In the inactive X chromosome, as DNA is methylated at cytosines in CpGs, methylated genes loose their transcriptional function. Cytosines are converted to uracils in bisulfite-treated DNA, but methylcytosines are not. The results of nucleotide sequencing of *HUMARA* in bisulfite DNA of the subjects demonstrated clones in which all cytosines, except for cytosines contained in CpGs, were detected as thymines, and clones in which all cytosines were converted to thymines. As uracils are regarded as thymines during PCR amplification, these data suggested that the bisulfite treatment completely changed unmethylated cytosines to uracils. On the other hand, as methylcytosines are not changed by bisulfite treatment, cytosines in the sequencing data designate methylated cytosines in bisulfite-treated DNA.

The results of the methylation status of CpGs revealed that not all CpGs were methylated even in methylated *HUMARA* alleles, and that the methylation pattern in CpGs varied clone by clone, both in the position of methylation in the CpGs and between subjects. Since CpGs that were methylated commonly in almost all of the subjects were limited (nucleotides 3 and 7), this suggested that there are CpGs which are more prone to methylation and those which are not. Silva and White (1988) described methylation patterns of certain genomic regions that appeared to be polymorphic between people and were inherited, suggesting either the persistence of certain methylation at all stages of development, or the encryption of methylation pattern information. With the noted exceptions of imprinted genes and several genes on the inactive X chromosome in females, CpGs within CpG islands are normally unmethylated while most CpGs outside CpG islands are methylated (Bird et al. 1985; Bird 1992). Therefore, it is necessary to determine the methylation status in the PCR region used for XCI evaluation.

One potential problem of the methylation-specific PCR assay used in this XCI study is mis-amplification of the original untreated DNA with primers. Because the efficiency of the DNA modification with sodium bisulfite is nearly 100% under our conditions (Wang et al. 1980; Clark et al. 1994), the primers were no longer complementary for the original genomic DNA sequence. Therefore, the chance of the mis-amplification is small. Another problem is the specificity of primers for template DNA. In the study of XCI evaluation, the M-PCR primers were designed for the region containing four CpGs and U-PCR primers were three bases upstream and involved five CpGs. If primers are designed on other sites including fewer CpGs, nucleotide mismatches likely occur. The results of methylation in CpGs revealed that not all CpGs were methylated even in methylated *HUMARA* alleles; nevertheless the difference between methylated alleles and unmethylated alleles was clear in each subject. Therefore, the XCI patterns, which were revealed by the adopted methylation-specific PCR method, appeared to be reliable.

In the study of XCI patterns in genomic DNA samples obtained from peripheral blood leukocytes of phenotypically normal women, it was revealed that the degree of XCI skewing differed between all individuals demonstrating an almost 'normal distribution'; many of the women showed near 1:1 in ratios of the heterozygous peak sizes, but 11–12% of the women showed excessive skewing of XCI, as determined by the ratio. This incidence of excessive skewing is similar to those of previous reports by Busque et al. (1996) and Kubota et al. (1999). In the women showing excessive skewing in our study, we could not determine which one of two X-chromosomes (paternally or maternally derived X-chromosomes) was predominantly inactivated. However, we can speculate the incidence of affected female carriers of X-linked recessive hereditary diseases. Unless uniparental X chromosomes are predominantly inactivated, either paternally or maternally derived X chromosomes are predominantly inactivated in equal chance in women showing excessive skewing of XCI. In X-linked recessive disease in which the maternally derived X chromosome contains a mutated gene, female carriers manifest clinical symptoms and signs when the paternally derived X chromosome is predominantly inactivated. Espinos et al. (2000) reported that a female patient with hemophilia B (factor IX deficiency) had an extreme bias in favor of inactivity of the normal paternal X chromosome in leukocytes, but her healthy

sister had a significant inactivation of the mutated chromosome inherited from her mother. To date, there have been no previous reports estimating incidences of which female carriers of X-linked recessive diseases manifest clinical symptoms and signs. Although such incidences might differ in each disease, it is speculated that the incidence of clinical troubles in female carriers may be 5–6% in general (the value is a half of the incidence of excessive skewing of XCI, 11–12%).

We must also consider the possibility that our data from peripheral blood leukocytes do not reflect the general status of XCI. Since peripheral blood leukocytes consist of two kinds of cell strains, mainly myeloid and secondly lymphoid cells, XCI data from peripheral blood leukocytes are affected by populations of the two strains, because the XCI status of the cells possibly differs from one another. However, the two strains are commonly different from pluripotent stem cells. XCI occurs coincidently with cellular differentiation from the late morula through to the blastocyst stage (Tan et al. 1993), and is generally stable throughout subsequent cell divisions (Heard et al. 1997). Therefore, in women whose pluripotent stem cells show nonrandom XCI, the peripheral leukocyte XCI pattern would be nonrandom.

Since Busque et al. (1996) reported the increased incidence of nonrandom XCI in older individuals, the other factor affecting XCI status in peripheral blood leukocytes is the age of our subjects. The aging phenomenon, where functioning bone marrows becomes limited over time, probably increases this incidence. However, as the mean age of our subjects was 28.6 years, the bone marrow in all our subjects likely maintained hematopoietic potency. Therefore, our data suggest that the XCI data from peripheral blood leukocytes in our population reflect that of XCI in the general population.

In conclusion, our data demonstrate that the amount and pattern of methylation in *HUMARA* CpGs varied in position and between individuals, and that the degree of XCI skewing in peripheral blood leukocytes of phenotypically normal women differed among individuals and showed an almost 'normal distribution' among the population.

References

Belmont JW (1996) Genetic control of X inactivation and processes leading to X-inactivation skewing. Am J Hum Genet 58:1101–1108

Bird A (1986) CpG-rich islands and the function of DNA methylation. Nature 321:209–213

Bird A (1992) The essentials of DNA methylation. Cell, 70:5–8

Bird A, Taggart M, Frommer M, Miller OJ, Macleod D (1985) A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. Cell 40:91–99

Busque L, Mio R, Mattioli J, Brais E, Blais N, Lalonde Y, Maragh M, Gilliland DG (1996) Nonrandom X-inactivation patterns in normal females: lyonization ratios vary with age. Blood 88:59–65

Clark SJ, Harrison J, Paul CL, Frommer M (1994) High sensitivity mapping of methylated cytosines. Nucleic Acids Res 22:2990–2997

Clark SJ, Harrison J, Frommer M (1995) CpG methylation in mammalian cells. Nat Genet 10:20–27

Espinos C, Lorenzo J, Casana P, Martinez F, Aznar JA (2000) Hemophilia B in a female caused by skewed inactivation of the normal X-chromosome. Haematologica 85:1092–1095

Fey MF, Liechiti-Gallati S, von Rohr A, Borisch B, Theilkas L, Schneider V, Oestreicher M, Nagel S, Ziemiecki A, Tobler (1994) Clonality and X-inactivation patterns in hematopoietic cell populations detected by the highly informative M27 beta DNA probe. Blood 83:931–938

Gale RE, Fielding AK, Harrison CN, Linch DC (1997) Acquired skewing of X-chromosome inactivation patterns in myeloid cells of the elderly suggests stochastic clonal loss with age. Br J Haematol 98:512–519

Heard E, Clerc P, Avner P (1997) X-chromosome inactivation in mammals. Ann Rev Genet 31:571–610

Herman JG, Graff J, Myohanen S, Nelkin BD, Baylin SB (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93:9821–9826

Kubota T, Nonoyama S, Tonoki H, Masuno M, Imaizumi K, Kojima M, Wakui K, Shimadzu M, Fukushima Y (1999) A new assay for the analysis of X-chromosome inactivation based on methylation-specific PCR. Hum Genet 104:49–55

Lanasa MC, Hogge AW, Hoffman EP (1999a) The X chromosome and recurrent spontaneous abortions: the significance of transmanifesting carriers. Am J Hum Genet 64:934–938

Lanasa MC, Hogge AW, Kubik C, Blancato J, Hoffman EP (1999b) Highly skewed X-chromosome inactivation is associated with idiopathic recurrent spontaneous abortion. Am J Hum Genet 65:252–254

Lyon MF (1961) Gene action in the X-chromosome of the mouse (*Mus musculus* L.). Nature 190:372–373

Pfeifer GP, Steigerwald SD, Mueller PR, Wold B, Riggs AD (1989) Genomic sequencing and methylation analysis by ligation mediated PCR. Science 246:810–813

Plenge RM, Hendrich BD, Schwartz C Arena JF, Naumova A, Sapienza C, Winter RM, Willard HF (1997) A promoter mutation in the XIST gene in two unrelated families with skewed X-chromosome inactivation. Nat Genet 17:353–356

Razin A, Riggs AD (1980) DNA methylation and gene function. Science 210:604–610

Riggs AD, Pfeifer GP (1992) X-chromosome inactivation and cell memory. Trends Genet 8:169–174

Sangha KK, Stephason MD, Brown CJ, Robinson WP (1999) Extremely skewed X-chromosome inactivation is increased in women with recurrent spontaneous abortion. Am J Hum Genet 65: 913–917

Silva AJ, White R (1988) Inheritance of allelic blueprints for methylation patterns. Cell 54:145–152

Tan SS, Williams EA, Tam PPL (1993) X-chromosome inactivation occurs at different times in different tissues of the post-implantation mouse embryo. Nat Genet 3:170–174

Uehara S, Tamura M, Nata M, Ji G, Yaegashi N, Okamura K, Yajima A (2000) X-chromosome inactivation in the human trophoblast of early pregnancy. J Hum Genet 45:119–126

Uehara S, Hashiyada M, Sato K, Sato Y, Fujimori K, Okamura K (2001) Preferential X-chromosome inactivation in women with idiopathic recurrent pregnancy loss. Fertil Steril 76:908–914

Vogelstein B, Fearon ER, Hamilton SR, Preisinger AC, Willard HF, Michelson AM, Riggs AD, Orkin SH (1987) Clonal analysis using recombinant DNA probes from the X-chromosome. Cancer Res 47:4806–4813

Wang RYH, Gehrke CW, Ehrlich M (1980) Comparison of bisulfite modification of 5-methyldeoxycytidine and deoxycytidine residues. Nucleic Acids Res 8:4777–4790