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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Genome-wide DNA methylation profiling in ectopic and eutopic of endometrial tissues

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Abstract

Purpose Endometriosis is a gynecological disease that causes the uterine lining to appear in other organs outside the uterus. As DNA methylation has an important role in this disorder, its profiling can reveal new information to improve the diagnosis and treatment of endometriosis patients.

Methods We conducted a genome-wide methylation profiling of ectopic and eutopic endometrial tissues from women with and without endometriosis using Infinium Human Methylation 450K BeadChip arrays. DNA methylation samples were collected from nine ectopic and nine eutopic endometrial tissues of endometriosis and six endometrial tissues of healthy controls.

Results Correlation heatmaps and the principal component analysis divided the samples into two clusters, one consisting of all ectopic samples and the other consisting of both eutopic and control samples unexpectedly without segregation between them. The assay identified a group of methylated genes that were overrepresented in biological processes, including abnormality in signaling, development, and adhesion of cells. Pathway analysis revealed disruption in HTLV infection pathways, PI3K-Akt, oxytocin, and relaxin signaling. Moreover, we found eutopic lesions are strongly associated with autoimmune disease.

Conclusions Our results confirmed the role of DNA methylation alternations in endometriosis development and pathogenesis. Our finding suggests aberrant DNA methylation can activate several signaling pathways including PI3k-AKT signaling, relaxin, and oxytocin which are associated with the pathogenesis of endometriosis.

Keywords Endometriosis · Epigenetics · Differentially methylated regions · Signaling pathways

Introduction

Endometriosis is a gynecological disease which is characterized by the ectopic presence of endometrial tissue outside the uterus. It is a benign estrogen-dependent disorder that causes chronic pelvic pain, pain with menstruation, abnormal uterine bleeding, and infertility and influences both women's health and life quality [1–3]. Despite several theories, the pathogenesis of endometriosis is unknown, and there is not any non-invasive biomarker resulting in a long delay between onset and diagnosis [4, 5]. Both genetics and epigenetic factors are involved in the disease [6, 7]. Several studies have indicated that DNA methylation, the most studied epigenetic modification, has a potential role in endometriosis [7, 8]. Hypermethylation of promoter and transcription start site (TSS) are among the hallmarks of gene silencing [9, 10]. Abnormal DNA methylation reported alternating the expression of several genes such as ERβ (ESR2) [11], steroidogenic factor-1 (SF-1) [12], homeobox 10A (HOXA10) [13], cyclooxygenase-2 (COX-2), aromatase [14], and other related genes which have an important role in endometriosis development.

During the last decade, there were numerous studies about different aspects of endometriosis [15–17]. Using high-throughput technologies, we have the opportunities to provide a comprehensive view of DNA methylation. Different studies

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have been focused on identifying global methylation alternations in endometriosis [18–20], but they did not survey the DNA methylation in all ectopic, eutopic, and control endometrium. Here, we profiled ectopic and eutopic endometriosis lesions and control tissues. We aimed to characterize genome-wide DNA methylation alternations in pairwise comparisons between ectopic and eutopic lesions and control samples. To our knowledge, this is the first study to profile the genome-wide DNA methylation in ectopic and eutopic endometrial tissues of Iranian women.

Materials and methods

Sample collection

Eutopic and ectopic endometrial tissue samples were isolated from nine women with endometriosis whom were in reproductive age (range 20–40). Endometrial biopsies of the control group were obtained from six healthy fertile women who were found not to have endometriosis during surgery. The samples were collected from women with regular cycle (28–32 days) during the proliferative phases, they had not used hormonal medication within 3 months before sampling, and none of them had visible endometrial hyperplasia or neoplasia and inflammatory disease at the time of clinical examination or laparoscopy. They underwent laparoscopy for benign gynecologic indications (tubal ligation, ovarian cystectomy, or hysterectomy). Ectopic biopsies were obtained during the laparoscopy, while eutopic biopsies were collected with the use of pipeline from the same group. The pathology of endometriosis was diagnosed with tissues analyzed by histopathology to confirm the presence of endometrial stroma and glands. Clinical stages of endometriosis were moderate or severe, as classified by the American Society for Reproductive Medicine (ASRM, 1997). Each of the women in the control group had given birth naturally. Just after surgical removal, tissue samples were divided into small pieces, placed later in an RNA solution, and stored at − 80C until being processed.

Illumina Infinium Human Methylation 450K

Whole-genome DNA methylation was analyzed using the Illumina Infinium Human Methylation 450K as follows [21]. DNA was whole-genome amplified, fragmented, and hybridized (500 ng) to the BeadChip arrays following the manufacturer's instructions. Bisulfite conversion and microarray manipulations were performed following standard procedures (Anger Core Facility, France). The BeadChips were scanned using the Illumina HiScan SQ scanner, and raw image data were imported into the GenomeStudio (v2011.1) methylation module (v1.9.2.), which was used to extract and transform the fluorescent signal intensities into beta values (range = 0 for the unmethylated site to 1 for a fully methylated site).

Preprocessing and normalization of methylation data

We performed data preprocessing and normalization using "minfi" package [22] developed into the R environment in Bioconductor. The raw data were then imported to minfi and quality control of data was done by checking QC sample plots. Probes containing SNP sites ($n = 65$) were filtered out by minfi package default (with $n = 485{,}512$ probes as input). Then, probes with p value > 0.01 in more than 50% of samples were selected to be removed ($n = 243$). Funnorm normalization method was used for background correction and adjusting probe types I and II. Probes containing CpG or SBE in probe body with parameter maf = 0 were dropped ($n = 17{,}539$). At least, beta values with the $\frac{m}{m+u+100}$ formula were calculated for each probe. The remaining probes ($n = 467{,}730$) with the beta value were considered as input to find differentially methylated regions (DMRs).

Heatmap of Spearman's correlation

The heatmap of Spearman's correlation with the use of "heatmap" package into R environment, after data preprocessing and normalization, was plotted to identify clusters between samples.

DMR identification

To identify DMRs, "seqlm" package [23] was used with a maximum 500-bp distance between each segmentation. DMRs for each two groups were filtered by false discovery rate, FDR < 0.05, methylated probes with more than three probes, and absolute methylation value ($\Delta\beta$) > 0.2 to be meaningful.

DMP identification

In eutopic compared with control samples, we found all DMRs had FDR > 0.05. Then, we selected regions with differentially methylated positions (DMPs) with an absolute methylation value ($\Delta\beta$) > 0.5.

Functional analysis

G:profiler web server (https://biit.cs.ut.ee/gprofiler/) was used to perform functional annotation chart and pathway analysis. Terms with q-value < 0.05 were considered statistically significant.

Differentially expressed genes

Differentially expressed genes between healthy women and patients with moderate/severe stages of endometriosis were identified using GEO2R tools (https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE51981). From differentially expressed genes, only genes with the absolute $\log_2$ fold change greater than 1 were selected.

Visualization

Visualization of the results was performed using "ggplot" package [24] in the R environment.

Results

Genome-wide DNA methylation profiling in endometrial tissues of endometriosis and control samples

Endometrial biopsy specimens were obtained from nine endometriosis patients and six control women referred to the Royan Institute. The institutional ethics committee of Royan Institute approved the study and all patients donating tissue for these studies signed a consent form. We first measured the pairwise correlation of DNA methylations among samples. To this end, the heatmap of Spearman's correlation coefficient

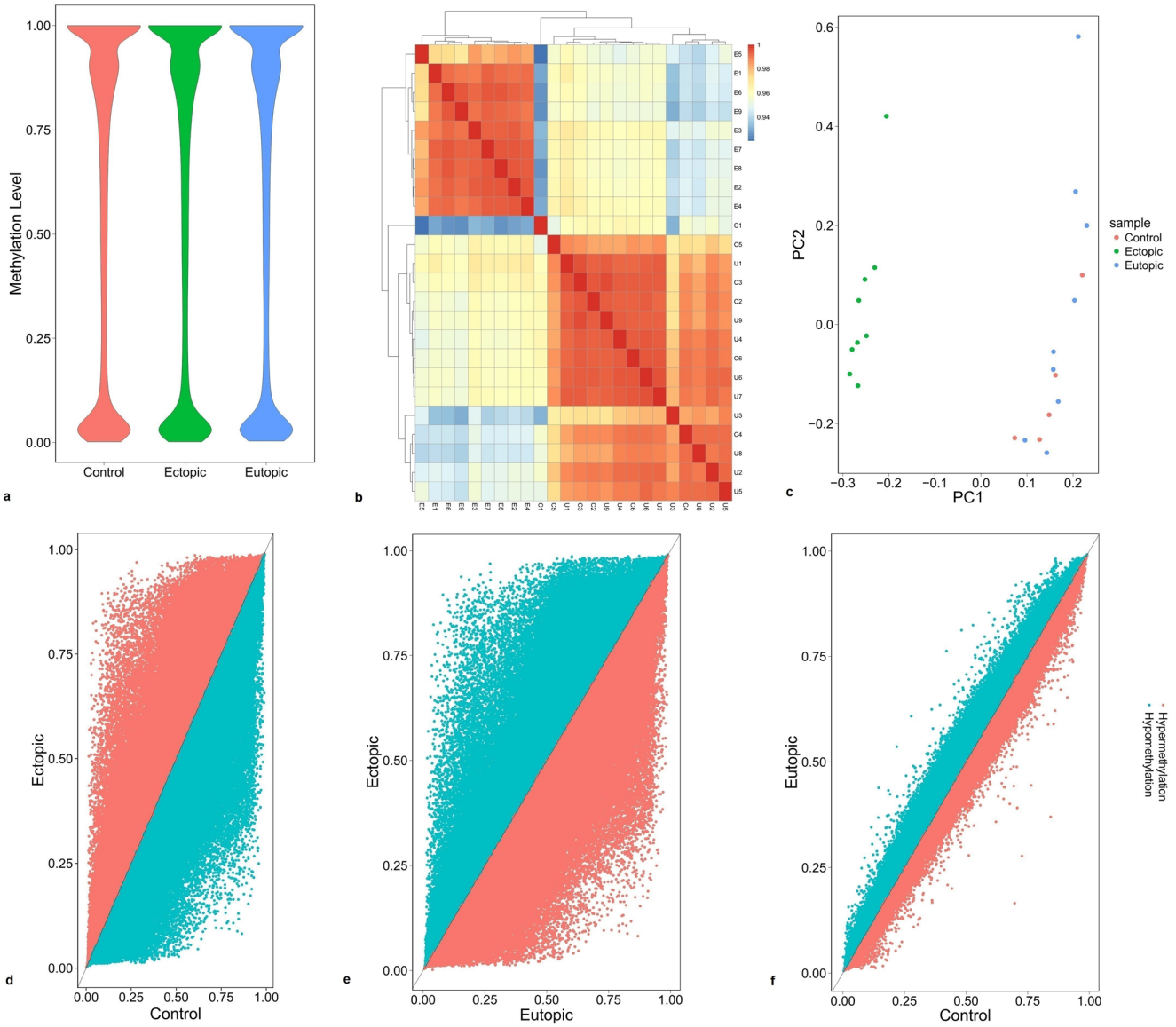

Fig. 1 Differential CpG methylated pattern in healthy and endometriosis lesions. The methylation distributions depicted as (**a**) violin plot showing the distribution of DNA methylation levels in different samples. (**b**) Heatmap of the Spearman's correlation between samples illustrated the similarities between samples which indicates the low correlation between C1 (one of control sample) and other samples (E, U, and C stands for ectopic, eutopic, and control, respectively). (**c**) PCA was illustrated. Also, scatter plots of ectopic comparing control (**d**) and eutopic (**e**) show more difference relative to eutopic and control comparison (**f**)

was generated using beta value that resulted from sample comparison. Heatmap graph demonstrates two main clusters by dividing the ectopic from the eutopic and control samples. As it is shown (Fig. 1b), one of the control samples with low correlation failed in correlation assessment and we found it as an outlier, so we removed it from further analysis. Consistent with the correlation heatmap, the principal component analysis (PCA) revealed that DNA methylation has more difference between ectopic and control than eutopic and control. Also, eutopic and control have closer DNA methylation patterns. The relationships between methylated CpG distribution and sample types are illustrated in the violin plot (Fig. 1a). In all cases, consistent DNA methylation distribution of CpGs were observed while methylated CpGs were more localized at methylation grade with a value of 1. Also, we examined methylation changes in β values. Similar DNA methylation distribution with more difference was revealed between eutopic and control in comparison with the ectopic samples (Fig. 1d, e). Additionally, we found a lower distribution of methylation in eutopic vs. control sample indicating low differences in control and eutopic samples (Fig. 1f). This is consistent with both violin plot and heatmap correlation. We aimed to find local DMRs among healthy controls and endometriosis lesions. For this purpose, we performed a comparative analysis and identified 1753 DMRs in ectopic vs. control and 2108 DMRs in the ectopic vs. eutopic (FDR < 0.05,

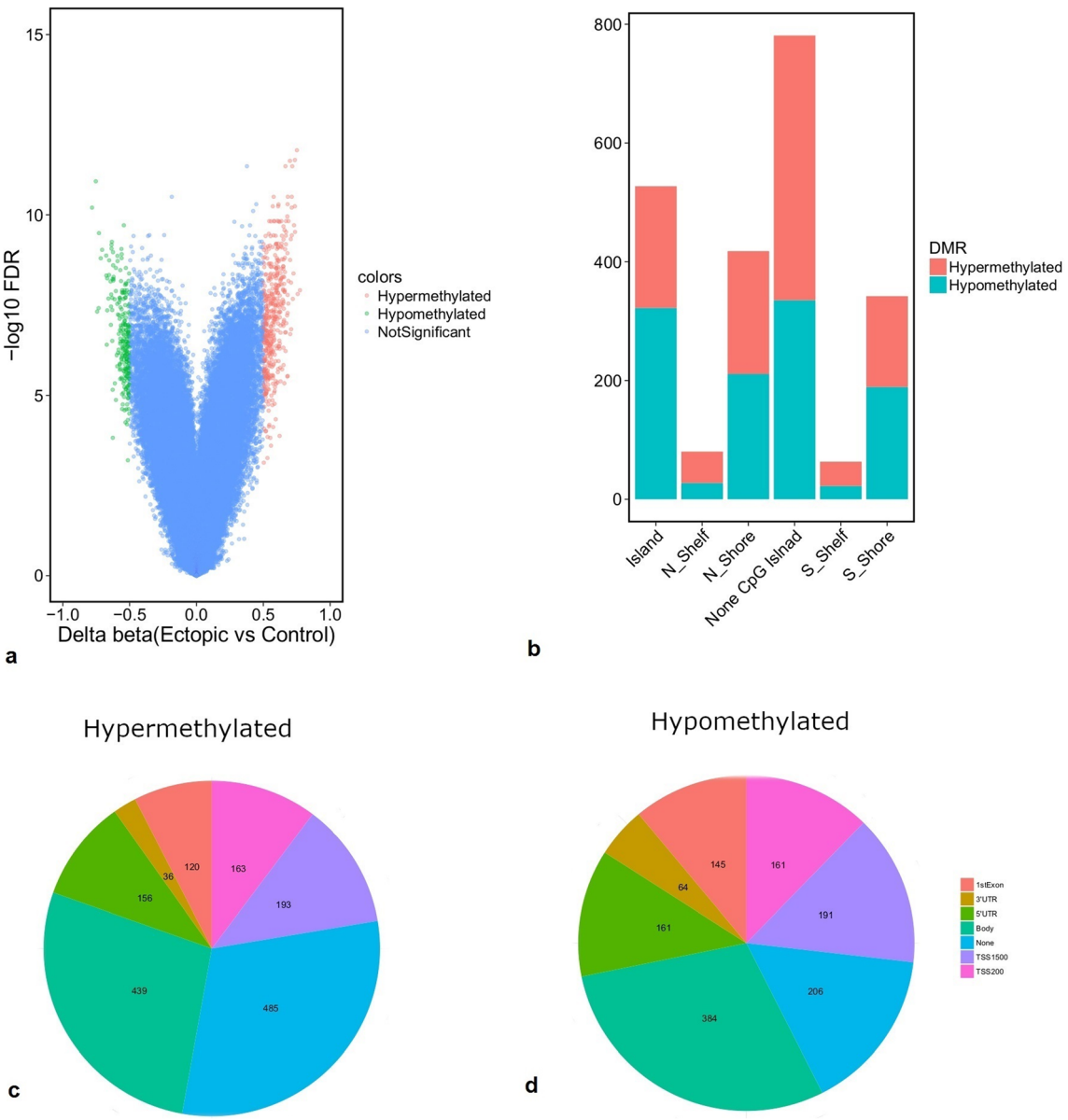

Fig. 2 DMR distribution of ectopic and control depicted in a volcano plot (**a**) where not significant regions are shown in blue color and hypermethylation and hypomethylation ones are shown in red and green. Pie charts of hyper- and hypomethylated CpGs depict genomic regions relative to gene sites (**c**, **d**) and bar plots show CpG context distributions (**b**)

absolute methylation degree > 0.2, and the number of methylated CpGs ≥ 3). However, we did not find any significant differentially methylated regions in eutopic vs. control comparisons, so we limited the analysis to DMPs in eutopic compared to control.

The volcano plot of DNA methylation between ectopic and control comparison is shown in Fig. 2a. Among all 2904 methylated CpGs, 1592 CpGs were hypermethylated and 1312 were hypomethylated. The most hypermethylated genes were SLC10A6, MACROD1, EMX2, and PRDM16 and the hypomethylated ones in ectopic were ZNF423, PPFIA1, ATP101A1, and PI3KCG. The genomic distribution of CpG sites (islands, shores, shelves) and gene structures and parts (3′ UTR, 5′UTR, first exon, TSS, body) influence the gene expression [9, 25]. In ectopic comparison to control samples, we identified a similar distribution of both hyper- and hypomethylated regions which most methylated regions were distributed in the gene body and intergenic parts. Promoter sites dedicate the third degree (Fig. 2c, d). Regions not located inside the CpG islands were more likely to undergo methylation. Shores and shelves are ordered after island parts in abnormal methylation, respectively (Fig. 2b).

The assessment distribution of the ectopic and eutopic comparison showed 1608 hypermethylated CpGs with most methylated genes as ADCY9, MACROD1, SLC10A6, and PRDM16 and 1684 hypomethylated CpGs that showed ZSCAN12L1, ZNF843, ZNF833, and ZNF808 with the most hypermethylation. Also, methylated CpG distribution is shown in Fig. 3. The differentially methylated CpGs had a similar methylation pattern in both island and gene structures

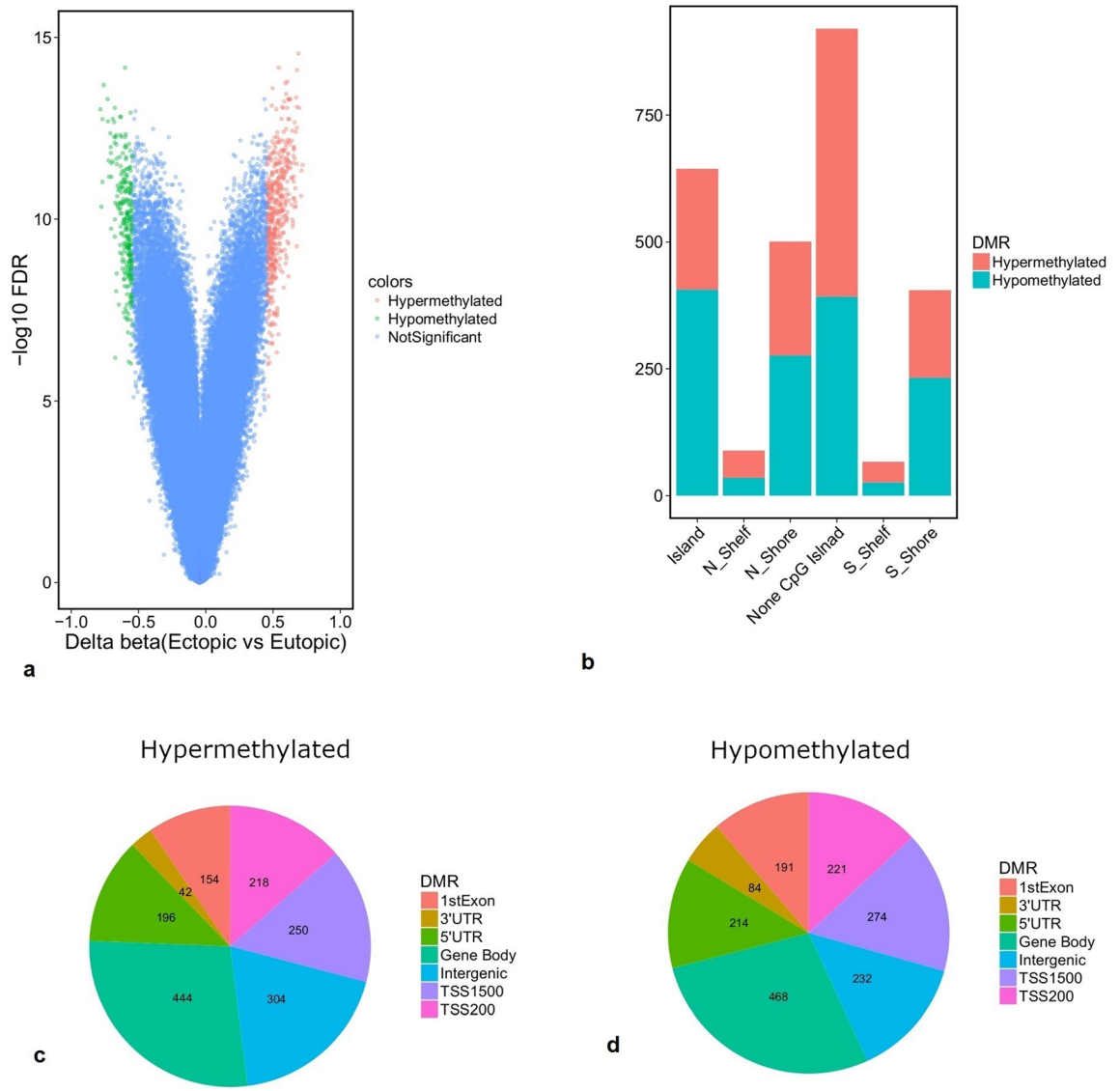

Fig. 3 DMR distribution of ectopic and eutopic depicted in a volcano plot (**a**) where not significant regions are shown in blue color and hypermethylation and hypomethylation ones are shown in red and green. Pie charts of hyper- and hypomethylated CpGs depict genomic regions relative to gene sites (**c**, **d**) and bar plots show CpG context distributions (**b**)

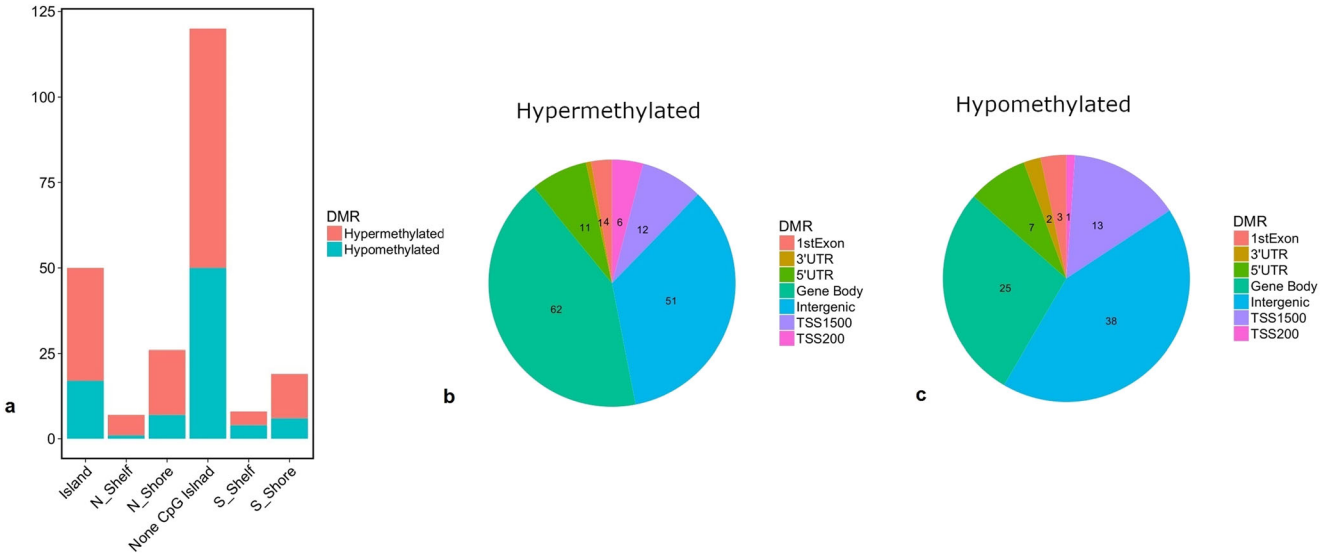

Fig. 4 Pie charts of hypermethylated (**b**) and hypomethylated (**c**) DMPs of eutopic and control comparisons depicted genomic regions relative to gene sites and bar plots (**a**) showed CpG context distributions

nearly with overmethylation in gene body and none of CpG islands and undermethylation in CGIs and shelves and 3′UTR. These observations indicate DNA methylation changes did not randomly occur and have a relation to genetic and CpG sites. As mentioned before, we did not find any significant DMRs (FDR < 0.05) in eutopic and control samples; it does not mean there are no differences between eutopic and control samples. There are a few genes with DMP which resulted in significant terms in the KEGG pathway analysis. Methylated genes associated with DMPs were categorized in 40 hypomethylated and 79 hypermethylated genes.

So, volcano plot was absent from eutopic and control sample study. CpG distribution in the gene context was surveyed based on DMP. Figure 4 shows how the regions with the differentially methylated position are distributed in eutopic in comparison to control. Also, we observed the most methylated regions are located in intergenic, gene body, and none of the CpG islands area.

Validation

To validate results and impact on methylation effects on gene expression, we focused on DNA methylation

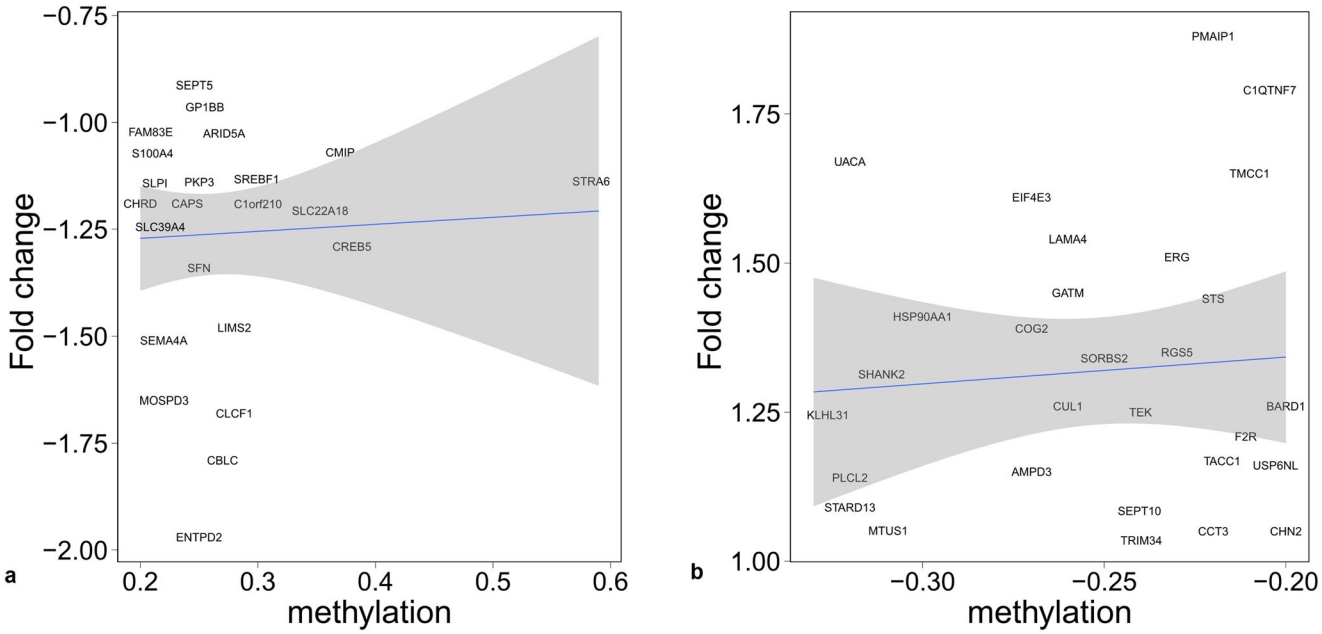

Fig. 5 Correlation of differentially methylated and differentially expressed genes in hypermethylated (**a**) and hypomethylated genes (**b**)

alternations of ectopic samples compared to control and mRNA expression data reported by Tamaresis et al. [26]. For this purpose, we calculated differentially expressed genes of a healthy patient and patient with endometriosis with absolute log fold change > 1 and adjusted p value ≤ 0.05. We then found the subscription between them and genes were methylated in the TSS regions. We reached 23 downregulated and 29 upregulated genes shared between them. Also, the KEGG pathway of shared genes showed consistent terms with endometriosis disease. To find the correlation between methylation and expression, we investigate the relevance of changes and their effect on expression. As Fig. 5 shows, hypermethylated genes resulted in a decrease of gene expression, and hypomethylated ones (Fig. 5b) were correlated with the upregulation of the genes.

GO and KEGG pathway analysis

To investigate possible biological functions of the identified differentially methylated regions, we performed Gene Ontology (GO) and pathway analysis of the genes associated with DMRs. The analysis revealed that 73% of the methylated genes in ectopic samples were related to the biological process, including developmental process, locomotion, regulation of signaling, and other terms (q value < 0.05) (Fig. 6a, b).

Pathway analysis of ectopic hypomethylated genes showed disruption in the PI3K-Akt signaling pathway (Fig. 7a). Alternations in ectopic vs. eutopic methylation were found in biological process terms associated with endometriosis development, such as biological adhesion, multicellular organismal process, response to a stimulus, cell proliferation, and the other terms (Fig. 6c, d). In pathway analysis, we found the terms relaxin signaling pathways, oxytocin signaling pathways, pancreatic secretion, HTLV-I infection, protein absorption, and digestion and the other terms illustrated in Fig. 7b, c. Indeed, we illustrated the main branch of the resulting biological terms and the subcategories of these branches had not been shown. On the other hand, genes associated with DMP (hypomethylated) occupied pathway terms in tuberculosis, inflammatory bowel disease (IBD), th17 cell differentiation, intestinal immune network of IgA production, Epstein-Bar virus infecting, toxoplasmosis, rheumatoid arthritis, leishmaniasis, etc. Go terms with at least 4 common genes were illustrated.

Discussion

Previous studies have reported abnormal DNA methylation changes as a mechanism of endometriosis development. Here, we used genome-wide methylation profiles to investigate alternations in the ectopic and eutopic lesions of patients compared to the endometrium of control women using the Infinium Human Methylation 450K BeadChip. The global methylation comparison showed a

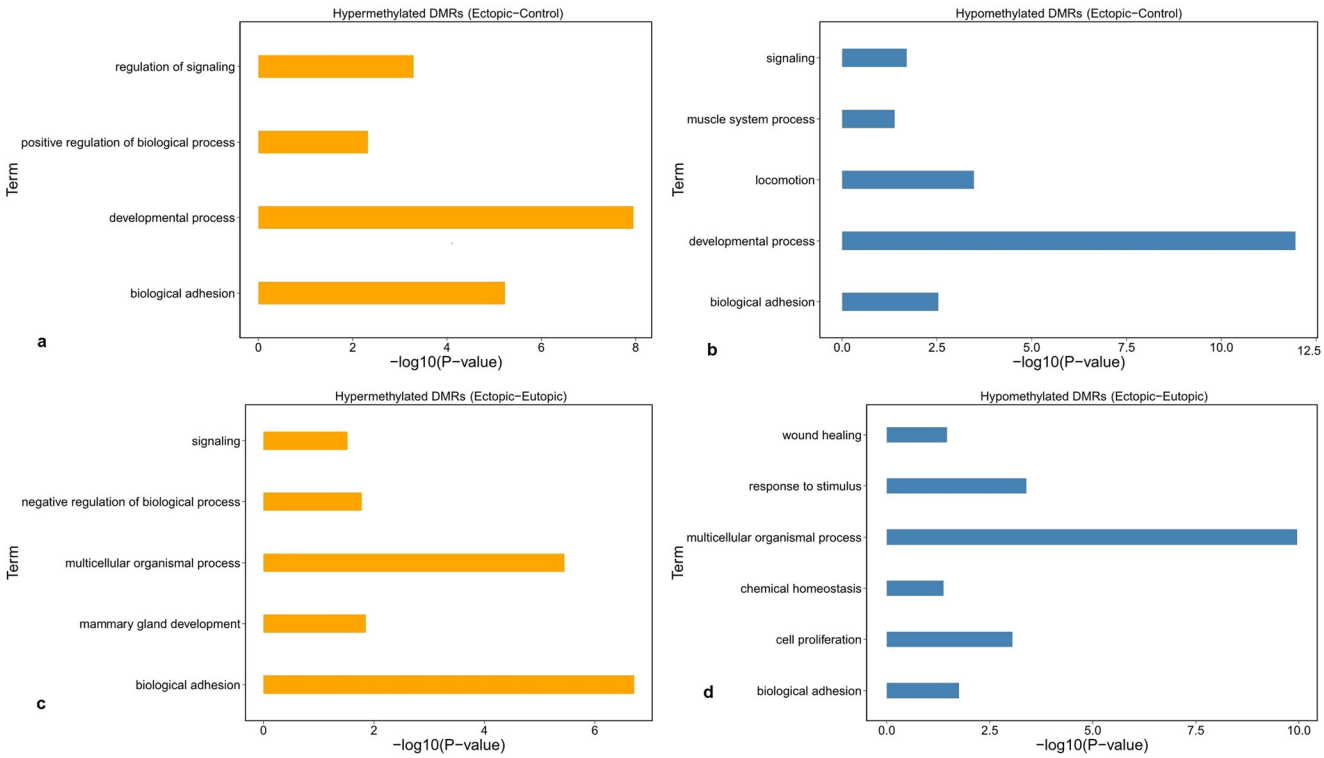

Fig. 6 Enrichment by gene ontology of hypermethylated (**a**) and hypomethylated (**b**) DMPs in ectopic compared to control and hypermethylated (**c**) and hypomethylated (**d**) in ectopic compared to eutopic

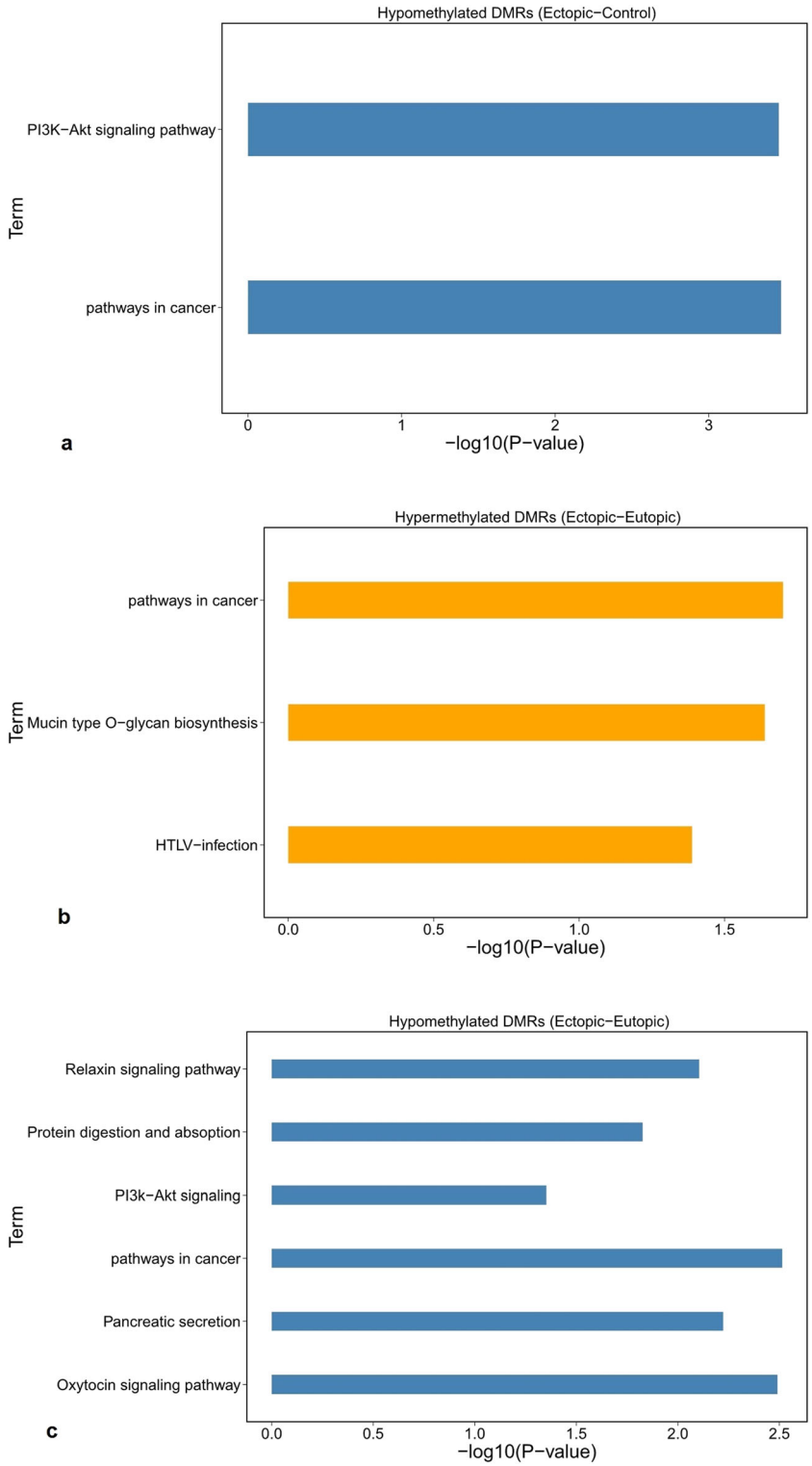

Fig. 7 Enrichment by pathway analysis of hypermethylated (**b**) and hypomethylated (**c**) genes in ectopic and eutopic comparison and hypomethylated ones in ectopic vs. control (**a**)

few significant DNA methylation differences between the endometria of patients and controls, while the large number of differentially methylated regions identified in control endometrial and ectopic lesions since variation between individual tissues is larger than within ones. Also, the endometrium is a heterogeneous glandular, composed of luminal, epithelial, and stromal cells with their own tissue type-specific DNA methylation signature [27], and therefore, the comparison between two really distinct tissues (lesions vs. endometrium) reflects tissue type differences. In the other site of views, the number of patients influences the significant changes

which are consistent with the results of Rahmioglu et al. [28].

Both hyper- and hypomethylated regions were observed in our comparisons. Abnormal methylations in regions relative to CpG islands and gene sites were potential targets associated with endometriosis development. The results of our study confirmed these regions are influenced by methylation changes, especially in intergenic and gene body sites. Also, the results revealed hypermethylation of the ESR1 promoter region, as we know endometriosis is an estrogen-dependent disease which steroid hormones regulate inflammatory environment leading to endometriosis development [29, 30]. Hypermethylation in candidate key genes (HOXC, HOXD, and WNT5) associated with proliferation and embryogenesis was observed too. Hox genes control the embryonic development by coding transcription factor while their dysfunction results in infertility by disrupting in embryonic development [31].

A study by Dyson et al. [32] compared ovarian endometrial stromal cells to eutopic endometrial stromal cells and found GATA family transcription factors with different methylations. We identified hypermethylation in GATA6 and hypomethylation in GATA2 and GATA3 in ectopic lesions which is consistent with the results of Dyson et al. On the other hand, in a recent study by Saare Merli, hypermethylation of SLC43A3 was noticed. We also found SLC10A6 with the most hypermethylation value in our results [33]. The other highly methylated genes were zinc finger family transcription factors, while methylation of zinc finger protein 681 was reported by Naqvi et al.

Gene ontology analysis showed methylated genes were enriched in the developmental process, multicellular organismal process, response to stimulus, and biological adhesions which indicate an abnormality in cell communication and signaling and its relevance in endometriosis establishment. Dysfunction of endometrial cells was proposed to be associated with the disruption in several pathways responsible for proliferation, angiogenesis, and cell growth. PI3K/Akt signaling pathways are involved in the growth and survival of cells [34]; its alternation plays a crucial role in endometrial cancer pathogenesis [34, 35]. Gene expression of women's endometrium with and without endometriosis showed activation of PI3K/Akt signaling pathways. Our results showed hypomethylation of receptor tyrosine kinase (RTK), extracellular matrix protein (ECM), and G protein-coupled receptors (GPCRs) in ectopic cells which lead to the upregulation of this stimulus contributing to the Akt activation. Overexpression of Akt is a key mechanism in promoting biological processes such as apoptosis, cell cycle, angiogenesis, and protein synthesis [36–38]. Another characteristic of endometriosis is adhesion. Hypermethylation of GALNT12, GALNT9, and GALNT6 in ectopic lesions activates core 2 of mucin-type *O*-glycan biosynthesis pathways which was reported to have a role in attenuating cell adhesion [39, 40]. An increase in the expression of GALNT leads to a chance for cell adhesion.

Though epigenetic abnormalities have a negative influence on women's lives and understanding these pathways' roles in endometriosis, especially focusing on pathways associated with endometriosis development may help to improve treatment modality. In this study, we only investigate DNA methylation effects of this disorder, but as we know, both genetics and epigenetics have influence on the endometriosis. Therefore, we think DNA methylation changes that correlated expression data could provide a better insight into this survey.

Conclusions

Emerging evidence indicates how the methylation influences the ectopic endometrium of women with endometriosis. So, understanding the methylation profiles in endometriosis patients can shed light on endometriosis development. Even though the etiology of endometriosis is unclear, the results of our analysis contribute to offering more information in this area. In this study, we profiled the DNA methylation of ectopic and eutopic lesions of patients referred to the Royan Institute. While DNA methylation changes between the endometria of women with and without endometriosis were similar, the numbers of DMRs between eutopic and endometrial tissue samples were considerable. The generalized results of our study show alternations in normal cell function such as proliferation, adhesion, signaling, and developmental process which can lead to endometriosis establishment. However, our results confirm that some of the prior findings provide new insights in endometriosis methylation changes and highlight several pathways as potential landmarks of the disease from an epigenetic perspective.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflicts of interest in the research.

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