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Epigenetic investigation of variably X chromosome inactivated genes in monozygotic female twins discordant for primary biliary cirrhosis

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Abstract

Primary biliary cirrhosis (PBC) is an autoimmune chronic cholestatic liver disease with a strong genetic susceptibility due to the high concordance in monozygotic (MZ) twins and a striking female predominance. Women with PBC manifest an enhanced X monosomy rate in peripheral lymphocytes and we thus hypothesized an X chromosome epigenetic component to explain PBC female prevalence. While most genes on the female inactive X chromosome are silenced by promoter methylation following X chromosome inactivation (XCI), approximately 10% of X-linked genes exhibit variable escape from XCI in healthy females. This study was designed to test the hypothesis that susceptibility to PBC is modified by one or more X-linked gene with variable XCI status. Peripheral blood mRNA and DNA samples were obtained from a unique cohort of MZ twin sets discordant and concordant for PBC. Transcript levels of the 125 variable XCI status genes were determined by quantitative RT-PCR analysis and two genes (*CLIC2* and *PIN4*) were identified as consistently downregulated in the affected twin of discordant pairs. Both *CLIC2* and *PIN4* demonstrated partial and variable methylation of CpG sites within 300 bp of the transcription start site that did not predict the XCI status. Promoter methylation of *CLIC2* manifested no significant difference between samples and no significant correlation with transcript levels. *PIN4* methylation showed a positive trend with transcription in all samples but no differential methylation was observed between discordant twins. A genetic polymorphism

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affecting the number of CpG sites in the *PIN4* promoter did not impact methylation or transcript levels in a heterozygous twin pair and showed a similar frequency in independent series of unrelated PBC cases and controls. Our results suggest that epigenetic factors influencing PBC onset are more complex than methylation differences at X-linked promoters and variably inactivated X-linked genes may be characterized by partial promoter methylation and biallelic transcription.

Keywords

autoimmune cholangitis; X chromosome inactivation; DNA methylation; gene regulation; gene promoter

Introduction

Clinical features of different autoimmune disease share more commonalities than differences including late onset, heritability, and female predominance¹. Primary biliary cirrhosis (PBC) is characterized by progressive destruction of intrahepatic bile ducts, high-titer serum anti-mitochondrial antibodies (AMA) and circulating autoreactive T cells². Similar to most autoimmune diseases, PBC manifests a 10:1 female predominance³ with a possible involvement of sex chromosomes⁴, as suggested by the preferential X chromosome loss observed in peripheral lymphocytes from women with PBC despite random X chromosome inactivation^{5, 6}. Despite a 50–100 fold higher risk for PBC among first degree relatives, the incomplete disease concordance among monozygotic (MZ) suggests that PBC results from combined effects of genetics and the environment^{2, 7}. Epigenetic modifications, particularly DNA methylation, have been shown to differ between MZ twins⁸ and appear as ideal candidates to explain the environmental influence on individual susceptibility to complex diseases such as PBC.

In females, the inactivation of one parental X chromosome is caused by epigenetic mechanisms, primarily promoter DNA methylation and histone modifications, resulting in dosage compensation. Of particular interest are the approximately 125 different X-linked genes that comprise almost 10% of the X chromosome and exhibit widely variable patterns of X chromosome inactivation (XCI) between healthy female individuals⁹. We therefore hypothesized that PBC susceptibility could be influenced by epigenetic differences of specific genes on the X chromosome, either by protective genes being silenced or susceptibility X chromosome genes variably escaping XCI.

In this study we investigated a unique cohort of MZ twins discordant or concordant for PBC for transcription differences of 125 X chromosome genes and the promoter methylation patterns of consistently differentially expressed genes. Two genes *CLIC2* and *PIN4* had consistently decreased transcript levels in the PBC-affected twins compared to healthy twins. Bisulfite sequencing of *CLIC2* and *PIN4* promoter regions revealed both genes to show partially variable methylation patterns that did not separate into active versus inactive alleles and did not significantly correlate with transcript levels. These results provide two novel genes that may be relevant to understanding PBC pathogenesis and suggest that additional epigenetic studies outside of the promoter regions are needed to understand the differential transcript levels between MZ twin pairs.

METHODS

Biological Samples

Peripheral blood samples were collected and processed from 5 MZ twin sets (one concordant and 4 discordant for PBC) identified in our previous study⁷. The age and pairing of the utilized subjects are illustrated in Table 1. Genomic DNA from whole blood was isolated using Qiagen Blood Mini kits (Qiagen, Valencia, CA) while Tempus tubes (Applied Biosystems, Foster City, CA) were utilized and mRNA isolated using an automated ABI 6100 Nucleic Acid PrepStation. Obtained mRNA was then translated into cDNA using previously described methods¹⁰. We used internal quality control thresholds obtained with species specific TaqMan assays to assess the quality of bulk samples. Stored DNA samples from 114 female patients with PBC and 118 age-matched controls, and 20 discordant pairs of sisters of similar age (within 5 years) were utilized for single nucleotide polymorphism (SNP) analysis. The protocol was approved by the IRB of the University of California at Davis and all subjects provided written informed consent.

Transcription analysis—RNA was extracted using a 6100 Nucleic Acid PrepStation (Applied Biosystems) according to the manufacturer's instructions. The cDNA was synthesized as described in Clay et al¹⁰. Real time (RT)-PCR was performed at the Lucy Whittier Molecular & Diagnostic Core Facility at UC Davis using Applied Biosystem TaqMan 384 low density arrays custom-designed to include primers for the 125 differentially inactivated genes, 5 housekeeping genes to maximize reliability (18S, B2M, GAPDH, HPRT1, RPLPO), and 3 copies of each of the internal controls androgen receptor (*AR* – fully inactivated) and *XIST* (fully escaping XCI). The assays were designed using AB Primer Express Software 3.0 with the sequences from the listed genebank accession numbers (Supplementary Table 1). Each PCR reaction contained 20X primers and probe for the respective TaqMan® system with a final concentration of 400 nM for each primer and 80 nM for the TaqMan® probe and commercially available PCR mastermix (Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 µl of the diluted cDNA sample in a final volume of 12 µl. The samples were placed in a 384 well plate and amplified in an automated fluorometer (ABI PRISM 7900 HTA FAST, ABI). ABI's standard amplification conditions were used: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Control wells without reverse transcriptase were included. Fluorescent signals were collected during the annealing temperature and CT values for were exported with a threshold of 0.06 and a baseline of 3–15 for the genes of interest. The intra-assay coefficient of variation of the threshold cycle (*C_T*) ranged from 0.1 to 3%. Final quantitation was done using the comparative Ct method, and is reported as relative transcription or the n-fold difference relative to a calibrator cDNA. In brief, the weakest expressing housekeeping gene, beta2-microglobin (B2M), was used to normalize the Ct values of the target genes (Δ CT). The Δ CT was then calibrated against the average of the healthy control for each target gene. The relative linear amount of target molecules relative to the calibrator, was calculated by 2 elevated to the absolute value of $\Delta\Delta$ Ct. Therefore, all gene transcription is expressed as an n-fold difference relative to the calibrator. The linearized value of the calibrator is 1. Eleven/125 analyzed genes did not provide sufficient amplification to obtain transcription data; only genes for which at least 3 twin pairs provided transcripts were included for consistency comparisons (genes not fulfilling this criterion are highlighted in Supplementary Table 2).

Bisulfite sequencing of PIN4, CLIC2 and AR—Bisulfite treatment of 500 ng of gDNA from each sample was performed using the Zymo Methylation Direct Kit (Zymo Research) following manufacturer's instructions. Converted DNA was stored at –20°C until

used. Converted bisulfite DNA was PCR amplified using primers for the promoter regions of *CLIC2*, *PIN4* and *AR* designed by online MethPrimer software (<http://www.urogene.org/methprimer/index.html>)¹¹. Primers for were as follows: *CLIC2* (Figure 1a) F: CTCAATCT CAGGGTCCACTTGAGTGC, R: CCAAAGGGGTGT GTATAAACTGCCTGG; *PIN4* (Figure 2a) F: GTCTGCCCAAGCTGTGCCTGCTTC, R: GAAGGGAGGGAACAGACAGTCC ATCT; *AR* F: GAGCT TTCCAGAATCTGTT CCAGAG, R: TAGAGGCCCCACAGGCTACCTGGTC. These primers were designed to regions without CpG sites to avoid amplification bias of methylated versus unmethylated sequences. PCR products were purified using a gel extraction kit (Qiagen, Valencia, CA) and then cloned into a pGEM-T easy plasmid and JM109 competent cells (Promega). Using blue/white color screening on ampicillin plates, 20 white transformed colonies from each sample and each PCR amplicon were sequenced and analyzed for the percentage of methylated cytosine at each sample.

***PIN4* genotyping**—The *PIN4* coupled polymorphism that removes two adjacent CpG sites is designated as SNP: ss10563357 (Rs7058353) according to NCBI dbSNP. Primers for *PIN4* genotyping were designed using Primer3 online software F: CAGACATCTTCAGCCCCATT, R:CTCTACCTTTCCCCGCTTTT. The polymorphism was genotyped in all twins DNA as well as in 114 unrelated PBC cases and 118 controls for further confirmation.

Statistical analysis—Consistency for transcription data among MZ twin sets was defined as a consistent up- or down-regulation of specific genes observed in 3 out of 4 affected twins compared to their healthy counterpart among discordant sets. We also analyzed the transcription profiles between concordant twins and 20 pairs of PBC discordant sisters as a stringent criterion for consistency (see example in Supplementary Fig. 1). SNP frequencies were compared between patients with PBC and controls using the chi-square test. All statistical analyses were performed using Intercooled Stata 8.0 (Stata Corporation, College Station, TX) and SAS (SAS Institute Inc., Cary, NC).

RESULTS

Gene transcription data

Normalized transcription data were analyzed by and sorted using linearized values from the weakest normalized calibrator to identify consistent genes that were either significantly up- or down-regulated in the affected twin compared to the healthy twin (Supplementary Table 2). While none of the variable XCI genes were consistently dysregulated in all 4 pairs of discordant PBC twin pairs, two transcripts (*CLIC2* and *PIN4*) were found to be consistently dysregulated in 3 out of 4 discordant sets while with similar transcription profiles in the concordant set of twins (Table 1).

CLIC2 promoter methylation

DNA methylation was examined for the *CLIC2* promoter that lacks a CpG island (<http://genome.ucsc.edu>, CpG island track). Bisulfite sequencing primers were designed to a 279 bp region 5' of the transcription start site containing 6 CpG sites (Figure 1A). *CLIC2* bisulfite sequencing data was calculated by 5 MZ twin sets, one concordant and 4 discordant for PBC, each with a minimum of 20 clones. Methylation patterns of individually sequenced clones are shown for a representative discordant twin pair in Figure 1B, with circles representing CpG sites that are methylated (filled) or unmethylated (open). Variable methylation patterns were observed between individual clones and individual CpG sites for all samples (Supplementary Figure 1). Overall promoter methylation was determined as the percentage of methylated CpG sites out of all possible CpG sites while site-specific

methylation was calculated for each of the 6 CpG sites in the *CLIC2* promoter region for each sample (Table 1). Percent methylation calculations were then grouped according to diagnosis (mean \pm SEM) and tested for significance by t-test. *CLIC2* showed partial promoter methylation ranging from 44 to 67% of available CpG sites and no significant differences in percent methylation for overall promoter methylation (Figure 1C) or site-specific methylation (Figure 1D) were observed between healthy and PBC twins. Transcript levels of *CLIC2* did not significantly correlate with overall promoter methylation (Figure 1E).

***PIN4* promoter genotyping and methylation**

PIN4 lacks a CpG island at its promoter, but bisulfite sequencing primers were designed to span 11–13 CpG sites covering 221 bp including part of exon 1 (Figure 2A). This promoter region contains two linked SNPs at predicted CpG sites 7 and 8. Both SNPs change the predicted CG to AG resulting in 11 CpGs rather than 13 CpGs for the less common allele (polymorphic sites are colored green and red in Figure 2B). *PIN4* alleles are defined as allele 1 (including 11 CpG sites) or allele 2 (13 CpG sites) based on ss10563357, dbSNP (Figure 2C–G). All twin pair DNA samples were genotyped and were homozygous for allele 1 except for twin set 54, 55 who were heterozygous for both alleles (Table 1). The genotype frequencies observed were consistent with the NCBI database with the majority of the population homozygous for allele 1, a small percentage heterozygous, and zero of the population homozygous for allele 2 (Table 2). *PIN4* promoter methylation was analyzed separately for the two alleles since the polymorphisms affect CpG sites. Only one of the PBC twin pairs was heterozygous for ss10563357, but no apparent or significant difference was observed in methylation patterns between the healthy and PBC twin (Figure 2B, E). Grouped overall percent methylation of both alleles were determined as the total number methylated sites out of the total possible sites (Figure 2C for allele 1, 2F for allele 2). Individual CpG site percent methylation of the two alleles in the *PIN4* promoter also showed no significant differences between healthy and PBC subjects (Figure 2D for allele 1, 2G for allele 2). Conversely, transcription analysis of *PIN4* demonstrated a trend for increased transcription correlating with increased methylation in allele 1 (Figure 2H) that was not statistically significant.

***CLIC2* and *PIN4* X chromosome inactivation status**

While the individual clones from bisulfite sequencing of both *CLIC2* and *PIN4* promoters did not appear to distinguish active and inactive X chromosome alleles, we tested this hypothesis formally using an approach previously described for promoters of XCI subject genes *MECP2* and *AR*¹². Bisulfite sequencing of *AR* was used as a control for XCI status in twins as this is a known gene subject to XCI in females and used for clinical XCI assays¹³. The XCI status of each allele was illustrated with the comparison of the number of methylated CpG sites per clone compared to the percentage of the total clones. X_A (active X) represents the frequency of bisulfite clones with no methylated CpG sites while X_I (inactive X) represents the bisulfite clones with greater than 50% methylation of the CpG sites in a given clone designated as the inactivated alleles. $X_?$ represents bisulfite clones with greater than 0% and less than 50% methylated CpGs that cannot be classified as activated or repressed (Figure 3). In addition to the MZ twin cohort, two healthy female and two healthy male controls with no familial history of PBC or other autoimmune disease history were included. Both males showed no XCI for *AR* as expected, with 90% of the clones having zero methylation and only 10% with one methylated site. Female controls showed the typical bi-modal distribution of X_A and X_I alleles of *AR* seen in Figure 3A, with very few clones in the $X_?$ category. The healthy and PBC female twins show a similar pattern of XCI status as the female controls and do not present aberrant XCI at the *AR* promoter. Figure 3B, C illustrates the XCI status of the *CLIC2* and *PIN4* promoter regions in which a bimodal

distribution into X_A and X_I alleles is not observed. Unlike *AR* that contains a CpG island and is always subject to XCI, *CLIC2* and *PIN4* both show approximately one third of bisulfite sequenced clones as of X_I alleles that cannot be designated as active or inactive.

DISCUSSION

PBC is an autoimmune disease commonly referred to as a model for understanding the pathogenesis of autoimmunity based on its uniform features including female prevalence and clinical presentation. While genetic factors are of seminal importance in determining disease susceptibility, these can be confirmed only in subgroups of patients¹⁴ and could not explain the imbalanced sex ratio. We previously observed that women with PBC manifest an enhanced rate of preferential X monosomy but random XCI in their peripheral lymphocytes compared to age-matched women^{5, 6}. In this study we thus tested the hypothesis that X-linked promoters, particularly those of variable XCI genes may be dysregulated through aberrations in promoter methylation and utilized the unique model of clinically discordant MZ twin pairs. From this analysis, we identified two candidate genes *CLIC2* and *PIN4* that exhibited decreased transcription in 3 out of 4 affected compared to healthy twins in discordant twin pairs. However, for both *CLIC2* and *PIN4* promoters, methylation was partial, variable and did not predict either XCI status or transcript levels. These results are informative both for future studies into understanding genetics and epigenetics of PBC as well as the epigenetic characteristics of genes that escape XCI in human females.

Although DNA methylation has been associated with a variety of diseases and disorders; specific patterns of DNA methylation in gene promoters and nonrepetitive genomic regions are remarkably similar between unrelated individuals^{15–18}. In addition, methylation profiles between male and female autosomes are also highly similar¹⁷. A recent large scale sequencing of the genome and epigenome of twin pairs with multiple sclerosis has also demonstrated that methylated CpG sites within CpG islands (MspI fragments) are nearly identical in the same tissue (CD4+ T cells) compared between twins, only slightly more variable between unrelated individuals, and most variable between different tissues or tumor versus normal tissue¹⁹. Moreover, Javierre and colleagues recently studied the methylation status of a cohort of MZ twins discordant for three autoimmune diseases: systemic lupus erythematosus (SLE), rheumatoid arthritis, and dermatomyositis; only MZ twins discordant for SLE featured widespread changes in the DNA methylation status of a significant number of genes²⁰. However, while most DNA methylation studies focus on CpG island and promoters, genome-wide epigenomic studies have reinstated that gene promoters are vastly de-enriched for DNA methylation, while methylation of intergenic and repetitive regions is extensive^{21–24}.

Several reports have linked epigenetics to disease phenotypes in humans. One example includes the incidence of discordant MZ twins for the X-linked dominant disorder Rett syndrome where discordance was due to differences in skewed XCI^{25, 26}. Methylation at one particular CpG site upstream of the *MECP2* promoter has been shown to be significantly increased in male autism subjects compared to controls²⁷. In schizophrenia samples, hypermethylation of the reelin gene (*RLN*) promoter CpG island has been observed²⁸. It is also noteworthy that discordance in MZ twin pairs for Beckwith-Wiedemann syndrome is due to differences in imprinting of the *KCNQ1OT1* gene²⁹. In addition, differences to methylation patterns to the imprinted gene *H19* correlates to discordance of MZ twins for Silver-Russell syndrome³⁰. While there is also evidence of epigenetic mechanisms in animal models for the autoimmune disease systemic lupus erythematosus^{31, 32} we note that cells to be investigated for epigenetic changes may include target or effector cell populations thus introducing an additional confounding factor.

While *CLIC2* and *PIN4* are dysregulated in most discordant PBC twin pairs, the mechanism behind the variant transcript levels does not appear to be promoter methylation. Genomic evidence has suggested that X-linked genes lacking a CpG island may not be ‘classically’ regulated by promoter methylation³³. In addition, PCR-based methylation analyses of the known XCI escape gene *MAOA*, encoding monoamine oxidase A, has shown a similar pattern of variable promoter methylation not associated with transcription or XCI status³⁴. The bisulfite sequencing based XCI status categorization approach performed here and described for XCI subject genes previously¹² may be useful for future studies investigating promoter methylation patterns for genes that escape XCI. While genetic polymorphisms within or close to promoters have been hypothesized to be responsible for the variability in XCI status of specific genes, our preliminary analysis of a known polymorphism creating two additional CpG sites in the *PIN4* promoter did not apparently affect overall promoter methylation or transcription. Perhaps polymorphic repetitive elements may be an unexplored genetic component affecting transcription and XCI status of X-linked genes in human.

CLIC2, chloride intracellular channel gene 2, was first suggested as a possible candidate for one of the many diseases linked to chromosomal region Xq28 because previous findings showed other human chloride channel genes had a direct association to a wide range of hereditary diseases³⁵. More recent work demonstrated that *CLIC2* modulated intracellular Ca²⁺ homeostasis through ryanodine receptors (RyR), suggesting that *CLIC2* may alter the calcium homeostasis of any tissue through RyR³⁶. *CLIC2* dysregulation could possibly be causing aberrations in calcium levels in the liver resulting in the degradation of the tissue, ultimately leading to disease.

PIN4 is part of the parvulin family of peptidylprolyl cis/trans isomerases and has been shown to be involved in mitotic regulatory mechanisms and cell proliferation^{37, 38}. More interesting, however, is a previous study has showing *PIN4* is involved in chromatin remodeling, an epigenetic mechanism involving ATP dependent changes to histone modifications³⁹. *PIN4* dysregulation could therefore affect chromatin remodeling altering transcription and regulation of multiple susceptibility or protective genes for PBC.

The major challenges for the future of epigenetic studies in human disease outcome is in identifying regions of the genome to investigate for DNA methylation differences relevant to gene transcription and to develop novel methods for identifying DNA methylation patterns that correlated with disease outcome. MZ twin studies of discordant disease are expected to continue to be of use in separating genetic from epigenetic variables in such studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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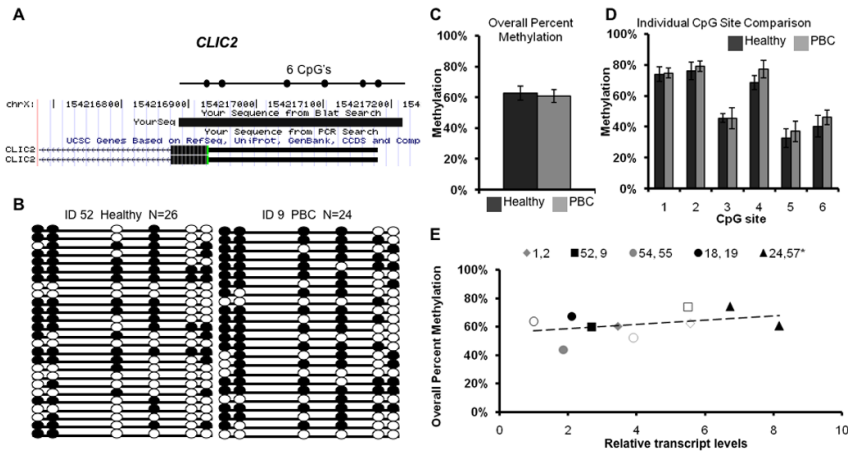


Figure 1. *CLIC2* bisulfite sequencing for promoter methylation analysis

A) Chromosomal sequence location of the region of the *CLIC2* promoter that was analyzed by bisulfite sequencing. This region contains 6 CpG sites (circles) within a 279 bp region overlapping the transcription start site as shown. **B)** Representative bisulfite sequencing data from a PBC discordant twin pair. Each circle represents a CpG site and each line represents an individually sequenced clone. Filled circles represent methylated sites (protected from bisulfite conversion) while unfilled circles represent unmethylated CpG sites (converted). **C)** Overall percent methylation was determined by the total number methylated sites out of the total CpG sites in all clones and graphed as mean \pm SEM for PBC (n=6) versus healthy (n=4) samples. **D)** Percent methylation was calculated for each individual site within the *CLIC2* promoter region for all clones and represented as mean \pm SEM for PBC (n=6) versus healthy (n=4) samples. **E)** Analysis of *CLIC2* promoter methylation versus transcription for each individual sample. Each twin pair is represented by an individual symbol, as shown, with filled symbols representing PBC and open symbols representing healthy individuals of each twin pair. No significant correlation was observed between *CLIC2* promoter methylation and transcription.

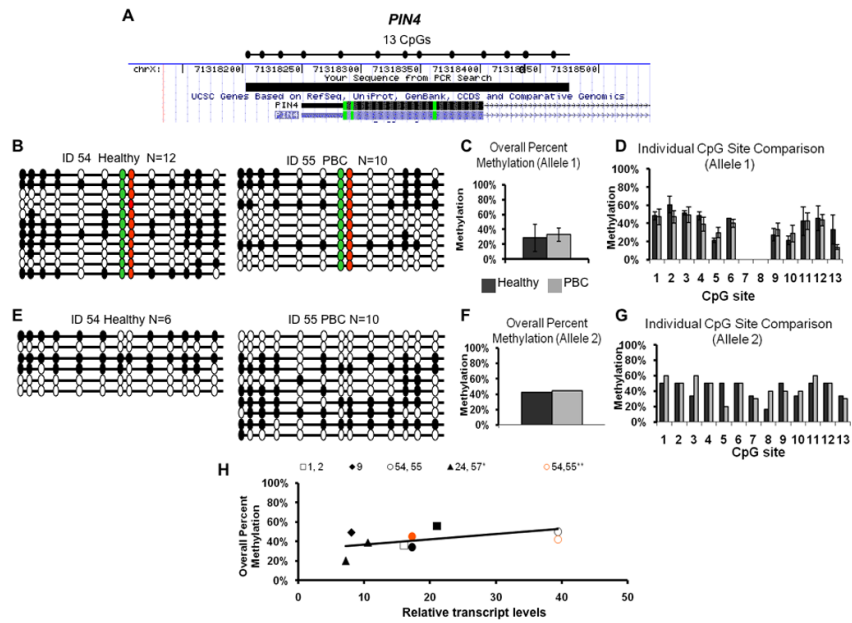


Figure 2. Bisulfite sequencing analysis of *PIN4* promoter methylation and genotype

A) Chromosomal sequence location of the region of the *PIN4* promoter that was analyzed by bisulfite sequencing. This region contains 11 or 13 CpG sites dependent on SNP: ss10563357. All samples were homozygous for allele 1 (11 CpG sites, AG at sites 7 and 8) except for one discordant twin set heterozygous for alleles 1 and 2 (allele 2 has 13 CpG sites). **B, E)** Bisulfite sequencing results for the heterozygous twin pair 54 and 55. Results are exhibited as in panel 1B except red and green filled circles represent CG sites absent in allele 1 and the two alleles are grouped separately as allele 1 (**B**) and allele 2 (**E**). DNA methylation patterns were variable between clones, alleles, and twins, but no apparent association was observed between PBC status or allele status and DNA methylation. **C, F)** Overall percent methylation of allele 1(**C**) or allele 2 (**F**) was determined and graphed as mean \pm SEM of all available samples. **D, G)** Site specific analysis of individual CpG sites in the *PIN4* promoter of allele 1(**D**) or allele 2 (**G**) was determined and graphed as mean \pm SEM of all available samples. **H)** Analysis of *PIN4* promoter methylation versus transcription for each individual sample. Each twin pair is represented by an individual symbol, as shown, with filled symbols representing PBC and open symbols representing healthy individuals of each twin pair; black symbols representing allele 1 and red symbols representing allele 2. Allele 1 of *PIN4* showed a trend for increased transcription with increased methylation that was not significant, while allele 2 did not show an apparent correlation between transcription and methylation.

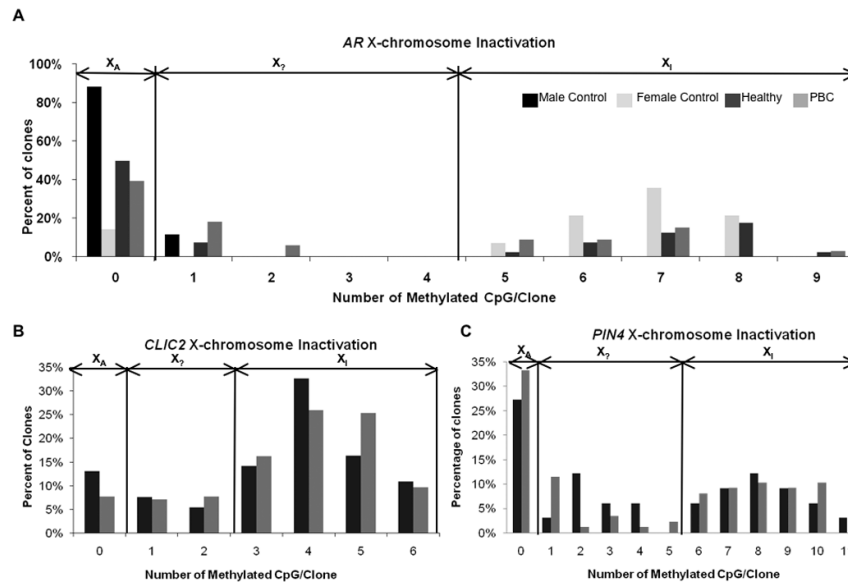


Figure 3. Determination of XCI allele status from bisulfite sequencing analyses

A–C) Each clone from bisulfite sequencing was analyzed for the number of CpG sites methylated in each clone. Histograms represent the distribution of percentage of clones based on degree of methylation. The bimodal peaks observed at all three loci are consistent with X chromosome inactivation. Clones with no sites methylated are designated as clear active alleles (X_A), clones with >50% of maximum number of methylated sites are designated as inactive (X_I), and the intervening clones as potentially undefined XCI status ($X_?$). **A)** The control X inactivated gene *AR* has a maximum of 9 possible methylation sites. Both male controls had no X_I clones, as expected, with 90% of the clones showing no methylated sites and only one clone showing one methylated CpG site. Female controls showed the typical bimodal distribution into active and inactive alleles. Individuals within the PBC twin pairs showed a similar bimodal distribution with no apparent differences between X_A and X_I alleles at the *AR* promoter. **B)** Unlike *AR*, the XCI allele distribution of *CLIC2* methylation patterns did not follow a bimodal distribution typical of promoters subject to XCI. Approximately 40% of clones at *CLIC2* fell into the $X_?$ status category that could not be designated as active or inactive. No apparent differences were observed between healthy and PBC individuals in the XCI status distribution. **C)** The *PIN4* methylation pattern also did not fit the typical XCI distribution pattern, as 20–30% of the clones fell within the $X_?$ category. Both healthy and PBC subjects showed similar frequencies of $X_?$ clones.

Table 1

Transcription and promoter methylation analyses of *CLIC2* and *PIN4* in PBC twin pairs

<i>CLIC2</i>		<i>PIN4</i>					Twin status	
Relative transcript level ^a	Overall % Methylation ^b	Relative transcript level ^a	Overall % Methylation Allele 1 ^c	<i>PIN4</i> genotype ^d	Sample ID	Age (years)		
5.80	62%	16.11	36%	1, 1	1	60	Healthy	Discordant
3.46	61%	21.11	56%	1, 1	2	60	PBC	Discordant
5.50	48%	22.47	n/a	1, 1	52	68	Healthy	Discordant
2.73	60%	8.11	49%	1, 1	9	68	PBC	Discordant
3.92	52%	39.4	50%	1, 2	54	63	Healthy	Discordant
1.88	44%	17.27	34%	1, 2	55	63	PBC	Discordant
1.00	63%	10.06	n/a	1, 1	18	64	Healthy	Discordant
2.11	61%	<LOD	n/a	1, 1	19	64	PBC	Discordant
6.73	55%	7.21	20%	1, 1	24	58	PBC	Concordant
8.17	67%	5.86	39%	1, 1	57	58	PBC	Concordant

^aln eared values, weakest normalized calibrator

^bnumber of methylated CpG sites out of total number of CpG sites assayed

^cnumber of methylated CpG sites out of 11 CpG sites assayed per clone in allele 1

^dallele 1 has 11 CpG sites in promoter region, allele 2 has 13 CpG sites

Table 2

PIN4 genotyping of SNP Rs7058353 in unrelated patients with PBC and controls and in 20 sets of sisters of similar age discordant for PBC. The presence of this SNP determines 11 CpG sites, instead of the original 13. Genotype and allele frequencies did not differ significantly between patients and related or unrelated controls.

	PBC	Controls
Unrelated subjects		
C/C	3/114	0/118
C/A	9/114	7/118
A/A *	102/114	111/118
A allele frequency	0.934	0.970
Siblings		
C/C	0/20	0/20
C/A	2/20	2/20
A/A *	18/20	18/20
A allele frequency	0.950	0.950

* Allele frequency in Caucasian-European population 0.966 (NCBI SNP database)