ABSTRACT

Objective: The aim of this study was to establish criteria that would indicate whether fertility preservation therapy would likely be safe for patients aged 40 years or less with endometrioid endometrial cancer based on their DNA methylation profile.

Methods: Forty-nine fresh-frozen tissue samples from patients with endometrial cancer from an initial cohort and 31 formalin-fixed paraffin-embedded tissue samples from a second cohort were subjected to genome-wide DNA methylation analysis using the Infinium MethylationEPIC BeadChip.

Results: Epigenomic clustering of early-onset endometrial cancer was correlated with the widely used recurrence risk classification. Genes showing differences in DNA methylation levels between the low-recurrence-risk category and intermediate- and high-risk categories were accumulated in pathways related to fibroblast growth factor and nuclear factor-κB signaling. DNA hypomethylation and overexpression of \textit{ZBTB38} were frequently observed in the low-risk category. Eight hundred thirty-one marker CpG probes showed area under the curve values of >0.7 on the receiver operating characteristic curve for discrimination of patients belonging to the low-risk category. By combining marker CpG sites, seven panels for placing patients into the low-risk category with 91.3% or more sensitivity and specificity in both the initial and second cohorts were established.

Conclusions: DNA methylation diagnostics criteria using up to 6 of 8 CpG sites for \textit{LPP}, \textit{FOXO1}, \textit{RNF4}, \textit{EXOC6B}, \textit{CCP1}, \textit{RREB1} and \textit{ZBTB38} may be applicable to recurrence risk estimation for patients aged 40 years or less with endometrial cancer, regardless of tumor cell content, even if formalin-fixed paraffin-embedded biopsy or curettage materials are used.

Keywords: DNA Methylation; Infinium Array; Endometrioid Carcinoma; Early Onset; Fertility Preservation; \textit{ZBTB38}
INTRODUCTION

Although endometrial cancer is typically a disease of post-menopausal women [1], around 2%-14% of cases occur in women aged 40 years or less [2-4]. Along with the increasing incidence of risk factors such as obesity in young women, early-onset endometrial cancer has also recently shown a marked increase [4]. The standard treatment for endometrial cancer is hysterectomy and bilateral salpingo-oophorectomy, but for younger patients fertility-sparing treatment needs to be considered. Currently, conservative treatment with hormones such as medroxyprogesterone acetate (MPA) is used for early-stage early-onset endometrial cancer [5]. However, we have reported previously that the recurrence rate after MPA therapy is 63% [6]. In order to balance fertility preservation and curability maximization, MPA therapy should be considered for younger patients with less-aggressive early-onset cancers for whom a favorable outcome can be expected even without hysterectomy. To enable such personalized therapy, any objective indicator of aggressiveness for early-onset endometrial cancers should be explored.

Our previous studies have revealed that mutations of the CTNNB1 gene or DNA methylation alterations of genes involved in Wnt signaling were frequent in early-onset endometrial cancer, whereas genetic and epigenetic alterations of FGF signaling genes were observed in late-onset endometrial cancer in women over 40 years of age [7]. In this context, objective indicators of aggressiveness for early-onset endometrial cancer should be explored based on molecular abnormalities in early-onset endometrial cancer itself. In our previous study, early-onset endometrioid endometrial cancers were divisible into clusters EA and EB based on their genome-wide DNA methylation profiles: those in cluster EA tended to show more frequent lymphovascular invasion and to be more frequently diagnosed as histological grade 3 than those in cluster EB [7]. Therefore, it is feasible that DNA methylation alterations might be a reliable indicator of cancer aggressiveness.

In the present study, to establish prognostication criteria that could be useful for identifying patients with early-onset endometrioid endometrial cancer who might be good candidates for fertility preservation therapy, we performed genome-wide DNA methylation analysis of 49 fresh frozen tissue samples in an initial cohort and 31 formalin-fixed paraffin-embedded (FFPE) tissue samples in a second cohort, totaling 80 samples of cancerous tissue, from patients aged 40 years or less.

MATERIALS AND METHODS

1. Patients and tissue samples
The initial and second cohorts included 49 and 31 tissue samples of primary endometrioid endometrial cancers obtained from patients aged 40 years or less who underwent hysterectomy.
at Keio University Hospital, respectively. Histological diagnosis and grading were based on the 2003 World Health Organization classification [8] and the Tumor-Node-Metastasis classification [9]. The clinical stage of the disease was based on the 2008 revised International Federation of Gynecology and Obstetrics classification [10]. Six patients in the initial cohort and 5 in the second cohort had received preoperative MPA therapy. Recurrence was diagnosed by clinicians on the basis of physical examination and imaging modalities such as computed tomography and positron emission tomography. The clinicopathological parameters of patients belonging to the initial and second cohorts are summarized in Table S1; no significant differences in any of these parameters were observed between the cohorts.

Tissues samples in the initial cohort were taken immediately after surgery and then frozen in liquid nitrogen for preservation in the Keio Women's Health Biobank in accordance with the “Japanese Society of Pathology Guidelines for the handling of pathological tissue samples for genomic research” [11]. Tissues samples in the second cohort were routinely fixed in 10% neutral buffered formalin for 1 to 3 days and embedded in paraffin in accordance with the same Guidelines [11]. This study was approved by the Ethics Committee of Keio University Hospital and was performed in accordance with the Declaration of Helsinki. All patients included in this study provided written informed consent for use of their materials.

2. Tissue preparation and bisulfite modification
From the fresh frozen tissue samples in the initial cohort, high-molecular-weight DNA was extracted using phenol-chloroform followed by dialysis. We performed microdissection of each sample of FFPE tissue in the second cohort: areas showing a tumor cell content of more than 80% were dissected out using a toothpick under a microscope, avoiding contamination with non-cancerous epithelial cells, myometrial cells and infiltrating inflammatory cells. Subsequently, the genomic DNA was extracted from the microdissected specimens using a GeneRead FFPE Kit (QIAGEN GmbH, Hilden, Germany). Five-hundred-nanogram aliquots of DNA extracted from fresh frozen tissue samples were subjected to bisulfite conversion using an EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). For FFPE tissue samples, 50- to 300-nanogram aliquots of DNA were used and DNA restoration was performed using an Illumina HD FFPE Restoration Kit (Illumina, San Diego, CA, USA), which includes a DNA polymerase, a repair enzyme, and a ligase, after bisulfite conversion as described previously [12].

3. Infinium assay
DNA methylation status at 866,895 CpG loci was examined at single-CpG resolution using the Infinium MethylationEPIC BeadChip (Illumina) [13]. After hybridization, the specifically hybridized DNA was fluorescence-labeled by a single-base extension reaction and detected using an iScan reader (Illumina) in accordance with the manufacturer’s protocol. The data were then assembled using GenomeStudio methylation software (Illumina). At each CpG site, the ratio of the fluorescence signal was measured using a methylated probe relative to the sum of the methylated and unmethylated probes, i.e., the so-called β-value, which ranges from 0.00 to 1.00, reflecting the methylation level of an individual CpG site. Methylome data for a proportion of the initial cohort samples had been included in our previous study focusing on epigenomic clustering of endometrial cancer [9]. The results of the Infinium assay have been deposited in the Gene Expression Omnibus (GEO) database (accession number: GSE178610; https://www.ncbi.nlm.nih.gov/geo/).
4. **Pathway analysis**

MetaCore™ software (version 19.3; Thomson Reuters, New York, NY, USA) is a pathway analysis tool based on a proprietary manually curated database of human protein-protein, protein-DNA and protein compound interactions. MetaCore pathway analysis by GeneGo was performed using genes showing significant differences in DNA methylation levels among recurrence risk classification categories. Such genes were considered significantly enriched in pathways for which the p-value was less than 0.01.

5. **Whole-exome sequencing**

Whole-exome analysis of genomic DNA was performed for the 47 tissue samples in the initial cohort for which genomic DNA was available even after methylome analysis. The fragmented genomic DNA was end-repaired and ligated with paired-end adaptors. The adaptor-ligated libraries were hybridized with biotinylated oligo RNA bait, SureSelect Human All Exon V6 (Agilent Technologies, Santa Clara, CA, USA) and sequenced on an Illumina NovaSeq 6000 (Illumina) using 150-bp paired-end reads in accordance with the manufacturer’s standard protocols.

After completion of the entire run, the quality of the sequencing reads was evaluated using FastQC software (version v0.11.7; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and low-quality reads were eliminated using Trimmomatic software (version 0.38) [14]. The reads were aligned against the reference human genome from JG2.1 (https://jmorp.megabank.tohoku.ac.jp/202112/downloads/#sequence) using the Burrows Wheeler Aligner Multi-Vision software package (version 0.7.17-r1188) [15]. Duplication reads were eliminated using Picard software (version 2.18.11; https://broadinstitute.github.io/picard/). Single-nucleotide variants (SNVs) and insertions/deletions (indels) were identified using the Genome Analysis Toolkit (GATK) (version 4.0.8.1; https://gatk.broadinstitute.org/hc/en-us) [16].

6. **Immunohistochemistry for ZBTB38**

Five-micrometer-thick sections of FFPE tissue specimens from 61 patients in the initial and second cohorts, for whom additional sections were available for immunohistochemistry, were deparaffinized and dehydrated. All sections were incubated with anti-human ZBTB38 (zinc finger and BTB domain-containing 38) rabbit polyclonal antibody (21906-1-AP, dilution 1:200; Proteintech, Rosemont, IL, USA). Before incubation, the sections were heated for 30 minutes at 95°C in a water bath using citrate buffer at pH 6 (Genostaff Co., Ltd., Tokyo, Japan) for antigen retrieval. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in methanol. Non-specific reactions were blocked with 2.5% normal horse serum (Vector Laboratories, Inc., Burlingame, CA, USA). The primary antibody incubation was conducted at 4°C overnight, followed by incubation with ImmPRESS® HRP horse anti-rabbit IgG polymer reagent (MP-7401, Vector Laboratories, Inc.) at room temperature for 30 minutes. The 3,3′-diaminobenzidine tetrahydrochloride was used as the chromogen. All sections were counterstained with hematoxylin.

Immunoreactivity for ZBTB38 was detected in the nucleus in myometrial cells of all examined cases and served as an internal positive control. As a negative control, the primary antibody was omitted from the reaction sequence. Various levels of somewhat weak immunoreactivity were frequently observed in the cytoplasm of both myometrial cells and cancer cells themselves. Since ZBTB38 is able to function as a methyl-CpG binding protein in nuclei, distinct nuclear immunoreactivity was considered as positive and the incidence of positivity in cancerous tissues was evaluated quantitatively. For each cancer, 5 areas including at least
200 (not more than 300) cancer cells were randomly counted. The incidence of positive nuclear immunoreactivity in each area was expressed as a percentage of all the cells counted.

7. Statistics
In the Infinium assay, the call proportions (p-values for detection of signals above the background <0.01) for 86,783 probes in all 80 tissue samples of the initial and second cohorts were less than 90%. Since such a low proportion may have been attributable to polymorphism at the probe CpG sites, these 86,783 probes were excluded from the present assay, as described previously [17,18]. The 727 probes containing missing β-values of more than 10% were also excluded. In addition, all 11,648 probes on chromosomes X and Y were excluded to avoid any gender-specific methylation bias, leaving a final total of 759,540 autosomal CpG sites.

Correlations between epigenomic clustering or ZBTB38 expression on one hand and recurrence risk classification on the other were examined by Fisher’s exact test. Differences in DNA methylation levels between sample groups were examined by Welch’s t-test. The receiver operating characteristic (ROC) curve was generated and the area under the curve (AUC) value and Youden index were calculated for each CpG site. Statistical analyses were performed using the programming language R and SPSS program version 20.0 (IBM Corp, Armonk, NY, USA). Differences at p-values of <0.05 were considered statistically significant.

RESULTS

1. Correlation between epigenomic clustering and the recurrence risk classification of early-onset endometrial cancer
A proportion of early-onset endometrial cancers (n=34) in the initial cohort were also included in our previous study and placed into Clusters EA (n=22) and EB (n=12) [7]. We then examined the correlation between this epigenomic clustering and recurrence risk classification, which has been established on the basis of histopathological features of hysterectomy specimens and is used as a reliable indicator for determination of follow-up strategy after hysterectomy [19]. Patients belonging to Cluster EA were significantly accumulated in intermediate- or high-recurrence-risk categories (p=0.011) (Table S2). These findings prompted us to establish prognostication criteria that would be useful for selection of patients with early-onset endometrial cancer who would be eligible for fertility preservation therapy. A flowchart describing the process up to the establishment of the prognostication criteria is shown in Fig. S1.

2. Identification of potential marker CpG sites for recurrence risk estimation in the initial cohort
In all 49 fresh frozen tissue samples of the initial cohort, 16,220 probes showed significant differences in DNA methylation levels between patients belonging to the low-risk category (n=24) and those belonging to the intermediate- and high-risk categories (n=25) (p-value <0.05 by Welch’s t-test, false discovery rate [FDR] <0.3 and Δβlow-intermediate or high value >0.1 or <-0.1). Using those 16,220 probes, ROC curves were generated to discriminate patients belonging to the low-risk category from those belonging to the intermediate- and high-risk categories. Among the probes, 11,747 showed AUC values of more than 0.7 for such discrimination. Scattergrams of DNA methylation levels on the representative probes in the low-risk category and the intermediate- and high-risk categories are shown in Fig. 1. Among 11,747 probes, 6,999
and 4,748 showed DNA hyper- and hypo-methylation, respectively, in the low-risk category relative to the intermediate- and high-risk categories. For each of 11,747 probes, the Youden index was calculated as a cutoff value for discrimination of patients belonging to the low-risk category from those belonging to the intermediate- and high-risk categories.

Fig. 1. Scattergrams of DNA methylation levels on representative probes from the 11,747 showing significant differences in DNA methylation levels between patients belonging to the low-risk category (L) (n=24) and those belonging to the intermediate- and high-risk categories (I+H) (n=25) (p-value <0.05 by Welch's t-test, FDR <0.3 and Δβ_L-I+H >0.1 or <−0.1) and AUC values of more than 0.7 for discrimination of patients belonging to L from those belonging to I+H. Probe IDs and gene symbols are shown at the top of each panel. AUC, area under the curve; FDR, false discovery rate; NA, not applicable (designed for within the intergenic region).
**Table 1.** Top 20 pathway maps in which the 325 genes whose DNA methylation alterations were associated with the recurrence risk classification in early-onset endometrioid endometrial cancer and could potentially result in mRNA expression alterations, were accumulated (p<0.01) as demonstrated using MetaCore™ software.

| Pathway maps | p     | FDR   | Included genes                                                                 |
|--------------|-------|-------|---------------------------------------------------------------------------------|
| Development_Schema: FGF signaling in embryonic stem cell self-renewal and differentiation | 6.22E-07 | 5.14E-04 | SOX1, NEFM, NXX6-1, TAL1, NEFH, CD34, PAX6 |
| Signal transduction_Additional pathways of NF-κB activation (in the cytoplasm) | 1.26E-06 | 5.23E-04 | ADCY5, ADCY8, NFKBIE, MAP3K8, PIK3R5, MAP3K14, PIK3R3, PRKAR1A |
| Development_FGF2 signaling during embryonic stem cell differentiation | 2.88E-05 | 5.96E-03 | ADCY5, ADCY8, NFKBIE, MAP3K8, PIK3R5, MAP3K14, PIK3R3, PRKAR1A |
| Immune response_Gastrin in inflammatory response | 8.73E-05 | 1.03E-02 | MEF2C, NFKBIE, IRS1, MAP3K14, GNA11 |
| Signal transduction_Additional pathways of NF-κB activation (in the nucleus) | 2.82E-04 | 2.33E-02 | ADCY5, ADCY8, NFKBIE, MAP3K14, PIK3R3, PRKAR1A |
| Development_Thyroliberin signaling | 1.56E-04 | 2.93E-02 | GNA11, ADCY5, ADCY8, IRS1, PIK3R3, PRKAR1A |
| Immune response_LTB1 signaling | 1.31E-04 | 2.93E-02 | GNA11, ADCY5, ADCY8, PIK3R5, IRS1, PRKAR1A |
| G-protein signaling_G-Protein beta/gamma signaling cascades | 6.21E-04 | 2.93E-02 | ADCY5, PRKAR1A, RGS3, PIK3R3, GNA11 |
| Development_Lipoxin inhibitory action on PDGF, EGF and LIF signaling | 5.76E-04 | 3.18E-02 | SOC52, ADCY5, ADCY8, GNA11, PRKAR1A |
| G-protein signaling_Regulation of p38 and JNK signaling mediated by G-proteins | 6.41E-04 | 3.31E-02 | PAK1, PIK3R5, MEF2C, GNA11 |
| Reproduction_Gonadotropin-releasing hormone (GnRH) signaling | 1.13E-03 | 4.47E-02 | ADCYAP1, PER1, ADCY5, ADCY8, GNA11, PRKAR1A |
| Signal transduction_AKT signaling | 1.24E-03 | 4.47E-02 | NFKBIE, IRS1, CD20, PIK3R3 |
| Impaired Lipoxin A4 signaling in CF | 1.24E-03 | 4.47E-02 | SOC52, ADCY5, ADCY8, GNA11, PRKAR1A |
| Development_Estrogen-independent activation of ESR1 and ESR2 | 1.24E-03 | 4.47E-02 | ADCY5, IRS1, PIK3R3, PRKAR1A |
| Immune response_TNF-R2 signaling pathways | 1.35E-03 | 4.47E-02 | NFKBIE, TNFRSF1B, MAP3K14, PIK3R3 |
| G-protein signaling_Regulation of Cyclic AMP levels by Gα | 1.35E-03 | 4.47E-02 | GNA11, ADCY5, ADCY8 |
| Chemotaxis_SDF-1/CXCR4-induced chemotaxis of immune cells | 1.59E-03 | 4.70E-02 | PAK1, PIK3R5, PIK3R5, PRKAR1A |
| CHD1_Correlations from Replication data_Causal network (positive correlations) | 1.59E-03 | 4.70E-02 | HDAC7, MEF2C, NFKBIE, PIK3R5, MAP3K14 |
| Translation_Opioid receptors in regulation of translation | 1.84E-03 | 5.03E-02 | PENK, PIK3R5 |
| Epigenetic alterations in ovarian cancer | 1.87E-03 | 5.03E-02 | PYCARD, ZIC4, DNMT3A, OCMC, WIFI |

FDR, false discovery rate; FGF, fibroblast growth factor; NF, nuclear factor.

*After elimination of pathways related solely to specific organs other than the uterus or specific diseases other than cancer, the top 20 processes are listed. All 84 statistically significant pathways are summarized in Table S3. Pathways relating to immune cells infiltrating the cancer stroma.

### 3. Signaling pathways affected by DNA methylation alterations associated with the recurrence risk classification

Among 11,747 probes showing AUC values of more than 0.7, 4,186 probes were located within CpG islands, island shores (2,000-bp regions adjacent to a CpG island) or island shelves (2,000-bp regions adjacent to an island shore) based on the University of California, Santa Cruz (UCSC) genome browser ([https://genome.ucsc.edu/](https://genome.ucsc.edu/)). Among those 4,186 probes, 2,314 were located around the transcription start site (TSS), i.e., TSS1500 (from 200 bp upstream of the TSS to 1,500 bp upstream of it), TSS200 (from the TSS to 200 bp upstream of it), the 5’ untranslated region, and the 1st exon or 1st intron, based on the RefSeq database ([http://www.ncbi.nlm.nih.gov/refseq/](http://www.ncbi.nlm.nih.gov/refseq/)). Among those 2,314 probes, significant inverse correlations between DNA methylation and mRNA expression levels (r<−0.2 and p<0.05) were confirmed on 538 probes using data for samples of cancerous and non-cancerous endometrial tissue (n=196) deposited in The Cancer Genome Atlas (TCGA) database ([https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tega](https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tega)). These 538 probes were designed for 325 genes, which were subjected to MetaCore pathway analysis and found to be significantly accumulated in 84 pathways (p<0.01). After excluding pathways solely relating to specific organs other than the uterus or specific diseases other than cancer, the top 20 pathways were selected and are summarized in Table 1. Four of the top 5 signaling pathways are clearly related to the fibroblast growth factor (FGF) and nuclear factor (NF)-κB signaling pathways. Representative pathway maps, e.g., Development_Schema: FGF signaling in embryonic stem cell self-renewal and differentiation (p=6.22×10⁻² and FDR=5.14×10⁻⁴) and "Signal transduction_Additional pathways of NF-κB activation (in the cytoplasm) (p=1.26×10⁻⁷ and FDR=5.23×10⁻⁴), are illustrated schematically in Fig. S2."
4. Validation of marker CpG sites for recurrence risk estimation in the second cohort

Among the 11,747 probes showing AUC values of more than 0.7 for discrimination of the low-risk category from the intermediate- and high-risk categories in the initial cohort, 843 probes again showed significant differences in DNA methylation levels between patients belonging to the low-risk category (n=16) and those belonging to the intermediate- and high-risk categories (n=15), even in the 31 FFPE tissue samples of the second cohort (p-value <0.05 by Welch's t-test, FDR <0.3 and Δβlow-intermediate or high value >0.1 or <−0.1). Among those 843 probes, 831 again showed AUC values of 0.7 or more for such discrimination in the second cohort. Among the latter 831 probes, 792 and 39 showed DNA hyper- and hypo-methylation, respectively, in the low-risk category relative to the intermediate- and high-risk categories.

For the 831 probes, designed for 556 genes, the annotation (TSS1500, TSS200, 5' untranslated region, 1st exon, 1st intron or gene body [2nd exon and downstream] based on the RefSeq database), CpG type (CpG islands, island shores or island shelves based on the UCSC genome browser), sensitivity was calculated in the initial cohort and the Youden index calculated in the second cohort are summarized in Table S4.

5. Establishment of the recurrence risk estimation criteria for early-onset endometrioid endometrial cancer

With the aim of allowing younger patients with less-aggressive cancers belonging to the low-recurrence-risk category to have fertility-sparing treatment, we attempted to establish criteria for estimation of recurrence. First, we tried to discriminate tissue samples belonging to the low-risk category from those belonging to the intermediate- and high-risk categories using each of 10 representative marker CpG probes showing high AUC values in both cohorts and included in Table S4 (Table 2). Using the Youden index based on data for the initial cohort, the sensitivity and specificity of each CpG site for such discrimination in both initial and second cohorts are summarized in Table 2. Although differences in the DNA methylation levels of only one marker, cg09618690, showed a marginal difference (p=0.0497) between patients belonging to the low-risk category from those belonging to the intermediate- and high-risk categories in the initial cohort, 843 probes again showed significant differences in DNA methylation levels between patients belonging to the low-risk category (n=16) and those belonging to the intermediate- and high-risk categories (n=15), even in the 31 FFPE tissue samples of the second cohort (p-value <0.05 by Welch's t-test, FDR <0.3 and Δβlow-intermediate or high value >0.1 or <−0.1). Among those 843 probes, 831 again showed AUC values of 0.7 or more for such discrimination in the second cohort. Among the latter 831 probes, 792 and 39 showed DNA hyper- and hypo-methylation, respectively, in the low-risk category relative to the intermediate- and high-risk categories.

For the 831 probes, designed for 556 genes, the annotation (TSS1500, TSS200, 5' untranslated region, 1st exon, 1st intron or gene body [2nd exon and downstream] based on the RefSeq database), CpG type (CpG islands, island shores or island shelves based on the UCSC genome browser), AUC values in both the initial and second cohorts, and the Youden index calculated in the initial cohort are summarized in Table S4.

Table 2. Representative marker CpG sites for discrimination of patients with early-onset endometrioid endometrial cancer (aged 40 years or less) belonging to the low-recurrence-risk category

| Gene ID   | Gene symbol | Annotation | CpG type | Youden index (cut-off value) | DNA methylation status | Initial cohort | Second cohort |
|-----------|-------------|------------|----------|----------------------------|------------------------|----------------|--------------|
| cg10179694 | LPP         | Gene body  | Open sea | 0.625                      | L/I+H                  | 0.932          | 0.850        |
| cg06342969 | SHOC2       | 5'UTR/1st intron | Open sea | 0.421                      | L/I+H                  | 0.877          | 0.846        |
| cg09618690 | FOXO1       | 1st intron  | Open sea | 0.220                      | L/I+H                  | 0.863          | 0.888        |
| cg13758817 | IMMT        | Gene body  | Open sea | 0.589                      | L/I+H                  | 0.853          | 0.875        |
| cg06197731 | NA          | Intergenic region | Open sea | 0.611                      | L/I+H                  | 0.839          | 0.896        |
| cg14617296 | RNF4        | Gene body  | Open sea | 0.537                      | L/I+H                  | 0.825          | 0.904        |
| cg24621312 | EXOC6B      | Gene body  | Open sea | 0.147                      | L/I+H                  | 0.808          | 0.925        |
| cg05418285 | CCPO1       | 5'UTR/1st intron | Open sea | 0.310                      | L/I+H                  | 0.795          | 0.938        |
| cg10298221 | RRE81       | Gene body  | Open sea | 0.652                      | L/I+H                  | 0.793          | 0.946        |
| cg06342969 | ZBTB38      | 5'UTR/1st intron | S_Shore | 0.466                      | L/I+H                  | 0.762          | 0.979        |

AUC, area under the curve.

1. Probe IDs for the Infinium MethylationEPIC BeadChip (Illumina).
2. Gene ID, not applicable (designed for the intergenic regions).
3. UTR, untranslated region; Gene body, second exon and downstream on the RefSeq database (http://www.ncbi.nlm.nih.gov/refseq/).
4. 5'-Shore, 2000 bp region 3' adjacent to CpG islands based on the UCSC Genome Browser (https://genome.ucsc.edu/).
5. Gene body, second exon and downstream on the RefSeq database (http://www.ncbi.nlm.nih.gov/refseq/).
6. S_Shore, when the DNA methylation level of the sample was lower than the cut-off value (Youden index), the patient was diagnosed as belonging to the low-risk category.
7. Sensitivity is defined as the ratio of the number of tissue samples diagnosed as belonging to the low-risk category based on the criteria, relative to the exact number of patients belonging to the low-risk category.
8. Specificity is defined as the ratio of the number of tissue samples not diagnosed as belonging to the low-risk category using the criteria employed, relative to the exact number of patients belonging to the low-risk category.
between patients who received MPA treatment (n=5) and those who did not (n=26) in the second cohort (0.137±0.088 vs. 0.252±0.178, respectively), none of the 10 markers showed any significant differences in the initial cohort and nine other markers showed no such differences even in the second cohort, indicating that the DNA methylation status of marker CpG sites was not affected by preoperative MPA therapy. To improve the diagnostic impact, we combined multiple CpG markers included in Table 2; 7 diagnostic panels showing a sensitivity of 91.7%–95.8% and a specificity of 91.3%–95.7% in both the initial and second cohorts were established (Table 3).

Table 3. Discrimination criteria using the combination of marker CpG sites for patients with early-onset endometrioid endometrial cancer (aged 40 years or less) belonging to the low-recurrence-risk category

| Combination of marker CpG sites | Number of CpG sites satisfying the criteria shown in Table 2 | Initial cohort | Second cohort |
|--------------------------------|-------------------------------------------------------------|----------------|---------------|
|                                | Sensitivity (%)†                                           | Specificity (%)†| Sensitivity (%)†| Specificity (%)†|
| cg09618690, cg10298221 and cg06197731 | Two or three                                               | 91.7            | 93.8           | 91.3            | 93.3           |
| cg10179694, cg09618690, cg20959189, cg10298221 and cg06197731 | Three or more                                              | 91.1            | 93.8           | 93.8            | 93.3           |
| cg10179694, cg09618690, cg20959189, cg24621312 and cg14617296 | Three or more                                              | 91.7            | 93.8           | 93.8            | 93.3           |
| cg09618690, cg20959189, cg06197731, cg24621312 and cg05418285 | Three or more                                              | 91.7            | 93.8           | 93.8            | 93.3           |
| cg09618690, cg20959189, cg06197731, cg24621312 and cg14617296 | Three or more                                              | 91.7            | 93.8           | 93.8            | 93.3           |
| cg09618690, cg10298221, cg06197731, cg24621312 and cg05418285 | Three or more                                              | 95.8            | 93.8           | 93.8            | 93.3           |
| cg10179694, cg09618690, cg20959189, cg06197731, cg24621312 and cg14617296 | Three or more                                              | 91.7            | 93.8           | 93.8            | 93.3           |

6. Mutation status of the MLH1, MSH2, MSH6, PMS2, POLE and TP53 genes and the recurrence risk estimation criteria

As a result of the whole-exome sequencing, non-synonymous SNVs, indels and mutations at the splicing donor or acceptor sites of the mismatch repair genes (the MLH1, MSH2, MSH6 and PMS2 genes), another gene showing DNA repair function, POLE, and TP53, a guardian of the genome, are summarized in Table S5. In order to determine the most effective criteria based on genomic status, tumors in the initial cohort subjected to whole-exome sequencing were divided into groups with and without mutations. The sensitivity and specificity for discrimination of patients belonging to the low-risk category were calculated for each group (Table S6). Although we cannot draw any definitive conclusions due to the reduced number of samples in each cohort after stratification based on the presence or absence of mutations, these data suggested that the criteria comprising cg09618690, cg10298221, cg06197731, cg24621312 and cg05418285 are suitable for patients positive for mutations of any of MLH1, MSH2, MSH6 or PMS2, whereas the criteria comprising cg09618690, cg20959189, cg06197731, cg24621312 and cg05418285 and those comprising cg09618690, cg20959189, cg06197731, cg24621312 and cg05418285 may be suitable for patients positive for TP53 mutations (Table S6).

7. Immunohistochemistry for ZBTB38 expression in early-onset endometrioid endometrial cancer

If recurrence risk prediction is possible using immunohistochemistry on FFPE specimens that are routinely prepared for pathological diagnosis, it would be easy to introduce into clinical settings. Therefore, we aimed to select a target gene for immunohistochemical examination from the 10 probe CpG sites designed for the 9 genes included in Table 2. The only gene for which the probe CpG site was located within the CpG islands, island shores and island shelves around the TSS, which are important regions for transcriptional regulation, was the ZBTB38 gene (cg20959189). In addition, since an inverse correlation between the DNA
methylation and mRNA expression levels of the ZBTB38 gene was confirmed using the TCGA database, we performed immunohistochemical examination of ZBTB38. Representative photos of cancer cells positive and negative for nuclear immunoreactivity are shown in Fig. 2. The median incidence of nuclear immunoreactivity in 305 cancerous areas examined was 0.544. When the incidence was 0.544 or more, the area was defined as showing a higher level of ZBTB38 protein expression, whereas the area showing an incidence of less than 0.544 was defined as showing a lower level of expression.

**DISCUSSION**

The present genome-wide DNA methylation analysis using pathological tissue specimens has shown that DNA methylation diagnostics criteria using the Youden index as a cutoff value for up to 6 of 8 CpG sites for LPP, FOXO1, RNF4, EXOC6B, CCPG1, RREB1 and ZBTB38 may be applicable for recurrence risk estimation in patients aged 40 years or less with endometrioid endometrial cancer, regardless of tumor cell content, even if formalin-fixed paraffin-embedded biopsy or curettage materials are used.

In the widely used recurrence risk classification of endometrial cancer, grade 1 or grade 2 endometrioid adenocarcinoma showing <1/2 myometrial invasion with no cervical involvement, no lymphovascular invasion and no distant metastasis is categorized into...
the low-risk group, whereas G3 endometrioid adenocarcinoma showing ≥1/2 myometrial invasion with spread to the uterine adnexa, serosa, or cardinal ligament, invasion of the vaginal wall, pelvic or para-aortic lymph node metastasis, vesical or rectal invasion, peritoneal dissemination and distant metastasis is categorized into the high-risk group [19]. Therefore, such recurrence risk classification can be performed only after hysterectomy based on a precise histopathological evaluation of all the surgically resected material. If molecular abnormalities responsible for generating aggressive features can be detected using biopsy or curettage specimens, recurrence risk estimation should be possible even before hysterectomy or even in patients for whom hysterectomy is not indicated.

In fact, epigenomic clustering of early-onset endometrial cancer [7] was significantly correlated with the recurrence risk classification (Table S2) in the initial cohort, and the DNA methylation levels of many candidate marker CpG sites showed high AUC values for discrimination of patients belonging to the low-risk category for whom MPA therapy might be indicated. Genes for which candidate marker probes had been designed, and whose DNA methylation can result in alterations of their expression, were accumulated in the FGF and NF-κB-related signaling pathways (Table 1, Fig. S2). Our previous study had shown that genetic and epigenetic alterations of FGF-related signaling pathways were not characteristic to early-onset endometrial cancer, but rather to late-onset endometrial cancer in patients aged 40 years or more, whereas such alterations of Wnt signaling were frequent in early-onset endometrial cancer [7]. It is feasible that early-onset endometrial cancer, which has FGF signaling abnormalities that are common in late-onset endometrial cancer, shows higher aggressiveness and higher recurrence risk as is the case for late-onset endometrial cancer. Moreover, it is well known that NF-κB signaling frequently affects the malignant potential of cancers in various organs through induction of the epithelial-mesenchymal transition [21,22]. Therefore, it is quite feasible that early-onset endometrial cancer with NF-κB signaling abnormalities would be categorized into the group with higher recurrence risk.

None of the 10 marker probe CpG sites designed for the 9 genes in Table 2 are included in Table 1. This is attributable to the manual curating system of the MetaCore™ software, which was used for pathway analysis in Table 1. However, some of our marker genes in Table 2 actually have a certain relationship to the FGF and NF-κB signaling pathways. For example, the SHOC2 gene encoding a scaffold protein linking RAS to downstream signal transducers was initially identified as a human homolog of soc-2 of Caenorhabditis elegans, which belongs to soc genes suppressing EGL-15 FGF receptor signaling activity [23]. The expression level of SHOC2 was critically reduced in cultured neural progenitor cells, whose differentiation is induced by removal of basic FGF [24]. On the other hand, FOXO1 is known to be one of the insulin signaling markers: increased expression of NF-κB was reportedly observed in brain tissue in a murine model of diabetes mellitus [25]. In addition, in silico analysis has identified FOXO1 as a transcription factor involved in hypoxia-stress responses and associated cell cycle control mechanisms, as well as regulating NFKBIA and CDKN1A [26]. With respect to the FGF pathway, when FGF21 treatment attenuates angiotensin II-induced cardiac hypertrophy and dysfunction, FGF21 markedly increases the deacetylase activity of SIRT1 and promotes the interaction of SIRT1 with FOXO1, resulting in decreased acetylation of these SIRT1 target proteins [27]. These previous findings suggest that our marker genes may be involved in the FGF and NF-κB signaling pathways even during carcinogenesis.

The number of candidate marker probes for which AUC values of more than 0.7 were validated even in the second cohort was restricted, i.e., 831 probes. Here, we used the second cohort
consisting of FFPE surgically resected materials for the validation study after screening the initial cohort comprising fresh frozen tissue samples. Usage of FFPE samples, especially such biopsy or curettage samples, is very feasible in a clinical setting, whereas a sufficient amount of high-quality genomic DNA can be extracted from fresh frozen tissue samples. The discrepancy of DNA methylation data between the initial and second cohorts may have been attributable to the differences between FFPE and fresh frozen tissues, including formaldehyde-induced cross-links, DNA fragmentation and deamination of cytosine bases [28].

Another reason for such discrepancy may be differences in the tumor cell content. Since bulk tissue used in the initial screening had a generally low tumor cell content, the Infinium assay would have potentially highlighted CpG sites at which DNA methylation abnormalities in fibroblasts, endothelial cells and infiltrating immune cells in the cancer stroma were correlated with the risk classification. Various growth factors are known to be produced by both cancer cells and stromal cells and to potentiate cancer cell proliferation and/or invasiveness in an autocrine and paracrine manner. Therefore, it is feasible that the DNA methylation status of both cancer cells and stromal cells would be altered, and that such alteration might determine tumor aggressiveness via alterations in the expression levels of such growth factors [29, 30]. On the other hand, before the validation study, we microscopically dissected out areas showing a tumor cell content of more than 80% from FFPE specimens in the second cohort. Therefore, CpG sites reflecting abnormalities of only non-cancerous stromal cells, and not those of tumor cells, were not verified in the second cohort.

Then, in the second cohort, we performed MetaCore pathway analysis using the 384 genes (523 probes) showing significant differences in DNA methylation levels between patients belonging to the low-risk category (n=16) and those belonging to the intermediate- and high-risk categories (n=15) (p-value <0.05 by Welch’s t-test, FDR <0.3 and Δβ_{low-intermediate or high value} >0.1 or <-0.1). Top 20 pathway maps for the second cohort are summarized in Table S7. When Table 1 in the initial cohort and Table S7 in the second cohort are compared, more pathways relating to immune cells infiltrating the cancer stroma are included in Table 1 for the initial cohort of fresh frozen tissue samples containing more stromal cells, than in Table S7 for the second cohort of microdissected FFPE samples. These findings indicate that the discrepancy of DNA methylation profiles between fresh frozen tissue samples and FFPE samples is attributable to differences in the stromal cell content.

After these identification processes, our risk prediction is expected to well work even for FFPE samples: we successfully identified effective marker CpG sites even with formaldehyde-induced cross-links, DNA fragmentation and deamination of cytosine bases after the validation study using FFPE samples. Moreover, our marker is useful for FFPE samples with varying tumor cell contents because they were identified using both fresh frozen tissue, which generally has a lower tumor cell content, and microdissected specimens for which the tumor cell content is generally higher.

ZBTB38 is a methyl-CpG binding protein [31]. It is feasible that altered expression of this “DNA methylation reader” in human cancers might disturb regulation of the epigenomic expression of a wide range of tumor-related genes. In fact, it has been reported that reduced expression of ZBTB38 is associated with an increased incidence of chromosomal abnormalities and more aggressive pathological features in prostate cancer [32]. Although the significance of ZBTB38 in endometrial cancer and regulation of the expression of ZBTB38 itself due to DNA methylation have never been reported previously, our DNA
methylation profiling has suggested that DNA hypomethylation in the low-risk category might result in higher expression and a less aggressive phenotype in cancers. In fact, our immunohistochemical examination revealed a higher incidence of nuclear immunoreactivity for ZBTB38 in the low-risk category (Fig. 2), suggesting that such examination using biopsy or curettage samples could be applicable as an auxiliary procedure for prediction of recurrence risk based on quantification of DNA methylation.

**Table 3** indicates that it would be possible to predict recurrence risk by quantifying only 6 validated marker CpG sites at most. Such a small number of quantification targets suggested that this approach would be feasible as a laboratory examination in a clinical setting, as well as being applicable to FFPE biopsy or curettage materials. We have developed a system for quantification of DNA methylation involving high-performance liquid chromatography, which is easy to introduce into the clinical laboratories of hospitals, for diagnosis of clinical samples using even small amounts of genomic DNA [33]. Through the use of such appropriate diagnostic approaches, we expect that our markers would be applicable for estimation of recurrence risk and the indications for fertility-preservation therapy based on biopsy and curettage samples from younger patients with early-onset endometrioid endometrial cancer. In addition, the present whole-exome sequencing suggests that simultaneous acquisition of genomic information would allow us to select more appropriate diagnostic panels and increase the accuracy of recurrence risk prediction. We intend to prospectively examine fertility-sparing outcomes with reduced recurrence after MPA therapy in patients aged 40 years or less with endometrioid endometrial cancer employing stratification based on our criteria.

**SUPPLEMENTARY MATERIALS**

**Table S1**
Correlation between epigenomic clustering of patients with early-onset endometrioid endometrial cancer (aged 40 years or less) based on DNA methylation profiles and clinicopathological parameters

Click here to view

**Table S2**
Correlation between epigenomic clustering and recurrence risk classification in the initial cohort of patients with early-onset endometrioid endometrial cancer

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**Table S3**
Pathway maps in which the 325 genes whose DNA methylation alterations were associated with the recurrence risk classification in early-onset endometrial cancer, and potentially resulted in mRNA expression alterations, were accumulated (p<0.01), as demonstrated using MetaCore™ software

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Table S4
Eight hundred thirty-one probes showing significant differences in DNA methylation levels between patients belonging to the low-recurrence-risk category and those belonging to the intermediate- and high-recurrence-risk categories (p-value <0.05 by Welch’s t-test, FDR <0.3 and Δβ_{low-intermediate or high} value >0.1 or <−0.1) and AUC values of more than 0.7 for discrimination of samples belonging to the low-recurrence-risk category in both the initial and second cohorts

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Table S5
Single-nucleotide variants, insertions/deletions and mutations at the splicing donor or acceptor sites of the MLH1, MSH2, MSH6, PMS2, POLE and TP53 genes in endometrial cancers based on whole-exome sequencing

Click here to view

Table S6
Correlations between mutation status of the MLH1, MSH2, MSH6, PMS2, POLE and TP53 genes and the recurrence risk estimation criteria

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Table S7
Top 20 pathway maps in which the 384 genes showing significant differences in DNA methylation levels between patients belonging to the low-risk category (n=16) and those belonging to the intermediate- and high-risk categories (n=15) in the second cohort (p-value <0.05 by Welch’s t-test, FDR <0.3 and Δβ_{low-intermediate or high} value >0.1 or <−0.1) were accumulated (p<0.01), as demonstrated using MetaCore™ software

Click here to view

Fig. S1
Flowchart describing the process up to establishment of the prognostication criteria.

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Fig. S2
Schematically illustrated representative pathway maps obtained using MetaCore™ software (version 19.3). Genes for which DNA methylation alterations were associated with the recurrence risk classification in early-onset endometrioid endometrial cancer, and would potentially result in mRNA expression alterations (red cycles), were accumulated in “Development_Schema: FGF signaling in embryonic stem cell self-renewal and differentiation” (p=6.22×10^{-7}) (A), “Signal transduction_Additional pathways of NF-κB activation (in the cytoplasm) (p=1.26×10^{-5}) (B).

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REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359-86.

2. Yamagami W, Aoki D. Annual report of the Committee on Gynecologic Oncology, the Japan Society of Obstetrics and Gynecology. J Obstet Gynaecol Res 2015;41:167-77.

3. Ota T, Yoshida M, Kimura M, Kinoshita K. Clinicopathologic study of uterine endometrial carcinoma in young women aged 40 years and younger. Int J Gynecol Cancer 2005;15:657-62.

4. Duska LR, Garrett A, Rueda BR, Haas J, Chang Y, Fuller AF. Endometrial cancer in women 40 years old or younger. Gynecol Oncol 2001;83:388-93.

5. Laurelli G, Di Vagno G, Scaffa C, Losito S, Del Giudice M, Greggi S. Conservative treatment of early endometrial cancer: preliminary results of a pilot study. Gynecol Oncol 2011;120:43-6.

6. Yamagami W, Susumu N, Makabe T, Sakai K, Nomura H, Kataoka F, et al. Is repeated high-dose medroxyprogesterone acetate (MPA) therapy permissible for patients with early stage endometrial cancer or atypical endometrial hyperplasia who desire preserving fertility? J Gynecol Oncol 2018;29:e21.

7. Makabe T, Arai E, Hirano T, Ito N, Fukamachi Y, Takahashi Y, et al. Genome-wide DNA methylation profile of early-onset endometrial cancer: its correlation with genetic aberrations and comparison with late-onset endometrial cancer. Carcinogenesis 2019;40:611-23.

8. Zaino R, Matias-Guiu X, Carinelli SG, Mutter GL, Ellenson LH, Peters WA 3rd, et al., editors. World Health Organization classification of female reproductive organs. Lyon: IARC Press; 2014. p. 125-35.

9. Brierley JD, Gospodarowicz MK, Wittekind C. Uterus-endometrium. In: TNM classification of malignant tumours. 8th ed. Hoboken, NJ: Wiley-Blackwell Press; 2017. p. 171-4.

10. Pecorelli S. Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. Int J Gynaecol Obstet 2009;105:103-4.

11. Kanai Y, Nishihara H, Miyagi Y, Tsuruyama T, Taguchi K, Katoh H, et al. The Japanese Society of Pathology Guidelines on the handling of pathological tissue samples for genomic research: Standard operating procedures based on empirical analyses. Pathol Int 2018;68:63-90.

12. Ohara K, Arai E, Takahashi Y, Fukamachi Y, Ito N, Maeshima AM, et al. Feasibility of methylome analysis using small amounts of genomic DNA from formalin-fixed paraffin-embedded tissue. Pathol Int 2018;68:633-5.

13. Bibikova M, Le J, Barnes B, Saedini-Melian S, Zhou L, Shen R, et al. Genome-wide DNA methylation profiling using Infinium® assay. Epigenomics 2009;1:177-200.

14. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014;30:2114-20.

15. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 2010;26:589-95.

16. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20:1297-303.

17. Fujimoto M, Arai E, Tsumura K, Yotani T, Yamada Y, Takahashi Y, et al. Establishment of diagnostic criteria for upper urinary tract urothelial carcinoma based on genome-wide DNA methylation analysis. Epigenetics 2020;15:1289-301.

https://ejgo.org
https://doi.org/10.3802/jgo.2022.33.e74
15/16
18. Tsumura K, Arai E, Tian Y, Shibuya A, Nishihara H, Yotani T, et al. Establishment of permutation for cancer risk estimation in the urothelium based on genome-wide DNA methylation analysis. Carcinogenesis 2019;40:1308-19.

PUBMED | CROSSREF

19. Yamagami W, Mikami M, Nagase S, Tabata T, Kobayashi Y, Kaneuchi M, et al. Japan Society of Gynecologic Oncology 2018 guidelines for treatment of uterine body neoplasms. J Gynecol Oncol 2020;31:e18.

PUBMED | CROSSREF

20. Cancer Genome Atlas Research Network, Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, et al. Integrated genomic characterization of endometrial carcinoma. Nature 2013;497:67-73.

PUBMED | CROSSREF

21. Mizraki S, Saghari S, Bassiri F, Raesi R, Zarrabi A, Hushmandi K, et al. NF-κB as a regulator of cancer metastasis and therapy response: a focus on epithelial-mesenchymal transition. J Cell Physiol 2022;237:2770-95.

PUBMED | CROSSREF

22. Li Y, Lin Z, Chen B, Chen S, Jiang Z, Zhou T, et al. Ezrin/NF-κB activation regulates epithelial-mesenchymal transition induced by EGF and promotes metastasis of colorectal cancer. Biomed Pharmacother 2017;92:140-8.

PUBMED | CROSSREF

23. Selfors LM, Schutzman JL, Borland CZ, Stern MJ. soc-2 encodes a leucine-rich repeat protein implicated in fibroblast growth factor receptor signaling. Proc Natl Acad Sci U S A 1998;95:6903-8.

PUBMED | CROSSREF

24. Moon BS, Kim HY, Kim MY, Yang DH, Lee JM, Cho KW, et al. Sur8/Shoc2 involves both inhibition of differentiation and maintenance of self-renewal of neural progenitor cells via modulation of extracellular signal-regulated kinase signaling. Stem Cells 2011;29:320-31.

PUBMED | CROSSREF

25. Bathina S, Das UN. Dysregulation of PI3K-Akt-mTOR pathway in brain of streptozotocin-induced type 2 diabetes mellitus in Wistar rats. Lipids Health Dis 2018;17:168.

PUBMED | CROSSREF

26. Gupta A, Ragumani S, Sharma YK, Ahmad Y, Khurana P. Analysis of hypoxiamiR-gene regulatory network identifies critical miRNAs influencing cell-cycle regulation under hypoxic conditions. MicroRNA 2019;8:223-36.

PUBMED | CROSSREF

27. Li S, Zhu Z, Xue M, Yi X, Liang J, Niu C, et al. Fibroblast growth factor 21 protects the heart from angiotensin II-induced cardiac hypertrophy and dysfunction via SIRT1. Biochim Biophys Acta Mol Basis Dis 2019;1865:1241-52.

PUBMED | CROSSREF

28. Wen X, Jeong S, Kim Y, Bae JM, Cho NY, Kim JH, et al. Improved results of LINE-1 methylation analysis in formalin-fixed, paraffin-embedded tissues with the application of a heating step during the DNA extraction process. Clin Epigenetics 2017;9:1.

PUBMED | CROSSREF

29. Oya Y, Hayakawa Y, Koike K. Tumor microenvironment in gastric cancers. Cancer Sci 2020;111:2696-707.

PUBMED | CROSSREF

30. Erdogan B, Webb DJ. Cancer-associated fibroblasts modulate growth factor signaling and extracellular matrix remodeling to regulate tumor metastasis. Biochem Soc Trans 2017;45:229-36.

PUBMED | CROSSREF

31. de Dieuleveult M, Miotto B. DNA methylation and chromatin: role(s) of methyl-CpG-binding protein ZBTB38. Epigenet Insights 2018;11:2516865718811117.

PUBMED | CROSSREF

32. de Dieuleveult M, Marchal C, Jouniot A, Letessier A, Miotto B. Molecular and clinical relevance of ZBTB38 expression levels in prostate cancer. Cancers (Basel) 2020;12:1106.

PUBMED | CROSSREF

33. Yotani T, Yamada Y, Arai E, Tian Y, Gotoh M, Komiyama M, et al. Novel method for DNA methylation analysis using high-performance liquid chromatography and its clinical application. Cancer Sci 2018;109:1690-700.

PUBMED | CROSSREF

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https://doi.org/10.3802/jgo.2022.33.e74