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Genome-wide characterization of aberrant DNA methylation patterns and the potential clinical implications in patients with endometrial cancer

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ABSTRACT

Aberrant DNA methylation has been implicated in the development of the majority of human cancers. However, the association of aberrant DNA methylation with the clinical characteristics of uterine corpus endometrial carcinoma (UCEC) has not been fully explored. We performed an integrative analysis in order to examine the genome-wide DNA methylation profile and the gene expression profile of 432 UCEC samples and 46 normal tissue samples. A total of 793 differentially methylated regions (DMRs) that were associated with 472 protein-coding genes were identified, including 622 hypermethylated DMRs and 171 hypomethylated DMRs. These DMRs were capable of differentiating UCEC from normal tissues with an accuracy of 99.07% using an unsupervised hierarchical clustering method. The genomic analysis suggested that the hypermethylated DMRs were located in CpG island regions nearer to the transcription start site (TSS) compared with the hypomethylated DMRs. Functional analysis for genes associated with DMRs revealed an enrichment of methylated genes that were involved in key cancer-related biological processes and pathways, such as cell adhesion, cell differentiation and the cAMP signaling pathway. Finally, we performed a correlation analysis of the methylation levels of DMRs and patient survival time, and identified 130 DMRs. These molecular markers could classify patients into high-risk and low-risk groups with significantly different overall survival. Taken together, the present study revealed the potential applications of the detection of aberrant DNA methylation as a valuable prognostic marker for UCEC. The current findings may aid the therapeutic exploitation of UCEC treatment strategies and improve our understanding regarding the regulation of methylation in UCEC.

1. Introduction

Endometrial cancer (also referred to as uterine corpus endometrial carcinoma or UCEC) is one of the most commonly diagnosed gynecological cancers. It is estimated that it will become the 4th most commonly diagnosed cancer and the 6th leading cause of cancer death among females in the USA [27]. In China, the incidence and mortality rate of UCEC exhibits an increasing trend [13]. Surgical treatment followed by adjuvant therapy (radiation therapy and/or chemotherapy) are the standard treatment options for patients with UCEC [15]. The prognosis of UCEC patients varies greatly. For example, UCEC patients who are diagnosed at an early-stage exhibit a favorable prognosis with a 5-year survival rate of 85–90%. However, the overall survival of UCEC patients with recurrent and advanced disease (stage III and IV) remains poor [15]. Therefore, the identification of reliable molecular markers may aid the development of personalized therapies and improve clinical outcome.

Recent advances in molecular profiling have increased our understanding of the molecular etiology of UCEC and have suggested that UCEC is a heterogeneous disease characterized by distinct molecular, genetic and clinical factors. Several altered molecular features have been identified in UCEC, including genomic alterations (somatic alterations, copy number alteration and microsatellite instability), altered gene expression pattern (protein-coding genes, miRNAs and long non-coding RNAs) and epigenetic deregulation (DNA methylation and histone modification) [3,16,24]. In previous years, several molecular biomarkers and/or molecular signatures focusing on the detection of mRNA, miRNA and long non-coding RNAs have been proposed as indicators of diagnosis and prognosis in UCEC [2,11,31,37]. DNA methylation is a major type of epigenetic mechanism and plays important roles in normal development and cell functions by regulating gene expression [23]. Aberrant DNA methylation has been implicated in the

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pathogenesis of several types of human cancer and may contribute to tumorigenesis. Recently, altered DNA methylation patterns have been observed to be involved in UCEC, implying their potential as novel biomarkers for the diagnosis and prognosis of UCEC [28]. However, a limited number of studies have explored DNA methylation by a genome-wide scale approach with regard to its clinical significance in predicting prognosis in UCEC.

In the present study, we investigated the DNA methylation profile of UCEC by a genome-wide approach in a large cohort of 432 UCEC samples and 46 normal tissue samples. We identified aberrant DNA methylation as a potential prognostic biomarker for predicting the overall survival of patients with UCEC.

2. Materials and methods

2.1. Data sources

Genome-wide DNA methylation profiling of 478 tissue samples (including 432 UCEC samples and 46 matched tumor-adjacent normal tissue samples) based on Illumina HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA) were derived from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/). Gene expression profiles of 582 samples generated using RNA-seq platforms were obtained from UCSC Xena (https://xena.ucsc.edu/), including 381 samples based on Illumina Genome Analyzer and 201 samples based on Illumina HiSeq 2000. The clinical information of the UCEC samples was available from the GDC Data Portal (https://portal.gdc.cancer.gov/), which included 431 primary tumors, 1 recurrent tumor and 46 solid normal tissues.

2.2. Preprocessing and normalization of methylation data

Raw methylation data files (IDAT files) were derived from the Illumina HumanMethylation450 BeadChip and were preprocessed using the 'minfi' package [1]. The data were further processed by subset-quantile within array normalization (SWAN) [18], including probe filtering, color bias correction, background subtraction and subset quantile normalization [8]. The probes with a detection P-value higher than 0.01 ($P > 0.01$) in one or more regions, or in locations of the X and Y chromosomes were excluded [9]. Furthermore, the probes with SNPs located in the 10 last bases and/or with two or more SNPs in the probe sequence were excluded from further analysis [9].

2.3. Identification of differentially methylated regions (DMRs)

Differentially methylated probes (DMPs) were detected using 'minfi' package as those with a Benjamini and Hochberg adjusted P-value that was lower than 0.001 ($P < 0.001$) and beta value higher than and/or equal to 0.25 ($B \geq 0.25$) [25]. DMRs were identified as differentially methylated regions between UCEC and adjacent normal tissues according to the following parameters: $B = 100$, cut off $= 0.25$, length $= 200$ bp and probe number $= \geq 5$. The methylation levels of DMRs were estimated using the average methylation levels of multiple CpG probes that were mapped to this DMR. Genomic distribution characteristics and functional interpretation of cis-regulatory regions of DMRs were analyzed using Genomic Regions Enrichment of Annotations Tool (GREAT, version 3.0.0) [19] and 'ChIPseeker' package (version 1.5.1) [33].

2.4. Clustering analysis

Hierarchical clustering of the methylation levels of DMRs was conducted for UCEC samples and adjacent normal tissue samples using the R function "hclust" with the Euclidean distance and complete linkage clustering method.

2.5. Statistical analysis

The differences in gene expression between UCEC and adjacent normal tissues were determined by a two-tailed Student's t-test. The genes with a P-value lower than 0.05 ($P < 0.05$) and a fold change higher than 2 (> 2.0) were considered to be differentially expressed. The correlation between DNA methylation levels and gene expression levels was measured using the Pearson correlation coefficient if a CpG was within 100 kb of the TSS of a gene according to a previous study [8]. The following formula was used:

$$\mathrm{PCC}(dmr,\ gene) = \frac{1}{n-1}\sum_{i-1}^{n}\left(\frac{e(dmr,\ i) - \overline{e(dmr)}}{\sigma(dmr)} \times \frac{e(gene) - \overline{e(gene)}}{\sigma(gene)}\right)$$

Where e(gene, i) represents the gene expression of sample i; $\sigma(gene)$ is the standardization of gene expression of n samples; e(dmr, i) represents the methylation levels of DMR in sample i; $\sigma(dmr)$ represents the standardization of the methylation levels of DMR of n samples. An association was considered significant if the P-value was smaller than 0.05. The differences in the overall survival between patient groups with high-methylated DMRs or low-methylated DMRs were assessed by Kaplan-Meier curves and the log-rank test method using 'Survival' package.

2.6. Functional enrichment analyses of DMRs

Gene ontology (GO) enrichment analysis for genes that were co-expressed with DMRs was performed to identify over-represented GO terms using the cluster Profiler [32] to the following three categories: biological processes, molecular function and cellular component. KEGG pathway enrichment analysis for genes co-expressed with DMRs was used to identify statistically significantly enriched pathways using the KOBAS 3.0 software [29].

3. Results

3.1. Identification of DMRs between UCEC and adjacent normal tissues

Following the removal of the probes that were affected by SNPs, 467,971 of 485,577 loci were retained for further analysis. Differential methylation analysis was performed to identify DMRs between UCEC and adjacent normal tissues using minfi package as described in the Materials and methods section. A total of 793 DMRs were identified in the UCEC samples compared with the normal tissue samples, including 622 hypermethylated DMRs and 171 hypomethylated DMRs. Unsupervised hierarchical clustering analysis indicated that UCEC and adjacent normal tissues could be clearly separated according to the methylation levels of the 793 DMRs, which was applicable to 99.07% (428/432) of the UCEC samples (Fig. 1A).

3.2. Genomic characteristics of differentially methylated regions between UCEC and adjacent normal tissues

We initially investigated the genomic distribution of 793 DMRs and demonstrated that both hyper- and hypomethylated DMRs were predominantly located at the gene regulatory or genetic regions, such as the 5′UTR, 1st exon and promoter regions (Fig. 2A). The proportions of the 793 DMRs that were measured by the location relative to the transcriptional start site (TSS) were also presented in Fig. 2B. The hypermethylated DMRs tended to accumulate in regions nearer to the TSS compared with the hypomethylated DMRs (Fig. 2B). We further analyzed the distribution features of the CpG island of 793 DMRs based on the CpG island annotations provided by Illumina under GPL13534 [5]. The analysis indicated that the majority of the 622 hypermethylated

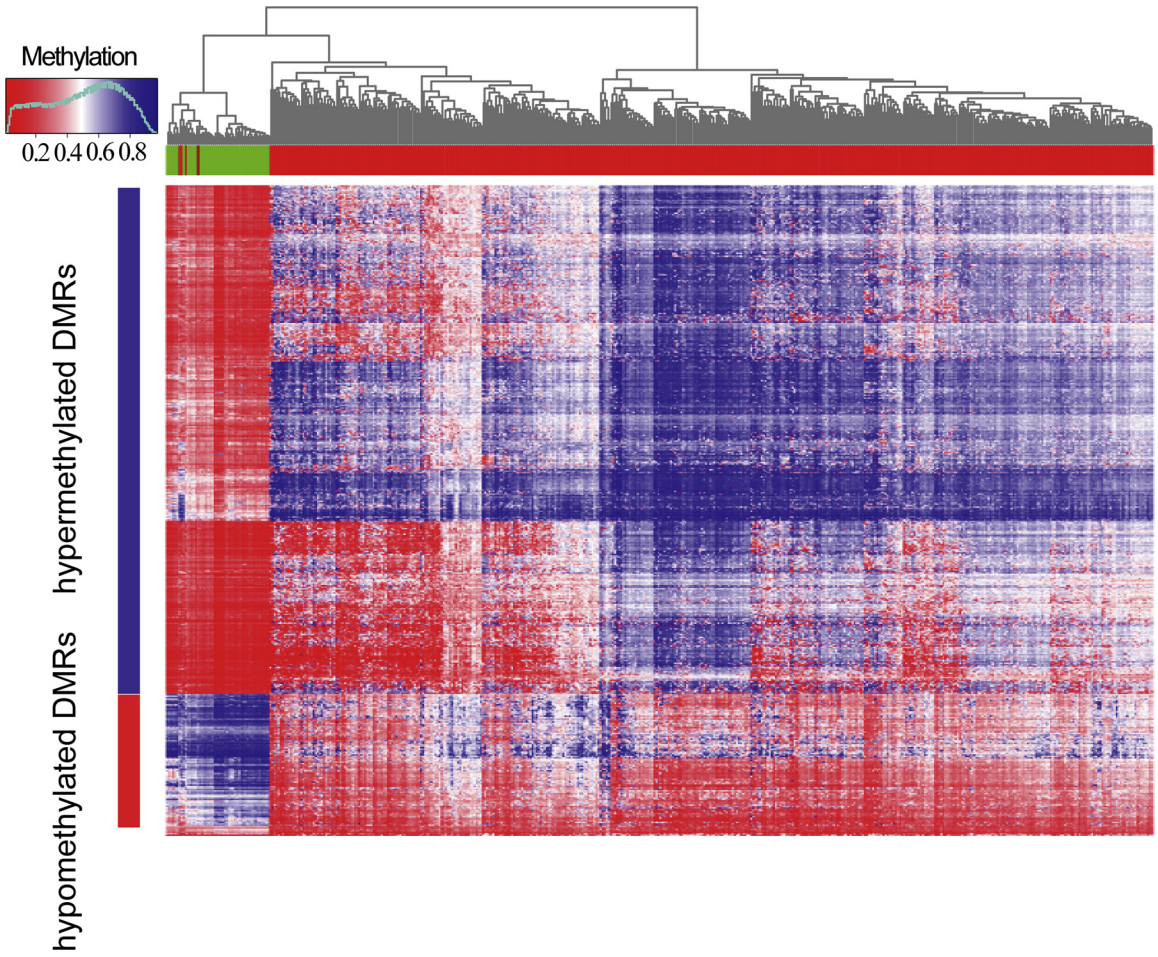

Fig. 1. Hierarchical clustering heatmap of 478 tissue samples based on the methylation levels of 793 DMRs. Green branches represent normal tissue samples and red branches represent the UCEC samples (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

DMRs were located in genomic regions with high GC content, such as CpG islands, followed by the “North Shore” and the “South Shore” regions, while 171 hypomethylated DMRs were mainly located in genomic regions with low GC content, such as “Open Seas”, followed by the CpG islands (Fig. 2C). To determine significant differences in evolutionary conservation between hypermethylated DMRs and hypomethylated DMRs, we computed the average evolutionary conservation score for hypermethylated DMRs and hypomethylated DMRs based on the base-wise conservation score derived from multiz alignment of 99 vertebrates. The data indicated no significant differences in evolutionary conservation between hypermethylated DMRs and hypomethylated DMRs (Fig. 2D).

3.3. Functional characterization of differentially methylated regions

To investigate which biological functions and pathways were involved in expression of the DMRs, we initially identified 472 genes that were associated with the hypermethylated DMRs and 117 genes that were associated with the hypomethylated DMRs. Subsequently, we performed functional enrichment analysis for genes associated with DMRs. The genes that were associated with DMRs were related to several GO terms including cell adhesion and cell differentiation (Fig. 3A), and four KEGG pathways including neuroactive ligand-receptor interaction, glutamatergic synapse, cAMP signaling pathway and Nicotine addiction (Fig. 3B).

3.4. Analysis of transcriptional regulatory patterns of differentially methylated regions

To investigate potential transcriptional regulatory patterns of differentially methylated regions, we analyzed the Pearson correlation of the expression levels of the genes under examination with the methylation levels of the DMRs for 464 samples. Based on the gene expression and DNA methylation profiles, we selected 38 gene-DMR pairs that ranked in the top 50% with regard to the methylation status for further analysis. Unsupervised hierarchical clustering analysis indicated that the majority of DMRs in gene-DMR pairs (86.84%, 33/38) were hypermethylated in UCEC compared with adjacent tissues and that their corresponding 38 genes in gene-DMR pairs were significantly downregulated in UCEC compared with adjacent normal tissues (Fig. 4A and B). For example, negative correlations were noted between DMR165 and IFFO1 ($P < 0.001$, Fig. 4C), and between DMR14 and SFN ($P < 0.001$, Fig. 4D). Positive correlations were noted between DMR312 and C17orf93 ($P < 0.001$, Fig. 4E).

3.5. Identification of potential methylation markers for survival prediction in UCEC patients

To further investigate whether these DMRs were associated with the clinical progression of UCEC and could be used as potential biomarkers for survival prediction, we sought to discern the association between DMRs and the clinical outcome of patients with UCEC. All patients with survival information were divided into two patient groups with high methylation or low methylation status using the median of methylation

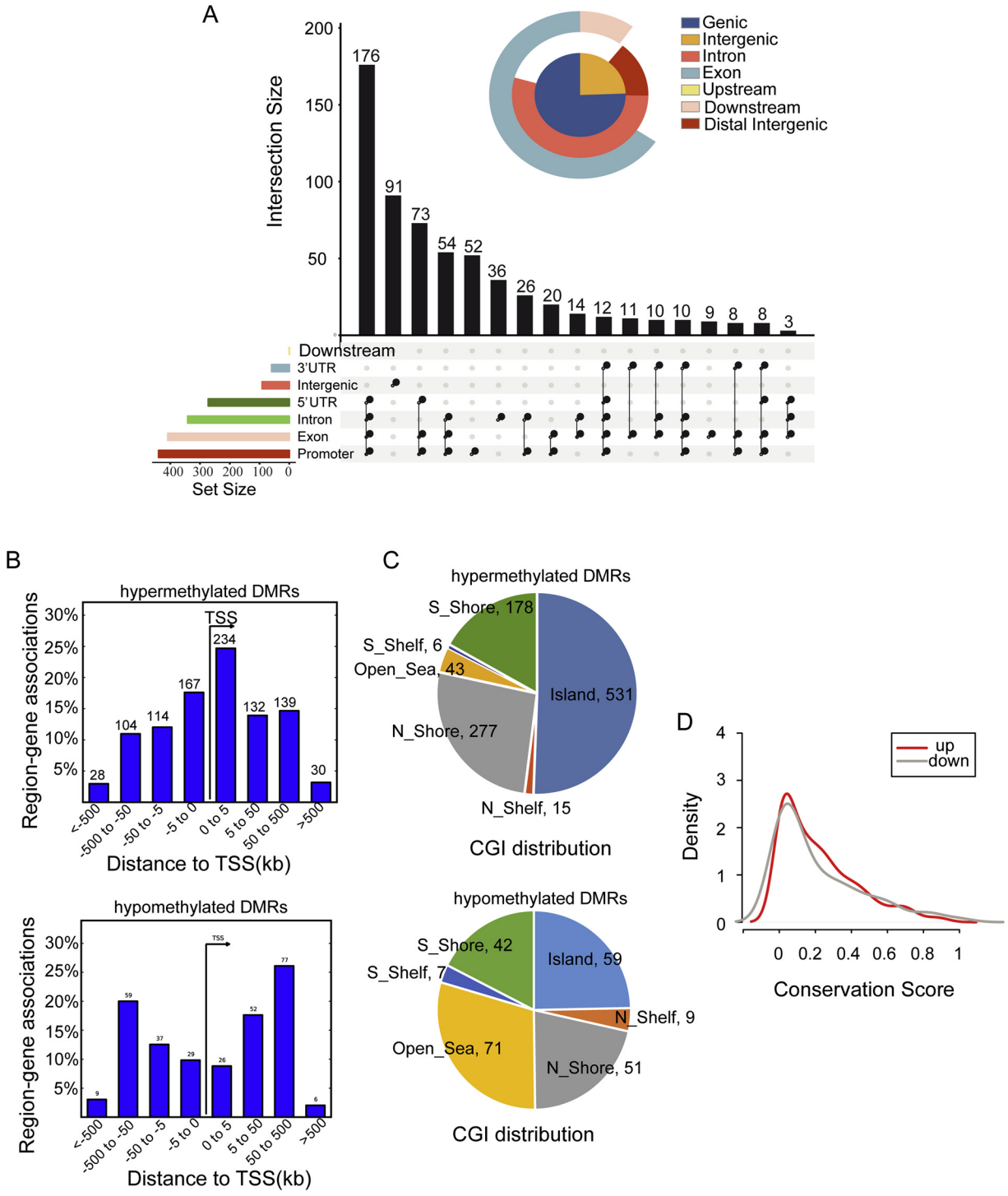

Fig. 2. Genomic distribution of 793 DMRs in CpG island and other parts of the genome. (A) The genomic distribution of 793 DMRs across different genomic regions. (B) The genomic distribution of hypomethylated and hypermethylated DMRs with regard to the TSS. (C) Pie charts of hypomethylated and hypermethylated DMRs according to the CpG islands. (D) Evolutionary conservation between hypermethylated DMRs and hypomethylated DMRs.

levels. Subsequently, we compared the differences in overall survival between the two patient groups with high methylation or low methylation status using Kaplan-Meier curves and the log-rank test method. Survival analysis demonstrated that 130 of 793 DMRs were significantly associated with patient overall survival, including 113 hypermethylated DMRs and 17 hypomethylated DMRs. Based on this evidence, the patients could be classified into high-risk and low-risk groups with significantly different overall survival rates. For example, patients with low-methylation of two hypermethylated DMRs (DMR165 and DMR312) exhibited longer overall survival rates than those with high methylation of the corresponding DMRs (P = 0.008 for DMR165 and P = 0.003 for DMR312, Fig. 5A and B), whereas low methylation of DMR120 strongly correlated with poor disease outcome (P = 0.011, Fig. 5C).

4. Discussion

Recent advances in omics have demonstrated that UCEC is a

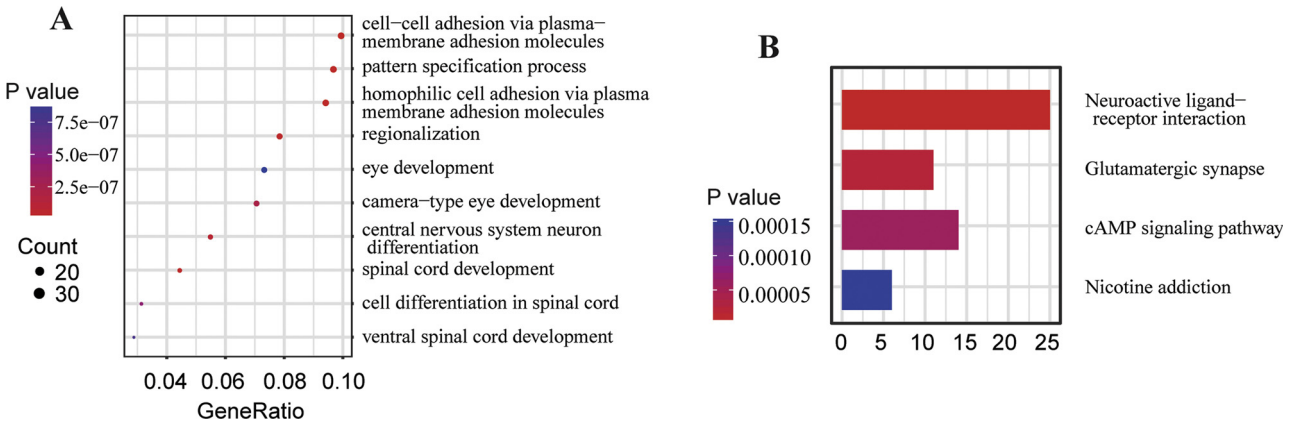

Fig. 3. Functional enrichment analysis. (A) Enriched GO terms. (B) Enriched KEGG pathways. The color represents the ranges of the enriched P value. The count represents the number of genes associated with the DMRs that match the KEGG pathway and is indicated by the circle area. The geneRatio represents the amount of genes associated with DMRs enriched in the GO category.

complex disease characterized by abnormal genetic and epigenetic changes, as well as environmental factors [28]. Despite numerous efforts to improve our understanding of the molecular mechanism of UCEC, its clinical outcome in patients is still not satisfactory and varies considerably due to the molecular heterogeneity of UCEC. Several studies have focused on identifying potential molecular biomarkers for the diagnosis and prognosis of patients with UCEC. It is well known that aberrant DNA methylation is the best-known epigenetic event

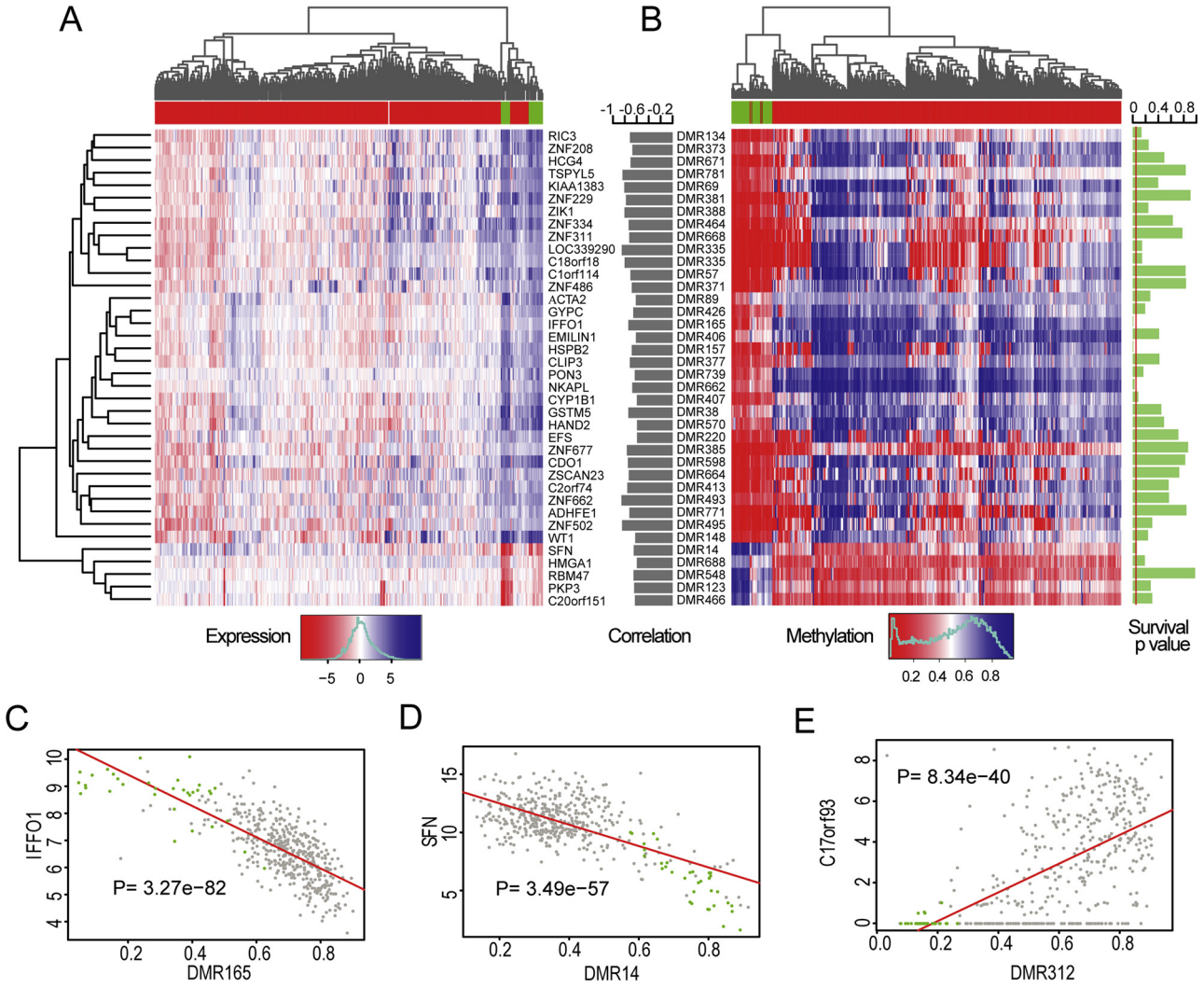

Fig. 4. Transcriptional regulatory patterns of DMRs. (A) Hierarchical clustering heatmap of 478 tissue samples based on the expression levels of 38 genes in gene-DMR pairs. (B) Hierarchical clustering heatmap of 478 tissue samples based on the methylation levels of DMRs in gene-DMR pairs. (C) Correlation of DMR165 and IFFO1. (D) Correlation of DMR14 and SFN. (E) Correlation of DMR312 and C17orf93.

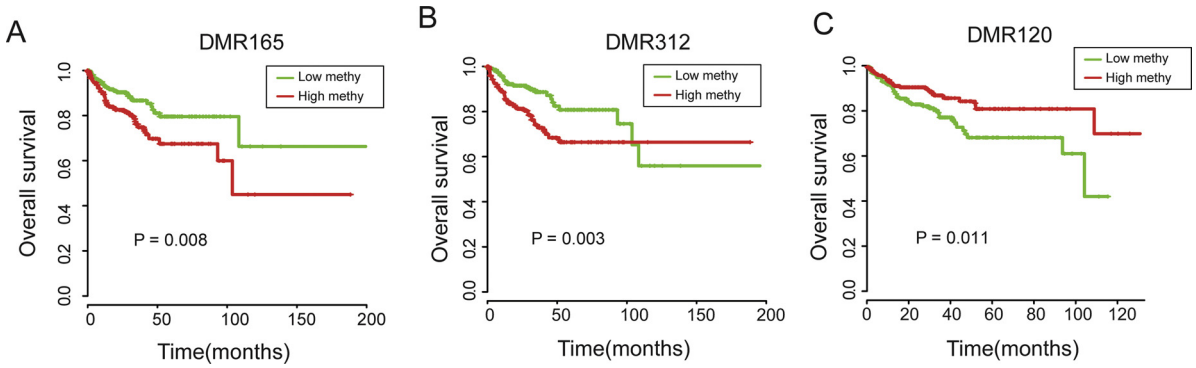

Fig. 5. Kaplan-Meier survival curves using 3 prognostic methylation markers for patients with UCEC. (A) The assessment of patient survival based on DMR165 methylation levels as determined by Kaplan-Meier curves. Patients with low-methylation levels of DMR165 exhibited longer overall survival than those with high-methylation levels of DMR165 (P = 0.008, log-rank test). (B) The assessment of patient survival based on DMR312 methylation levels as determined by Kaplan-Meier curves. Patients with low-methylation levels of DMR312 exhibited longer overall survival than those with high-methylation levels of DMR312 (P = 0.003, log-rank test). (C) The assessment of patient survival based on DMR120 methylation levels as determined by Kaplan-Meier curves. The patients with low-methylation levels of DMR120 exhibited poor clinical outcome of the disease than those with high-methylation levels of DMR120 (P = 0.011, log-rank test).

contributing to cancer development and progression [4,26]. Furthermore, it has been suggested that aberrant DNA methylation is cell-specific and cancer-type-specific and that it is easily detected in the plasma or serum of cancer patients [10]. This indicates its potential as a novel promising biomarker for the diagnosis and prognosis of patients with UCEC [12,17,20,30,40].

In the present study, we performed differential methylation analysis in a large cohort of 432 UCEC samples and 46 normal tissue samples and observed a significant difference in DNA methylation between UCEC and normal tissue samples. The data provided further supporting evidence that aberrant DNA methylation contributed to the development of UCEC. We identified 793 differentially methylated regions (DMRs) in UCEC vs. normal tissues, which is highly significant for the detection of UCEC. A significant difference in the genomic distribution between hyper- and hypomethylated DMRs was noted, implying their different functional roles in the development of UCEC. Hypermethylated DMRs were found preferentially in CpG islands. Previous studies have suggested that promoter CpG islands are usually unmethylated in somatic cells, whereas they frequently gain methylation during cancer development leading to the repression of the associated tumor suppressor genes, which is a hallmark of cancer [6,14].

To explore the potential functional roles of these molecular markers, 589 protein-coding genes associated with hypermethylated and hypomethylated DMRs were obtained by integrating analysis of methylation and gene expression profiles. The GO and pathway enrichment analysis of protein-coding genes associated with DMRs indicated that hypermethylated DMRs were frequently enriched in several GO terms and KEGG pathways compared with hypomethylated DMRs. Functional analysis of hypermethylated DMRs identified a group of biological processes and pathways that were functionally involved in cell adhesion, neuroactive ligand-receptor interaction, the glutamatergic synapse and cAMP signaling. It is well known that various cell adhesion molecules function as tumor suppressors and that their repression, which is caused by hypermethylation of CPG islands, contributes to the development of UCEC [21]. Previous studies have suggested that the cyclic AMP (cAMP) signaling pathway is an important cell signaling pathway, and alterations in the activation of this pathway have been implicated in various types of cancer [7]. The data of the present study demonstrated that aberrant DNA pattern methylation resulted in the perturbation of the core related biological processes and signaling pathways contributing to UCEC. Therefore, we further investigated the prognostic value of these DMRs and identified 130 DMRs that were associated with overall survival. These regions could be considered as candidate prognostic markers for patients with UCEC. Previous studies have reported specific genetic or molecular factors (including genomic alterations, altered miRNA or long non-coding RNA expression levels) that could affect the clinical outcome of patients with UCEC [16,22,34–39]. Altered DNA methylation is considered an early event during UCEC development and progression. This process exhibits higher clinical sensitivity and dynamic range compared with other types of prognostic markers [13]. Therefore, the prognostic DMRs identified in the current study may be useful in improving risk stratification in combination with other genetic and molecular markers that are used to determine prognosis in UCEC.

In conclusion, we conducted a genome-wide analysis of DNA methylation and compared the gene expression profiles in 432 UCEC samples and 46 normal tissue samples. We identified aberrant DNA methylation regions in UCEC compared with normal tissues, which have been implicated in specific key cancer-signaling pathways and also appeared to have underlying implications for UCEC carcinogenesis. In addition, we analyzed genomic characteristics of these aberrant DMRs using publicly available expression and epigenetic profiles, and identified those that were associated with overall patient survival. The selected DMRs may be used as prognostic markers for UCEC. However, the prognostic value of these candidate prognostic markers needs to be further validated in additional independent datasets. Nevertheless, the present findings overall revealed the potential of aberrant DNA methylation detection as a candidate prognostic and therapeutic application in UCEC. The data may further improve our understanding with regard to the regulation of gene methylation in UCEC.

Funding

Not applicable.

Availability of data and material

The data used and analyzed during the current study are available from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/), UCSC Xena (https://xena.ucsc.edu/) and GDC Data Portal (https://portal.gdc.cancer.gov/).

Authors' contributions

GL conceived and designed the experiments; YW, DDL, XJ and HWS performed the experiments and analyzed the data; YW wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This research was completed in compliance with the Helsinki Declaration. The data collection and analysis were carried out without disclosing patients' identities.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

Not applicable.

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