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Promoter hypermethylation of *CIDEA*, *HAAO* and *RXFP3* associated with microsatellite instability in endometrial carcinomas

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Abstract

Objective—DNA promoter methylation is an epigenetic phenomenon for long-term gene silencing during tumorigenesis. The purpose of this study is to identify novel hypermethylated loci associated with clinicopathologic variables in endometrioid endometrial carcinomas.

Methods—To find hypermethylated promoter loci, we used differential methylation hybridization coupling with microarray and further validated by combined bisulfite restriction analysis and MassARRAY assay. Methylation levels of candidate loci were corrected with clinicopathologic factors of endometrial carcinomas.

Results—Increased promoter methylation of *CIDE*, *HAAO* and *RXFP3* was detected in endometrial carcinomas compared with adjacent normal tissues, and was associated with decreased gene expression of all three genes. In a clinical cohort, promoter hypermethylation on *CIDEA*, *HAAO* and *RXFP3* was detected in 85, 63 and 71% of endometrial carcinomas, respectively (n=118, P<0.001) compared with uninvolved normal endometrium. Methylation status of *CIDEA*, *HAAO* and *RXFP3* had significant association with microsatellite instability in tumors (P<0.001). Furthermore, methylation levels of *HAAO* were further found to relate to disease-free survivals (P=0.034).

Conclusions—Hypermethylation of *CIDEA*, *HAAO* and *RXFP3* promoter regions appears to be a frequent event in endometrial carcinomas. Hypermethylation at these loci is strongly associated with

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The authors have no conflicts of interest to declare.

microsatellite instability status. Moreover, *HAAO* methylation predicts disease-free survival in this cohort of patients with endometrioid endometrial cancer.

Keywords

Endometrial carcinoma; Hypermethylation; CIDEA; HAAO; RXFP3

Introduction

The 2009 estimated new cases and death for uterine corpus (endometrium) cancer are 42,160 and 7,780, respectively [1]. Most uterine cancers are endometrial adenocarcinomas, among which endometrioid endometrial carcinoma is the most common histological subtype. Tumorigenesis is a multistep process in which genetic defects are progressively accumulated [2]. These DNA changes, including mutations, loss of heterozygosity and gene amplification, can lead to inactivation of tumor suppressor genes and activation of oncogenes, all of which contribute to uncontrolled growth of transformed cells. More recently, epigenetic defects have been found to be equally important in cancer development. These molecular alterations do not involve changes in primary DNA sequences, but are frequent events observed in tumors [3, 4], including endometrial cancer.

Although tumor suppressor genes can be inactivated by deletions and/or mutations in cancer cells, epigenetic mechanisms including aberrant methylation of CpG sites within the promoter region also contribute to gene silencing [3,4]. In the past, candidate gene approaches were used to identify potential biomarkers for endometrial cancer. Promoter hypermethylation of hormone-receptor genes, *ERα* or *PR*, is usually associated with loss of their expression in more advanced stages of this disease [5,6]. This hypermethylation event may also occur early in endometrial tumorigenesis [7]. Recently, hypermethylation of more tumor suppressor genes has been reported in endometrial tumors. Aberrant methylation is frequently correlated with clinicopathologic features of endometrial cancer patients. For example, hypermethylation of *RAR-β2* was found in 75% of endometrial hyperplasia samples and 92% of carcinomas [8]. Functional analysis has implicated that this methylation-mediated silencing may contribute to high proliferative activities of endometrial hyperplasia without differentiation [8]. Hypermethylation of *RASSF1A* is frequently associated with tumors of advanced stage disease (FIGO stage III and IV), lymph node involvement, and high grade [9,10]. Reduced expression of *GSTP1* as a result of hypermethylation of its promoter is found to be associated with myometrial invasion potential of endometrial carcinoma [11]. Taken together, these previous studies have demonstrated that hypermethylated CpG islands are potential biomarkers for early detection and disease recurrence of endometrial cancer.

Promoter hypermethylation of *MLH1*, one of DNA mismatch repair genes, contributes frequently to microsatellite instability (MSI) in sporadic endometrial carcinomas [12,13]. MSI is a phenomenon of the accumulation of insertions and/or deletions at short DNA repeats, caused by the loss of DNA mismatch repair. The impact of MSI on outcomes in patients with endometrial cancer is still inconclusive [14–18]. The contradictory findings could be explained by the heterogeneity of patient population and by the methodologies to assess DNA mismatch repair [15]. We previously showed that MSI+ tumors without *MLH1* methylation were associated with younger age but the combined MSI/*MLH1* methylation status did not predict overall survival (OS) or disease-free survival (DFS) [15].

Herein, we report that the expression of *CIDEA*, *HAAO* and *RXFP3* was lost and their promoters were hypermethylated in endometrial cancer when compared with adjacent normal tissues. Endometrial cancer cells exposed to inhibitors of DNA methylation and/or of histone deacetylation, reactivated *CIDEA*, *HAAO* and *RXFP3* gene expression. We further show that

CpG methylation of all three genes was associated with microsatellite instability. Particularly, hypermethylation of *HAAO* is related to disease-free survival. This study provides novel hypermethylated loci corrected with MSI+ phenotype in endometrial cancer.

Materials and methods

Endometrial specimens and cell lines

Tissue specimens (118 tumors and 22 uninvolved controls) were obtained as part of our ongoing work and were described in a previous report [19]. All participants consented to both molecular analyses and follow-up studies, and the protocols were approved by the Human Studies Committee at Washington University and the Ohio State University. Tumor specimens and adjacent normal tissues were collected from primary endometrioid endometrial carcinomas at the time of hysterectomy. Normal controls were procured from women (pre-menopausal, age<50, except one case), also undergoing hysterectomy. All specimens were evaluated by at least one pathologist and confirmed the diagnoses from hematoxylin and eosin-stained tissue sections. Tumor specimens had high neoplastic cellularity (mean 74%, median 80%) while normal tissues did not contain any malignant portion by direct microscopic visualization. The presence of MSI and *MLH1* methylation status was determined and reported previously [15, 20]. Tumor characteristics were summarized in Supplementary Table S1, including patient age, tumor grade and stage, and menopausal status. Standard methods were used to extract DNA and RNA from tumors, corresponding non-neoplastic tissues and normal controls.

Human endometrial cancer cell lines, AN3CA, ECC-1, HEC1A, Ishikawa, KLE, RL95-2 and SK-UT-1B, were routinely maintained in our laboratory [19]. For epigenetic studies, these cells were treated with 5-aza-2′-deoxycytidine (DAC, 5 μM, Sigma) for 48 h and/or trichostatin A (TSA, 0.5 μM, Sigma) for 24 h. DNA and RNA from treated and untreated cells were isolated as described previously [19].

Differential methylation hybridization (DMH) analysis

DMH was done to profile global methylation of two pools of DNA from endometrial cancer samples (10 samples/pool) and a reference control pooled from two normal endometrial DNA samples. A CpG island microarray (Agilent) contains probes spanning all the 27,800 islands annotated to the human reference sequence (NCBI Build 36.1, UCSC hg18). Tumor and control amplicons were labeled with Cy5 and Cy3, respectively, and cohybridized onto microarray slides using our established protocols [20]. Normalized Cy5/Cy3 hybridization intensities were calculated, and candidate loci with ratios >1.5 were identified as hypermethylated in tumors compared with normal endometrial tissues according to previously published methods [20].

Reverse transcription and quantitative PCR (RT-qPCR)

Total RNA (1 μg) was reverse transcribed with the Superscript III reverse transcriptase (Invitrogen). PCR was performed as described previously [19]. Specific primers for amplification are available upon request. The relative expression of a coding gene in cells was determined by comparing the threshold cycle (Ct) of the gene against the Ct of *GAPDH*.

Combined bisulfite restriction analysis (COBRA)

Genomic DNA (500 ng) was treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research) following manufacturers' recommended protocols. COBRA was used to evaluate methylation of *CIDEA*, *HAAO* and *RXFP3*. Target sequences were amplified by PCR, and the products were digested with *Aci*I, (New England Biolabs) to identify methylated sequences. Primer sequences and PCR conditions are available upon request. Digested and

non-digested PCR products were resolved on 2% agarose gels stained with ethidium bromide. DNA fragments digested by *Aci*I were scored as "methylated" in a given sample.

MassARRAY analysis

To quantify methylation levels of the CpG islands of *CIDEA*, *HAAO* and *RXFP3* in clinical samples, the high-throughput MassARRAY platform (Sequenom) was carried out as described previously [19]. Briefly, bisulfite-treated DNA was amplified with primers and the PCR products were spotted on a 384-pad SpectroCHIP (Sequenom) and followed by spectral acquisition on a MassARRAY analyzer. Methylation data of individual units (1–4 CpG sites per unit) were generated by the EpiTyper software (Sequenom).

Statistical and survival analyses

The mRNA expression and methylation levels of the paired tumors and adjacent normal endometrial tissues were compared by using paired two-sample *t*-test. A significance was assigned as * if $P \leq 0.05$. The marginal relationship between continuous methylation levels of *CIDEA*, *HAAO* and *RXFP3* and a relevant categorical clinicopathologic covariate including MSI was examined using the *t*-test for binary variables or ANOVA for categorical variables. Linear model was performed to examine the effect of a clinicopathologic covariate on methylation levels after adjusting for others. Overall survival (OS) and disease-free survival (DFS) were defined in a previous report [19]. The Kaplan-Meier analysis, and univariate and multivariate Cox proportional hazard models were used to evaluate the effect of continuous or dichotomized methylation levels on the survival outcomes as described previously [19]. All tests were two-sided and all analyses were performed using R 2.8.1.

Results

CIDEA, HAAO and RXFP3: three novel methylation markers in endometrial carcinoma

In initial microarray data analysis, we identified 16 loci with increased levels of promoter methylation in tumors compared with normal endometrial tissues (normalized Cy5/Cy3>1.5) (Fig. 1A, Table S2). Among these loci, the expression of five (*CD34*, *LTBP2*, *LZTS2*, *OGT* and *SSTR1*) have been reported with cancer development [21–25]. The expression of six other (*CIDEA*, *CLDN3*, *G0S2*, *HAAO*, *HOXA6* and *SOX7*) have previously been shown to be regulated by DNA methylation [26–31]. All 16 loci were evaluated by COBRA in six endometrial cancer cell lines, one pooled sample derived from two noncancerous endometrial samples, as well as seventeen primary endometrial tumors (Fig. 1A). Promoter hypermethylation of *CIDEA* was detected in all six endometrial cancer cell lines, ECC-1, HEC1A, Ishikawa, KLE, RL95-2, and SK-UT-1B (Fig. 1B, left panel). Hypermethylation of *HAAO* and *RXFP3* was also found in five out of six cancer cell lines (Fig. 1C, 1D, left panels). Based on COBRA (see Fig. 1A), *CIDEA*, *HAAO* and *RXFP3* were selected for further analysis because these three genes were predominantly hypermethylated in cancer cell lines and primary tumors, but not in normal specimens. In addition, our unpublished MassARRAY analysis indicated that *HOXA6* was methylated in both normal and cancer specimens (data not shown). Optimized methylation assays could not be developed for *CD34* and *SSTR1*, due to poor PCR amplification or high GC-content of their CpG islands. These three loci, therefore, were not analyzed in the present study.

Since the hypermethylation of *CIDEA*, *HAAO* and *RXFP3* was observed in endometrial cancer cells (Fig. 1A) and the 5′-end of these loci has a canonical CpG island (Fig. S1), we determined whether these mRNAs can be reactivated in endometrial cancer cell lines. When these cancer cells were treated with a demethylating agent, DAC (5 μmol/L), a histone deacetylase inhibitor, TSA (0.5 μmol/L), or their combination, reactivation of *CIDEA*, *HAAO* and *RXFP3* was observed in these cells (Fig. 1B–1D, right panels). These results suggest that the silencing of

CIDEA, *HAAO* and *RXFP3* is mediated through promoter hypermethylation in endometrial cancer.

Loss expression and hypermethylation of CIDEA, HAAO and RXFP in endometrial primary tumors

Since the expression of these genes has not been reported in endometrial cancer, we first examined their mRNA expression in 31 paired specimens. RT-qPCR results demonstrated that mRNA levels of all three genes were lower in tumors compared with the adjacent normal counterparts ($P<0.05$; Fig. 2A). We confirmed an inverse relationship between mRNA expression and DNA methylation in 21-paired samples randomly chosen from the aforementioned specimens (n=31) (Fig. 2). The detailed methylation level of each CpG unit (1–4 CpG sites per unit) was showed in Supplementary Fig. S1. These results suggest that *CIDEA*, *HAAO* and *RXFP3* loss their expression in endometrial carcinoma due to promoter hypermethylation.

Methylation status of CIDEA, HAAO and RXFP3 in a large cohort of endometrioid endometrial carcinomas and its association with clinicopathologic variables

To determine if hypermethylation of *CIDEA*, *HAAO* and *RXFP3* genes is associated with clinicopathologic variables of endometrioid endometrial carcinomas, we performed MassARRAY in 118 clinical tumor samples and 22 uninvolved controls. Quantitative methylation levels of each CpG unit were shown in Fig. 3A, 4A and 5A. The mean methylation level of each specimen was used to compare the differences between tumor and normal groups. Extensive promoter methylation of *CIDEA*, *HAAO* and *RXFP3* was found in more than 85, 63 and 71% of the primary tumors relative to those of uninvolved controls, respectively ($P<0.001$; Fig. 3B, 4B, 5B).

Statistical analysis by *t*-test further revealed that hypermethylation of *CIDEA* ($P=0.0078$), *HAAO* ($P=0.0001$) and *RXFP3* ($P<0.0001$) was significantly associated with MSI status and *MLH1* methylation (Fig. 3C, 4C, 5C). After adjusted for other clinical covariates by linear modeling, the association of the three loci with MSI status was still significant ($P<0.001$, Table S3). Linear model analysis also indicated that *CIDEA* methylation was associated with tumor grade whereas *RXFP3* methylation was correlated with tumor recurrence, body mass index and tumor grade (Table S3).

We also evaluated the association between DNA methylation and patient survival. On univariate analysis, *RXFP3* hypermethylation was significantly correlated with disease-free survival (Cox hazard ratio, 0.11 (0.01~0.93), $P=0.042$), but not with overall survival (Cox hazard ratio, 0.27 (0.05~1.55) $P=0.14$) (Table S4).). The Kaplan-Meier survival analysis on methylation levels (dichotomized by median) indicated that patients with *HAAO* hypermethylation had poor disease-free survival (Fig. 4D left panel; $P=0.038$, log-rank test). However we did not observe the methylation of *CIDEA* and *RXFP3* correlated with disease-free or overall survival by the Kaplan-Meier survival analysis (Fig. 3D, 5D). Detailed survival analysis of all three genes was shown in Supplementary Table S5. On multivariate Cox analysis, *HAAO* methylation (dichotomized by median) was also significantly associated with disease-free survival (DFS, $P=0.03$) and age was also a risk factor for DFS, while stage was not (Table S5).

Discussion

Our previous report identified two hypermethylation markers, *SESN3* and *TITF1*, but their methylation status did not predict overall or disease-free survival within endometrioid endometrial cancers [20]. In this study, we found three additional cancer-specific methylation

markers, *CIDEA, HAAO* and *RXFP3*, through an evaluation of promoter microarrays containing ~27,800 CpG islands. Both COBRA and MassARRAY assays confirmed that hypermethylation of all three loci was frequent (≥63%) in endometrial carcinomas but was infrequent in normal tissues.

CIDEA is a member of the cell death-inducing DFF45-like effector (CIDE) family. DFF45 is a subunit of the DNA fragmentation factor which is cleaved by active caspase-3 during apoptosis. CIDEA was found to induce apoptosis in mammalian cells, which was inhibited by DFF45 [32]. CIDEA also plays important roles in energy homeostasis. In an animal model, the absence of CIDEA expression may result in lean phenotypes, insulin resistance, and resistance to diet-induced obesity in mice [33]. HAAO, known as 3-Hydroxyanthranilate-3,4-dioxygenase, is an enzyme to catalyze the biosynthetic pathway from tryptophan to quinolonate. While its function in cancer development is still unclear, we demonstrate that *HAAO* is hypermethylated in ovarian cancers with high sensitivity and specificity as a biomarker [29]. RXFP3, formerly called GPCR135 or SALPR, belongs to the relaxin family peptide receptors and can be activated by relaxin-3, a member of the insulin superfamily [34]. Upon ligand stimulation, RXFP3 activates extracellular signal-regulated kinase signaling via various pathways including protein kinase C [34]. The function of RXFP3 related to cancer is also unknown. Our findings have shown by RT-qPCR that *RXFP3* is expressed in normal endometrium and a small subset of endometrial cancers (see Fig. 2C). The loss of *RXFP3* expression in tumors is inversely associated with its promoter hypermethylation.

In this study we found that the hypermethylation of *CIDEA, HAAO* and *RXFP3* is associated with MSI+ phenotype. This observation is consistent with previous report that endometrial carcinomas with MSI+ had significantly more epigenetic alterations than MSI- tumors [35]. Promoter hypermethylation of *MLH1* contributes frequently to MSI in sporadic endometrial carcinomas [12,13]. Our previous report showed that MSI+ tumors without *MLH1* methylation were associated with younger age while the combined MSI/*MLH1* methylation status did not predict OS or DFS [15]. In the current study, the methylation status of *HAAO* may predict DFS in patients with endometrial cancer. To definitively prove its clinical significance in predicting patient survivals, methylation analysis of a large cohort, such as the GOG-210 trial, is needed to validate this finding. Methylation markers identified in current study and others [5–15,20] could be specific and informative for endometrial cancer only. Future development of endometrial cancer methylator phenotypes could hold great promise to improve risk assessment, diagnosis, and prognosis.

In conclusion, our findings provide three novel and cancer-specific hypermethylation markers. The methylation levels of all three loci are correlated with MSI status. Although MSI could be either a cause or a consequence of DNA methylation, high-throughput studies can be developed to establish the relationship between MSI status and DNA hypermethylation. This type of omics study may find that loss of DNA mismatch repair together with epigenetically mediated silencing of these genes can be common alterations that contribute to tumorigenesis. As such, the combined epigenetic and genetic alternations can be feasible options for predicting survival rates in cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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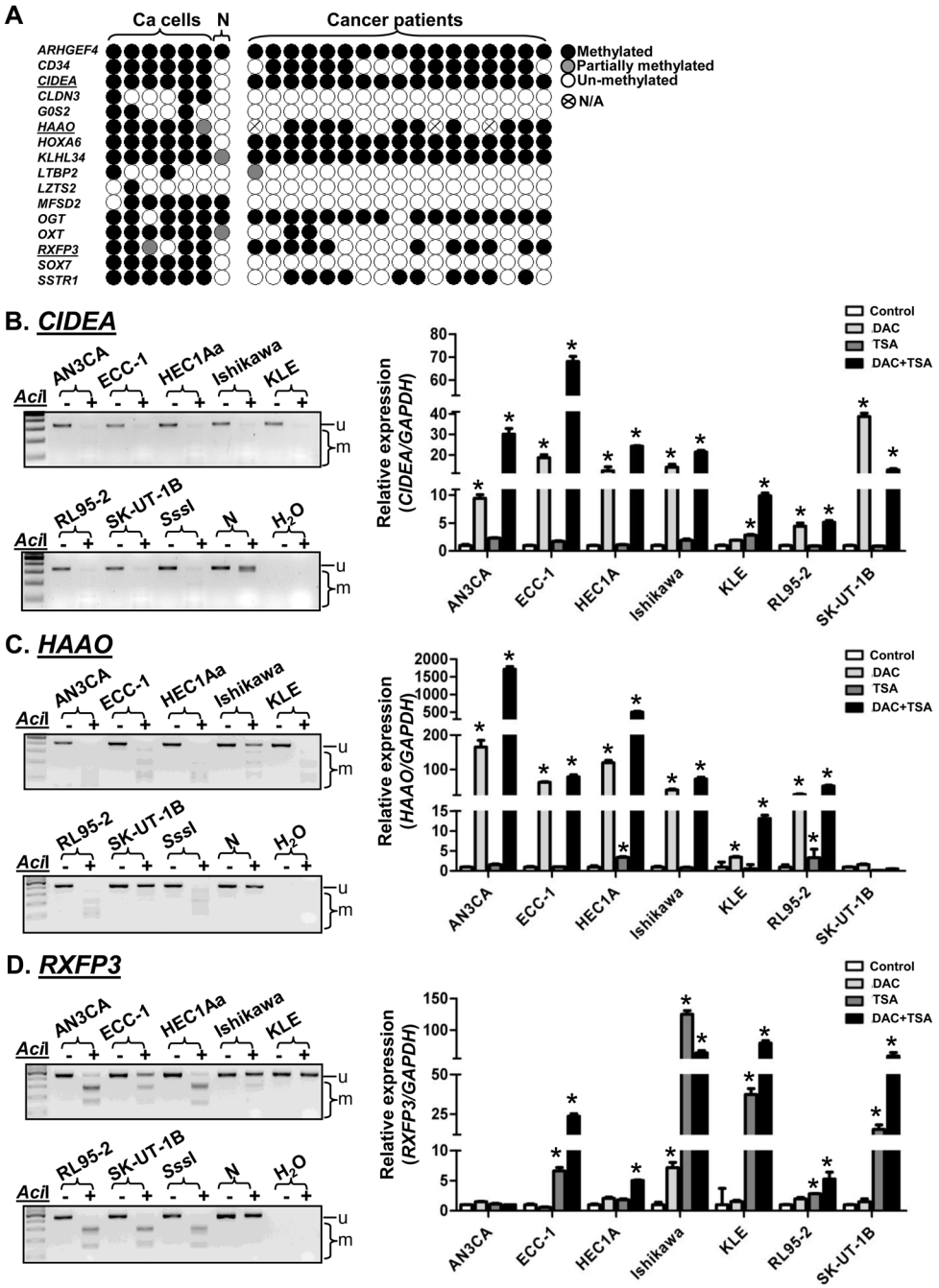

Fig. 1.

CIDEA, *HAAO* and *RXFP3* are novel hypermethylated markers in endometrial cancer. (A) Summary of the methylation status of sixteen loci by COBRA in endometrial cancer cell lines as well as in primary tissues. (B–D, left panels) COBRA in endometrial cancer cell lines. u, unmethylated band; m, methylated bands; *Sss*I, 100% methylated control; N, one pooled sample derived from two noncancerous endometrial tissues as negative control; +, *Aci*I restriction enzyme added; −, without *Aci*I. (B–D, right panels) Relative expression levels of *CIDEA*, *HAAO* and *RXFP3* mRNA in endometrial cancer cell lines treated with DAC and/or TSA in relation to untreated controls was determined by RT-qPCR analysis. *GAPDH* was used

as internal control gene. *Error bar*, SD; *, $P<0.05$ compared with untreated control of the same cell type.

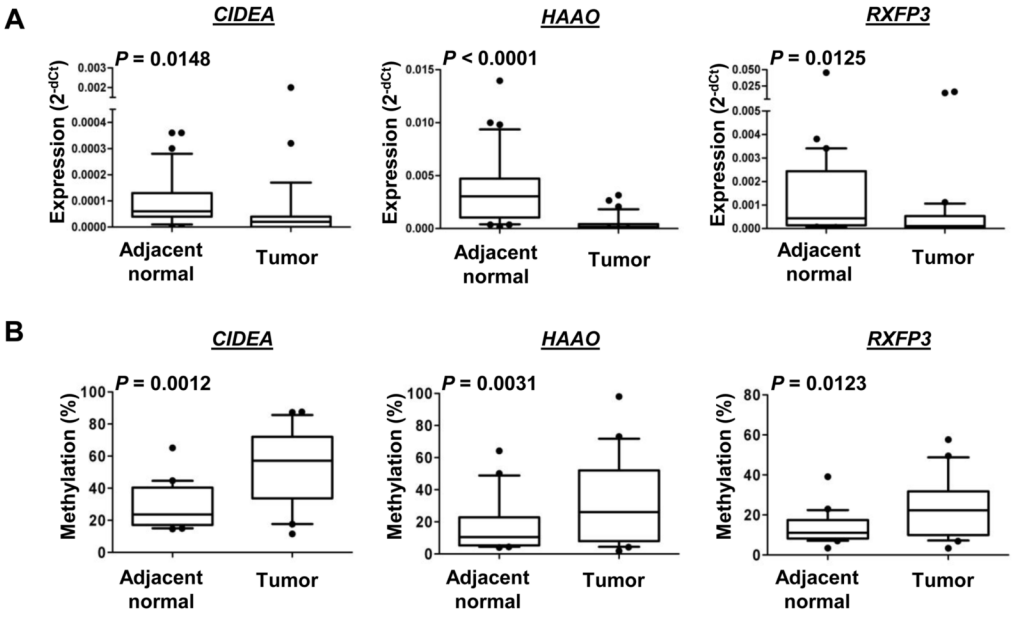

Fig. 2.

CIDEA, *HAAO* and *RXFP3* are loss of mRNA expression and are hypermethylated in endometrial tumors. (A) Box plots indicating the relative expression levels of *CIDEA*, *HAAO* and *RXFP3* mRNA in 31 paired endometrial tissues. *GADPH* served as internal controls. *P* value estimated from paired two-sample *t*-test. (B) Box plots pointing the quantitative DNA methylation analysis using MassARRAY in 21 paired samples of endometrial tissues. Measured methylation levels of the samples were corrected using a standard curve. *P* value estimated from paired two-sample *t*-test.

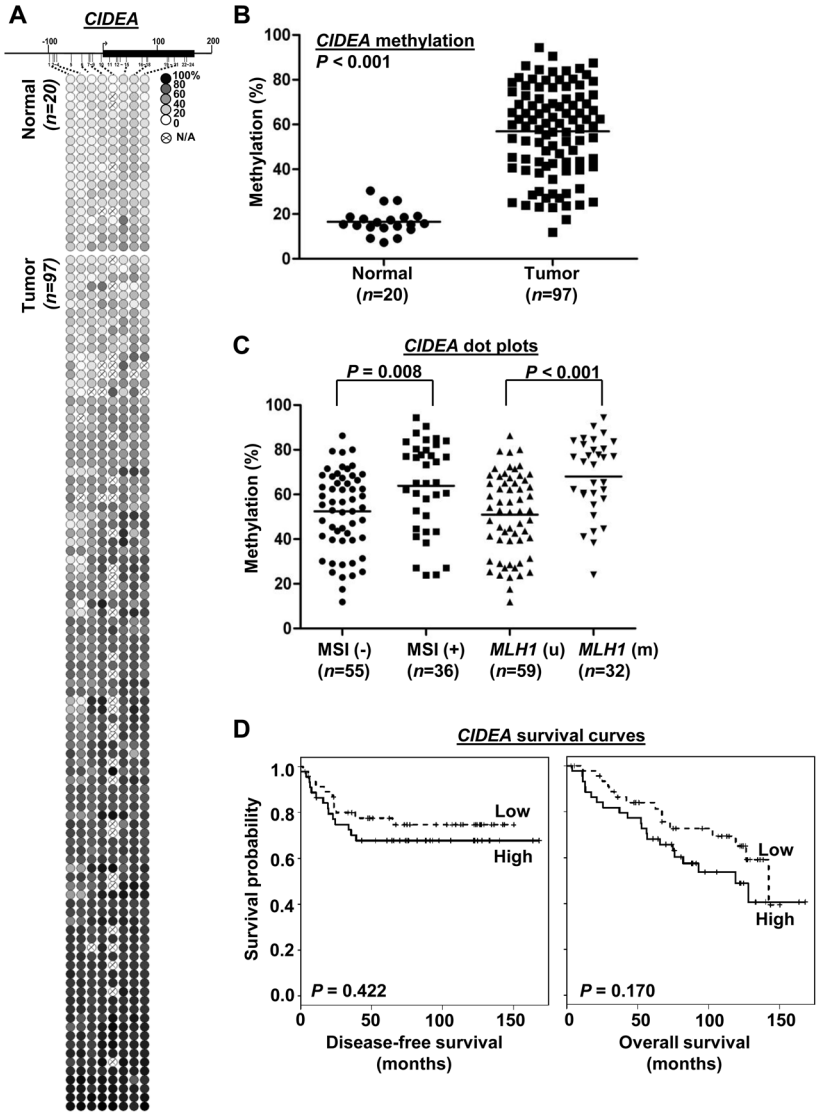

Fig. 3.

Methylation of *CIDEA* CpG island and clinicopathologic covariates analyses in primary endometrial carcinomas. (A) Methylation profiles of 20 normal endometrial tissues and 97 primary tumors created following MassARRAY analysis. Each row represents a sample and each column represents a CpG unit. Color-coding depicts the degree of methyltion with dark black being 100% and white being 0%; N/A, not analyzable. (B) Dot plots pointing that *CIDEA* hypermethylation was in endometrial tumors. Each dot represents the mean of each specimen on the all CpG sites in Fig. 3A. *Horizontal lines*, mean values. *P* value was calculated by two-sample *t*-test. (C) Dot plots indicating the level of *CIDEA* promoter methylation was correlated with MSI and moderately associated with *MLH1* methylation status. *P* values were calculated by two-sample *t*-test. (D) Kaplan-Meier curves for disease-free and overall survivals. Samples were grouped according to the mean level of methylation for the all CpG units of *CIDEA* while methylation was dichotomized into low/high group based on the median methylation level. Vertical bars represent excluded cases. *P* values were estimated from Log-rank test.

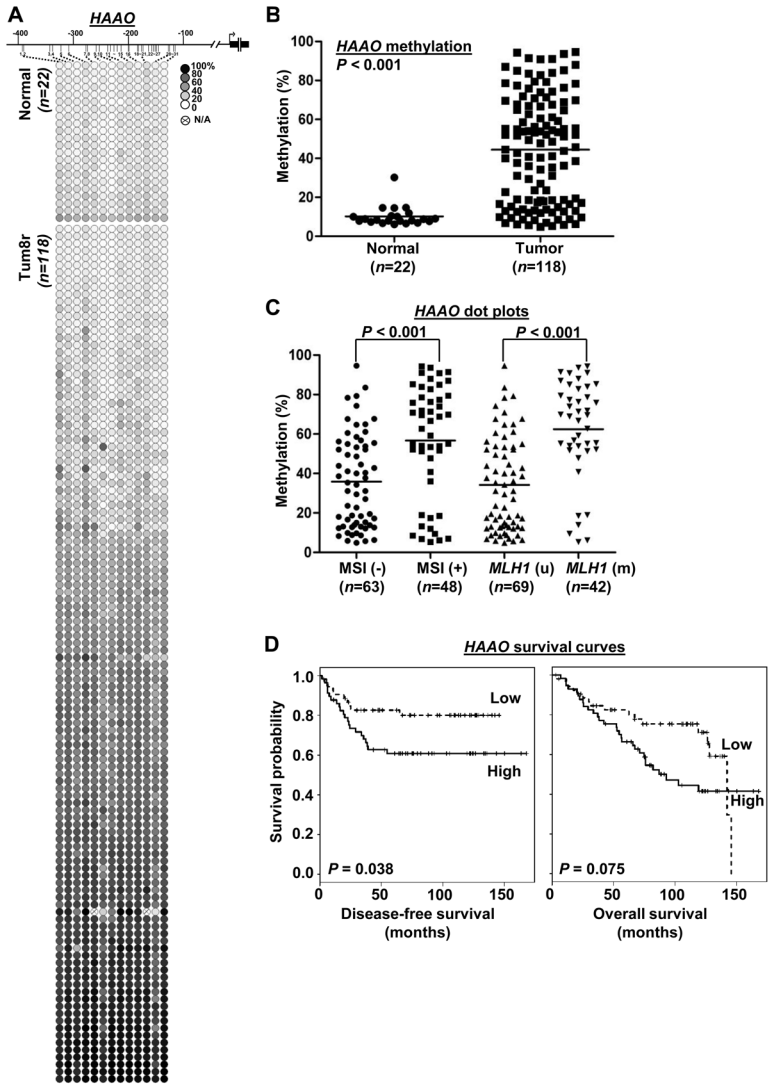

Fig. 4.

Methylation of *HAAO* CpG island and clinicopathologic covariates analyses in primary endometrial carcinomas. (A) Methylation profiles of 22 normal endometrial tissues and 118 primary tumors created following MassARRAY analysis. (B) Dot plots pointing that *HAAO* hypermethylation was found in endometrial tumors. (C) Dot plots indicating the level of *HAAO* promoter methylation was positively correlated with MSI status. (D) Kaplan-Meier curves indicate *HAAO* methylation was associated with disease-free survival. (A–D) Assays were described as in Fig. 3.

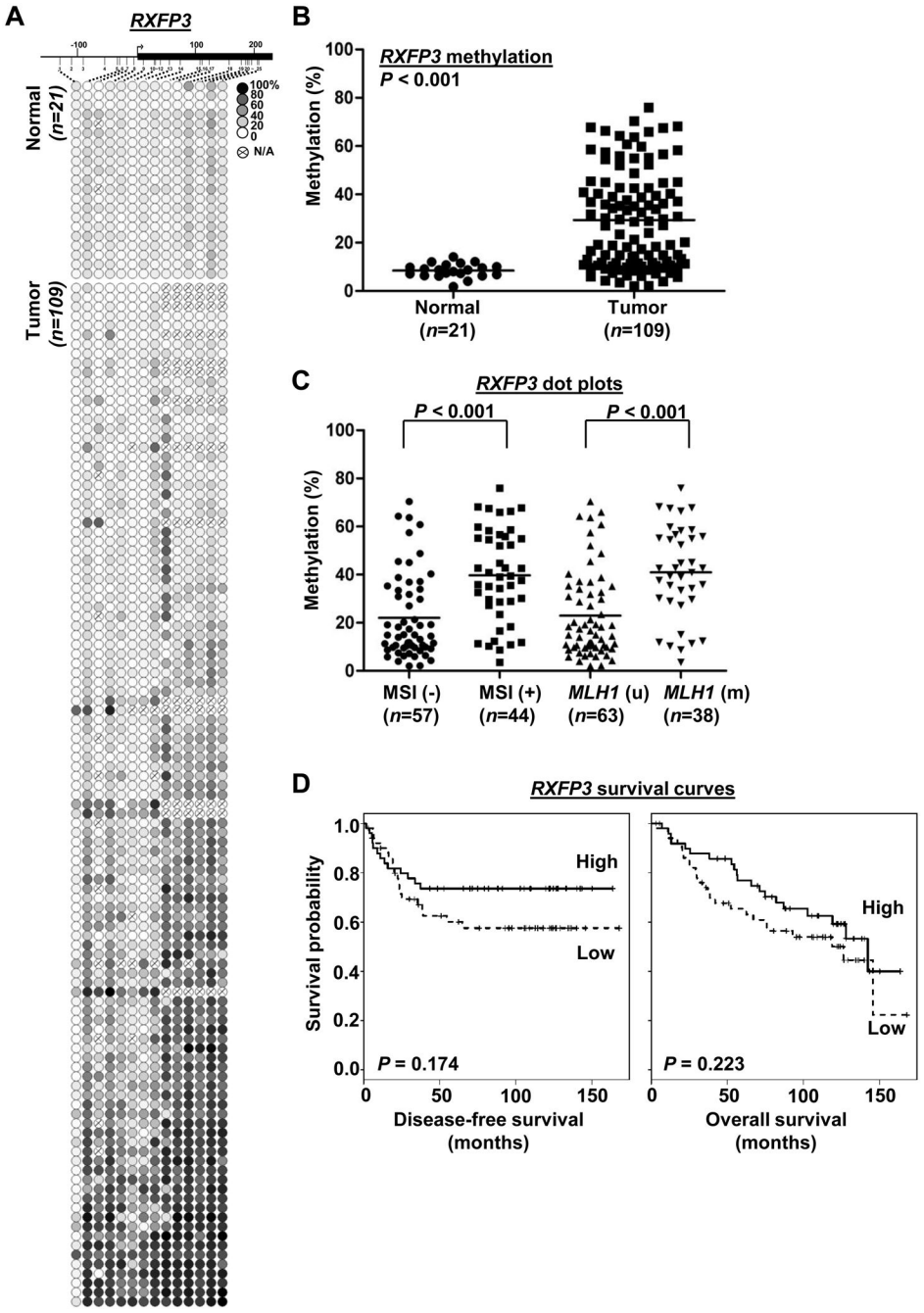

Fig. 5.

Methylation of *RXFP3* CpG island and clinicopathologic covariates analyses in primary endometrial carcinomas. (A) Methylation profiles of 21 normal endometrial tissues and 109 primary tumors created following MassARRAY analysis. (B) Dot plots pointing that *RXFP3* hypermethylation was found in primary endometrial tumors. (C) Dot plots indicating the level of *RXFP3* promoter methylation was positively correlated with MSI status. (D) Kaplan-Meier curves disease-free and overall survivals. (A–D) Assays were described as in Fig. 3.