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Research Article

Identification of Differentially Methylated Genes as Potential Biomarkers for Early Diagnosis and Prognosis of Endometrial Cancer

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Abstract:

Objective: To identify methylated gene loci associated with uterine corpus endometrial carcinoma (UCEC) and evaluate their diagnostic and prognostic potential.

Methods: mRNA expression and DNA methylation data for UCEC were obtained from TCGA. Differential expression and methylation analyses were performed on eight candidate genes. Hypermethylated CG loci were screened based on logFC > 0.4 and assessed for diagnostic performance (sensitivity, specificity, AUC). The correlation between methylation and patient survival was analyzed using Kaplan-Meier curves. Methylation status was validated in normal (293T) and UCEC cell lines (ISHIKAWA, KLE).

Results: Six genes (AJAP1, GALR1, ZNF486, ZSCAN12, ZSCAN23, and CDO1) were significantly downregulated and hypermethylated in UCEC tumors. Key hypermethylated CG loci demonstrated high specificity (>95%) and AUC values (>80%) for distinguishing tumors and were significantly associated with patient survival. Experimental validation confirmed significantly elevated methylation levels for AJAP1, CDO1, GALR1, and ZSCAN12 in UCEC cell lines.

Conclusion: The identified methylation sites in AJAP1, GALR1, ZNF486, ZSCAN12, ZSCAN23, and CDO1 are closely associated with UCEC pathogenesis and prognosis, showing promise as diagnostic and prognostic biomarkers.

Keywords Endometrial Carcinoma, Methylation, Screen, Diagnosis

Introduction

Uterine corpus endometrial carcinoma (UCEC) is one of the most common malignant tumors of the female reproductive system, with its morbidity and mortality rates increasing worldwide[1]. Despite advances in early diagnosis and therapeutic approaches, the clinical management of UCEC still faces many challenges due to its complex molecular mechanisms and heterogeneity[2-4]. Efficient and precise biomarkers for early screening and individualized treatment of tumors are lacking[5-7]. Therefore, exploring new molecular markers, especially gene methylation markers that are closely related to tumorigenesis, progression, and prognosis, is important for early diagnosis, prognostic assessment, and development of therapeutic strategies for UCEC.

DNA methylation is an important epigenetic modification that regulates gene expression without altering gene sequences[8]. Many studies have shown that the methylation pattern of DNA in tumor cells is aberrant; in particular, the promoter regions of tumor suppressor genes are often hypermethylated, leading to silencing their expression[9,10]. Therefore, gene methylation is considered one of the key events in tumorigenesis, and changes in methylation levels are often closely associated with tumorigenesis, progression, and metastasis[11,12]. Gene

methylation has been proposed as a potential diagnostic and prognostic marker in UCEC. However, systematic analyses of specific genes and their methylation sites remain limited[13-15].

In this study, for the first time, we systematically integrated mRNA expression and DNA methylation data from patients diagnosed with UCEC into The Cancer Genome Atlas (TCGA) database, identifying differentially expressed genes and their hypermethylated sites that are closely related to the development of endometrial cancer from a multilevel, genome-wide perspective. This study provides a comprehensive molecular map for understanding the epigenetic regulatory mechanisms of endometrial cancer. This study attempts to address the limitations of previous database analyses and verifies the hypermethylation status of AJAP1, CDO1, GALR1, and ZSCAN12 genes in endometrial cancer cell lines and normal cell lines through the qMSP assay, which clearly supports the reliability of the results of the biosignature analysis and greatly enhances the credibility and persuasiveness of the study. By pinpointing specific CG loci, this study identified gene-level differences and delved into the regulation of methylation at the locus level, providing a basis for the development of methylation-targeted early diagnostic tools.

This study aimed to screen differentially methylated genes in UCEC by mining TCGA data and evaluate their potential for early diagnosis and prognosis prediction, with the expectation of providing a theoretical basis and practical guidance for clinical diagnosis, prognosis assessment, and individualized treatment of endometrial cancer.

Results

Differential expression analysis

Differential expression analysis of eight genes (AJAP1, CELF4, GALR1, IRX2, ZNF486, ZSCAN12, ZSCAN23, and CDO1) in TCGA UCEC data showed that between the tumor and normal groups, AJAP1, GALR1, ZNF486, ZSCAN12, ZSCAN23, and CDO1 had significantly low mRNA levels ($p < 0.05$), while the differences in CELF4 and IRX2 were not significant (**Figure 1**). Data analysis in **Table 1** and Figure 2 showed that in patients with UCEC, the CDO1 AUC was 95.4%, with 100% specificity and a positive predictive value (PPV). AJAP1 and ZSCAN12 had high diagnostic ability, with an AUC of 88.4% and 88.9%, respectively, performing well in terms of sensitivity and PPV. ZNF486 showed high positive predictive ability (100%) but a lower negative predictive value (NPV). In contrast, CELF4 (AUC, 57.0%) and IRX2 (AUC, 55.1%) showed lower sensitivity and poorer diagnostic performance.

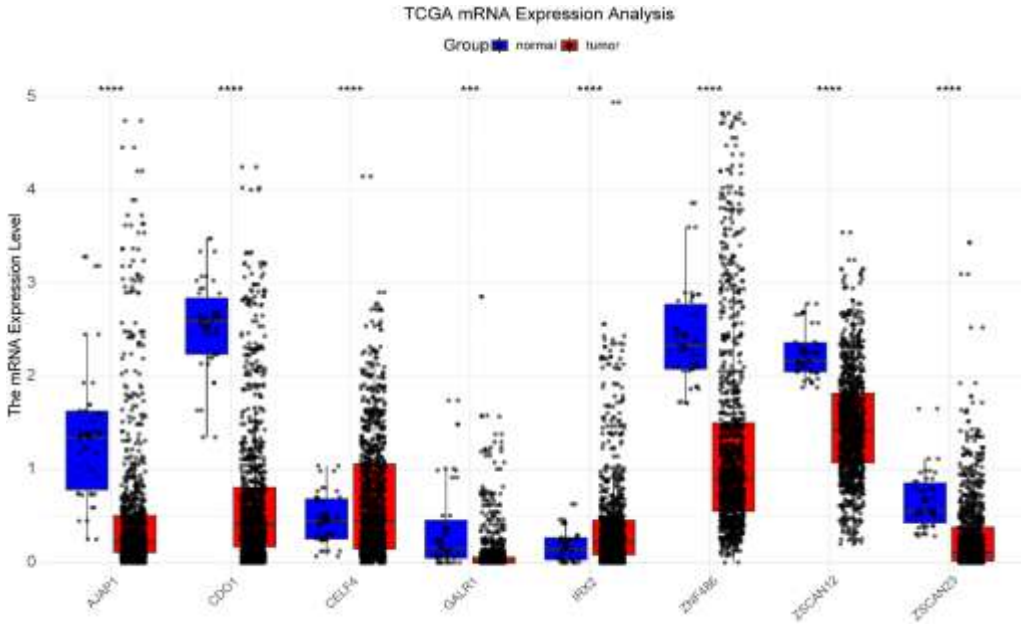

Figure.1 TCGA mRNA Expression Analysis
TCGA: The Cancer Genome Atlas

Table 1 Performance of different genes for classifying UCEC and normal tissues.

Gene	AUC	Sensitivity	Specificity	PPV	NPV
AJAP1	88.4	83.3	88.6	99.1	25.2
CDO1	95.4	86.4	100.0	100.0	31.8
CELF4	57.0	34.2	94.3	98.9	17.8
GALR1	68.3	72.9	62.9	96.6	12.9
IRX2	55.1	24.2	91.4	97.8	97.1
ZNF486	86.9	78.4	100.0	100.0	22.7
ZSCAN12	88.9	78.5	97.1	99.8	22.4
ZSCAN23	82.3	68.2	97.1	99.7	16.3

AJAP1: Adherens Junction Associated Protein 1; CDO1: Cysteine Dioxygenase Type 1; CELF4: CUGBP Elav-Like Family Member 4; GALR1: Galanin Receptor 1; IRX2: Iroquois Homeobox 2; ZNF486: Zinc Finger Protein 486; ZSCAN12: Zinc Finger and SCAN Domain Containing 12; ZSCAN23: Zinc Finger and SCAN Domain Containing 23; UCEC: uterine corpus endometrial carcinoma; PPV: Positive Predictive Value; NPV: Negative Predictive Value

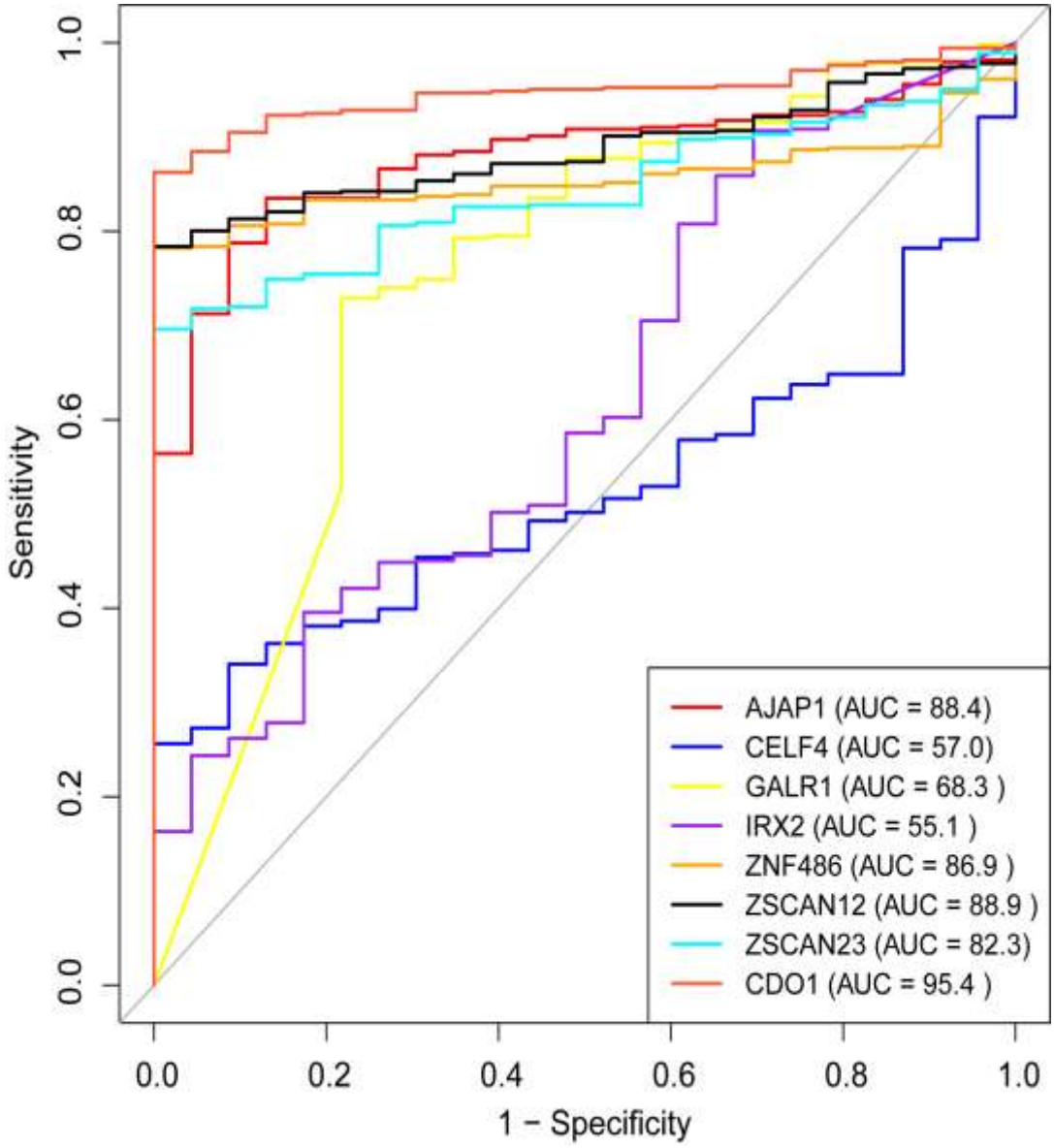

Figure.2 ROC curves of the mean of the copy number of genes on TCGA datasets.
ROC: Receiver Operating Characteristic.

Differential Methylation Analysis of CG Sites

Further analysis of the methylation levels of specific CG sites of these genes revealed that each gene contained multiple differentially methylated CG sites, all of which were significantly higher in the tumor group than in the normal group (**Supplementary Figure 1**). **Supplementary Figure 2** shows the locations of individual CG sites in the genes.

Clinical Performance Analysis

Based on differential methylation analysis, CG sites with more concentrated methylation positions were selected, and multiple hypermethylated sites were screened based on a logFC value > 0.4. The AUC values of each AJAP1 CG site exceeded 85%, with the highest being 92.7% (cg15484532), which showed high accuracy with a specificity of 100.0%, indicating extremely high reliability in excluding non-target samples; however, the Positive Predictive Value (NPV) was low (as low as 29.3) (**Table 2, Figure 3**). The CDO1 AUC values ranged from 96.7 to 97.1 (AUC of 97.1 for cg23180938); specificity and PPV were both 100.0%, whereas the NPV was relatively high (NPV of cg16707405 was 71.9%). The GALR1 AUC values were low, ranging from 81.8% to 85.5%, indicating moderate diagnostic performance, while specificity remained at 100.0% and sensitivity was low (e.g., cg03502002's sensitivity was only 61.6%). IRX2 AUC values ranged from 97.1 to 98.9% (cg26333652 AUC 98.9), sensitivity and specificity were close to or reached 100.0%, and the NPV was as high as 75.4% (cg26333652). Both ZNF486 and ZSCAN12 showed high diagnostic performance, with AUC values ranging from 94.5% to 96.0%; specificity and PPV remained at 100.0%, and sensitivity and Negative Predictive Value (NPV) were slightly lower than those of IRX2. ZSCAN23 had an AUC value of 84.4% (cg00651523), sensitivity of 75.2%, and specificity of 97.8% (**Table 2, Figure 3**).

Table 2 Performance of different methylation CGs for classifying UCEC and normal tissues.

Gene	CpG site	AUC	Sensitivity	Specificity	PPV	NPV
AJAP1	cg11835068	91.7	83.6	100.0	100.0	39.3
	cg13495205	92.4	87.0	95.7	99.5	44.0
	cg15484532	92.7	85.2	100.0	100.0	41.8
	cg17525406	85.6	74.3	100.0	100.0	29.3
CDO1	cg12880658	96.9	95.1	100.0	100.0	68.7
	cg16707405	96.9	95.8	100.0	100.0	71.9
	cg02792792	96.7	95.3	100.0	100.0	70.0
	cg14470895	96.9	95.3	100.0	100.0	70.0
	cg23180938	97.1	94.7	100.0	100.0	66.7
	cg08516516	97.1	94.0	100.0	100.0	63.9
	cg11036833	97.1	94.9	100.0	100.0	67.6
	cg07405021	96.6	93.3	100.0	100.0	61.3
GALR1	cg03502002	81.8	61.6	100.0	100.0	21.7
	cg04534765	81.8	68.1	95.7	99.3	24.2
	cg10390058	85.5	74.8	100.0	100.0	30.0
IRX2	cg05903444	98.5	96.3	97.8	99.8	73.8
	cg09524455	97.1	91.4	100.0	100.0	55.4
	cg26333652	98.9	96.5	100.0	100.0	75.4
	cg08235864	97.5	94.4	97.8	99.8	65.2
	cg08204280	97.6	94.9	97.8	99.8	67.2
	cg26504021	91.7	84.5	100.0	100.0	40.7
	cg04992127	96.9	94.4	97.8	99.8	65.2
ZNF486	cg02427230	95.2	91.0	100.0	100.0	54.1
	cg03570035	94.7	91.0	100.0	100.0	54.1
	cg08471835	94.5	91.2	100.0	100.0	54.8
	cg15748470	95.0	91.2	100.0	100.0	54.8

ZSCAN12	cg25666433	95.1	92.6	100.0	100.0	59.0
	cg02622316	94.9	91.9	100.0	100.0	56.8
	cg23164203	94.9	91.0	100.0	100.0	54.1
	cg27577527	95.4	91.4	100.0	100.0	55.4
	cg25060829	95.4	91.2	100.0	100.0	54.8
	cg07660236	96.0	87.5	100.0	100.0	46.0
ZSCAN23	cg00651523	84.4	75.2	97.8	99.7	29.6

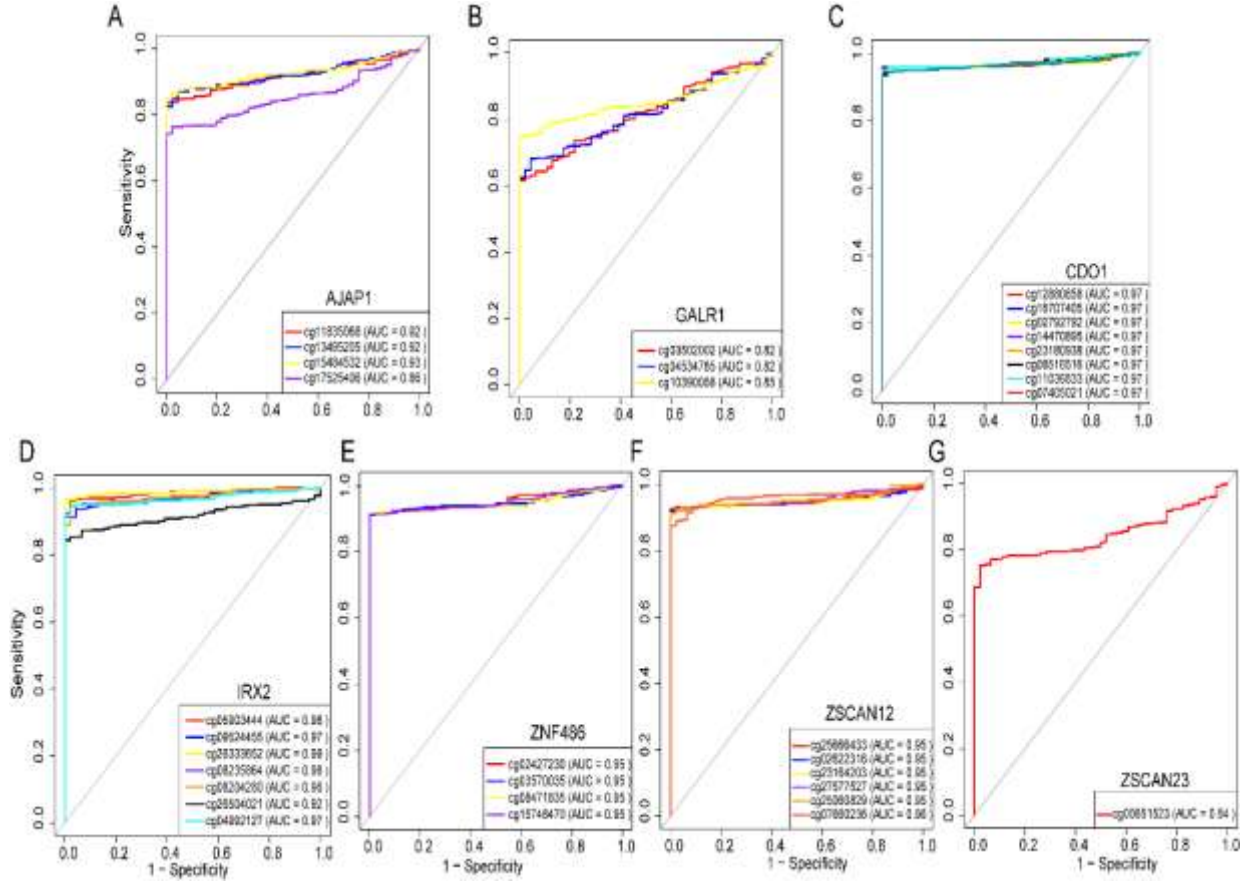

Figure. 3 ROC curves of CpGs on TCGA datasets. A. AJAP1; B. GALR1; C. CDO1; D. IRX2; E. ZNF486; F. ZSCAN12; G. ZSCAN23.

Methylation and Survival

Since methylation of the screened CG loci was increased in tumor tissues, their potential as prognostic factors for overall survival (OS) was investigated using univariate Cox regression and Kaplan–Meier analysis. After dichotomization using the optimal cut-off value, the four methylation sites of AJAP1 (cg11835068, cg13495205, cg15484532, and cg17525406) were strongly associated with the OS of patients with UCEC in univariate Cox regression and Kaplan–Meier survival curve analyses. Patients in the hypomethylated group had a significantly increased risk of death (**Table 3**) and a significantly lower survival rate than those in the hypermethylated group (**Figure 4**) (all HR values > 1, $p < 0.05$).

Table 3 Hazard ratios and 95% confidence intervals for mean methylation at CpG sites in specific genomic regions in relation to overall survival.

	Genomic region	N CpG loci	**β-value, mean (SD)**	Overall survival	
				HR (95% CI)	p-value
AJAP1	cg11835068	Chr1	0.450	8.93(4.09-30.17)	<0.01
	cg13495205	Chr1	0.620	7.31(3.6-21.88)	<0.01
	cg15484532	Chr1	0.503	7.22(3.43-23.86)	<0.01

	cg17525406	Chr1	0.475	9.07(4.14-30.65)	<0.01
CDO1	cg12880658	Chr5	0.593	1.59(1.31-2.24)	<0.01
	cg16707405	Chr5	0.589	1.72(1.42-2.35)	<0.01
	cg02792792	Chr5	0.577	4.51(1.28-2.02)	<0.01
	cg14470895	Chr5	0.604	1.55(1.29-2.11)	<0.01
	cg23180938	Chr5	0.608	1.58(1.3-2.2)	<0.01
	cg08516516	Chr5	0.524	1.65(1.35-2.32)	<0.01
	cg11036833	Chr5	0.539	1.63(1.34-2.25)	<0.01
	Cg07405021	Chr5	0.692	1.54(1.27-2.17)	<0.01
GALR1	cg03502002	Chr18	0.479	9.35(4.16-33.21)	<0.01
	cg04534765	Chr18	0.390	16.6(5.44-105.46)	<0.01
	cg10390058	Chr18	0.429	12.9(5.14-54.26)	<0.01
IRX2	cg05903444	Chr5	0.623	1.55(1.28-2.18)	<0.01
	cg09524455	Chr5	0.427	1.73(1.37-2.59)	0.029
	cg26333652	Chr5	0.755	1.52(1.29-2.01)	<0.01
	cg08235864	Chr5	0.504	1.61(1.33-2.23)	<0.01
	cg08204280	Chr5	0.454	1.46(1.23-1.96)	<0.01
	cg26504021	Chr5	0.558	5.65(3-15.34)	0.017
	cg04992127	Chr5	0.449	1.53(1.26-2.17)	<0.01
ZNF486	cg02427230	Chr19	0.541	1.46(1.24-1.96)	<0.01
	cg03570035	Chr19	0.538	1.52(1.28-2)	<0.01
	cg08471835	Chr19	0.529	1.63(1.36-2.2)	<0.01
	cg15748470	Chr19	0.524	1.38(1.2-1.75)	<0.01
ZSCAN12	cg25666433	Chr6	0.520	1.65(1.33-2.38)	0.012
	cg02622316	Chr6	0.550	1.6(1.32-2.24)	<0.01
	cg23164203	Chr6	0.506	1.52(1.27-2.1)	<0.01
	cg27577527	Chr6	0.602	3.85(2.27-9.2)	0.244
	cg25060829	Chr6	0.561	1.5(1.25-2.06)	<0.01
	cg07660236	Chr6	0.659	1.78(1.39-2.7)	0.044
ZSCAN23	cg00651523	Chr6	0.446	5.43(2.9-14.72)	0.025

CI: Confidence interval; HR: Hazard Ratio

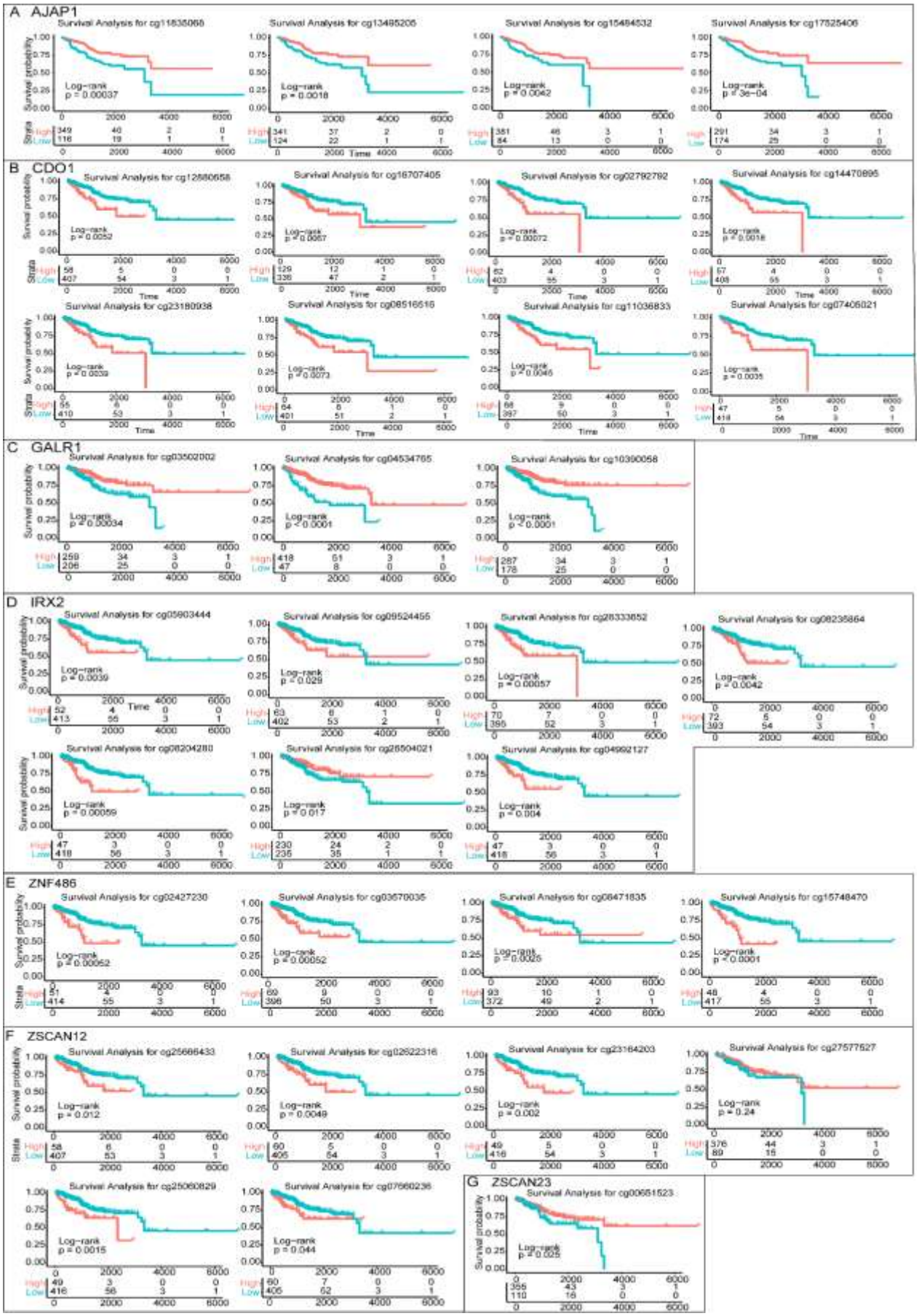

Figure. 4 Overall survival based on CG island phenotype (best cut-off value). A. AJAP1; B. CDO1; C. GALR1; D. IRX2; E. ZNF486; F. ZSCAN12.

Multiple methylation sites in CDO1 (cg12880658, cg16707405, cg02792792, cg14470895, cg23180938, cg08516516, cg11036833, and cg07405021) showed significant prognostic relevance. Patients in the hypermethylated group had a significantly increased risk of death (**Table 3**), and Kaplan–Meier survival curves showed that patients in the hypomethylated group had a significantly lower survival rate than those in the hypermethylated group (Figure 4) (all HR values were greater than 1, $p < 0.05$).

The three methylation sites of the GALR1 gene (cg03502002, cg04534765, and cg10390058) exhibited significant prognostic relevance. The risk of death significantly increased in the hypomethylated group (**Table 3**), and the Kaplan–Meier survival curves showed that patients in the hypomethylated group had a significantly lower survival rate than those in the hypermethylated group (**Figure 4**) (all HR values > 1, $p < 0.05$).

Multiple methylation sites of the IRX2 gene (cg05903444, cg09524455, cg26333652, cg08235864, cg08204280, and cg04992127) were strongly associated with OS in patients with UCEC. The risk of death was significantly increased in patients in the hypermethylated group (**Table 3**), and the Kaplan–Meier survival curves showed that patients in the hypermethylated group had significantly lower survival rates than those in the hypomethylated group (**Figure 3**) (HR values > 1, $p < 0.05$) (except for cg26504021).

Multiple methylation sites on ZNF486 (cg02427230, cg03570035, cg08471835, and cg15748470) showed significant prognostic correlations. Patients in the hypermethylated group had a significantly increased risk of death (Table 3), and their survival was significantly lower than that of the hypomethylated group (**Figure 4**) (all HR values > 1, $p < 0.05$).

Multiple methylation sites (cg25666433, cg02622316, cg23164203, cg25060829, and cg07660236) of ZSCAN12 (cg27577527 were not statistically significant) had prognostic significance in terms of the OS of patients with UCEC. Patients in the hypermethylated group had a significantly higher risk of death (**Table 3**), and Kaplan–Meier survival curves showed that patients in the hypermethylated group had significantly lower survival rates than those in the hypomethylated group (**Figure 4**) (all HR values were greater than 1, $p < 0.05$).

A significant prognostic correlation exists between the methylation site of ZSCAN23 (cg00651523) and OS of patients with UCEC. Patients in the hypomethylated group had a significantly increased risk of death (**Table 3**) and a significantly lower survival rate than those in the hypermethylated group (**Figure 4**) (all HR values > 1, $p < 0.05$).

Identification of Specific Methylation Markers

qMSP primers were successfully designed and validated for four of the seven potential biomarkers. cg17525406, cg07405021, cg03502002, and cg07660236 were associated with AJAP1, CDO1, GALR1, and ZSCAN12, respectively. Methylation-specific priming tests for AJAP1, CDO1, GALR1, ZSCAN12, and the C-less DNA control were performed using qMSP cancer cells and normal cells. The methylation levels of AJAP1, CDO1, GALR1, and ZSCAN12 were significantly elevated and statistically significant ($p < 0.05$) in endometrial cancer cell lines (**Figure 5**).

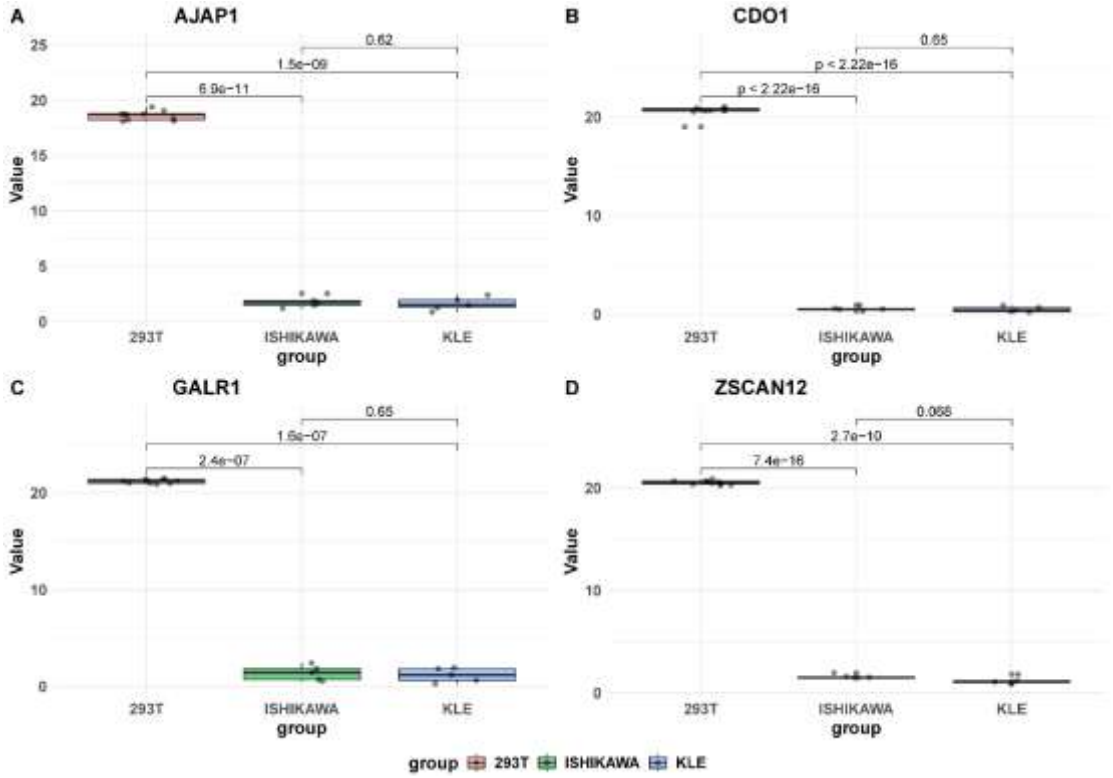

Figure. 5 Quantitative methylation-specific PCR of AJAP1, CDO1, GALR1, and ZSCAN12 in tumor and non-tumor cell samples. The methylation levels of AJAP1, CDO1, GALR1, and ZSCAN12 were significantly higher in tumor cell samples than in non-tumor cell samples (Wilcoxon test, p < 10-4 for each biomarker). PCR of AJAP1 (A), CDO1 (B), GALR1 (C) and ZSCAN12 (D).

Discussion

In this study, differential expression analysis showed that six genes, AJAP1, GALR1, ZNF486, ZSCAN12, ZSCAN23, and CDO1, exhibited significantly lower expression in UCEC tumor tissues ($p < 0.05$), whereas differences in the expression of CELF4 and IRX2 were not significant. These results suggest that these genes play an important role in the genesis and progression of UCEC and that their low expression may be associated with the malignant properties of the tumor[16-22].

In the clinical performance evaluation, CDO1 showed a very high diagnostic ability with an AUC value of 95.4% and a specificity and PPV of 100%, indicating its potential as a powerful molecular marker for the early screening of UCEC. AJAP1 and ZSCAN12 showed high AUC values and exhibited good sensitivity and PPV. In contrast, CELF4 and IRX2 showed weaker diagnostic abilities, with AUC values of 57.0% and 55.1%, respectively, suggesting that they may be poor molecular markers of UCEC. Therefore, greater emphasis should be placed on the detection of AJAP1, ZSCAN12, and CDO1 in clinical applications. In the clinical performance analysis, the methylation sites of genes such as AJAP1 and IRX2 exhibited high AUC values, especially in multiple CG sites of AJAP1, with AUC values of more than 85% and a specificity of 100.0%. These hypermethylated loci have a strong diagnostic ability and help improve the early diagnosis rate of UCEC. However, a few genes, such as GALR1, with relatively low AUC values (81.8–85.5%), have poor sensitivities that may affect the detection of certain positive cases. Therefore, future studies should further explore improvements in diagnostic sensitivity and accuracy by combining multi-gene testing.

Further differential methylation analyses revealed that each gene contained multiple differentially methylated CG sites and that all sites had significantly higher methylation levels in the tumor group than in the normal group. These findings support the critical role of methylation in the development of UCEC. By screening for hypermethylated sites with logFC values > 0.4, we identified specific CG sites with high diagnostic power in clinical practice.

In the Cox regression analysis and Kaplan–Meier survival curves, we observed that different methylation sites of seven genes, AJAP1, GALR1, ZSCAN23, CDO1, IRX2, ZNF486, and ZSCAN12, were closely associated with the OS of patients with UCEC.AJAP1, GALR1. The risk of death was significantly increased in patients in the hypomethylation groups of the three genes AJAP1, GALR1, and ZSCAN23, and the survival period was significantly lower than that in the hypermethylation group, suggesting that these genes may exhibit oncogene-like behaviors of abnormal activation or transcriptional upregulation in UCEC. Patients in the hypermethylation groups of the four genes CDO1, ZNF486 and ZSCAN12 had a significantly increased risk of death and a significantly shorter survival period than those in the hypomethylation group, reflecting that these genes have tumor suppressive functions under normal circumstances. This suggests that the CG sites are key nodes of epigenetic regulation and potential targets for therapeutic intervention.

The methylation levels of the four genes, AJAP1, CDO1, GALR1, and ZSCAN12, were verified experimentally and were highly consistent with the results of the BioBuzz data analysis; that is, the methylation levels of these four genes were significantly higher in UCEC cells than in normal cells. This result verified the reliability of TCGA data analysis and indicated that these methylated sites may play a key role in UCEC occurrence. PCR validation experiments provided strong experimental support for the results of the BioBelief analysis in this study and further enriched our understanding of the mechanism of UCEC methylation regulation. Future studies should validate these results using larger samples and functional experiments exploring the specific roles of these methylation events in tumor biology.

This study revealed potential methylation sites of several genes as diagnostic and prognostic markers for UCEC. Using these methylation markers, physicians can accurately screen patients with UCEC at an early stage and effectively assess their survival and prognostic risk. The methylation sites of genes such as AJAP1, CDO1, ZSCAN12, and IRX2 have high specificity and diagnostic ability, which can help to improve the early diagnosis rate of UCEC. Especially for high-risk groups, a combination of screening for methylation markers can lead to better individualized treatment plans. Methylation markers can provide a basis for targeted therapy and immunotherapy of UCEC[23-25]. They can provide personalized guidance for patients' treatment plans[26].

Although this study revealed the potential of methylation markers for multiple genes in UCEC, it had certain limitations. First, the data in this study were mainly based on TCGA database, and future multicenter clinical validation is needed to ensure the broad applicability of the results. Second, this study did not explore the correlation between these methylation sites and other clinical features (e.g., tumor staging and typing); future studies could further evaluate the relationship between methylation markers and clinical features to improve their prognostic predictive ability. Additionally, methylation alterations may only be early events in tumorigenesis, and future studies are needed to explore the specific mechanisms of methylation during tumor development and how to improve patient outcomes through targeted demethylation therapy.

Conclusions

In this study, the systematic analysis of the methylation sites of several genes revealed their potential in the diagnosis, prognosis, and clinical application of UCEC, with genes such as ZSCAN12 and CDO1 having high accuracy and sensitivity as diagnostic markers of UCEC and their methylation levels being closely related to the OS of patients, which has a high prognostic value. Future studies should further validate the clinical applicability of these methylation markers in patients with UCEC and explore their application in personalized therapies.

Materials and Methods

Data Sources and Preprocessing

The UCEC dataset (n = 596) from TCGA was used in this study. The mRNA expression and methylation data of patients with UCEC were obtained from TCGA database, and the samples included tumor and normal control groups. After the data were downloaded, normalization and missing value filling were performed to ensure data quality and consistency.

Differential Expression Analysis

Eight UCEC-associated genes were selected — AJAP1, CELF4, GALR1, IRX2, ZNF486, ZSCAN12, ZSCAN23, and CDO1. First, the mRNA expression data of these genes were subjected to differential expression analysis using the limma package, which compares gene expression levels between the tumor and normal groups. For differential expression analysis, we set a false discovery rate < 0.04 as a statistically significant criterion.

Methylation Differential Analysis

Based on the results of the differential expression analysis, we further examined the methylation data of the selected genes. Methylation levels were then compared between those of the tumor and normal groups using the limma package. These values were expressed as β-values, and |logFC| > 0.4 was chosen as the screening criterion for differentially methylated sites to ensure that the results were biologically significant and statistically meaningful. By analyzing the methylation data, we identified hypermethylated CG sites for each gene.

CG loci Screening and Clinical Performance Analysis

After identifying the differentially methylated genes, the methylation levels of the CG sites of these genes were analyzed. Based on the methylation data for each gene, CG sites with more concentrated methylation positions were selected for further analysis. We screened CG sites with logFC values > 0.4 and evaluated their clinical performance by calculating the sensitivity, specificity, and area under the curve (AUC) values. All clinical performance analyses were performed using the ROCR package.

Survival Analysis

To explore the relationship between methylated loci and the prognosis of patients with UCEC, we performed a survival analysis of differentially methylated CG loci. Patient survival data were analyzed using the survival package, and the correlation between methylation levels and patient survival was assessed using Kaplan–Meier survival curves and log-rank tests. The results of the survival analysis provided multiple survival analysis plots demonstrating the impact of methylation sites on the prognosis of patients with UCEC.

Experimental Confirmation

The human normal cell line 293T (CVCL_0063) and the UCEC tumor cell lines ISHIKAWA(CVCL_2529) and KLE(CVCL_1329) were obtained from Hunan Fenghui Biotechnology Co., Ltd, China. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA) and 1% penicillin–streptomycin at 37 ℃ in 5% CO_2. After the cells reached 80% confluence, genomic DNA was extracted using a DNA Kit (Xiangmei Note: No. 20170149, HOOMYA, China) according to the manufacturer's instructions, and 500 ng was subjected to bisulfite conversion treatment to ensure that unmethylated cytosine (C) was converted to uracil (U), whereas methylated cytosine (5 mC) remained unchanged. The converted DNA was used immediately for subsequent fluorescence quantitative methylation-specific PCR (qMSP)assays or stored at –20°C for subsequent experiments.

Polymerase Chain Reaction (PCR) amplification was performed on an ABI 7500 Real-Time PCR system using DNA polymerase FastAmpli Premix IV (Zhuhai Baorui Biotechnology Co., Ltd., China). The primer and probe sequences were provided in **Supplementary Table 1**. The threshold value of each gene was set to 20,000, and the data were analyzed and exported to calculate methylation levels as follows:

$$\triangle CT = CT_{\text{-Target}} - CT_{\text{-reference}} \quad (1)$$

where $CT_{\text{-Target}}$ is the CT value of the target gene, and $CT_{\text{-reference}}$ is the CT value of the internal reference gene.

Statistical Analysis

All statistical analyses were performed using R 4.3.3 software, and the relevant packages included ‘limma’, ‘ggplot2’, and ‘survival’. The t-test was used for differential expression analysis, and the linear model of the ‘limma’ package was used for differential methylation analysis. Survival analysis was performed using the log-rank test, and all p-values were set to two-sided significance tests with a significance level of $p < 0.05$.

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Contributors

Peng Gan, Meizhen Hu: conceptualization, methodology, laboratory work, writing original draft, software analysis. Xiaozhu Pei: supervision, methodology, investigation, writing–review and editing. Xiaofan Fan: coordinated the evaluation of liquid-based cytology specimens, review and editing. Hong Tao: conceived the original trial, supervision, funding acquisition, project administration, writing—review and editing. Meizhen Hu: software analysis, data curation, review and editing. Hong Tang, Hongtao Li and Xi Luo: investigation, laboratory work, review and editing. Peng Gan, Meizhen Hu, Xiaofan Fan: coordinated clinical implementation in the original trial, review and editing. Hong Tao: investigation, review and editing. Xiaozhu Pei and Hong Tao: conceptualization, supervision, funding acquisition, investigation, project administration, guarantor, writing, review and editing. Peng Gan and Meizhen Hu contributed equally to this manuscript.

Competing Interests

All authors declare no conflicting interests.

Data Availability Statement

Data are available on reasonable request. Limited data may be available on reasonable request from the corresponding author.

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Abbreviations

AJAP1: Adherens Junction Associated Protein 1;

CDO1: Cysteine Dioxygenase Type 1;

CELF4 : CUGBP Elav-Like Family Member 4;

GALR1: Galanin Receptor 1;

IRX2 : Iroquois Homeobox 2;

ZNF486 : Zinc Finger Protein 486;

ZSCAN12 : Zinc Finger and SCAN Domain Containing 12;

ZSCAN23 : Zinc Finger and SCAN Domain Containing 23

UCEC : Uterine corpus endometrial carcinoma

TCGA : The Cancer Genome Atlas

AUC : Area under the curve

DMEM : Dulbecco's Modified Eagle Medium

qMSP : quantitative Methylation-Specific PCR

FBS : Fetal bovine serum

PCR : Polymerase Chain Reaction

PPV : Positive Predictive Value

NPV : Negative Predictive Value

FDR : False discovery rate

HR : Hazard Ratio