Genome-wide DNA methylation analysis of uterosacral ligaments in women with pelvic organ prolapse

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Abstract. Pelvic organ prolapse (POP) is an increasingly serious health problem that impairs quality of life and is caused by multiple additive genetic and environmental factors. As the uterosacral ligaments (ULs) provide primary support for the pelvic organs, it was hypothesized that disruption of these ligaments (as a result of aberrant methylation) may lead to a loss of support and eventually contribute to POP. In the present study, whether there are any aberrant methylations in the ULs of patients with POP compared to those of controls was investigated. Genomic DNA was isolated from the ULs of five women with POP and four women without POP, as controls, undergoing hysterectomy for benign conditions. An Illumina Infinium Methylation EPICBeadChips Infinium Human Methylation 850 K bead array was used to investigate the total methylation in the ULs. There were 3,723 differentially methylated CpG sites ($\Delta\beta<0.14$; $P<0.05$), including 3,576 hypermethylation and 147 hypomethylation sites in the ULs of patients with POP compared with the normal controls. There were more hypermethylated CpG sites, but a high ratio of hypomethylation between CpG islands and the N-shelf; in the gene structure, there was more hypermethylation than hypomethylation in TSS1500 and the 5' untranslated region. Gene ontology analysis demonstrated that these differentially methylated genes were associated with 'cell morphogenesis', 'extracellular matrix', 'cell junction', 'protein binding' and 'guanosine triphosphatase activity'. Several significant pathways were identified, including 'focal adhesion' and 'extracellular matrix-receptor interaction pathway'. This study provides evidence that there are differences in genome-wide DNA methylation between ULs in menopausal women with and without POP, and that epigenetic mechanisms may partly contribute to POP pathogenesis.

Introduction

Pelvic organ prolapse (POP) is an increasingly serious health problem that impairs quality of life with a diverse clinical spectrum, including herniation of the uterus, bladder and/or rectum. This condition occurs with a high prevalence in post-menopausal women, with a lifetime risk of surgery for POP or urinary incontinence of 11% (1). This figure is expected to increase with the aging society.

Currently, despite the growing prevalence of POP, no consensus exists among researchers regarding its etiology and pathogenesis. There is no doubt, however, that it is a multi-factorial disorder associated with a genetic predisposition. In addition, multiple additive genetic, environmental and lifestyle factors contribute to the pathogenesis of POP. Epidemiological studies have identified several risk factors, including age (2), race (3,4), parity (5,6) and obesity (5,7,8). However, these factors fail to explain the pathogenesis of POP in nulliparous women and to answer the question of why POP does not develop in all women exhibiting high-risk factors.

Recently several gene arrays for POP were revealed. Differential expression signatures were identified in 81 genes and specific extracellular matrix (ECM)-associated genes may participate in the pathology of POP, according to RNA-Seq data (9). Ak *et al* (10) reported, for the first time, that certain genes serve a role in the cell cycle, proliferation and embryonic development, along with cell adhesion processes during the development of POP, using gene chip microarrays, and a genome-wide association study (11) identified promising single nucleotide polymorphisms associated with POP. Recently published genome-wide linkage analysis (12) provided evidence for two additional loci in relation to symptomatic POP and whole-exome sequencing identified a novel gene, WNK1, that influences susceptibility to POP (13).

Generally, epigenetic regulation of gene transcription occurs by three main mechanisms: DNA methylation, histone modification and microRNA (miRNA) expression (14). DNA methylation, the most common epigenetic mechanism, leads to changes in gene expression without alteration of the DNA sequence. Aberrant (hyper or hypo-) methylation is believed

to be greatly influenced by environmental risk factors. Klutke *et al* (15) first reported that methylation in the promoter region may suppress lysyl oxidase (LOX) gene expression in women with POP, but the DNA methylome of POP has never been characterized.

Since the uterosacral ligaments (ULs) provide primary support for the uterus and the upper vagina, it was hypothesized that the disruption of these ligaments may lead to a loss of support and eventually contribute to POP. In the present study, whether there is any aberrant methylation in the ULs of patients with POP compared to the controls was investigated.

Materials and methods

Tissue collection. Approval from the institutional review board was obtained from the Beijing Obstetrics and Gynecology Hospital Ethics Committee. Informed consent was obtained from all individual participants included in the study. A total of nine postmenopausal women, with five POP and four non-POP controls, undergoing hysterectomy for benign conditions were included, from January 2015 to June 2017. The clinicopathological characteristics of these patients are presented in Table I. In order to eliminate the intermixing factors between the experimental group and the control group, strict limits on inclusion and exclusion criteria were set for the uterine ligament samples. Exclusion criteria were as follows: Women with a history of connective tissue disorders, endometriosis, prior pelvic reconstructive surgery and cancer. Inclusion in the POP group required uterine prolapse beyond the hymen (stage 3 or stage 4) with/without cystocele and/or rectocele. Patient characteristics assessed included: Age, parity, body mass index (BMI), menopause, duration of menopausal period, history of hormone replacement therapy (HRT), smoking history and history of hypertension. The ULs were obtained during the procedures, providing ~1 g of tissue per sample.

DNA extraction. DNA was isolated using an OMEGA TISSUE DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA), according to the manufacturer's protocol. DNA was quantified by spectrophotometry (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Genomic DNA (500 ng) was treated with bisulfate using an EZ DNA Methylation Gold kit (Zymo Research Corps., Irvine, CA, USA), according to the manufacturer's protocol.

Microarray experiments. Microarray experiment correction for multiple testing was done in order to separate the most significant differences from the background noise. Prior to microarray experiments, clustering test was done between the two groups. POP and control subjects were separated (Data not shown). The methylation of DNA was assayed on an Illumina Infinium Methylation EPICBeadChip (Illumina, Inc., San Diego, CA, USA) using the Illumina HD methylation assay kit from Shanghai Biotechnology Corporation (Shanghai, China). Each of these arrays contained 853,307 probes, covered CpG islands, RefSeq genes, ENCODE open chromatin, ENCODE transcription factor binding sites and FANTOM5 enhancers. Bisulfite-converted DNA was analyzed on an EPICBeadChip following the manufacturer's protocol. GenomeStudio

methylation module V.1.9.0 (Illumina, Inc.) was used to extract the image intensities.

Statistical analysis and microarray data analysis. Clinical characteristics were analyzed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance for the t-test was determined to be at the level of P<0.05. Values are expressed as the mean and standard deviation.

The CpG probe signal intensities were normalized using Subset-Quantile Within Array Normalization in the minfi packages from R Bioconductor (16) for background correction and subtraction. Methylation values for individual CpG sites were obtained as β-values, calculated as the ratio of the methylated signal intensity to the sum of the methylated and unmethylated signals following background subtraction. The β-values were reported as a DNA methylation score ranging from 0 (non-methylated) to 1 (completely methylated). Initially, probes located on the sex chromosome were excluded. Differentially methylated CpGs were selected using an algorithm in IMA Bioconductor. In the present study, the mean-difference β-value ($\Delta\beta$) between the two sample groups for each CpG site was assessed. Specifically, a probe was considered to be differentially methylated if the absolute $\Delta\beta$ was higher than 0.14 and the statistical test was significant (P<0.05 was considered to indicate a statistically significant difference). The interaction network was generated using the Search Tool for the Retrieval of Interacting Genes (STRING) database (string-db.org) to analyze genes from the experiments and text mining.

Results

Clinical characteristics of all participants. A total of 9 subjects were recorded, including five patients with POP and 4 controls. ULs were obtained from a total of 9 hysterectomies. The overall grade of prolapse was 3-4 and the degree of uterine prolapse was grade 3-4 beyond the hymen. Clinical characteristics of all participants included in the study are presented in Table I.

Differential methylation and expression profiling in POP. DNA methylation analysis identified 3,723 differentially methylated CpG sites; 0.4% of the total sites in POP ULs were detected, compared to the controls (Fig. 1A). Surprisingly, the hypermethylated CpG site accounted for ~96.1% (3,576), which was markedly higher than the number of hypomethylated ones (147 CpG sites, 3.9%, Fig. 1B). Notably, in methylated CpG content and neighborhood context (Shore: 0-2 k distance around the island; Shelf: 2 to 4 k distance around the island), there was a difference between hypermethylation and hypomethylation; hypermethylated CpG site percentages were 4.4, 3.2, 6.8, 2.9, 5.9 and 76.9% in Island, N-Shelf, N-Shore, S-Shore, S-Shelf and Open Sea, respectively. However, hypomethylated CpG site percentages were 8.2, 6.1, 5.4, 4.8, 6.1 and 69.4% in Island, N-Shelf, N-Shore, S-Shore, S-Shelf and Open Sea, respectively. In particular, more hypomethylated CpG sites were detected in Island and N-Shelf (Fig. 1C and D). Hypermethylated and hypomethylated CpG sites are presented in Fig. 1C and D in chromosomal locations. Finally, probes from the X and Y chromosomes were excluded due to the

Table I. Clinical characteristics.

Characteristics	POP (n=5)	CON (n=4)	P-value
Age (years)			
Range	60.60±5.23 (55-66)	61.25±6.65 (53-68)	NS
BMI (kg/m^2)	24.84±2.17	26.80±5.74	NS
Pregnancy (n)			
Range	3.6±1.14 (2-5)	2.75±0.05 (2-3)	NS
Parity (n)	2.00±0.00	1.75±0.50	NS
Patients (n) without vaginal delivery	0	0	-
Patients (n) one vaginal delivery	0	1	-
Patients (n) two vaginal deliveries	5	3	-
Menopause (n)	5	4	-
During of menopausal period (years)			
Range	10.20±4.82 (5-16)	11.00±7.79 (4-20)	NS
Smoking (n)	0	0	-
Suffering from hypertension (n)	1	2	-

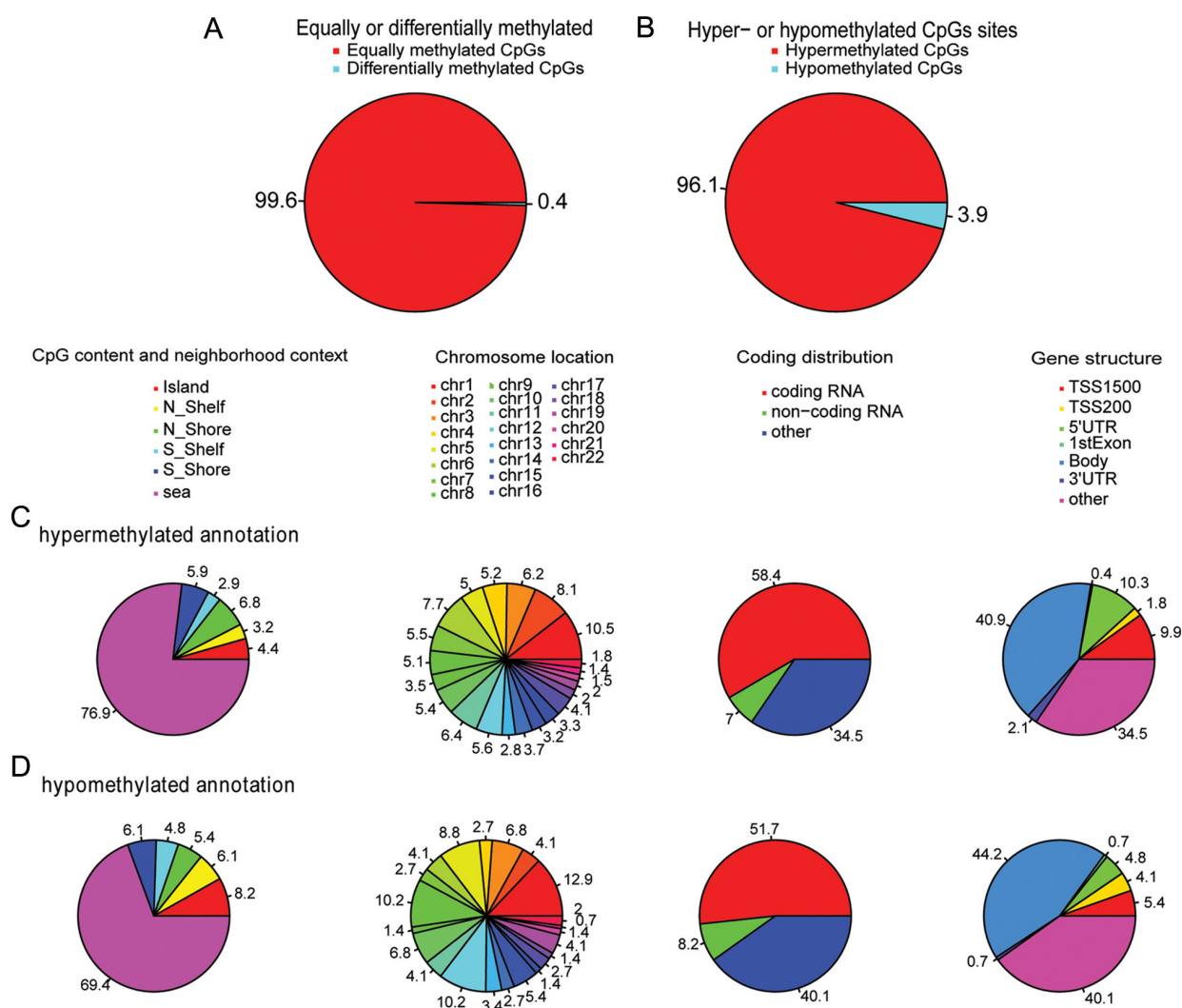NS, not significant; BMI, body mass index; POP, pelvic organ prolapse; CON, control.

Figure 1. Characteristic methylation patterns between pelvic organ prolapse uterine ligament and control subjects. (A) The ratio of equally or differentially methylated CpG sites are presented as a pie chart. (B) The ratio of hypermethylated or hypomethylated CpG sites. (C) Hypermethylated annotation of differentially methylated CpG sites. (D) Hypomethylated annotation of differentially methylated CpG sites. UTR, untranslated region.
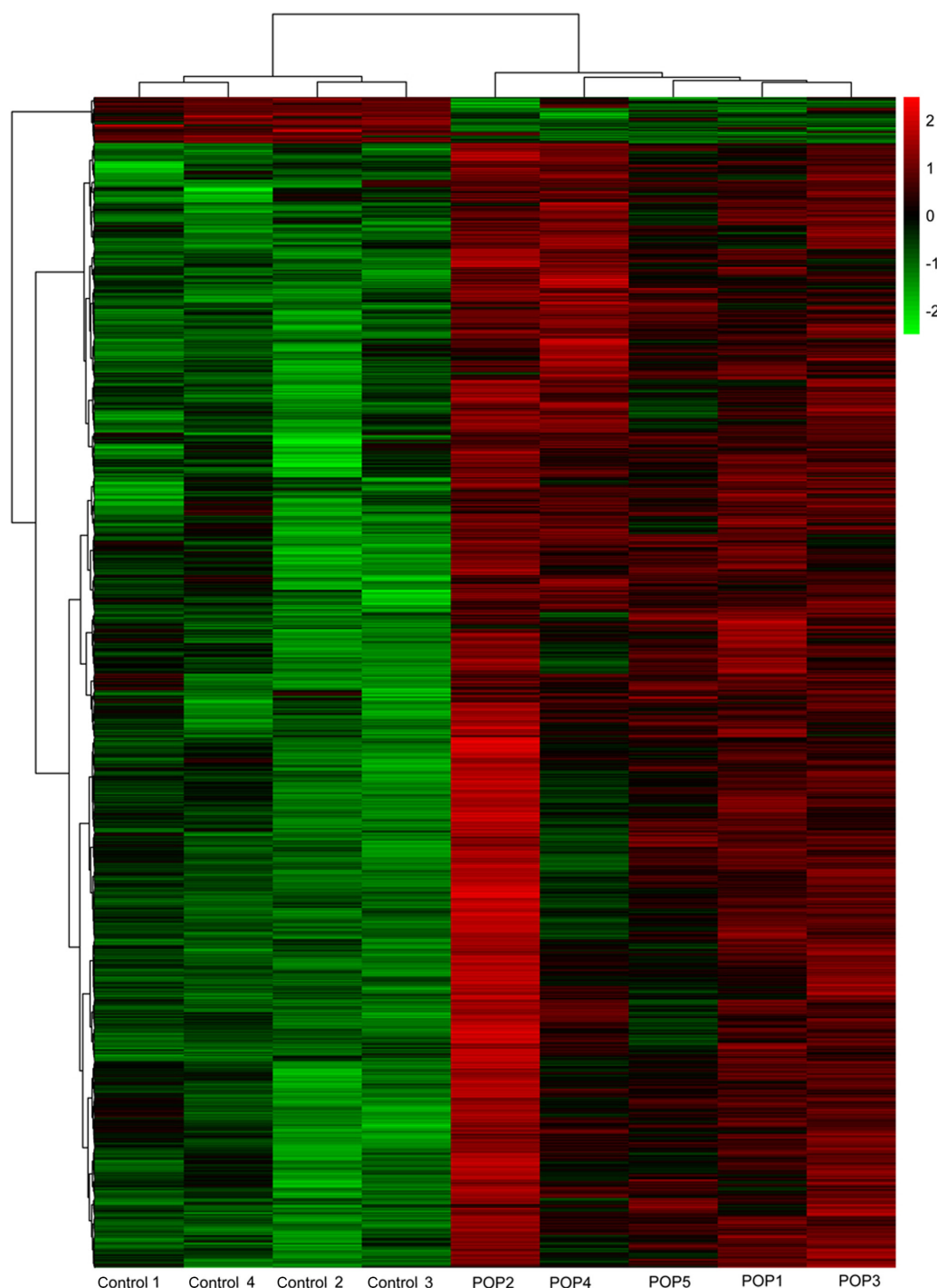
Figure 2. The heat map demonstrates an overview of hierarchical clustering of the differentially methylation in POPs and control subjects. Hypermethylations are presented in red, hypomethylation is labeled green. POP, pelvic organ prolapse.

partial homology of the X and Y chromosomes, and the fact that the 9 samples were females. As for gene structure, there was significant difference (TSS1500, TSS200, 5'UTR, 1st Exon, Body, 3'UTR and others) between hypermethylation and hypomethylation; in particular, there was greater hypermethylation residing in TSS1500 and 5'UTR compared with hypomethylation. Detailed differences in gene structure are presented in Fig. 1C and D.

Heat maps of differential methylation are presented in Fig. 2. Total methylation of POP samples was markedly different from that of the controls; however, in the POP group,

samples POP4 and POP5 were more similar to each other, and were slightly different from the other three POP samples.

GO analysis of the differentially methylated genes in POP. To begin defining the functional significance of the extensive changes in DNA methylation in POP, GO analysis was used. According to the functions of the differential genes, the top 20 in each category were listed, respectively, in molecular function, biological process and cellular component (Fig. 3). These differentially methylated genes were widely associated with cell morphogenesis, the extracellular matrix, cell junctions,
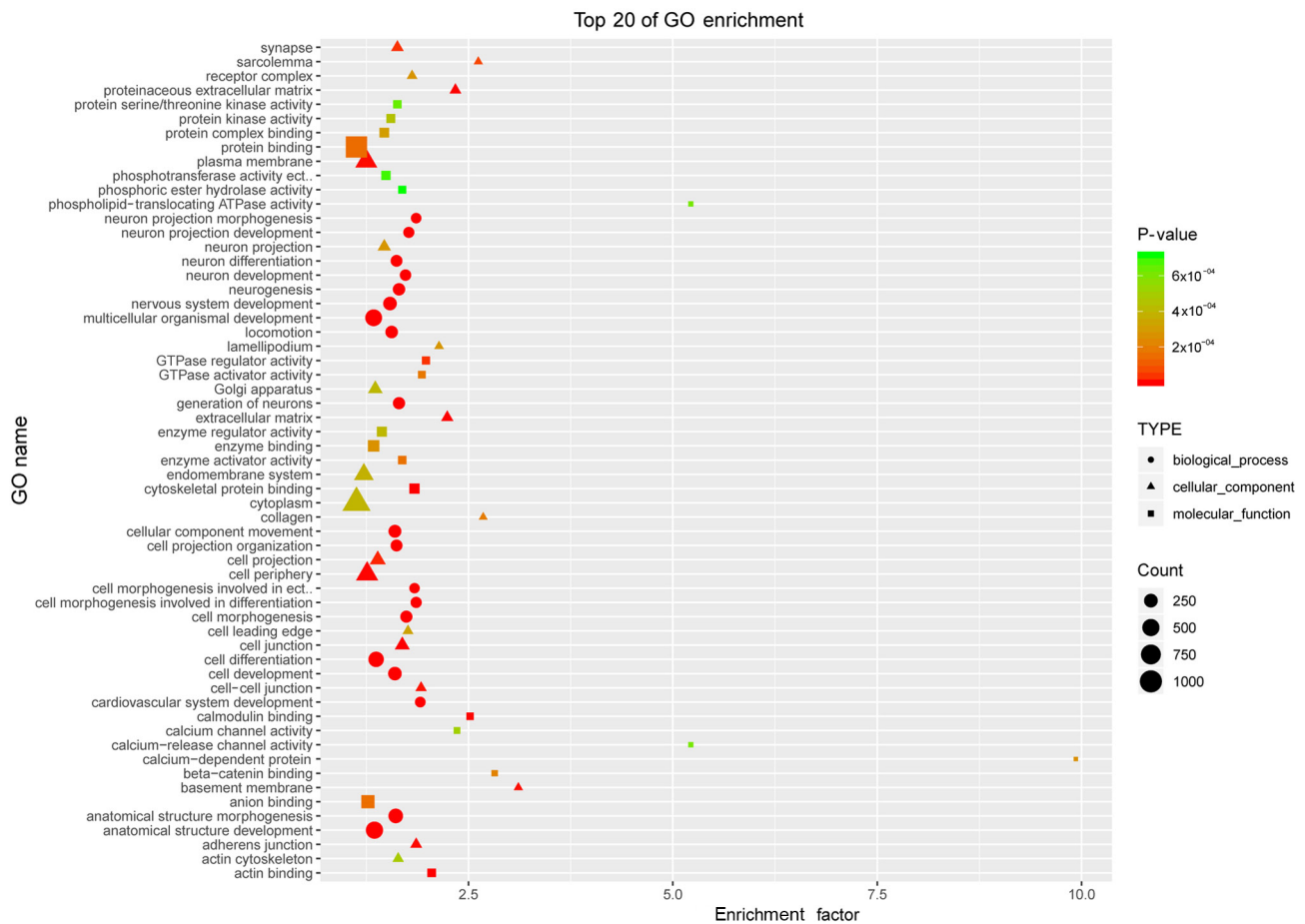
Figure 3. GO analysis of differential methylated genes (β>0.14; P<0.05). X-axis, enrichment factor; Y-axis, GO category. The red represents small P-values and the green represents the larger ones. A round node represents a biological process, the triangle represents a cellular component and a square represents a molecular function. The top 20 GO terms are presented. GO, Gene Ontology; ATPase, adenosine triphosphatase; GTPase, guanosine triphosphatase.

protein binding and guanosine triphosphatase (GTPase) activity.

KEGG analysis of the differentially methylated genes in POP. To further investigate key pathways associated with these distinct genes, the interaction network of the significant pathways associated with POP was built according to the KEGG database. The analysis in the present study demonstrated that these differentially methylated genes were widely involved in various cellular pathways. There were a total of 206 pathways associated with POP and the top 20 included 'ErbB signaling pathway', 'pathways in cancer', 'focal adhesion' and 'ECM-receptor interaction' (presented in Fig. 4). Notably, 'focal adhesion' and 'ECM-receptor interaction' were enriched in the top 3 and top 4 of differentially methylated profiling in the top 20 enriched pathways. The two pathways of the differentially methylated genes are listed in Table II.

Network analysis in POP. The STRING was used to provide information regarding predicted and experimental interactions of proteins. Differentially expressed genes were demonstrated to be enriched via methylation profiling and literature mining in the focal adhesion and ECM-receptor interaction pathways (Fig. 5); furthermore, the nodes with higher degrees of interaction were considered as hub nodes. The top 5 nodes were

integrin (IGTB1; degree=43), fibronectin (FN1; degree=39), protein tyrosine kinase (PTK2; degree=38), cluster of differentiation (CD)44 (degree=37) and integrin (IGTA1; degree=37).

Discussion

Emerging evidence indicates that epigenetic mechanisms may partly contribute to the pathogenesis of POP (15). However, until now there had been no reports on genome-wide DNA methylation profiling in POP, except the LOX methylation. Klutke *et al* (15) measured promoter methylation in the LOX gene in women with POP and found a total of 66 methylated CpG sites in the POP group and only one methylated CpG site in the non-prolapse control group. In the present study, it was reported that there were 3,723 differentially methylated CpG sites, 0.4% of the total sites in POP ULs compared with the controls in menopausal women.

In general, increased DNA methylation means higher levels of gene expression (17). Over the past decades, a number of studies have revealed that a considerable percentage of CpG site methylation varies with age (18), giving rise to genome-wide hypomethylation with site-specific incidences of hypermethylation. Notably, tumors have a unique methylation pattern with high levels of hypomethylation (19). In the present study, the five menopausal women with POP and the four without POP demonstrated a unique methylation pattern with
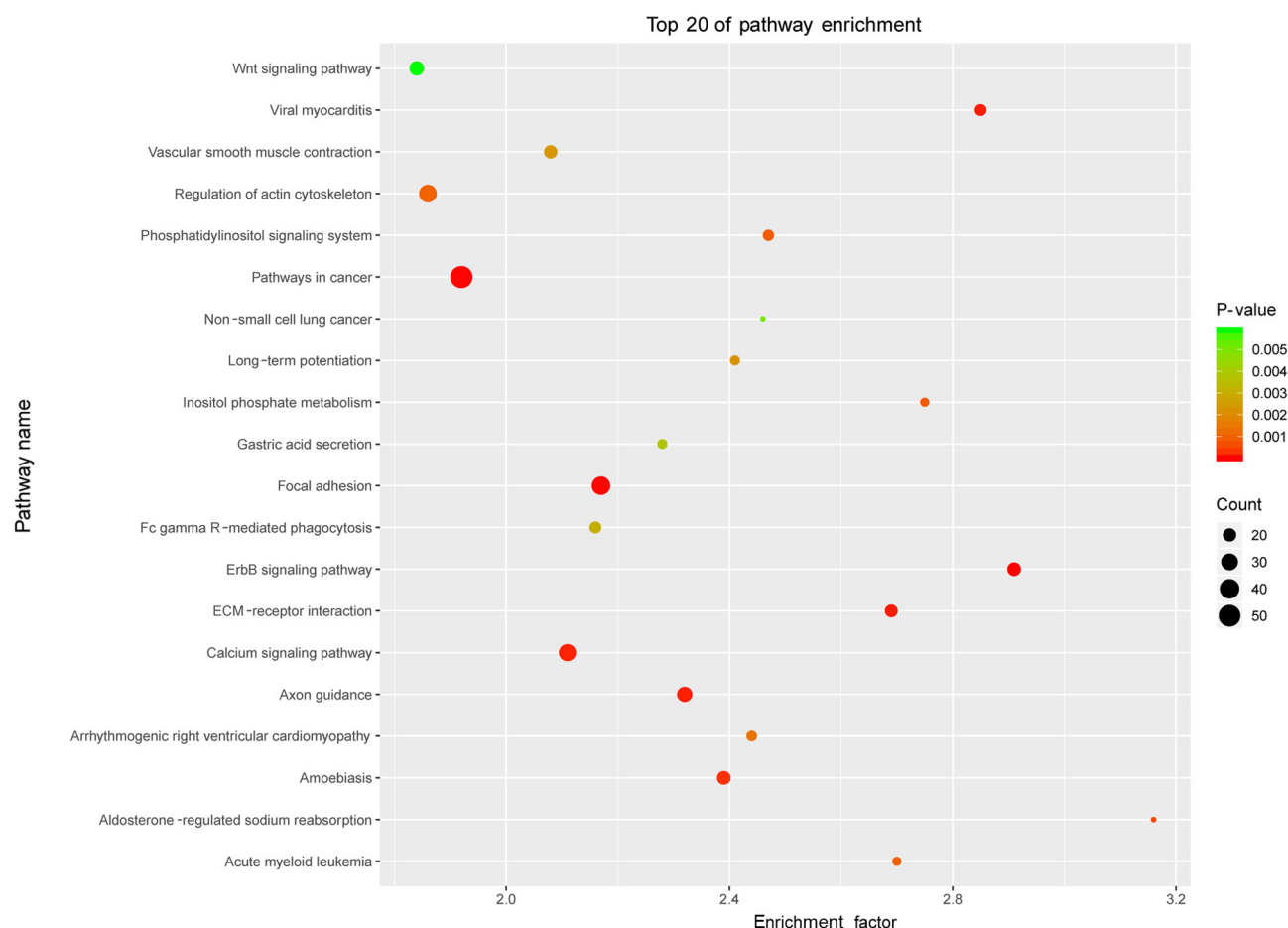
Figure 4. KEGG analysis of the differentially methylations. KEGG analysis of differential methylated genes (β>0.14; P<0.05). The red represents the smaller P-values and the green means the larger ones. X-axis represents the enrichment factor; the Y-axis, represents the KEGG category. The top 20 KEGG terms are presented. KEGG, Kyoto encyclopedia of Genes and Genomes; ECM, extracellular matrix.

low levels of hypomethylation, which may partly be associated with aging. The age range of the nine subjects was between 53 and 68 years old and therefore all were menopausal women. As for the sample limitations, age-associated variations in methylation should be further investigated in the future.

DNA methylation is a key epigenetic process involved in the regulation of gene expression. There is no doubt, however, that POP is a multifactorial disorder with a genetic predisposition, determined by interactions between additive genetic, environmental and lifestyle factors. As presented in the heat map, hypermethylation and hypomethylation were obviously different between POPs and controls, demonstrating their contribution to the pathogenesis of POP. Notably, with respect to the methylation within the POP group, POP4 and POP5 were more similar to each other and slightly different from the other three. Analyzing the clinical characteristics, it was demonstrated that the duration of menopause in the two cases was ~15 years, but it was five, six and ten years in the other three. These two POP cases were older than the other three also; there were no differences in parity or BMI. Therefore, age and duration of menopause may be highly associated with methylation.

For DNA methylation profiling, gene ontology analysis was used to separately assess molecular function, biological processes and cellular components. In biological processes, the enriched terms were 'anatomical structure morphogenesis', 'anatomical structure development', 'cell morphogenesis', 'locomotion involved in differentiation', 'cellular component movement' and 'locomotion', which was likely due to the pelvic organs moving from their normal anatomical position, accounting for prolapse. In the cellular component category, the terms were 'proteinaceous extracellular matrix', 'cell junction', 'cell periphery', 'adherens junction', 'basement membrane', 'cell-cell junction' and 'collagen', indicating that POP may be associated with the ECM. In the molecular function category, the terms were 'cytoskeletal protein binding', 'actin binding', 'calmodulin binding', 'GTPase regulator activity', 'calcium channel activity', 'calcium-release channel activity' and 'protein binding'. Similarly, previous evidence has demonstrated that several families, including collagen (20-22), LOX (15,23-25) and fibulin (25), have important roles in the synthetic metabolism and pathogenesis of POP; however, in the catabolism of the pathogenesis of POP, matrix metalloproteinase (26-30) and tissue inhibitors of metalloproteinase serve vital roles (27,30). In the regulation of anabolic and catabolic processes, the homeobox gene family is key (31,32). Notably, in the present study, it was demonstrated that specific key members of the families mentioned above were detected to be DNA methylated in the molecular function, biological process and cellular component categories.

Table II. KEGG analysis of distinct methylated genes in pathways. KEGG analysis of distinct hepermethylated genes and hypomethylated genes in focal adhesion and ECM-receptor interaction pathways.

Pathway ID	Description	P-value	Hypermethylated Genes	Hypomethylated Genes
hsa04510	Focal adhesion	3.38×10^{-05}	COL5A1 LAMB1 ITGA9 PDK1 FYN VWF AKT3 COL1A2 VEGFC MAPK9 ZYX BCAR1 CRK PARVB PIK3R1 PIK3CB THBS2 FN1 ITGAV COL11A2 PRKCA LAMB3 SOS1 CAPN2 LAMA2 DOCK1 RASGRF1 TNR COL6A3 PTK2 MYLK3 COL4A1 ITGB5 COL5A2 FIGF	ITGA8
hsa04512	ECM-receptor interaction	1.18×10^{-04}	COL5A1 LAMB1 SDC2 ITGA9 VWF COL1A2 THBS2 FN1 ITGAV COL11A2 LAMB3 LAMA2 TNR COL6A3 COL4A1 ITGB5 CD44 COL5A2	ITGA8

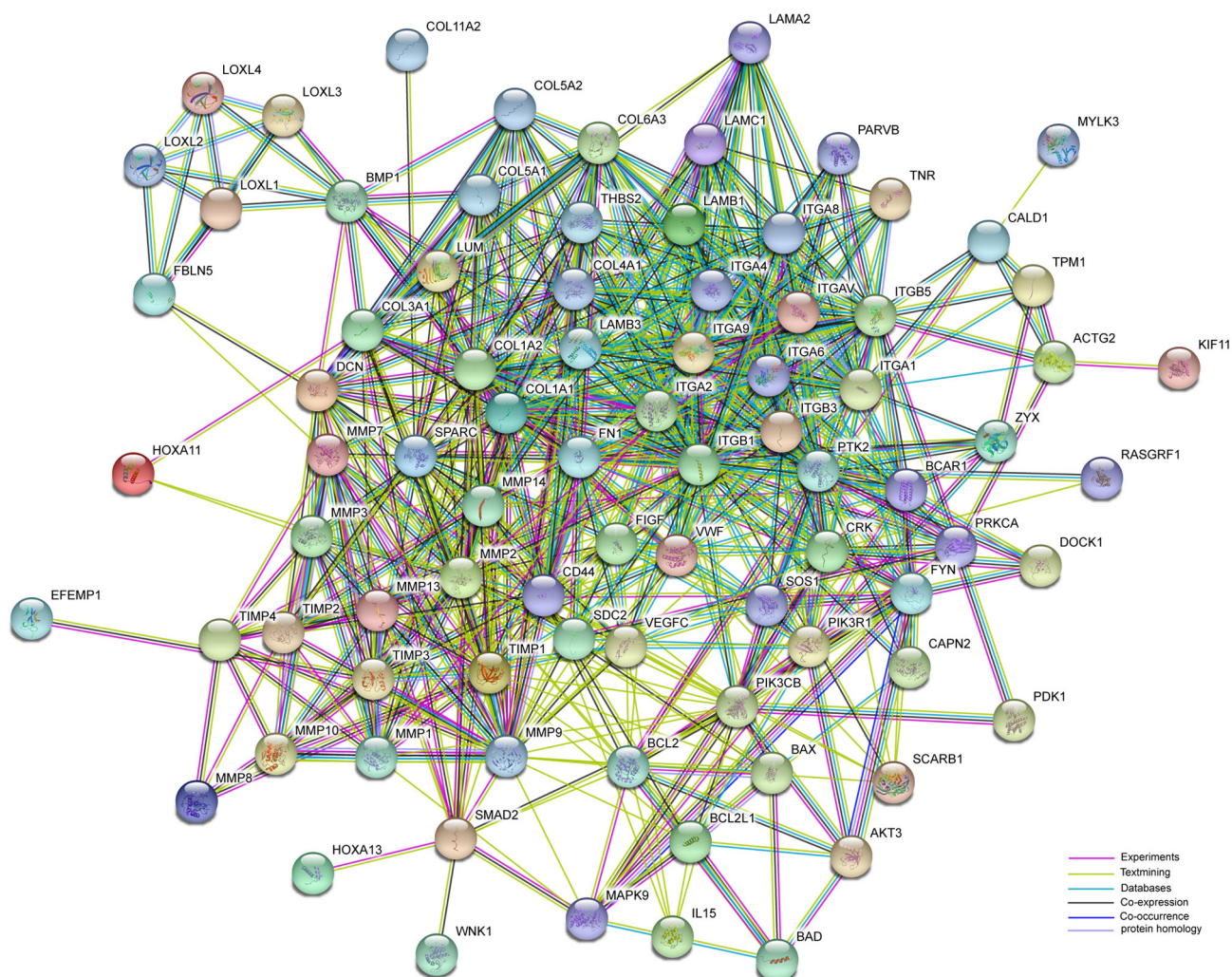KEGG, Kyoto encyclopedia of genes and genomes; ECM, extracellular matrix.

Figure 5. Protein-protein interaction network of differentially expressed genes using STRING. Network nodes represent proteins; edges represent protein-protein associations. Colored lines between the proteins indicate the various types of interaction evidence in STRING. STRING, Search Tool for the Retrieval of Interacting Genes.

KEGG analysis of expression profiling suggested that the 'focal adhesion' and 'ECM-receptor interaction' pathways may potentially serve direct or indirect roles in POP; however, to date, no studies have linked them to POP. Notably, the two pathways contained only one hypomethylated gene, ITGA8; however, ITGAV was hypermethylated in the two pathways.

A hypomethylated gene tends to indicate a high expression level, with hypermethylation meaning lower expression, in accordance with Kufaishi's studies (33,34) in vaginal cells derived from premenopausal women with and without severe POP. Li *et al* (35) demonstrated that mechanical strain could activate the phosphoionositide 3 kinase/protein kinase B signaling pathway in POP. Chen (36) reported that smooth muscle cells could activate the TGFER2/ALK5/mothers against decapentaplegic homolog 2 (Smad)2 and Smad3 signaling pathways, which may indicate a potential approach for the management of POP. In these two pathways certain genes, which were differentially methylated in POP were demonstrated.

Integrins are transmembrane receptors facilitating cell-ECM adhesion and activating signal transduction pathways. Not only do they bind cells to the ECM, they also regulate the cell cycle, organize the intracellular cytoskeleton and translocate novel receptors to the cell membrane (37). Several types of integrins exist and one cell may have multiple different types on its surface. Integrins interact with the ECM to generate large molecular complexes by focal adhesions. The study of Rhee *et al* (38) revealed that POP is associated with alterations in the sphingosine-1-phosphate/Rho-kinase signaling pathway, which could be reduced by a Rho-kinase inhibitor. Apart from ITGAV, there were two other genes, which were hypermethylated, ITGA9 and ITGB5, which require further investigation. In the network, ITGAV (degree=32), ITGA9 (degree=27) and ITGA8 (degree=25) were highly connected. This suggested that the integrin family may include hub genes in POP. Fibronectin is the main part of the ECM and while rarely mentioned with regard to POP, may be another important hub gene.

In the present study, there were certain limitations. All the ULs were from menopausal women and premenopausal women were not included. The number in each group was relatively small. Since microarray experiments use large numbers of variables (genes) in a small number of samples, standard power analysis determination according to standard hypothesis testing cannot be applied (39). Pavlidis *et al* reported (39) that 8-15 subjects in each group is sufficient to obtain near-maximal levels of power in array studies. Gene expression and functional tests were not performed. Although this issue was not the subject of this study, the products of these genes and their functions should be further investigated in future studies.

In conclusion, the present study demonstrated and elaborated upon the differences in genome-wide DNA methylation between POP ULs and controls. Epigenetic mechanisms may partly contribute to the pathogenesis of POP.

Acknowledgements

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LZ analyzed and interpreted the data, wrote the paper. DL helped editing the paper. LZ, PZ, AD, YH, CL and DL collected the tissues.

Ethics approval and consent to participate

The institutional review board was obtained from the Beijing Obstetrics and Gynecology Hospital Ethics Committee. The study was approved in written by Ethics Committee. Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare they have no competing interests.

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