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Epigenetic Repression of *microRNA-129-2* Leads to Overexpression of *SOX4* Oncogene in Endometrial Cancer

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Abstract

Genetic amplification, mutation, and translocation are known to play a causal role in the up-regulation of an oncogene in cancer cells. Here we report an emerging role of microRNA, the epigenetic deregulation of which may also lead to this oncogenic activation. *SOX4*, an oncogene belonging to the SRY-related high-mobility-group-box family, was found to be over-expressed ($P < 0.005$) in endometrial tumors ($n = 74$) compared with uninvolved controls ($n = 20$). This gene is computationally predicted to be the target of a microRNA, *miR-129-2*. When compared to the matched endometria, the expression of *miR-129-2* was lost in 27 of 31 primary endometrial tumors that also showed a concomitant gain of *SOX4* expression ($P < 0.001$). This inverse relationship is associated with hypermethylation of the *miR-129-2* CpG island, which was observed in endometrial cancer cell lines ($n = 6$) and 68% of 117 endometrioid endometrial tumors analyzed. Reactivation of *miR-129-2* in cancer cells by pharmacological induction of histone acetylation and DNA demethylation resulted in decreased *SOX4* expression. In addition, restoration of *miR-129-2* by cell transfection led to decreased *SOX4* expression and reduced proliferation of cancer cells. Further analysis found a significant correlation of hypermethylated *miR-129-2* with microsatellite instability and *MLH1* methylation status ($P < 0.001$), and poor overall survival ($P < 0.039$) in patients. Therefore, these results imply that the aberrant expression of *SOX4* is, in part, caused by epigenetic repression of *miR-129-2* in endometrial cancer. Unlike the notion that promoter hypomethylation may up-regulate an oncogene, we present a new paradigm in which hypermethylation-mediated silencing of a microRNA de-represses its oncogenic target in cancer cells.

Keywords

DNA methylation; epigenetics; *miR-129-2*; *SOX4*

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Supplementary data: The supplementary data include 6 figures and 3 tables.

Introduction

The *SRY-related high-mobility group box 4* gene, or *SOX4*, is known to be over-expressed in prostate, hepatocellular, lung, bladder and medulloblastoma cancers with poor prognostic features and advanced disease status (1-6). Its oncogenic potentials have been demonstrated in knock-in cells leading to aberrant transformation while the proliferation and metastatic capability has been greatly reduced in knock-out cancer cells (1-4,7). Functional analysis has shown that *SOX4* belongs to the TCF/LEF family of transcription factors that mediate transcription responses to Wnt signaling (2,8). More recently, a genome-wide chromatin immunoprecipitation study has further uncovered additional transcriptional targets of *SOX4* that are associated with TGF β , Hedgehog, and Notch pathways, microRNA processing, and tumor metastasis (7,9).

Genetic mechanisms leading to aberrant expression of *SOX4* have been explored in cancer cells. *SOX4* is mapped to chromosome 6p22, a region frequently amplified in lung, bladder, endometrial cancers (2,10-12). Somatic mutations have also been found in the exon region of this intronless gene in lung cancer (2). However, there is no experimental evidence to demonstrate positive correlation between these reported genetic alterations and the aberrantly increased *SOX4*. One emerging mechanism is microRNA (miRNA)-mediated oncogene expression.

miRNAs, a class of small non-coding RNAs (18-25 nt), are known to form imperfect pairing at the 3'-end of untranslated regions (UTR) of a target locus, resulting in mRNA degradation or translational inhibition (13). Through this post-transcriptional regulatory mechanism, miRNAs control a variety of physiological processes in normal cells, and deregulation of miRNAs may promote tumorigenesis (14). Increasing evidence indicates that epigenetic perturbations may contribute to abnormal miRNA expression in cancer cells (15). One well-studied epigenetic phenomenon is DNA methylation frequently observed in the promoter CpG island regions of genes (16). While promoter hypermethylation is associated with transcriptional silencing of coding genes for tumor suppressor functions, promoter hypomethylation can be related to activation of oncogenes in cancer cells (16,17). We therefore speculate whether methylation alteration may commonly occur in non-coding miRNAs, resulting in de-regulation of its target genes in cancer cells.

Here we report, for the first time, that the *SOX4* oncogene is also over-expressed in endometrial cancer. A miRNA, *miR-129-2*, was computationally predicted and functionally validated to be an upstream regulator of *SOX4*. A CpG island encompassing the *miR-129-2* locus was found to be hypermethylated in endometrial cancer cell lines and primary tumors. We further demonstrate that this methylation-mediated silencing has a causal role for *SOX4* activation in endometrial cancer.

Materials and Methods

Endometrial specimens and cell lines

Tissue specimens (117 tumors and 8 uninvolved controls) were obtained as part of our ongoing work on characterizing molecular alterations in endometrioid endometrial carcinomas. All participants consented to both molecular analyses and follow-up studies, and the protocols were approved by the Human Studies Committee at the Washington University and the Ohio State University. Clinicopathological variables of tumors, including age, stage, grade, microsatellite instability (MSI) and *MLH1* methylation, were summarized in Supplementary Table 1 and reported in our previous study (18). Human endometrial cancer cell lines, AN3CA, HEC1A, Ishikawa, KLE, RL95-2 and SK-UT-1B were routinely maintained in our laboratory (19), and ECC-1 cells were obtained from ATCC. For epigenetic studies, these cells were

treated with 5-aza-2'-deoxycytidine (DAC, 0.5 μ M, Sigma) for 48 h and/or trichostatin A (TSA, 5 μ M, Sigma) for 24 h. DNA and RNA from treated and untreated cells were isolated using standard protocols (20).

Endometrial tissue microarray

Tissue microarray slides, each containing a total of 74 endometrial tumors and 20 normal specimens, were obtained from US Biomax. Patients' characteristics were summarized in Supplementary Table 2. These slides were pre-processed prior to immunohistostaining. Antigen retrieval was performed by heat-induced epitope retrieval, in which the slides were placed in Dako TRS solution (pH 6.1) for 25 min at 94°C. Slides were then placed on a Dako autostainer with primary antibody (SOX4, 1:50, Abcam) and incubated for 60 min at room temperature. Staining was visualized with DAB chromogen, and slides were then counterstained and dehydrated through graded ethanol solutions. Images were digitally scanned with iScan™ (BioImagene) and analyzed with the BioImagene TissueMine™ software for discriminating immunohistochemically stained cancer cells from the surrounding stromal tissue. Intensities of nuclear staining were measured as segmented images then quantified. The algorithm reported the number and percentage of positively stained and non-stained nuclei.

Cell transfection

ECC-1 and Ishikawa (3×10^6) cells were transfected with mature *miR-129-2* mimics (*miR-129-3p* and *-5p*, 2.5 nM, Ambion), pre-miR negative control (#1, 2.5 nM, Ambion), *SOX4* siGenome SMART pool siRNA (2.5 nM, Dharmacon), siGenome non-targeting siRNA pool (#1, 2.5 nM, Dharmacon) and plasmids using the Cell Line Nucleofector Kit (Lonza) according to manufacturer's instructions.

Reverse transcription and quantitative PCR (RT-qPCR)

Total RNA (1 μ g) was reverse transcribed with the Superscript III reverse transcriptase (Invitrogen). PCR was performed as described previously (20). Specific primers for amplification were listed in the Supplementary Table 3. 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* (20).

For detecting mature miRNA molecules (i.e., *miR-129-3p* and *miR-129-5p*), reverse transcription was performed following the Applied Biosystems Taq Man MicroRNA Assay protocol. This sensitive system has been designed to specifically detect mature miRNAs that are distinct from their precursors. In addition, the assay can often distinguish between miRNA targets that differ by only a single nucleotide (21). All reactions were performed in triplicate. The expression of *miR-129-2-3p* or *-5p* was normalized using *RNU48* or *U6*. The expression relative to *RNU48* or *U6* was determined using the $2^{-\Delta\text{CT}}$ method.

Western blot analysis

Whole cell protein lysates were extracted with the M-PER Mammalian Protein Extraction Reagent (Pierce). Western blot analysis was conducted using antibodies against SOX4 (Abcam) and β -actin (Santa Cruz).

Cell proliferation assay

Cell proliferation was monitored using the CellTiter 96® Aqueous solution (Promega). Endometrial cancer cells (3,000/well) transfected with either *miR-129-2*, negative control miRNA, *SOX4* siRNA or non-targeting siRNA pool were seeded in 96-well plates. Cell proliferation was documented every 24 h following the manufacturer's protocol. To measure

cell proliferation, 20 μ L of MTS labeling reagent was added to each well and incubated at 37°C for 1 h. Absorbance was measured at 490 nm in the ELISA reader (Molecular Devices).

3'-UTR reporter assay

The full-length 3'-UTRs of *SOX4* and *UBE2F*, generous gifts from Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center, New York), were cloned into the Psicheck 2 dual luciferase reporter vector (Promega). ECC-1 or Ishikawa cells were transfected with reporter constructs and a *miR-129-2* and/or its antagomir targeting endogenous *miR-129-2* (Ambion). Cells were lysed at 24 h after transfection and the ratio of *Renilla* to firefly luciferase was measured using the dual luciferase assay (Promega). Normalized *Renilla* to firefly ratios were determined in the presence or absence of *miR-129-2* inhibition on *SOX4* UTR luciferase activities.

Combined bisulfite restriction analysis (COBRA)

Genomic DNA (500 ng) was treated sodium bisulfite using the EZ DNA Methylation kit (Zymo Research) following manufacturers' recommended protocols. COBRA analysis was used to evaluate methylation of *miR-129-2*. Target sequences were amplified by PCR, and the products were digested with *AciI*, (New England Biolabs) to identify methylated sequences. Primer sequences and PCR conditions are presented in Supplementary Table 3. Digested and non-digested PCR products were resolved on 2% agarose gels stained with ethidium bromide. DNA fragments digested by *AciI* were scored as "methylated" in a given sample.

MassARRAY analysis

To quantify methylation levels of the *miR-129-2* CpG islands in clinical samples, the high-throughput MassARRAY platform (Sequenom) was carried out as described previously (22). Briefly, bisulfite-treated DNA was amplified with primers for the *miR-129-2* CpG island. 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 (1-3 CpG sites per unit) were generated by the EpiTyper software (Sequenom).

Statistical and survival analyses

Student's *t* or Wilcoxon test was used to compare the immunohistostaining, cell proliferation, invasion, apoptosis, and RT-qPCR results in different treatment groups. A significance was assigned as * if $P < 0.05$. The relationship between methylation levels of *miR-129-2* and relevant categorical clinicopathological covariates was performed using the Wilcoxon rank sum test for binary variables. The Kruskal Wallis test was used followed by a pairwise Wilcoxon rank sum test. Overall survival (OS) was defined as the time interval from the date of diagnosis to the date of death or latest follow-up if alive. Recurrence-free survival (RFS) was defined as the time interval from surgery to recurrence, disease progression or latest follow-up. The Kaplan-Meier product limit method was used to estimate the empirical survival functions for categorical covariates accompanied with *P* value from the Log-rank test. Univariate Cox proportional hazard models were used to assess effect of a continuous covariate on survival outcomes. Multivariate Cox proportional hazard models were fitted to examine the potential predictive effect of covariates of interest on survival outcomes after adjustment for confounding factors. All tests were two-sided and all analyses were performed using R.

Results

SOX4 is over-expressed in endometrioid endometrial carcinomas

To determine whether SOX4 is aberrantly expressed in endometrial tumors, we conducted tissue microarray analysis in a panel of 74 endometrioid endometrial carcinomas and 20

uninvolved controls (see representative immunohistostaining images in Fig. 1A, left). The nuclear staining intensities of *SOX4* were significantly higher in tumor sections than those of normal tissue sections ($P < 0.005$, Fig. 1A, right). This finding is consistent with the RT-qPCR results in which the levels of *SOX4* mRNA were higher in tumors ($n=31$) compared to the adjacent normal counterparts ($P < 0.001$, Fig. 2D, left).

In a knock-out study, we assessed the role of *SOX4* in endometrial cancer cells. ECC-1 and Ishikawa cells were transiently transfected with *SOX4* siRNA or a non-targeting control. Both protein and mRNA levels of *SOX4* were found to be reduced to $\leq 50\%$ in transfectants (Fig. 1B & C), resulting in attenuation of cell growth (Fig. 1D). This *SOX4* knock-out, however, had no effects on cell invasion or migration (data not shown).

miR-129-2* is a negative regulator of *SOX4

Because miRNA may have a potential role in mediating oncogene repression (14), we searched potential target sequences at the 3'-UTR of *SOX4* using three software programs PicTar, TargetScan, and miRanda (23-25). Putative binding sites were found in a microRNA locus, *miR-129-2*, which is the precursor of two mature forms, *miR-129-3p* and *-5p* (Fig. 2A & B). To further validate this computational finding that *miR-129-2* may negatively regulate *SOX4*, we assessed the expression of 3'-UTR of *SOX4* in luciferase reporter assays (Fig. 2C). The expression of the *SOX4* reporter was significantly reduced to $\leq 55\%$ in *miR-129-3p*- or *-5p*-transfected ECC-1 and Ishikawa cells, but not in control cells. Transfection with either *miRNA* did not affect the reporter activity of a negative control gene, *UBE2F*, which has no known *miR-129-2* binding sites on its UTR. Moreover, inhibition of *miR-129-3p* or *-5p* by antagomirs slightly enhanced the expression of *SOX4*, suggesting that this gene is a direct target of *miR-129-2*. We additionally confirmed this inverse relationship between *miR-129-3p* and *SOX4* mRNA and expression using the aforementioned 31-paired samples (Fig. 2D).

Methylation-mediated silencing of *miR-129-2* derepresses *SOX4* expression

Since the expression of *miR-129-2* is frequently lost in endometrial tumors (see Fig. 2D) and the 5'-end of this locus has a canonical CpG island (Fig. 3A), we determined whether this down-regulation is mediated by epigenetic mechanisms. Hypermethylation of this promoter CpG island was detected in six endometrial cancer cell lines, ECC-1, HE1A, Ishikawa, KLE, RL95-2, and SK-UT-1B, based on a COBRA assay (Fig. 3B). When these cells were treated with a demethylating agent, DAC (0.5 μ M), a histone deacetylase inhibitor, TSA (5 μ M), or in combination, reactivation of *miR-129-3p* was observed in 4 (Ishikawa, KLE, RL95-2, and SK-UT-1B) of the 6 cell lines analyzed treated with DAC (Fig. 3C). More profound effects of this re-expression were found in all these cell lines treated with TSA and the combination (Fig. 3C & Supplementary Fig. S1). These results suggest that the loss of *miR-129-2* expression is associated with promoter hypermethylation in endometrial cancer cells. Interestingly, these epigenetic treatments might lead to the suppression of *SOX4* in endometrial cancer cells. The effect occurred at the mRNA level in ECC-1, HEC1A, KLE, RL95-2 and SK-UT-1B cells (Fig. 3D, left). This suppression, however, was more prominent at the post-translational level for Ishikawa and RL95-2 cells (Fig. 3D, right). In addition, we showed that the expression of four other *miR-129-2* targets, *EIF2C3*, *PLCG1*, *RAB21* and *STAT5B*, was repressed in cancer cells treated with DAC and/or TSA (Supplementary Fig. S2). Taken together, the observation indirectly indicates that these epigenetic treatments may lead to reactivation of *miR-129-2*, which in turn represses the expression of *SOX4*.

To further prove this inverse relationship, we conducted a functional knock-in study in two endometrial cell lines, ECC-1 and Ishikawa, harboring the epigenetically silenced *miR-129-2*. Transient transfection of these cells with either *miR-129-3p* or *miR-129-5p* resulted in reduction of both *SOX4* mRNA and protein (Fig. 4A & B) (Note: Transfection efficiency

was examined by measuring each mature microRNA, see Supplementary Fig. S3). Moreover, the knock-in of one of these mimics, *miR-129-5p*, greatly reduced the proliferation of these cancer cells ($P < 0.05$, Fig. 4C). In addition, the expression of three SOX4-regulated genes (*DHX9*, *FZD5* and *SEMA3C*) (3, 9) was partially reduced in ECC-1 cells treated with DAC and/or TSA or ectopically transfected with *miR-129-3p* or *-5p* (Supplementary Fig. S4). Taken together, these *in vitro* studies suggest that *miR-129-2* negatively regulates SOX4 and that promoter hypermethylation of this miRNA derepresses its expression.

Hypermethylation of *miR-129-2* is associated with shorter patient survival, MSI, and *MLH1* methylation in endometrial tumors

To confirm our *in vitro* findings in primary tumors, we first studied methylation patterns of the *miR-129-2* CpG island (see Fig. 3A) in eight tumors with matched normal endometria by COBRA and quantitative MassARRAY. We showed that tumors were hypermethylated compared with the corresponding adjacent normal tissues (Supplementary Fig. S5). We then extended this methylation study to 117 (34 recurrent and 83 non-recurrent) primary endometrioid endometrial tumors and 8 uninvolved controls. Quantitative analysis indicated that 80 of these primary tumors exhibited extensive methylation in 14 units (1-3 CpG sites per unit) of the *miR-129-2* CpG island relative to those of uninvolved controls ($P < 0.0005$) (Fig. 5A). This methylation survey in the patient cohort also uncovered a pattern in which methylation accumulation may begin at the flanking regions and progressively invade to the core of this CpG island, consistent with the so-called methylation spread theory (26).

The mean methylation level of each CpG unit was used to compare between the recurrent and non-recurrent group. More accumulation of *miR-129-2* methylation was found in the former group, but the sample size of this cohort could be too small to reach statistical significance ($P = 0.066$, Fig. 4B). Detailed analysis of individual CpG units is shown in Supplementary Fig. S6. We also evaluated the association between *miR-129-2* hypermethylation and patient survival. On univariate analysis, *miR-129-2* hypermethylation was correlated with shorter overall survival (Cox Hazard Ratios, 1.02; $P = 0.018$), but not with recurrence-free survival (Cox Hazard Ratios, 1.02; $P = 0.067$) when the mean methylation level of normal controls was used to set the threshold. The Kaplan-Meier survival analysis indicated that patients with this hypermethylation had poor overall survival (Fig. 5C, $P = 0.039$, Log-rank test). Statistical analysis further revealed that *miR-129-2* hypermethylation was significantly associated with MSI and *MLH1* methylation ($P < 0.0001$, Fig. 5D). Endometrial tumors exhibiting the MSI phenotype usually have high replication error rates and genomic instability (18,27). This defect is attributed, in part, to epigenetic repression of *MLH1* that is responsible for DNA mismatch repair in normal cells (27).

Discussion

In addition to genetic alterations, promoter hypomethylation has been recognized as an epigenetic mechanism that contributes to oncogene activation in cancer cells (16,17). In this case, a demethylation event is supposed to occur in an inactive, methylated promoter, leading to transcriptional reactivation of an oncogene. However, experimental proof for genuine promoter hypomethylation is frequently difficult and inconclusive because the outgrowth of a subpopulation of cancer cells may confound this epigenetic observation. For example, the oncogene of interest may have never been silent in a minor population of cancer-initiating cells while the majority of other cells display promoter hypermethylation of the gene. The increased expression of this oncogene may simply result from rapid expansion of these few cells that eventually take over the whole population during tumor progression. If this scenario indeed occurs, it cannot be a *bona fide* epigenetic event for oncogene activation.

In this study, we demonstrate that promoter hypermethylation can be directly associated with the activation of an oncogene. Specifically, this epigenetic event occurs in an upstream regulator, *miR-129-2*, that was shown to negatively regulate *SOX4* oncogene in both knock-in and -out assays. *miR-129-2* is embedded in a canonical CpG island on chromosome 11, which was found to be frequently hypermethylated in endometrial cancer. This epigenetic event results in *miR-129-2* silencing, which in turn derepresses *SOX4* expression. While we still cannot rule out hypomethylation of the *SOX4* promoter CpG island as one of the causes, our present observation conclusively suggest that promoter hypermethylation of *miR-129-2* is a common mechanism leading to the *SOX4* over-expression in endometrial cancer.

It should be noted that a second CpG island is found 1.2-kb upstream from the first one analyzed in this study. In addition, the 5'-ends of two transcripts, *BG120451* and *BI964058*, are located in this upstream region. It is possible that these transcripts are the primary RNAs for *miR-129-2* (28). Future mapping of putative transcription start sites located in these 5'-end areas will provide insight into the transcriptional control of this microRNA locus. Additional methylation analysis may further determine the role of this second CpG island in the silencing of *miR-129-2* during endometrial tumorigenesis.

We have additionally searched the miRBase database and found that the expression of *SOX4* may be regulated by at least 16 putative miRNAs, including *miR-129-1* located on chromosome 7. Similar to *miR-129-2*, the *miR129-1* precursor produces mature *miR-129-5p*, which negatively regulates *SOX4* as described in this study. Since there is no known CpG island located near or within the *miR-129-1* locus, it remains to be determined whether this miRNA is transcriptionally silenced by other epigenetic mechanisms (e.g., EZH2-mediated histone modifications, 29) in endometrial cancer.

Five (*miR-203*, *miR-335*, *miR-219-2*, *miR-596*, and *miR-618*) of the other 15 miRNAs are located close to CpG islands based on our computational analysis (data not shown). Among these loci, *miR-335* is the only one currently reported to be lost in primary breast tumors of recurrent patients (7). However, it remains to be determined whether promoter hypermethylation plays a role in the silencing of this locus. Future studies will therefore be important to study the coordinate regulation of these miRNAs on *SOX4* repression. It is also possible that concurrent hypermethylation of these loci contributes to a CpG island methylator phenotype (30) and may improve the statistical power for predicting disease recurrence in our endometrial patient cohort (see Fig. 5B).

Extending from our present observation, epigenetically mediated silencing of other miRNAs that lead to tumor progression has recently been reported in the literature (15,31-33). For example, *ABL1* was showed to be a direct target of *miR-203* (32). This miRNA was genetically and epigenetically down-regulated in leukemia cells expressing *ABL1* or *BCR-ABL1* fusion protein (32). Restoration of *miR-203 in vitro* led to reduced *ABL1* and *BCR-ABL1* expression and decreased proliferation of malignant cells (32). Taken together, these and our studies clearly indicate that epigenetic silencing of tumor-suppressive miRNAs can be important constituents in cancer epigenomes and that the event is as significant as hypomethylation of oncogenes and hypermethylation of tumor suppressor genes.

In conclusion, our findings support a comprehensive screening of miRNA regulators at the 3'-UTR regions of all known oncogenes. High-throughout functional studies can be developed to establish the inverse relationship between these tumor-suppressive miRNA loci and their target oncogenes. This type of omics study may find that epigenetically mediated silencing of these miRNAs can be as common as genetic alterations that contribute to oncogene activation in cancer cells. As such, the combined epigenetic and miRNA-based therapies can be feasible options for future treatments in cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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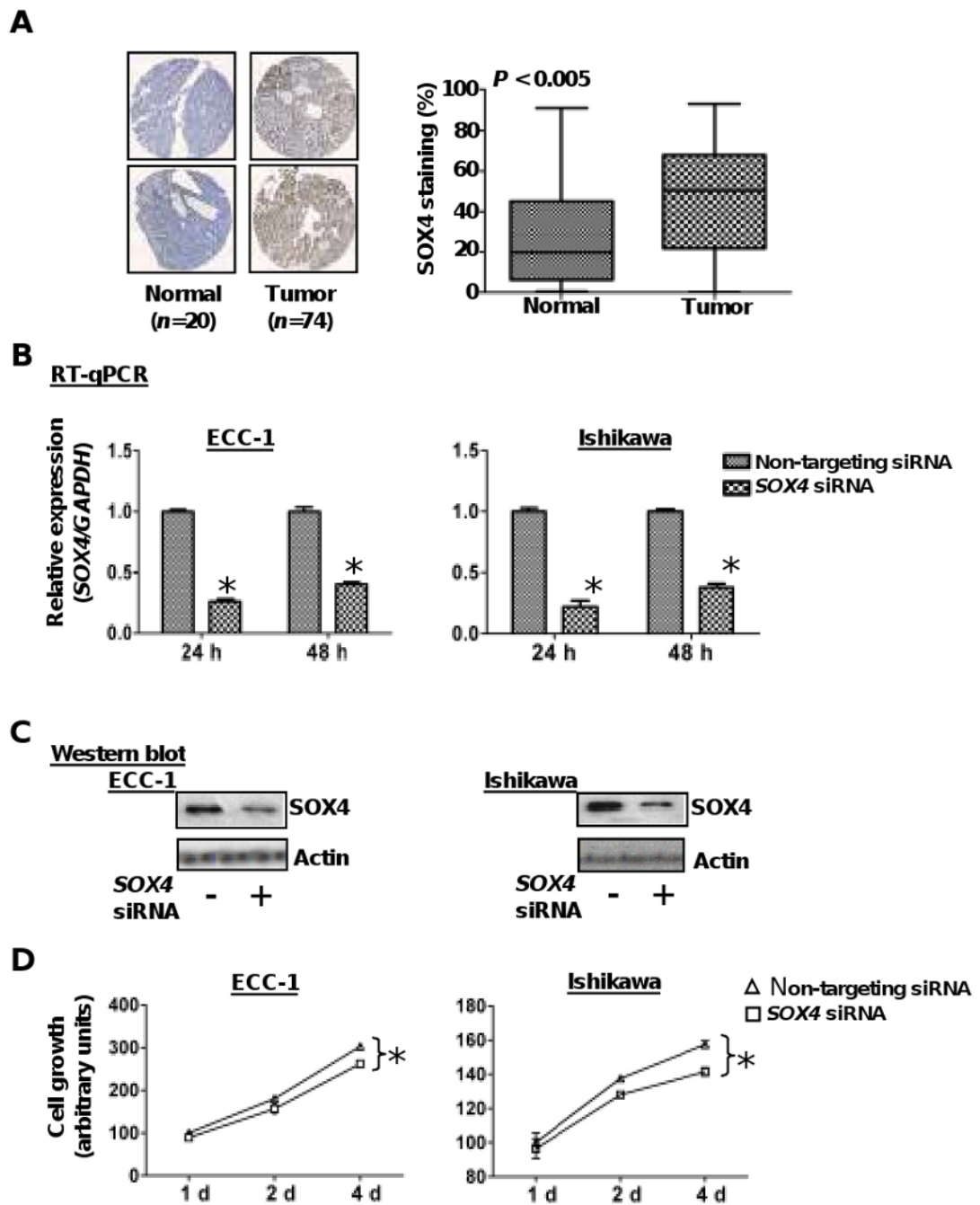


Figure 1.

SOX4 is overexpressed in endometrial tumors. **A**, representative photographs of endometrial tissue microarrays (1.5mm core diameter) which underwent immunohistochemical staining for *SOX4* and were scored for nuclear staining by the TissueMine software. Boxplots of *SOX4* nuclear staining for normal tissue ($n=20$) and endometrial tumors ($n=74$) $P < 0.005$. **B & C**, relative expression levels of *SOX4* mRNA and protein in ECC-1 and Ishikawa cells after transient transfection with *SOX4* siRNA or a pool of non-targeting siRNA oligonucleotides for 24 and/or 48 h. *GADPH* and β -actin served as internal controls for RT-qPCR and western blotting respectively. Error bars represent SD among triplicates; *, $P < 0.05$. **D**, cellular proliferation was measured by MTS assay in endometrial cancer cells transfected with *SOX4*

siRNA or non-targeting siRNA. Transfectants (3,000/well) were placed in 96-well plates and proliferation was measured every 24 h. Each point represents the mean value of at least triplicates. *, $P < 0.05$.

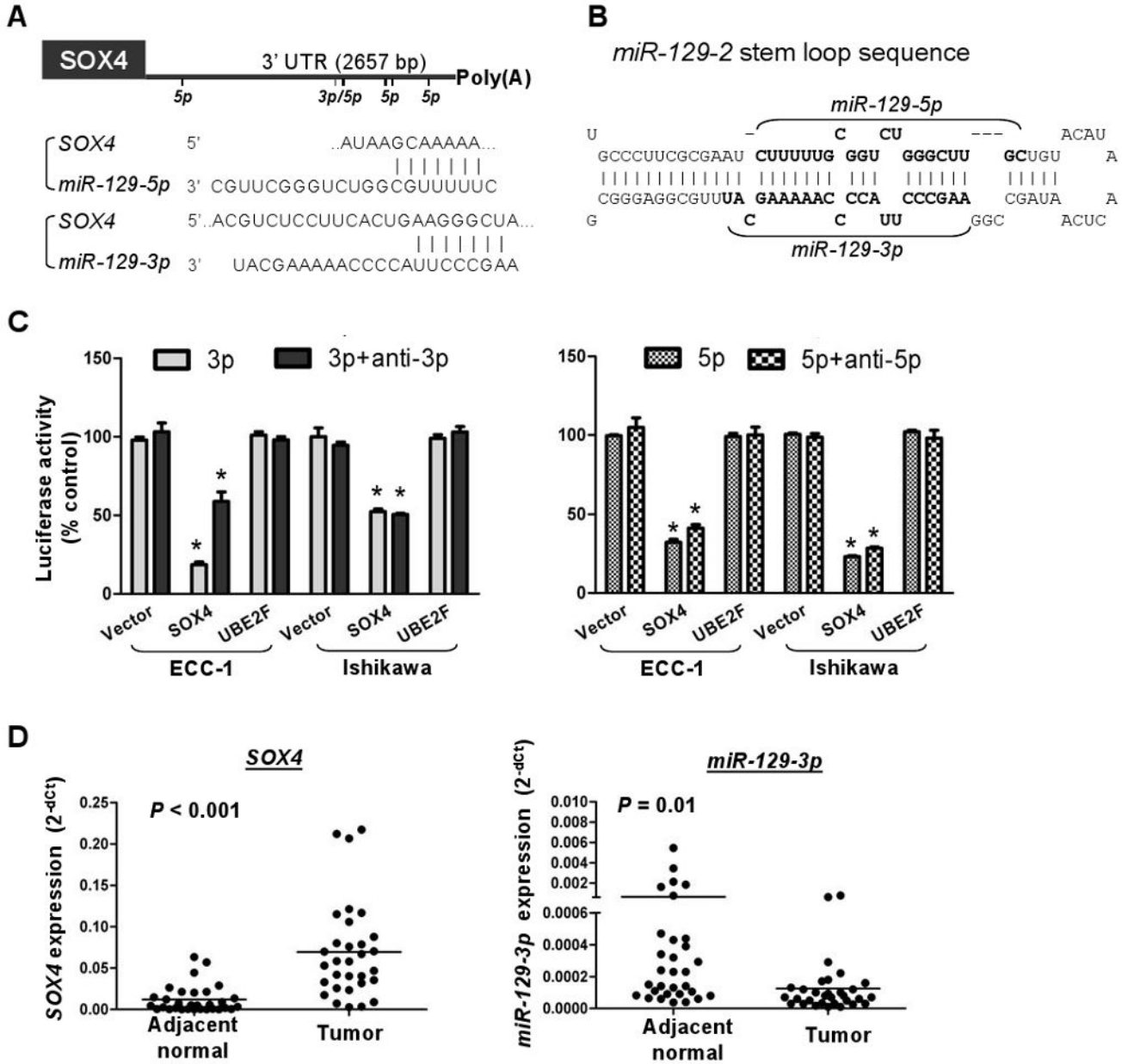


Figure 2. *miR-129-2* directly targets *SOX4*. **A**, bioinformatic analysis of *miR-129-3* and *-5p* (the mature forms of *miR-129-2*) predicted binding sites in the *SOX4* 3'-UTR (bars under line). The predicted pairing of mRNA target region (*top*) and miRNA (*bottom*) is as indicated, where a line (“|”) indicates hydrogen bonding. **B**, predicted secondary structure of *miR-129-2*. The stem loop structure of *miR-129-2* is a precursor to two mature miRNAs: *miR-129-3p*, and *miR-129-5p* (bold text). **C**, *miR-129-3p* and *-5p* suppressed the expression of a luciferase vector with the *SOX4* 3'-UTR. A luciferase expression vector with the 3'-UTR of *SOX4* and *UBE2F* or an empty vector was transfected into ECC-1 and Ishikawa cells. At the same time, anti-*miR-129-3p* or *-5p* (anti-3p or anti-5p) and/or *miR-129-3p* or *5p* (3p or 5p) were introduced. Twenty-four hours after the transfection, the cells were harvested and assayed for luciferase activity. *Renilla* luciferase was used for normalization to empty vector for the

transfection efficiency. *Error bars, SD; *, P < 0.05 compared with empty vector. D*, dot plots showing an inverse relationship between *SOX4* (*left*) and *miR-129-3p* (*right*) mRNA expression in 31 pairs of endometrial tumors and adjacent normal tissues. Horizontal bars represent mean expression levels. Significant differences were determined using Student's *t* tests.

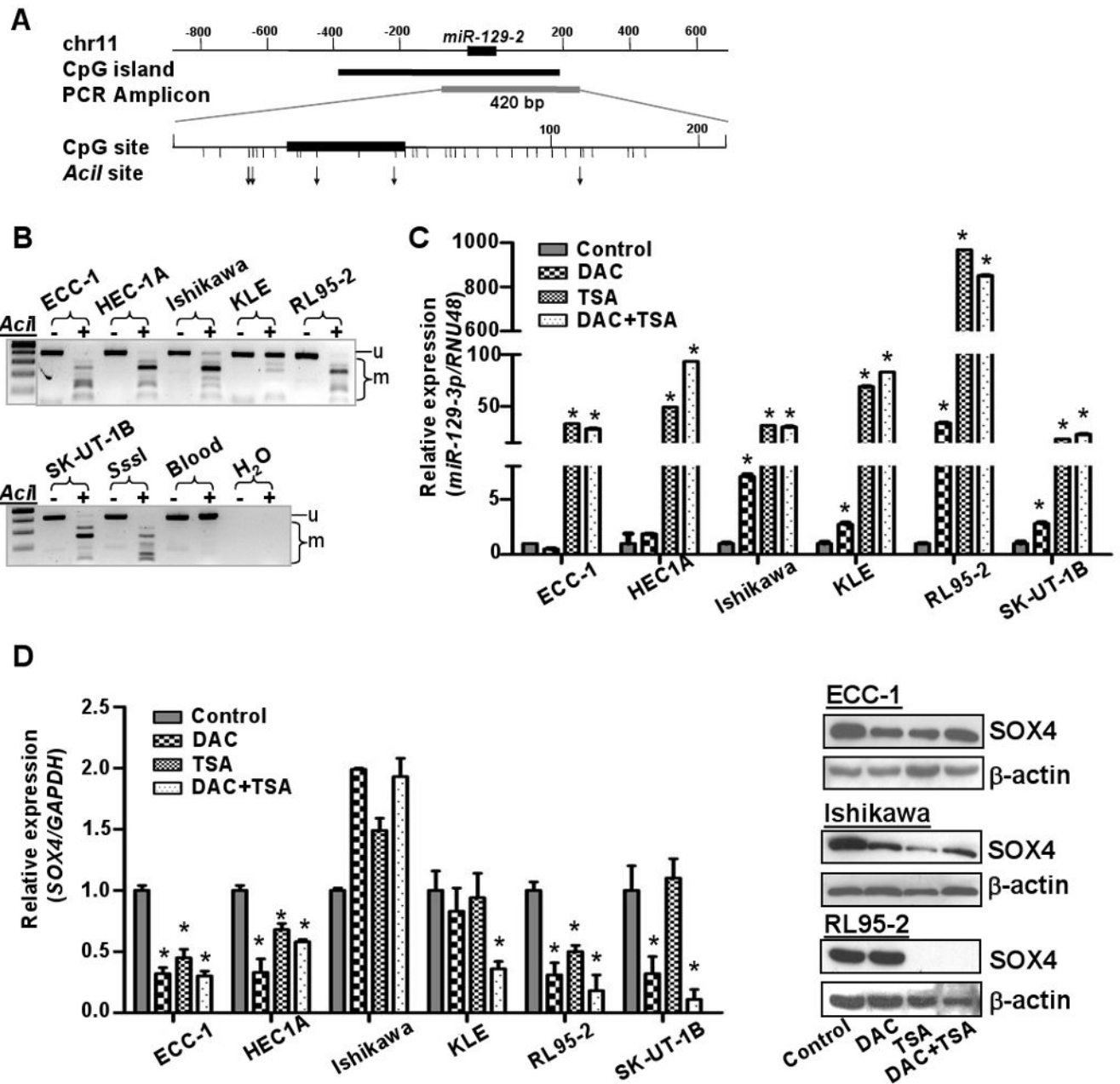


Figure 3. Reactivation of *miR-129-2* in cancer cells by pharmacological induction of hyperacetylation and DNA demethylation lead to reduced *SOX4* expression at both the mRNA and protein levels. **A**, genomic map of *miR-129-2* CpG island and amplicon. *Bar* under line, CpG site; \downarrow , *Acil* cutting sites. **B**, COBRA analysis in endometrial cancer cell lines. u, unmethylated band; m, methylated bands; *SssI*, 100% methylated control; Blood, a mix of 4 normal peripheral blood samples as negative control; +, *Acil* restriction enzyme added; -, without *Acil*. **C**, relative expression levels of *miR-129-3p* in endometrial cancer cell lines treated with DAC and/or TSA in relation to untreated controls was determined by RT-qPCR analysis. *RNU48* was used as internal control gene. *Error bar*, SD; *, $P < 0.05$ compared with untreated control of the same

cell type. **D**, relative expression levels of *SOX4* mRNA (*right*) and protein (*right*) indicating a down-regulation of *SOX4* in endometrial cancer cells after treatment with DAC and/or TSA. *GAPDH* or β -actin was used as internal or loading control, respectively.

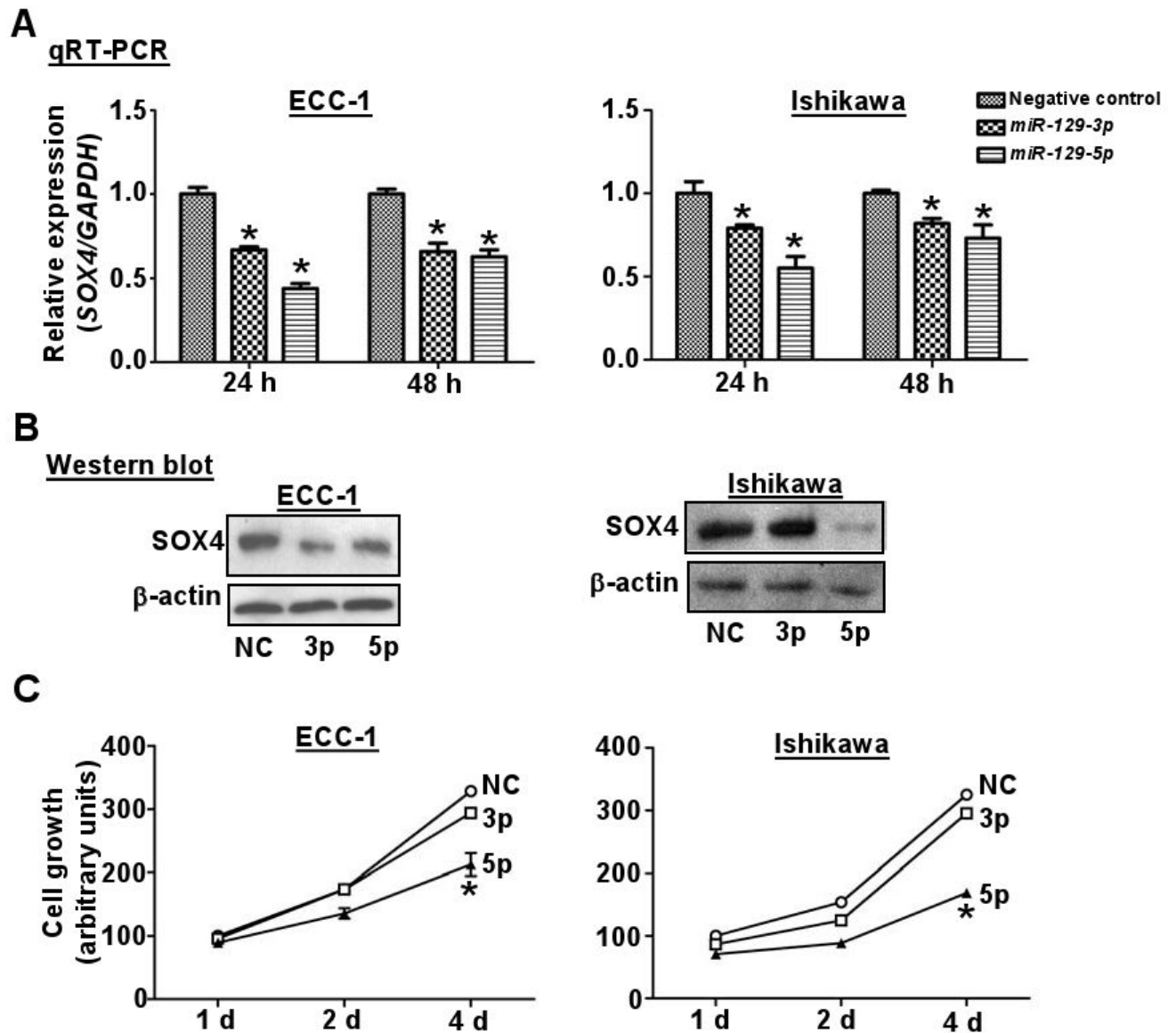


Figure 4.

Functional analysis of *miR-129-2* in endometrial cancer cell lines. **A & B**, relative levels of *SOX4* mRNA (**A**) or protein (**B**) expression in ECC-1 and Ishikawa cells after transient transfection with miRNAs or negative control (NC) RNA oligonucleotides for 24 or 48 h. *GADPH* and β -actin served as an internal control of mRNA or protein, respectively. *Error bars*, SD from triplicates; *, $P < 0.05$ compared with NC at the same time point. **C**, cellular proliferation by MTS assay in the endometrial cancer cell lines ECC-1 and Ishikawa transfected with *miR-129-3p* (3p), *miR-129-5p* (5p), or negative control (NC). Proliferation measured as described in Fig. 1C.

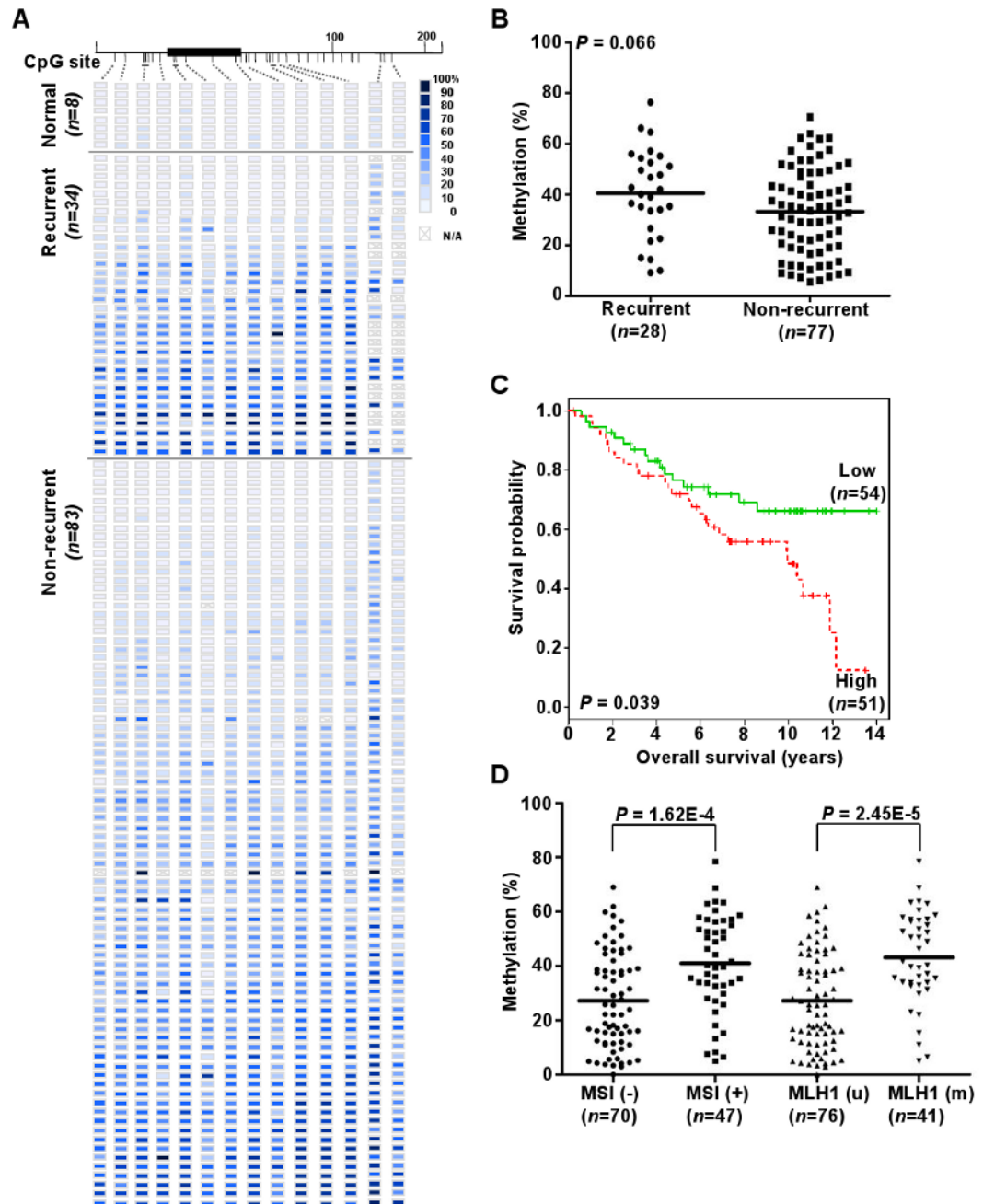


Figure 5.

Methylation of *miR-129-2* CpG island and clinic-pathological covariates analyses in primary endometrioid endometrial tumors. **A**, methylation profiles of 8 normal endometrial tissues, 34 recurrent, and 83 non-recurrent primary tumors created following MassARRAY analysis. Each row represents a sample and each column represents a CpG unit. Color-coding depicts the degree of methylation with dark blue being 100% and white being 0%; N/A, not analyzable. **B**, dot plots point that *miR-129-2* hypermethylation is moderately correlated with recurrent diseases. The mean of normal specimens in Fig. 5A was set as a threshold for analysis. Each dot represents the mean of each specimen on the first five CpG sites in Fig. 5A. Horizontal lines, mean values. P value was calculated by Wilcox test. **C**, Kaplan-Meier curves for overall

survival. Samples were grouped according to the mean level of methylation for the first five CpG units of *miR-129-2*, when the mean exceeded the mean of normal specimens. Vertical bars represent excluded cases. *P* value estimated from Log-rank test. **D**, dot plots indicating the level of *miR-129-2* promoter methylation is positively correlated with MSI and *MLH1* methylation status.