EXPERIMENTAL AND THERAPEUTIC MEDICINE 16: 4983-4990, 2018

Endothelial PAS domain protein 1 gene hypomethylation is associated with colorectal cancer in Han Chinese

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Received March 13, 2018; Accepted August 23, 2018

DOI: 10.3892/etm.2018.6856

Abstract. Endothelial PAS domain-containing protein 1 (EPAS1) serves a role in angiogenesis, which is important for the development of tumors, including colorectal cancer (CRC). The current study aimed to estimate whether *EPAS1* methylation was associated with CRC. A two-stage association study of *EPAS1* methylation and CRC was conducted. In the first phase, *EPAS1* methylation was evaluated in the tumor and adjacent non-tumor tissue samples from 41 patients with sporadic CRC in Jiangsu province, China. The diagnostic value of methylation of *EPAS1* for CRC in the second phase was evaluated in 79 patients with sporadic CRC and 22 normal individuals in Zhejiang province, China. The methylation assay was performed using a quantitative methylation-specific polymerase chain reaction (qMSP) method. The percentage of methylated reference (PMR) was used to quantify the methylation level. The first-stage results indicated that *EPAS1* promoter methylation was significantly lower in CRC tumor tissues compared with 5-cm-para-tumor tissues (median PMR, 0.59 vs. 1.22%; P=0.027) and 10-cm-para-tumor tissues (median PMR, 0.59 vs. 1.89%; P=0.001). In addition, the second-stage results indicated that *EPAS1* promoter methylation was significantly lower in tumor tissues compared with 5-cm-para-tumor tissues (median PMR, 1.91 vs. 6.25%; $P=3x10^{-7}$) and normal intestinal tissues from healthy controls (median PMR, 1.91 vs. 28.4%; $P=5x10^{-7}$). Receiver Operating Characteristic curve analysis of the second-stage data indicated that the highest area under the curve of *EPAS1* hypomethylation was 0.851 between Zhejiang CRC tissues and Zhejiang normal intestinal tissues (sensitivity, 95.5%; specificity, 60.8%).

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Abbreviations: EPAS1, endothelial PAS domain-containing protein 1; CRC, colorectal cancer; qMSP, quantitative methylation-specific polymerase chain reaction; PMR, percent of methylated reference; CpG, cytosine-phosphate-guanine; ROC, receiver operating characteristic

Key words: colorectal cancer, DNA methylation, endothelial PAS domain-containing protein 1, quantitative methylation-specific polymerase chain reaction

Introduction

In 2015, ~376,300 cases of colorectal cancer (CRC) were diagnosed in China (1). CRC is regarded as one of the most common cancers with high morbidity and mortality (2). Despite the improvement of diagnostic technologies, screening tools and clinical therapy, CRC remains a global challenge for public health due to the absence of a 'gold standard' for early diagnosis (2).

The molecular carcinogenic mechanisms of CRC have not been completely elucidated, however, CRC appears to be driven by the accumulation of abnormal genetic and epigenetic alternations in both oncogenes and tumor-suppressor genes (3). Cytosine modification, including DNA methylation, is one of the basic molecular mechanisms involved in the initiation and progression of CRC (2,4-6). Therefore, DNA methylation or epigenetic alterations may be promising markers for the early detection of CRC (4,7).

The endothelium PAS domain protein 1 (EPAS1) gene product is one of the important subunits of oxygen-induced hypoxia inducible factor (HIF) α, which regulates the primary transcriptional response to hypoxic stress (8). Hypoxia is one of the main factors promoting tumor angiogenesis (9,10). In addition, angiogenesis is considered a prerequisite for a range of biological processes, including tumorigenesis and tumor progression (11,12). HIF-2α/EPAS1 serves a role in tumor angiogenesis of different types of cancer, including lung cancer (13,14), renal carcinoma (15,16), liver cancer (17), pheochromocytoma (18-20) and CRC (8,21).

In a previous study including 39 patients with CRC and 43 normal controls, the expression of *EPAS1* in the blood of patients with CRC was significantly increased, and was subsequently decreased after surgical resection of the tumor, returning to a normal level (21). Another study revealed significantly increased levels of *EPAS1* methylation and significantly lower levels of *EPAS1* mRNA expression in 120 primary colon adenocarcinoma tissues compared with paracancerous tissues (8).

In the current study, quantitative methylation-specific polymerase chain reaction (qMSP) was used to measure *EPAS1* methylation in 120 Chinese patients with CRC and 22 healthy controls in two-stage experiments to assess whether the methylation of *EPAS1* could be used as a biomarker for the diagnosis of CRC.

Patients and methods

Study subjects. The first phase of the association study involved 41 patients with CRC, from whom frozen tumor tissues and adjacent tissues 5 and 10 cm away from the tumor lesions were collected. These patients with CRC were recruited from the Third Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (Nanjing, China) between August 2011 and March 2015 and their average age was 64.03±11.39 years (range, 21-86 years). Of the 41 patients, 28 were male, 12 were female and 1 was missing information. The second phase of the association study was conducted to verify the role of *EPAS1* methylation in CRC. The second phase of the association study involved 79 CRC tumor tissues, 79 paired adjacent tissues 5 cm away from the tumor lesions and 22 healthy human intestinal tissues. Patients involved in the second phase of the present study were recruited from the Zhejiang Cancer Hospital (Hangzhou, China) and Shaoxing First People's Hospital (Shaoxing, China) between August 2011 and January 2015. The average age of the 79 patients with CRC in the second phase of the present association study was 60.27±11.74 years. Of the 79 patients, 51 were male and 28 were female. All patients were diagnosed by pathological examination. No radiotherapy or chemotherapy was performed prior to surgery. The age and sex data of healthy controls were not available. All clinical data were extracted between August 2011 and March January 2015 from medical records for subsequent analysis. The Human Research Ethics Committee of Ningbo University (Ningbo, China) granted approval for the present study. Each participant completed the written informed consent form.

DNA extraction and bisulfite conversion. DNA was extracted from frozen tissues using E.Z.N.A.® Tissue DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's protocol. DNA concentrations measurement and bisulfite treatment were performed as previously described (22).

SYBR-Green-based qMSP. qMSP was performed as previously described (23,24). The following thermocycling conditions were used: Initial denaturation at 95˚C for 10 min; 45 cycles of 95˚C for 20 sec, 58˚C for 20 sec and 72˚C for 30 sec; melting curve analysis at 95˚C for 15 sec, 58˚C for 1 min and 60˚C for 1 min; and a final cooling stage at 40˚C for 10 min. The primer sequences for *EPAS1* (95 bp) were forward, 5'-GTTATAGATAGCGTTTGTAGAC-3' and reverse, 5'-GATTACCACATTCCCGATA-3'; and the primer sequences for *ACTB* (133 bp) were forward, 5'-TGGTGA TGGAGGAGGTTTAGTAAGT-3' and reverse, 5'-AACCAA TAAAACCTACTCCTCCCTTAA-3'. The percentage of methylated reference (PMR) of *EPAS1* for each sample was calculated using the $2^{-\Delta\Delta Cq}$ quantification approach, where $\Delta\Delta Cq$=sample DNA (Ct_{EPAS1}-$Ct_{ACTB\ control}$)-fully methylated DNA (Ct_{EPAS1}-$Ct_{ACTB\ control}$) (25).

Bioinformatics analysis. The genomic position of the amplified fragment was obtained from University of California Santa Cruz genome browser according to human (GRCh37) assembly (genome.ucsc.edu). To evaluate the association between mRNA expression and *EPAS1* methylation, data in the TCGA colorectal adenocarcinoma cohort with 372 samples were downloaded from cBioPortal (www.cbioportal.org).

Statistical analysis. All data were analyzed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Due to the skewed distribution of methylation levels, data were presented as the median (interquartile range). Friedman test and Wilcoxon nonparametric test were used to assess the difference in methylation between samples. Mann-Whitney U nonparametric test was used to assess the difference in methylation between groups. Contingency correlation test was used to evaluate the association between *EPAS1* methylation and clinical features. Spearman's rank correlation coefficient was used to assess the correlation between *EPAS1* methylation and gene expression. Pearson χ^2 test or Fisher's exact test were used to assess the difference in clinical features between different sampling locations. The receiver operating characteristic (ROC) analysis was used to evaluate the diagnostic value of *EPAS1* promoter methylation for CRC. Two-tailed $P<0.05$ was considered to indicate a statistically significant difference. All figures were plotted using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Sequencing results were analyzed using Chromas LITE 2.1.1 software (Technelysium Pty, Ltd., Brisbane, Australia).

Results

DNA methylation analysis. To assess the association between methylation of *EPAS1* and CRC, a two-stage association study was conducted. Two cytosine-phosphate-guanine (CpG) sites were identified in the 95 bp fragment of *EPAS1* (hg38; chr2:46 526691-46526785) (Fig. 1A). The tested *EPAS1* fragment was expected to be 95 bp (Fig. 1B). Sequencing results indicated that the amplified fragment matched the target sequence (Fig. 1C).

Association between EPAS1 hypomethylation in patients with CRC and clinical features. The results of the present study indicated that the *EPAS1* methylation was not associated with sex, age, TNM stage, differentiation, tumor size or lymph node metastasis (all $P>0.05$) (Table I). Methylation levels in the tissues from 41 patients with CRC (Jiangsu, China) and 79 patients with CRC (Zhejiang, China) were examined. The results indicated that *EPAS1* promoter methylation levels in CRC tissues (Jiangsu, China) were significantly lower compared with those of the 5-cm-para-tumor tissues (median PMR, 0.59 vs. 1.22%; $P=0.027$) (Fig. 2A) and 10-cm-para-tumor

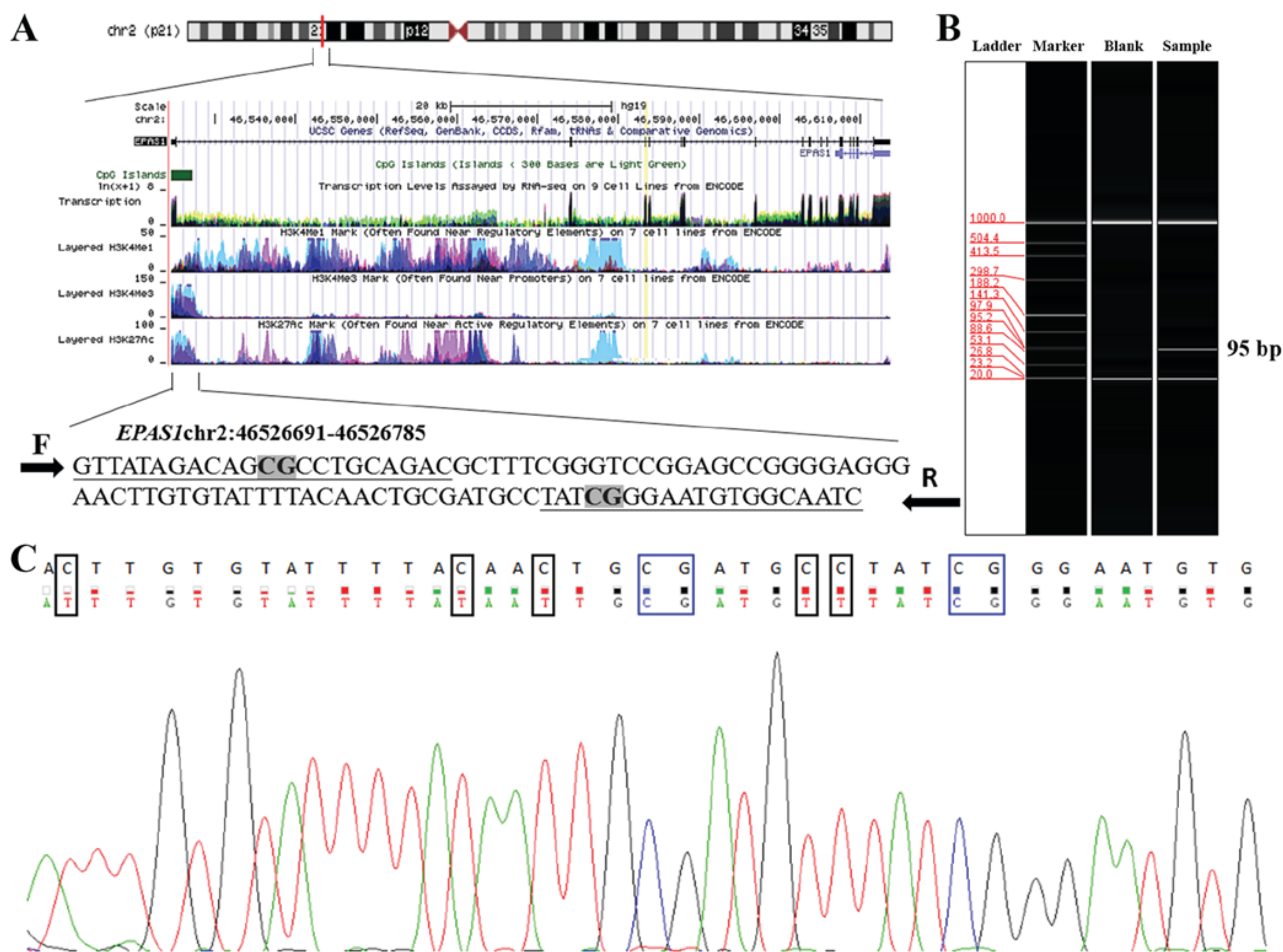

Figure 1. Characteristics of the target sequence in *EPAS1*. (A) Target sequences in *EPAS1* promoter region. The genomic position of the amplified fragment from University of California Santa Cruz genome browser according to human (GRCh37) assembly. The primers are underlined and two CpG sites are presented in bold fond with grey highlight. (B) Capillary electrophoresis for the amplified fragment (95 bp). (C) Sanger sequencing results. The top row of the sequence is the original sequence of the fragment. The bottom row of the sequence is the converted sequence; CG dinucleotides that remained unaltered are in blue boxes; and C nucleotides with corresponding converted T nucleotides are in black boxes. F, forward primer; R, reverse primer; *EPAS1*, endothelial PAS domain-containing protein 1; CpG, cytosine-phosphate-guanine.

tissues (median PMR, 0.59 vs. 1.89%; P=0.001) (Fig. 2A). Second-stage experiment was used to further validate the role of *EPAS1* methylation in CRC. The results indicated that *EPAS1* promoter methylation was significantly lower in CRC tissues (Zhejiang, China) compared with 5-cm-para-tumor tissues (median PMR, 1.91 vs. 6.25%; P=$3x10^{-7}$) (Fig. 2B) and normal intestinal tissues from healthy controls (median PMR, 1.91 vs. 28.84%; P=$5x10^{-7}$) (Fig. 2B). Additionally, a significantly lower *EPAS1* promoter methylation was found in the paired 5-cm-para-tumor tissues (Zhejiang, China) compared with normal intestinal tissues of healthy controls (median PMR, 6.25 vs. 28.84%; P=0.028) (Fig. 2B). A negative correlation between mRNA expression and *EPAS1* methylation was identified in 372 TCGA colorectal adenocarcinoma samples (r=-0.329, P=$8x10^{-11}$) (Fig. 3).

The present study demonstrated that *EPAS1* methylation levels of tumor tissues from patients from Zhejiang province were significantly higher compared with tumor tissues from Jiangsu province (median PMR, 1.91 vs. 0.59%; P=$4x10^{-7}$) (Table II). Significantly higher *EPAS1* methylation levels were observed in the adjacent non-tumor tissues from patients from Zhejiang province compared with patients from Jiangsu province (median PMR, 6.25 vs. 1.22%; P=$4x10^{-7}$) (Table II). However, the difference in *EPAS1* methylation levels between tumor and non-tumor tissues (D=PMR_{tumor}-$PMR_{non-tumor}$) was not significant between Jiangsu province and Zhejiang province (P=0.066) (Table III). To clarify the differences in methylation status between the two provinces, the present study further investigated the association between sample locations and the clinical phenotypes of patients with CRC. The results indicated that there was a significant difference in the age at diagnosis between Zhejiang (57/79; 72.2%) compared with Jiangsu (21/41, 51.2%) (χ^2=4.541; P=0.033) (Table III). In addition, there was statistically significant difference in differentiation between Jiangsu and Zhejiang province (P=0.002) (Table III). However, there were no significant differences in *EPAS1* methylation levels between age subgroups and between differentiation subgroups in both provinces (P>0.05) (Table II).

ROC curve analysis. ROC curve analysis was used to measure the diagnostic value of *EPAS1* hypomethylation for CRC. The second-stage association results indicated

Table I. Association between *EPAS1* hypermethylation and clinicopathological characteristics of patients with CRC in the two-stage experiment.

	First-stage experiment				Second-stage experiment			
Clinical characteristic	N	*EPAS1* hypomethylation	*EPAS1* hypermethylation	P-value	N	*EPAS1* hypomethylation	*EPAS1* hypermethylation	P-value
Total cases	41	31	10		79	57	22	
Sex[a]				1.000				0.534
Male	28	21	7		51	38	13	
Female	12	9	3		28	19	9	
Age, years[a]				0.859				0.534
≤65	21	16	5		57	40	17	
>65	19	14	5		22	17	5	
Differentiation[a]				NA				0.440
Poor	0	NA	NA		14	9	5	
Moderate + well	41	31	10		63	47	16	
TNM stage[b]				NA				0.945
I+II	NA	NA	NA		40	29	11	
III+IV	NA	NA	NA		39	28	11	
Tumor size, cm[a]				0.859				0.289
≤5	21	16	5		40	31	9	
5	19	14	5		39	26	13	
Lymphatic metastasis[a]				1.000				0.314
Yes	20	15	5		36	28	8	
No	20	15	5		43	29	14	

Data are presented as the median (interquartile range). [a]The information for 2 patients was lost. [b]The TNM stage information in the first-stage experiment was not available. Cutoff value of *EPAS1* hypermethylation is set at the mean value. Contingency correlation test was used to evaluate the association between *EPAS1* methylation and clinical features. PMR, percentage of methylated reference; N, number; NA, not available; *EPAS1*, endothelial PAS domain-containing protein 1; CRC, colorectal cancer.

Table II. Subgroup analysis by age and differentiation.

	Tumor PMR (%)			Non-tumor PMR (%)		
Variable	Jiangsu	Zhejiang	P-value	Jiangsu	Zhejiang	P-value
Total	0.59 (0.35, 1.19)	1.91 (0.79, 4.90)	4x10^{-7}	1.22 (0.63, 2.11)	6.25 (2.35, 29.63)	4x10^{-7}
Age, years						
≤65	0.83 (0.38, 1.54)	1.91 (0.83, 5.22)	0.008	1.06 (0.56, 1.92)	7.35 (7.24, 30.43)	3x10^{-6}
>65	0.48 (0.28, 1.24)	1.83 (0.65, 4.02)	0.009	1.53 (0.69, 2.27)	3.90 (2.33, 24.86)	2x10^{-4}
P-value	0.236	0.577		0.630	0.418	
Differentiation						
Poor	NA	2.39 (1.28, 6.03)	NA	NA	9.16 (2.32, 39.14)	NA
Moderate + well	0.59(0.35, 1.19)	1.89 (0.79, 4.72)	2x10^{-4}	1.22 (0.60, 2.18)	6.19 (2.35, 29.63)	8x10^{-9}
P-value	NA	0.235		NA	0.530	

Mann-Whitney U nonparametric test were used to assess the difference in methylation between groups. NA, not available; PMR, percentage of methylated reference.

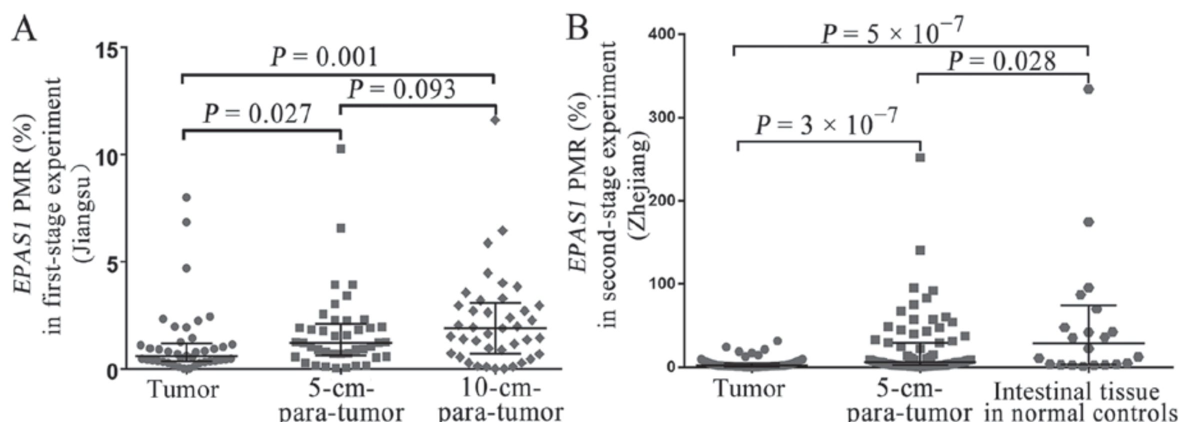

Figure 2. Comparisons of *EPAS1* methylation levels between patients and normal controls. (A) Comparisons of methylation levels between tumor tissues, 5-cm-para-tumor tissues and 10-cm-para-tumor tissues (Jiangsu, China). (B) Comparisons of methylation levels between tumor tissues, 5-cm-para-tumor tissues and normal intestinal tissues of healthy controls (Zhejiang, China). *EPAS1*, endothelial PAS domain-containing protein 1; PMR, percentage of methylated reference.

that *EPAS1* hypomethylation yielded a significant AUC of 0.731 (95% CI, 0.653-0.808) with a sensitivity of 58.2% and a specificity of 78.5% between cancer tissues and 5-cm-para-tumor tissues (Fig. 4A); a significant AUC of 0.851 (95% CI, 0.760-0.942) with a sensitivity of 95.5% and a specificity of 60.8% between CRC tissues and normal intestinal tissues of healthy controls (Fig. 4B); and a significant AUC of 0.654 (95% CI, 0.522-0.786) with a sensitivity of 63.6% and a specificity of 65.8% between 5-cm-para-tumor tissues and normal intestinal tissues of healthy controls (Fig. 4C). All of the above data supported the hypothesis that hypomethylation of *EPAS1* may be a potential diagnostic biomarker for CRC.

Discussion

CRC is regarded as a threat to human health (2), and the study of methylation in the context of CRC is a field of growing interest. In the present study, *EPAS1* promoter methylation significantly decreased according to the results of both the first-stage and the second-stage association tests. These results

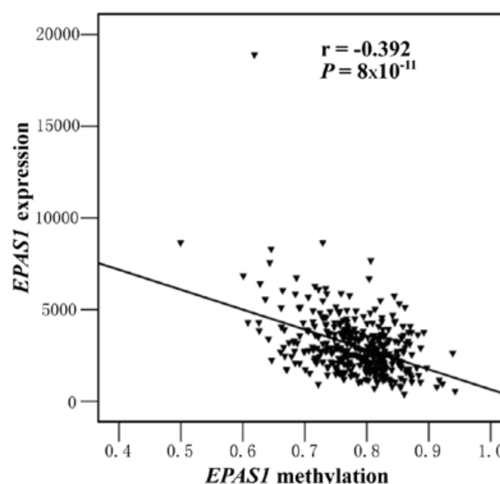

Figure 3. Bioinformatics analysis of the correlation between *EPAS1* methylation and expression. An inverse correlation was identified between *EPAS1* methylation and mRNA expression in 372 colorectal cancer samples (r=-0.329, P=8x10^{-11}). *EPAS1*, endothelial PAS domain-containing protein 1.

Table III. Association between sampling location and clinical characteristics.

Clinical characteristic	Number	Jiangsu	Zhejiang	P-value
Total cases	120	41	79	
Sex[a]				0.553[b]
Male	79	28	51	
Female	40	12	28	
Age, years[a]				0.033[b]
≤65	78	21	57	
>65	41	19	22	
Tumor size[a]				0.847[b]
<5 cm	61	21	40	
≥5 cm	58	19	39	
Differentiation[a]				0.002[c]
Low and none	14	0	14	
High and medium	103	40	63	
Lymph node metastasis[a]				0.842[b]
Negative	55	19	36	
Positive	64	21	43	
EPAS1 methylation				0.066[b]
Hypomethylation	91	27	64	
Hypermethylation	29	14	15	

[a]The information for 1 patient was lost. [b]Data analyzed using Pearson χ^2 test. [c]Data analyzed using Fisher's exact test. Hypermethylation was determined to be present if PMR detected in the tumor tissue was higher than in the matched normal sample. Hypomethylation was determined to be present in the inverse case. *EPAS1*, endothelial PAS domain-containing protein 1.

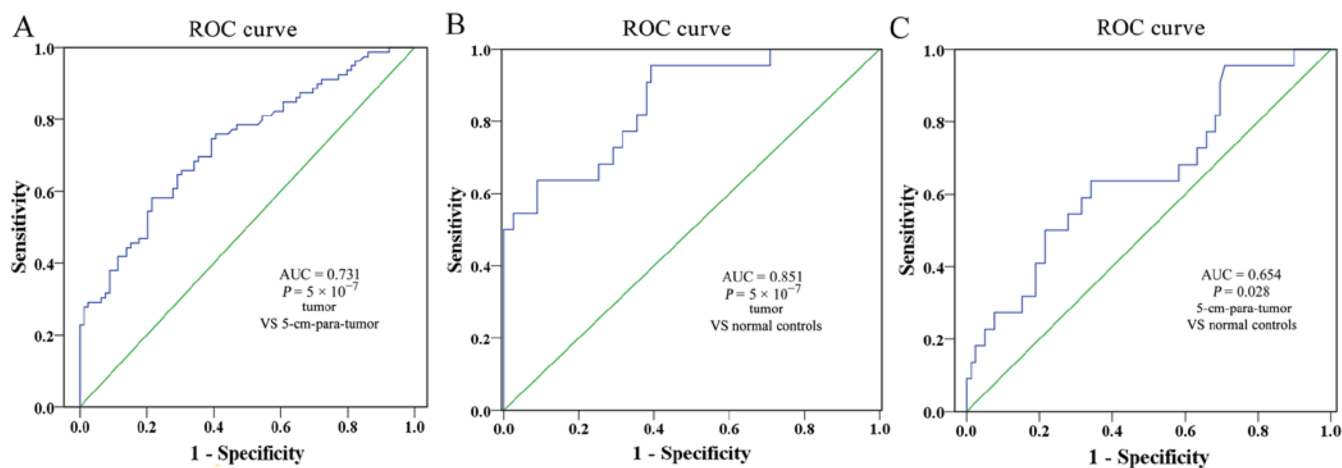

Figure 4. ROC curves for the diagnostic value of *EPAS1* hypomethylation in the second-stage experiment. (A) ROC curve for the diagnostic value of *EPAS1* hypomethylation between CRC tissues and paired non-cancer tissues (Zhejiang, China). (B) ROC curve for the diagnostic value of *EPAS1* hypomethylation between CRC tissues and normal intestinal tissues of healthy controls (Zhejiang, China). (C) ROC curve for the diagnostic value of *EPAS1* hypomethylation between paired non-cancer tissues and normal intestinal tissues of healthy controls (Zhejiang, China). ROC, receiver operating characteristic; AUC, area under the curve; *EPAS1*, endothelial PAS domain-containing protein 1; CRC, colorectal cancer.

led to a hypothesis that *EPAS1* hypomethylation may be associated with the development of CRC. In the subsequent experiment, *EPAS1* hypomethylation yielded an AUC of 0.851 (sensitivity, 95.5%; specificity, 60.8%) to distinguish the CRC tumor tissues from normal intestinal tissues, suggesting that *EPAS1* hypomethylation could serve as a promising diagnostic biomarker for CRC.

Numerous studies demonstrated that *EPAS1* served a role in angiogenesis of human cancer (26-28) at the post-transcriptional level (27). Yoshimura *et al* (11) demonstrated that EPAS1 was overexpressed in aggressive colorectal carcinoma and exhibited a significant direct correlation with tumor microvessel count. Cho *et al* (16) identified that EPAS1 was bound by TP2399 in the PAS B domain, which diminished its ability to bind to

ARNT, causing tumor regression in preclinical kidney cancer models. This means that EPAS1 might be an important factor in the processes of tumorigenesis and cancer progression. In human breast cancer cells, methyl-CpG-binding domain protein 3 can remove the methylation of CpG sites near the promoter of *EPAS1*, and, therefore, significantly increase the expression of *EPAS1* (27). In epithelial cells, DNA (cytosine-5)-methyltransferase 3A (DNMT3A) can silence the expression of *EPAS1* (29,30). DNMT3A deficiencies were reported in primary tumors and malignant cells, leading to demethylation of *EPAS1* promoter (31) and resulting in the growth of cancer cells under hypoxia (31-34). Re-introducing DNMT3A can restore the silencing of *EPAS1,* and prevent cell proliferation and viability under hypoxia, inhibiting tumor occurrence (8,31). In addition, our data mining of The Cancer Genome Atlas database discovered that there was an inverse *EPAS1* methylation-expression correlation in different cancers. Therefore, the present study further hypothesized that angiogenesis induced by increased expression of *EPAS1* could be a reason underlying the occurrence of CRC. Future studies should verify the function of *EPAS1* in CRC.

Although several types of biomarkers have been studied in CRC (35,36), a biomarker for early diagnosis remains to be identified. Carcinoembryonic antigen (CEA), a biomarker used globally, did not exhibit the desired diagnostic value according to one study (37). However, Peng *et al* (38) reported an AUC of 0.690 for CEA in the detection of CRC. In addition, there are two main methods widely used in clinical diagnosis of CRC, including fecal occult blood test (FOBT) and colonoscopy (37). However, FOBT is susceptible to bias resulting from external factors including drugs and diet, and colonoscopy is associated with significant costs and can often cause pain (37). The present qMSP-based study indicated an AUC of 0.851 (sensitivity, 95.5%; specificity, 60.8%) between CRC tissues and normal intestinal tissues of healthy controls.

A previous study indicated that patients with CRC exhibited significantly elevated *EPAS1* expression in blood, which decreased significantly to normal levels following surgical resection of the tumor (21). Further analyses of *EPAS1* methylation in blood samples of patients with CRC before and after surgery should be conducted to further verify the diagnostic value of *EPAS1* hypomethylation.

Benign colorectal disease can gradually progress to advanced adenoma and, subsequently, to invasive adenocarcinoma (39-41). In addition, aberrant methylation patterns exhibit a malignant potential in hyperplastic polyps (39-42). Numerous studies have indicated that there were significant differences in DNA methylation levels between CRC tissues, benign colorectal disease tissues and healthy intestinal tissues (43-45). However, methylation data for benign colorectal disease were not available in the present study. Further investigation is necessary to determine whether *EPAS1* methylation serves a role in benign colorectal disease.

Plasma circulating DNA (cell-free DNA) of patients with cancer may originate from circulating tumor cells, which can indicate the occurrence of micrometastasis and invasion of cancer cells (46,47). The present study indicated that the levels of *EPAS1* methylation in the tumor tissues were lower compared with the adjacent non-tumor tissues. The detection of *EPAS1* methylation in plasma cf-DNA could be performed in the future to avoid variation resulting from differences in tissue sampling sites, surgical techniques and so on.

In conclusion, the present study suggested that *EPAS1* hypomethylation may be a diagnostic biomarker for CRC. Further study is necessary to clarify the molecular mechanisms by which *EPAS1* hypomethylation may exert its role in carcinogenesis.

Acknowledgements

Not applicable.

Funding

The research was supported in part by K.C. Wong Magna Fund in Ningbo University (to SD) and grant no. U01CA217078 in Northwestern University (to WZ).

Availability of data and materials

Not applicable.

Authors' contributions

SD and RP conceived and designed the experiments. JY, DW, CZ, XY, JZ, HY, JD, BW, YM and YZ performed the experiments. RP, CZ and HY analyzed the data. RP, WZ and SD contributed to the completion of figures and tables, and writing of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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