

Significant association of *EED* promoter hypomethylation with colorectal cancer

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Abstract. Colorectal cancer (CRC) is one of the most common and serious types of malignancy worldwide. The embryonic ectoderm development (*EED*) gene is important to maintain transcriptional repressive states of genes over successive cell generations. The present study aimed to investigate the association between *EED* methylation and CRC. A total of 111 CRC tissue samples, 111 paired para-tumor tissues and 20 colorectal normal tissues were obtained for *EED* methylation assay, which was performed using a quantitative methylation-specific polymerase chain reaction. The percentage of methylated reference was calculated to represent the DNA methylation level. A dual-luciferase reporter gene assay was used to detect the gene promoter activity of a *EED* fragment. The current results revealed a significant difference in the *EED* methylation levels among tumor, para-tumor and normal colorectal tissues (tumor vs. para-tumor vs. normal, 5.03 ± 4.61 vs. 8.65 ± 11.50 vs. 40.12 ± 45.31 ; $F=45.014$; $P<0.0001$). The dual-luciferase reporter gene assay demonstrated that the transcriptional activity of recombinant pGL3-*EED* plasmid was significantly higher compared with that of the pGL3-Basic control vector (fold-change, 3.15; $P=0.014$), which suggests the *EED* fragment can promote gene expression. In conclusion,

the present study demonstrated that *EED* hypomethylation may be an important factor associated with CRC.

Introduction

Colorectal cancer (CRC) is one of the most common and serious malignancies in the world, particularly in developed countries (1). In England and Wales between 1991 and 1998, the 5-year survival rate of CRC in the initial stages was 60-95%, markedly decreasing to 35% in stages where lymph node metastases are detected (2). The lifetime risk of CRC is ~5%, and almost 45% of patients succumb to CRC, despite treatment (1). In China, although CRC is not the leading cause of cancer-associated mortality, the incidence and mortality rates of CRC have been increasing over the past 20 years, and they have been predicted to continue to increase if no effective intervention occurs (3).

The initiation and progression of human cancer depends on both genetic alterations and epigenetic changes (4). The carcinogenesis of CRC has been revealed to be involved in epigenetic alterations, including DNA methylation and histone modifications (5,6). Gene silencing caused by aberrant promoter methylation acts as one of the most significant epigenetic mechanisms (7). In CRC, methylation alterations frequently occur in chromosomes 1, 5, 6, 8, 11, 13, 18, 19, 21 and 22 (8). Differentially methylated DNA regions have been identified in both primary tumor tissues and blood samples (9), and more recently in cell-free DNA (10).

The embryonic ectoderm development (*EED*) gene, encodes a member of the Polycomb-group (PcG) family, which has been demonstrated to maintain the transcriptional repressive states of genes over successive cell generations (11). PcG genes have been revealed to be highly mutated in a number of human diseases (12). In addition, PcG proteins have been identified to be associated with cancer development (13) and proposed as potential targets for cancer therapeutic strategies (14). *EED* has been identified to mediate the repression of gene activity through histone deacetylation (15). *EED* hypermethylation has been detected in cholangiocarcinoma tumors (16). Furthermore, high *EED* expression levels have been demonstrated to be associated with CRC (17). However,

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to the best of our knowledge, no *EED* methylation in CRC has been reported. In light of previous findings, the present study aimed to investigate the association between *EED* methylation and CRC.

Materials and methods

Tissue specimens. In the past 5 years, a total of 111 CRC tissue samples, 111 paired para-tumor tissues and 20 colorectal normal tissues were collected from patients diagnosed at Shaoxing People's Hospital (Shaoxing, China), Zhejiang Province Cancer Hospital (Hangzhou, China) and Nanjing Chinese Medicine Hospital (Nanjing, China). Clinical diagnosis was determined on the basis of colonoscopy findings and histological assessment. The types of cancer were staged according to the seventh edition of the American Joint Committee on Cancer staging system (18). The mean age of the patients was 60.96 ± 11.66 years and the cohort included 72 males and 39 females. All individuals were of Han Chinese ethnicity from Eastern China. The specimens were freshly obtained and stored at -80°C . The study protocol was approved by the Ethical Committees of Shaoxing People's Hospital (Shaoxing, China), Zhejiang Province Cancer Hospital (Hangzhou, China), Nanjing Chinese Medicine Hospital (Nanjing, China) and Ningbo University (Ningbo, China). The number of institutional review board approval was IRB-2018-28. Written informed consent was obtained from all participants.

DNA methylation assay. DNA extraction and bisulfite conversion were performed as described previously (19). Quantitative methylation-specific polymerase chain reaction (qMSP) was used to detect the methylation levels. qMSP was performed as described in our previous studies (20–22). The percent of methylated reference (PMR) was used to represent gene methylation (23,24). The genomic position and function annotations of *EED* were obtained from the University of California Santa Cruz genome browser (GRCh37/hg19; <http://genome.ucsc.edu/index.html>). The primer sequences of *EED* were 5'-GAGGCGGAGGAATATGTT-3' for the forward primer and 5'-TCACTACTCACTTCTACTTCT-3' for the reverse primer. The primer sequences of *ACTB* were 5'-TGGTGATGGAGGAGGTTTAGTAAAGT-3' for the forward primer and 5'-AACCAATAAAACCTACTCCTCCCTTAA-3' for the reverse primer. The current study used qMSP with internal negative and positive controls, and a reference gene control for the methylation assay of *EED*.

The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) data analysis. *EED* methylation and *EED* expression datasets were retrieved from the online resource [<http://www.cbiportal.org/>; colorectal Adenocarcinoma (TCGA, Provisional)] to analyze the association between *EED* methylation and *EED* expression in patients with CRC. The dataset GSE32323 (25) was downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/gds/>) to provide data regarding *EED* expression with and without 5'-AZA-deoxycytidine treatment in the cell lines COLO320, HT29 and RKO.

Dual-luciferase reporter gene assay. 293T cells, obtained from the Chinese Academy of Sciences cell bank (Shanghai,

China), were cultured as previously described (26). The fragment of *EED* (-300 bp to +390 bp) was chemically synthesized according to the manufacturer's protocol (TransLipid HL Transfection Reagent; TransGen Biotech, Co., Ltd., Beijing, China) and digested with *Xho*I and *Kpn*I (New England BioLabs, Inc., Ipswich, MA, USA). The fragment was then purified by Cycle Pure kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and the target DNA fragment was cloned into a pGL3 vector (Promega Cooperation, Madison, WI, USA) using DNA Ligation kit (Takara Bio, Inc., Otsu, Japan). The empty pGL3 basic vector was used as the negative control and the pGL3 promoter vector (both Promega Cooperation, Madison, WI, USA) was used as the positive control, which contained an SV40 promoter upstream of the luciferase gene. The plasmid transfection and the detection of luciferase activity were performed as previously described (23,27).

Statistical analysis. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analysis. Data that were not normally distributed are presented as the median (inter-quartile range) and variables that were normally distributed are presented as the mean \pm standard deviation. Mann-Whitney U test, Kruskal-Wallis and unpaired Student's t-test were applied to analyze the baseline characteristics among the 111 patients with CRC. Association of PMR difference with the clinical characteristics in 111 paired samples was performed by χ^2 test. Analysis of variance test was applied to compare *EED* methylation levels among tumor, para-tumor and normal colorectal samples, and the Bonferroni correction was used for the post hoc test. To analyze the results of the dual-luciferase reporter assay, an analysis of variance and Bonferroni's correction were also used. Spearman's correlation test was applied to evaluate the correlation between *EED* mRNA expression level and methylation in 369 patients with CRC from TCGA data portal. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

In the present study, a total of 111 CRC tumor tissues, 111 paired para-tumor tissues and 20 colorectal normal tissues were obtained to investigate the role of *EED* promoter methylation in CRC.

Characteristics of the target sequence on the *EED* promoter region. The genomic region and target sequence of *EED* are presented in Fig. 1A. One CpG site was located in the primers of the tested fragment (hg19, chr11:85956259–85956348). The capillary electrophoresis result revealed a pMSP product with a length of 90 base pairs, which was expected, and Sanger sequencing demonstrated that the amplified fragment matched the target sequence (Fig. 1B).

Baseline characteristics of the methylation levels among patients. Subsequently, no significant difference was identified between *EED* methylation levels and clinical characteristics, including sex, age, tumor location, tumor size, differentiation and lymph node metastasis (Table I). However, when the PMR difference value between methylation levels of tumor and para-tumor samples was used to perform the χ^2 test, the only

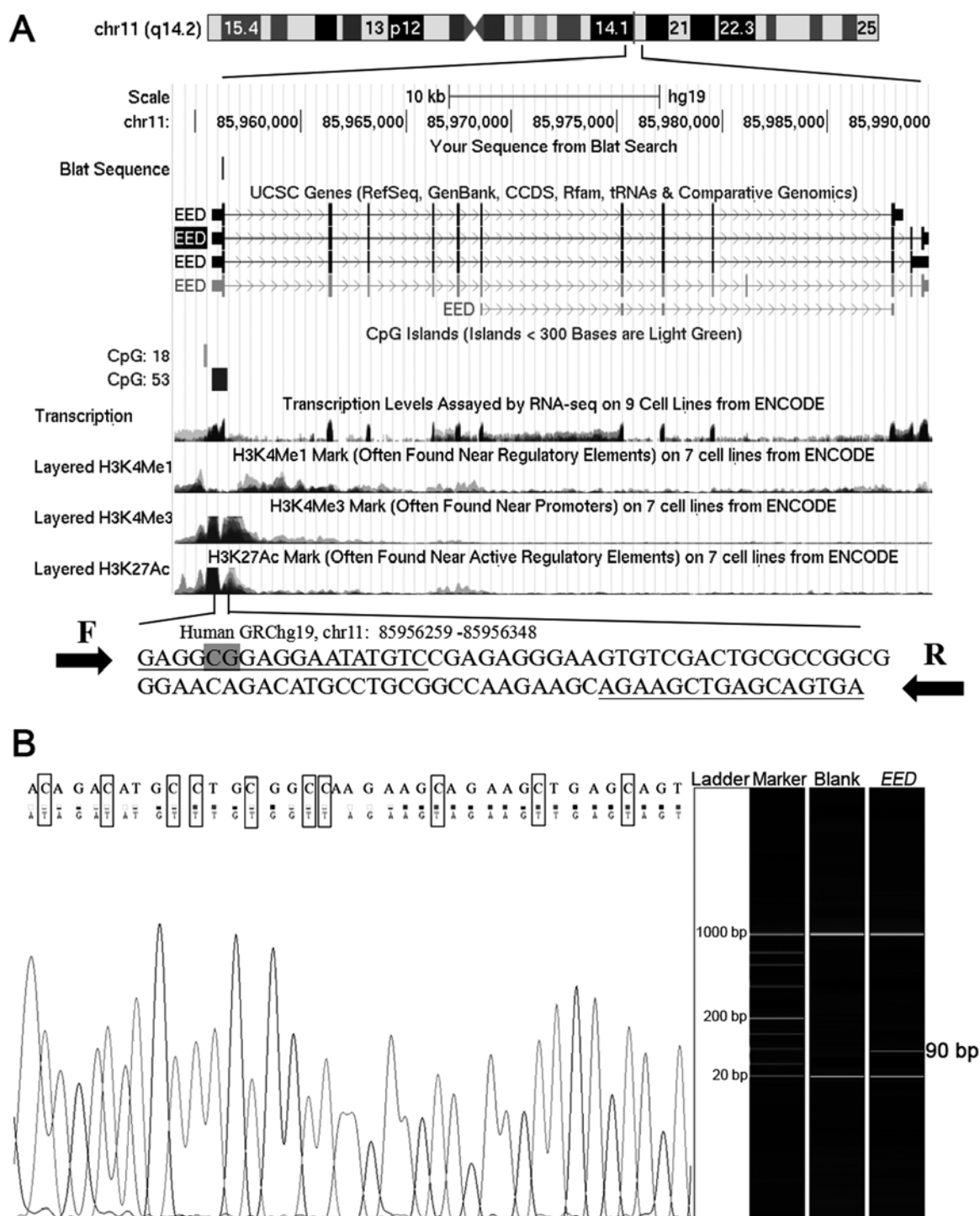


Figure 1. Characteristics of the target sequence on the *EED* promoter region. (A) The target sequence is located on the CpG island of the *EED* gene. F and R indicate the forward and reverse primers, respectively. One CpG site on the forward primer was highlighted in grey. (B) The electrophoresis result and the sequencing validation of a representative quantitative methylation-specific polymerase chain reaction product. The fragment length was 90 base pairs, as expected. The top and lower rows indicate the original and converted sequences, respectively. The framed bases indicate the cytosines that were replaced by thymines in the bisulfite-treated DNA. EED, embryonic ectoderm development; UCSC, University of California Santa Cruz.

significant association of PMR difference with clinical characteristics was identified for tumor location ($P=0.039$; Table II).

Notably, a significant difference of *EED* methylation levels among tumor, para-tumor and normal colorectal tissues was revealed (tumor vs. para-tumor vs. normal: 5.03 ± 4.61 vs. 8.65 ± 11.50 vs. 40.12 ± 45.31 ; $F=45.014$; $P<0.0001$; Fig. 2). Following Bonferroni's correction the results were as follows:

Tumor vs. para-tumor, $P=0.237$; tumor vs. normal tissue, $P<0.0001$; and para-tumor vs. normal tissue, $P<0.0001$ (Fig. 2). Previous studies have demonstrated that bisulfite sequencing PCR and qMSP can yield similar conclusions (28,29). Bisulfite sequencing PCR is a reliable and accurate method; however, it is labor intensive and therefore only applicable for methylation in a limited number of samples.

Table I. Baseline characteristics among 111 patients with colorectal cancer.

Clinical characteristics	No. (n=111)	Percentage of methylated reference in tumor	P-value
Sex			0.587 ^a
Male	72	4.73 (2.52,6.67)	
Female	39	4.41 (1.40,6.31)	
Age, years			0.165 ^a
≤65	74	4.79 (2.55,6.63)	
>65	37	3.33 (1.42,6.08)	
Tumor location			0.929 ^b
Colon	46	4.70 (1.91,6.38)	
Rectum	56	4.43 (2.50,6.62)	
Colon and rectum	9	4.78 (1.30,7.69)	
Tumor size, cm			0.673 ^a
≤6	96	4.42 (2.21,6.62)	
>6	15	5.76 (2.06,5.94)	
Differentiation			0.222 ^c
High and medium	15	7.58±8.86	
Low and none	96	4.63±3.44	
Lymph node metastasis			0.435 ^a
Positive	56	4.70 (2.60,6.55)	
Negative	55	4.44 (1.44,6.53)	

Data that do not conform to normal distribution are presented as median (lower quartile,upper quartile). Variables that conform to normal distribution are presented as the mean ± standard deviation. ^aNon-parametric test (Mann-Whitney U test) was applied. ^bNon-parametric test (Kruskal-Wallis) was applied. ^cUnpaired Student's t test was applied.

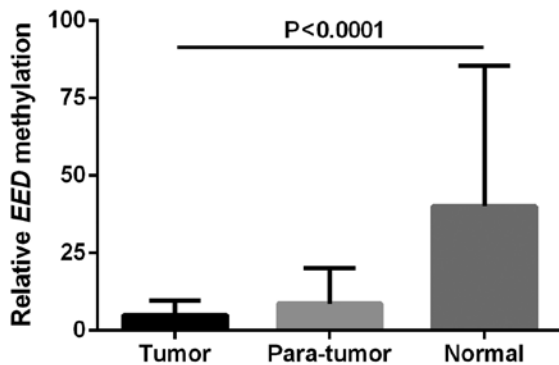


Figure 2. Comparisons of *EED* methylation levels among tumor tissues, paired para-tumor tissues and normal colorectal tissues. Analysis of variance was applied among tumor, para-tumor and normal samples. Data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference. *EED*, embryonic ectoderm development.

Diagnostic value of *EED* methylation. A further estimation of the diagnostic value of *EED* methylation for CRC revealed an area under the curve (AUC) of 0.574 (95% CI, 0.498-0.650) with a sensitivity of 33.3% and a specificity of 89.2% between tumor and para-tumor tissues (median PMR, 4.54 vs. 4.90%; $P=0.056$; Fig. 3). Furthermore, an AUC of 0.855 (95% CI, 0.729-0.981) with a sensitivity of 80% and a specificity of 89.2% was identified between tumor tissues and normal colorectal tissues (median PMR, 4.54 vs. 18.97%; $P<0.0001$;

Fig. 3). In addition, an AUC of 0.787 (95% CI, 0.657-0.917) with a sensitivity of 65% and a specificity of 86.5% was revealed between para-tumor tissues and normal colorectal tissues (median PMR, 4.90 vs. 18.97%; $P<0.0001$; Fig. 3).

Characteristics of *EED* expression and methylation. A dual-luciferase reporter gene assay was performed to evaluate whether the *EED* fragment (-300 bp to +390 bp) was able to regulate gene expression. The dual-luciferase assay demonstrated that the transcriptional activity of recombinant pGL3-*EED* plasmid was significantly higher compared with the pGL3-Basic control vector (pGL3-*EED* vs. pGL3-Basic vs. pGL3-Promoter: 2.05 ± 0.19 vs. 0.65 ± 0.02 vs. 30.27 ± 0.26 ; $F=16589.76$; $P<0.0001$; pGL3-*EED* vs. pGL3-Basic: fold-change, 3.15; $P=0.014$; pGL3-*EED* vs. pGL3-Promoter: $P<0.0001$; pGL3-Basic vs. pGL3-Promoter: $P<0.0001$; Fig. 4), which indicates that the *EED* fragment is able to promote gene expression.

Furthermore, *EED* mRNA expression and methylation data of patients with CRC were obtained from TCGA online database to investigate their correlation. Spearman correlation test revealed that *EED* mRNA expression level was inversely correlated with methylation ($r=-0.248$; $P<0.0001$; Fig. 5). In addition, a further analysis of GEO data (GSE32323) demonstrated that *EED* expression level in three CRC cell lines (COLO320, HT29 and RKO) was increased following 5'-AZA-deoxycytidine treatment (average fold-change, 1.44;

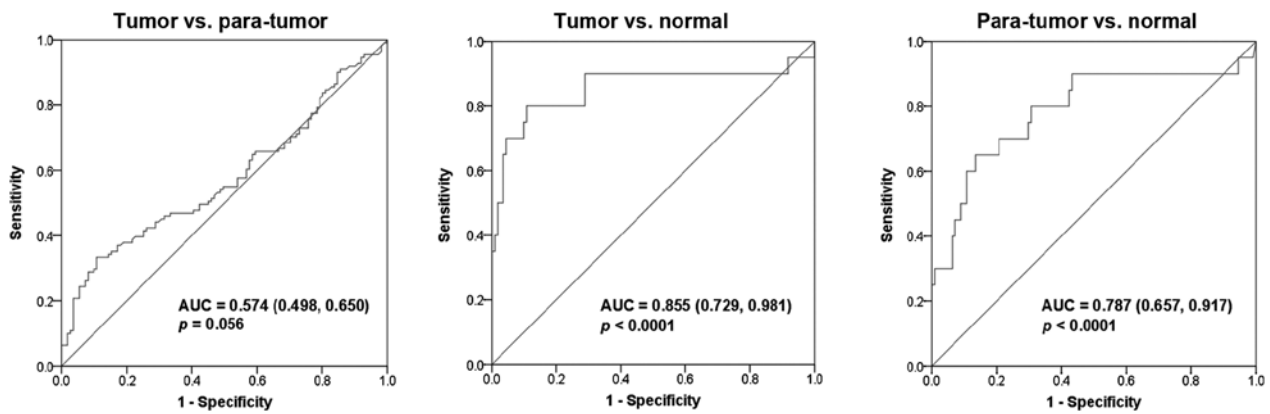


Figure 3. Receiver operating characteristic curves of *EED* methylation as a diagnostic marker for colorectal cancer. The AUC of *EED* methylation yielded a sensitivity of 33.3% and a specificity of 89.2% between tumor and para-tumor tissues, a sensitivity of 80% and a specificity of 89.2% between tumor tissues and normal colorectal tissues, and a sensitivity of 65% and a specificity of 86.5% between para-tumor tissues and normal colorectal tissues. $P < 0.05$ was considered to indicate a statistically significant difference. AUC, area under the curve; *EED*, embryonic ectoderm development.

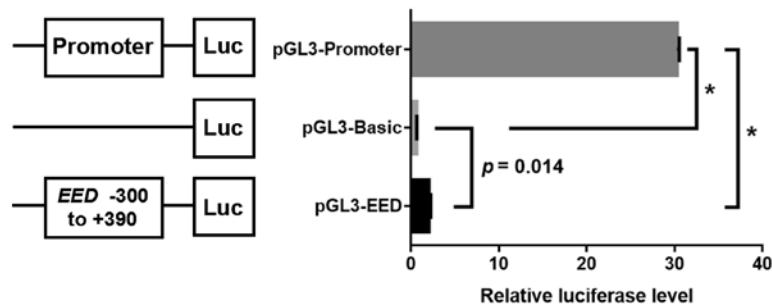


Figure 4. Dual-luciferase reporter assay in the 293T cell line. The pGL3-Basic and pGL3-Promoter vectors were used as negative and positive controls, respectively. The relative luciferase level is displayed for pGL3-Promoter, pGL3-Basic and pGL3-EED. The pGL3-EED indicates the recombinant *EED* fragment ligated to the pGL3-Basic vector. An analysis of variance and Bonferroni's correction were applied for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference. $P < 0.0001$. *EED*, embryonic ectoderm development; Luc, luciferase.

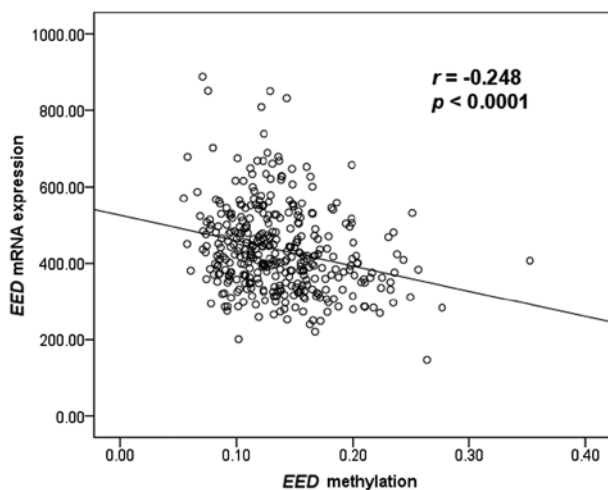


Figure 5. Correlation between *EED* mRNA expression level and methylation in 369 patients with colorectal cancer from The Cancer Genome Atlas data portal. Spearman's correlation test was applied. $P < 0.05$ was considered to indicate a statistically significant results. *EED*, embryonic ectoderm development.

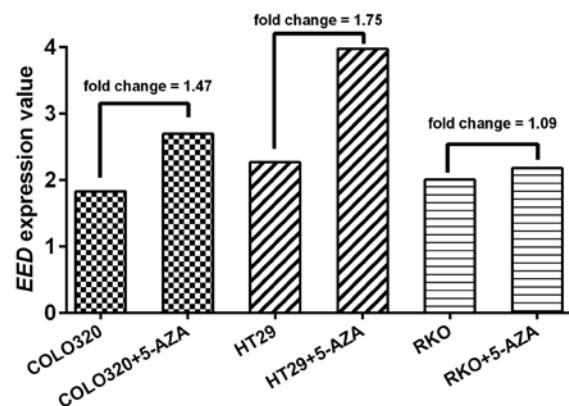


Figure 6. Expression value changes with and without 5-AZA treatment in COLO320, HT29 and RKO cell lines. The data were obtained from the Gene Expression Omnibus database (GSE32323) and expression was presented as a fold-change. The *EED* expression level was higher in three colorectal cell lines (COLO320, HT29 and RKO) following 5-AZA treatment. 5-AZA, 5'-AZA-deoxycytidine; *EED*, embryonic ectoderm development.

Discussion

Fig. 6). In summary, the present results indicate that *EED* hypomethylation is likely to upregulate *EED* expression and eventually increase the risk of CRC.

In the current study, *EED* methylation levels were detected in 111 pairs of CRC tumor and para-tumor tissues from patients with CRC and 20 colorectal tissues from normal controls. The

Table II. Associations between the PMR differences and clinical characteristics.

Clinical characteristics	No. (n=111)	P-value
Sex		0.643
Male	72	
Female	39	
Age, years		0.590
≤65	74	
>65	37	
Tumor location		0.039
Colon	46	
Rectum	56	
Colon and rectum	9	
Tumor size, cm		0.071
≤6	96	
>6	15	
Differentiation		0.119
High and medium	15	
Low and none	96	
Lymph node metastasis		0.286
Positive	56	
Negative	55	

Data were analyzed by χ^2 test. PMR difference value between methylation levels of tumor and para-tumor samples was used to perform this test. PMR, percentage of methylated reference.

results revealed that *EED* hypomethylation was significantly associated with the risk of CRC. In addition, the dual-luciferase reporter gene assay demonstrated that the *EED* fragment exhibited promoter activity. Further bioinformatics analyses revealed that *EED* methylation was inversely correlated with *EED* expression and demethylation treatment was identified to upregulate *EED* expression.

EED is a key component of the polycomb repressive complex 2 (PRC2) (30), which can mediate epigenetic silencing of genes associated with worse survival of patients with colon cancer (31). Higher expression of *EED* and two other PRC2 components has been demonstrated to contribute to the progression of CRC (17).

Polymorphism of *EED* gene has been identified to be associated with the lymph node metastatic process of CRC (32). In addition, *EED* expression has been demonstrated to be regulated by interleukin-22 and signal transducer and activator of transcription 3 (33). *EED* mRNA levels are significantly higher in CRC tissues compared with non-cancerous tissues (17). The dual-luciferase reporter gene assay performed in the current study demonstrated an enhanced transcriptional activity of the cloned *EED* fragment. Furthermore, data from TCGA and GEO databases suggested that *EED* methylation was associated with decreased expression levels of *EED*. This supports the hypothesis that *EED* hypomethylation may promote CRC via upregulation of *EED* expression. The present study

identified that *EED* expression may be regulated by the methylation of its promoter, which may promote understanding of the role of *EED* in CRC.

Previous studies have revealed that DNA cytosine modifications serve an important role in cancer biology, and provide promising biomarkers for cancer diagnosis and prognosis evaluation (10,34). Several epigenetic biomarkers have been studied for the diagnosis of CRC, including *SEPT9* (35), *BMP3*, *NDRG4* (36) and *hMLH1* (37). The ROC curves generated in the current study demonstrated that *EED* hypomethylation is a good biomarker for the diagnosis of CR. Tumor vs. para-tumor revealed a sensitivity of 33.3% and a specificity of 89.2%. Tumor vs. normal tissue analysis demonstrated a sensitivity of 80% and a specificity of 89.2%. Finally, para-tumor vs. normal tissue revealed a sensitivity of 65% and a specificity of 86.5%.

However, there were a number of limitations of the present study. Firstly, the GEO analyses to investigate the effect of demethylation agent on *EED* expression involved data from only three cell lines. Furthermore, a comparison of *EED* mRNA expression level between 17 paired tumor and non-cancerous tissues from TCGA database yielded an insignificant result (4.45 ± 0.58 vs. 4.32 ± 0.50 ; $P=0.393$), although this discrepancy may be due to different ethnic samples and a small sample size. Future studies are required to confirm the current findings and further address the functional roles of *EED* methylation in CRC. In conclusion, the present results demonstrated that *EED* hypomethylation might be an important risk factor associated with CRC.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

SD and WZ made substantial contributions to the conception and design. XY and RP analyzed and interpreted the data, drafted and revised the manuscript, and agreed to be accountable for all aspects of the work. JZ, BW, JY and YJ contributed to the interpretation of data and completion of figures and tables. SZ, YS, CZ and JD contributed to performing the experiments and analyzing the data. All the authors have read and approved the final manuscript.

Ethical approval and consent to participate

Informed consent was provided by all participants prior to their inclusion within the study. The study was approved by

the Ethical Committees of the Shaoxing People's Hospital, Zhejiang Province Cancer Hospital, Nanjing Chinese Medicine Hospital and Ningbo University.

Patient consent for publication

All patients have provided informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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