

Hypermethylation of protocadherin γ subfamily A12 and solute carrier family 19 A 1 promoters contributes to the occurrence and metastasis of colorectal cancer

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Received March 11, 2016; Accepted April 3, 2017

DOI: 10.3892/ol.2018.8393

Abstract. The development of colorectal cancer (CRC) involves genetic and epigenetic modifications, and aberrant DNA methylation within gene promoters is a primary mediator of epigenetic inheritance in CRC. The present study evaluated whether promoter methylation of four CRC candidate genes [protocadherin γ subfamily A12 (*PCDH- γ -A12*), solute carrier family 19 A 1 (*SLC19A1*), cAMP responsive element binding protein (*CREB*) and cylindromatosis (*CYLD*)] contributed to the risk and metastasis of CRC by screening a total of 42 CRC and 42 adjacent normal tissue samples. DNA methylation was measured by methylation-specific polymerase chain reaction (MSP). Polymerase chain reaction (PCR) products were bisulfite converted and validated by sequencing. The χ^2 test was employed to assess the association between promoter methylation and a series of clinicopathological characteristics. The promoters of *PCDH- γ -A12* and *SLC19A1* were observed to be more frequently methylated in CRC tissues than normal tissues. In addition, significantly higher methylation of the *PCDH- γ -A12* and *SLC19A1* promoters was also observed in CRC tissues with lymph metastasis compared with those without lymph metastasis. In addition, no association was observed between *CREB* and *CYLD* methylation and the

occurrence and metastasis of CRC. These results suggest that the hypermethylation of the *PCDH- γ -A12* and *SLC19A1* promoters may contribute to the occurrence and metastasis of CRC in the Han Chinese population.

Introduction

Colorectal cancer (CRC) is one of the most common digestive malignancies, and it arises through well-defined sequential multi-step carcinogenesis that transforms normal glandular epithelium into invasive adenocarcinomas (1,2). The development of CRC involves genetic and epigenetic modifications. Aberrant DNA methylation within gene promoters is a primary mediator of epigenetic inheritance in CRC (3,4).

DNA methylation typically occurs in CpG islands and it refers to the enzymatic addition of a methyl group to the 5' position of cytosine by DNA methyltransferases to produce 5-methyl cytosine. Methylation of CpG islands in the gene promoter region may induce chromatin conformational modifications that inhibit access of transcriptional machinery, altering gene expression levels (5,6). Therefore, promoter methylation is commonly associated with gene silencing and promoter demethylation with gene expression (7,8).

There are complex changes of DNA methylation in a number of carcinomas, and particularly in CRC (9,10). Numerous genes are aberrantly methylated in CRC patients, including adenomatous polyposis coli (*APC*), *WNT5A*, mutL homolog 1 (*MLH1*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and Ras association domain-containing protein 1 (11-15). Aberrant DNA methylation of gene promoters in CRC is involved in its occurrence, progression, diagnosis, staging, prognosis and response to chemotherapy (16).

The protocadherin gamma subfamily A12 (*PCDH- γ -A12*) gene encodes a cell surface adhesion protein that serves essential roles in cell-cell and cell-matrix interactions and tumor metastasis (17,18). The solute carrier family 19 A 1 (*SLC19A1*) gene encodes a membrane protein that is involved in the regulation of intracellular concentrations of folate (19). *SLC19A1* gene mutation is associated with the risk

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Key words: colorectal cancer, methylation-specific polymerase chain reaction, metastasis, promoter, protocadherin γ subfamily A12, solute carrier family 19 A 1

of CRC (20). The cAMP responsive element binding protein (*CREB*) gene encodes a transcription factor that induces the transcription of genes in response to hormonal stimulation of the cAMP pathway (21,22). P300/CREB binding protein genes promote cancer progression in colon cancer cell lines with microsatellite instability (23). Cyldromatosis (*CYLD*) encodes a cytoplasmic protein with three cytoskeletal-associated protein-glycine-conserved domains, and it regulates cell proliferation, apoptosis, cell movement and cell differentiation (24-27). *CYLD* is downregulated or lost in colon carcinoma cell lines compared with primary human colonic epithelial cells. The functional relevant loss of *CYLD* expression may contribute to tumor development and progression, and it may provide a new target for therapeutic strategies (28).

Promoter methylation of the *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* genes has been demonstrated to regulate their gene expression levels, and hypermethylation of these promoters has been observed in acute lymphoblastic leukemia (29), breast cancer (30,31) and malignant melanoma (32). However, hypermethylation of the *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* promoters has not been investigated in CRC. In light of the previous findings, the aim of the present study was to investigate whether *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* gene promoter methylation contributed to the risk of CRC.

Materials and methods

Tissue sample collection. In this study, CRC patients who had not received radiotherapy, chemotherapy, targeted therapy or dendritic cell/cytokine-induced killer therapy prior to surgery were recruited between June 2012 and April 2013 (Table I). CRC samples, normal adjacent tissue samples and matched metastatic lymph node samples were collected at the time of surgery from 42 primary sporadic CRC patients at the Department of Gastrointestinal Surgery in the Affiliated Hospital of Ningbo University, China. Tissues were immediately preserved in liquid nitrogen at -80°C following removal from the body and stored at -80°C until use. Normal adjacent tissues were collected from at least 5 cm away from the edge of the tumor, and there were no obvious tumor cells, as evaluated by a pathologist. Tumor stage was determined according to Dukes' staging system, and cellular differentiation was graded according to Broders' grading system. Informed consent was given by all subjects. The Human Research Ethics Committee of Ningbo University approved all aspects of the study.

DNA isolation and bisulfite modification. Genomic DNA was isolated using a QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany). The concentration and quality of genomic DNA were determined using the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The concentration of DNA was above 30 ng/μl and the purity of DNA was at the A260/A280 ratio of 1.7-1.9. DNA was bisulfite-treated with the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA). Following the completion of bisulfite modification, all unmethylated cytosines in CpG islands were converted to uracil, while methylated cytosines remained unchanged.

Methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing. The methylated and unmethylated primers (Table II) were designed using the Primer Premier 6.0 program (Premier Biosoft International, Palo Alto, CA, USA). MSP was performed in a total volume of 20 μl containing 2 μl bisulfite modified DNA, 1 μmol each of forward and reverse primers, 10 μl Premix Taq (Takara Biotechnology Co., Ltd., Dalian, China) and 7 μl double-distilled water with the following cycling parameters: 10 min of denaturation at 95°C followed by 55 cycles of 30 sec at 95°C, 45 sec at 72°C and a final extension for 10 min at 72°C. Polymerase chain reaction (PCR) products were then loaded and electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized under UV illumination. In order to confirm the result of methylation- and unmethylation-specific PCR, PCR products randomly obtained from the group were sequenced bidirectionally by Invitrogen (Thermo Fisher Scientific, Inc.) with the same primers used for MSP.

Statistics. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) statistical software package (version 16.0; SPSS, Inc., Chicago, IL, USA), and the results were obtained using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). All analyses were two-sided, and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Methylation rates of promoters in CRC vs. normal tissues. A total of 42 pairs of CRC and adjacent normal tissues were examined, and representative results of the agarose gel electrophoresis were selected (Fig. 1). The results revealed that the methylation rates of *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* promoters in CRC were 83.33% (35/42), 78.57% (33/42), 26.19% (11/42) and 14.29% (6/42), while the methylation rates of these promoters in normal tissues were 57.14% (24/42), 45.24% (19/42), 11.90% (5/42) and 11.90% (5/42). *PCDH-γ-A12* and *SLC19A1* gene promoters were more frequently methylated in CRC tissues than in normal tissues (83.33% vs. 57.14%, $P = 0.009$ and 78.57% vs. 45.24%, $P = 0.002$), while there was no significant difference in methylation rates of *CREB* and *CYLD* gene promoters between CRC tissues and normal tissues (26.19% vs. 11.90%, $P = 0.095$ and 14.29% vs. 11.90%, $P = 0.746$; Table III).

Methylation rates of promoters in lymph vs. non-lymph metastasis CRC tissues. In addition, the methylation rates of *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* promoters in lymph metastasis CRC tissues were 100.00% (21/21), 95.24% (20/21), 33.33% (7/21) and 19.05% (4/21), while the methylation rates of these promoters in non-lymph metastasis CRC tissues were 66.67% (14/21), 61.90% (13/21), 19.05% (4/21) and 9.52% (2/21). *PCDH-γ-A12* and *SLC19A1* gene promoters were more frequently methylated in lymph metastasis CRC tissues than non-lymph metastasis CRC tissues (100.00% vs. 66.67%, $P = 0.013$ and 95.24% vs. 61.90%, $P = 0.024$), while there was no significant difference in the methylation rate of *CREB* and *CYLD* gene promoters between lymph metastasis CRC tissues and non-lymph metastasis CRC tissues (33.33% vs. 19.05%, $P = 0.292$ and 19.05% vs. 9.52%, $P = 0.659$; Table IV).

Bisulphite sequencing of *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* genes. In order to confirm the results of the PCR-based methylation analysis describe above, high-resolution bisulfite genomic sequencing was performed in the stochastic samples derived from the methylation PCR experiments. In agreement with the MSP results, CpG dinucleotides of the *PCDH-γ-A12* and *SLC19A1* promoters in the samples demonstrated extensive hypermethylation, whereas the *CREB* and *CYLD* promoters were unmethylated at these CpG dinucleotides (Fig. 2).

Correlation between methylation status of promoters and clinicopathological factors. The correlation between the methylation status of the *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* gene promoters and the clinicopathological characteristics of CRC is shown in Table V. There was no significant difference in clinicopathological factors, including sex, age, tumor-node-metastasis stage, lymph node status, metastasis status, tumor location, differentiation status, tumor size and histological grade. There was also no correlation between the methylation status of the *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* gene promoters and the serum levels of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9).

Discussion

Cancer develops through a multi-step process which results from the progressive accumulation of genetic and epigenetic alterations (33). **Epigenetic modifications, which have a fundamental role in the regulation of gene expression, involve DNA methylation, specific histone modifications and non-coding RNA interventions (34,35).** As one of the main epigenetic modifications, DNA methylation of promoters often downregulates gene transcription, while DNA demethylation of promoters activates gene expression. DNA methylation-mediated tumor suppressor gene silencing may contribute to tumor progression (7,36). Aberrant DNA methylation of gene promoters has become a promising biomarker for the early diagnosis of diseases (37-41).

In the colon, aberrant DNA methylation arises extremely early, initially in normal-appearing mucosa, and it may be part of the age-associated field defects observed in sporadic CRC (42). Hypermethylation in CpG islands has been demonstrated to be a novel mechanism of tumor suppressor gene silencing (7,8). A number of genes have now been demonstrated to be hypermethylated in colorectal tumors, including *APC* (11), *MLH1* (43) and O⁶-methylguanine DNA methyltransferase (44). For example, the inactivation of the cyclin-dependent kinase inhibitor *PI6/CDKN2A/INK4a* by methylation leads to the disruption of cell-cycle regulation and potentially provides a growth advantage to affected cells (45).

PCDH-γ-A12 is a member of the protocadherin γ gene cluster, which includes 22 genes divided into 3 subfamilies (subfamily A, B and C) (46). The exon of *PCDH-γ-A12* encodes the extracellular region, which includes six cadherin ectodomains and a transmembrane region. These cadherin-like cell adhesion proteins most likely serve a critical role in the establishment and function of specific cell-cell connections in the brain and cancer (18). The hypermethylation of *PCDH-γ-A12* induces the downregulation of *PCDH-γ-A12* gene transcription by rendering the chromatin structure inaccessible to

Table I. Clinical profiles of the colorectal cancer patients.

Characteristics	Subgroup	Patients, n
Gender	Male	28
	Female	14
Age (years)	≤60	16
	>60	26
TNM stage	1, 2	21
	3, 4	21
Lymph metastasis	Yes	21
	No	21
Distant metastasis	Yes	8
	No	34
CEA	≥5.0 ng/ml	15
	<5.0 ng/ml	27
CA19-9	≥37 U/ml	9
	<37 U/ml	33
Tumor location	Colon	26
	Rectum	16
Differentiation	Poor	10
	Moderate	32
	Good	0
Tumor size	<5 cm	28
	≥5 cm	14
Histological classification	Adenocarcinoma	40
	Mucinous adenocarcinoma	2
	Undifferentiated carcinoma	0

TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

the transcription machinery in a variety of tumors including bladder cancer, breast cancer, acute lymphoblastic leukemia and non-small cell lung cancer (17,29,47,48). The present study in CRC provides new evidence for the contribution of *PCDH-γ-A12* promoter hypermethylation to the occurrence and metastasis of CRC.

SLC19A1 encodes a membrane protein that is a transporter of folate, and is involved in the regulation of intracellular concentrations of folate. *SLC19A1* is also a major transporter of antifolate drugs used for certain types of cancer chemotherapy, including methotrexate (MTX) (30). The expression of *SLC19A1* is downregulated following exposure to MTX in breast cancer, and a reverse correlation was identified between the promoter methylation and mRNA levels of *SLC19A1*. A variant of the *SLC19A1* gene is associated with metastatic colorectal cancer (20). The present study in CRC adds new evidence for the contribution of *SLC19A1* promoter hypermethylation to the occurrence and metastasis of CRC.

Certain studies have focused on the correlation between colorectal cancer clinical features and the methylation of certain genes, including *p15*, *APC* and *E-cadherin*, suggesting that the inactivation of certain tumor suppressor genes through aberrant promoter methylation of CpG islands may serve a role in the development of colorectal cancer (49,50). Multiple

Table II. List of all primers used.

Gene	Subgroup	Sense (5'-3')	Antisense (5'-3')	Size (bp)
<i>PCDH-γ-A12</i>	M	ATTAAGGTGGTGGCGGTGGAT	GACGCCGACGCTCCTATCAA	449
	U	AAGGTGGTGGTGGTGGATAG	ACCAACACTCCTATCAAAC	443
<i>SLC19A1</i>	M	TTGTTGTAGCGGTGTTGGAAG	TCCGCCGCAACCTACGAAT	361
	U	TTTGTGTAGTGGTGTGGAAG	TTCCACCACAACCTACAAAT	363
<i>CREB</i>	M	CGGCGGTAAAGAGTAGAGTTA	GCGTCACTCACCAACACT	492
	U	TGGTGGTTAAGAGTAGAGTTA	TCACTCACCAACACTCCAC	489
<i>CYLD</i>	M	AGTTGGTGGTAGCGTAGCG	CATTCACTAACCTCGAACGA	495
	U	TGGTGGTAGTGTAGTGT	TCACTAACCTCAAACAACA	489

PCDH-γ-A12, protocadherin γ subfamily A12; *SLC19A1*, solute carrier family 19 A 1; *CREB*, cAMP responsive element binding protein; *CYLD*, cylindromatosis; M, methylated; U, unmethylated.

Table III. Methylation status of *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* genes in colorectal cancer and normal tissues.

Gene	Group	Total	M	U	M%	χ^2	P-value
<i>PCDH-γ-A12</i>	Cases	42	35	7	83.33	6.891	0.009
	Controls	42	24	18	57.14		
<i>SLC19A1</i>	Cases	42	33	9	78.57	9.894	0.002
	Controls	42	19	23	45.24		
<i>CREB</i>	Cases	42	11	31	26.19	2.779	0.095
	Controls	42	5	37	11.90		
<i>CYLD</i>	Cases	42	6	36	14.29	0.105	0.746
	Controls	42	5	37	11.90		

PCDH-γ-A12, protocadherin γ subfamily A12; *SLC19A1*, solute carrier family 19 A 1; *CREB*, cAMP responsive element binding protein; *CYLD*, cylindromatosis; M, methylated; U, unmethylated.

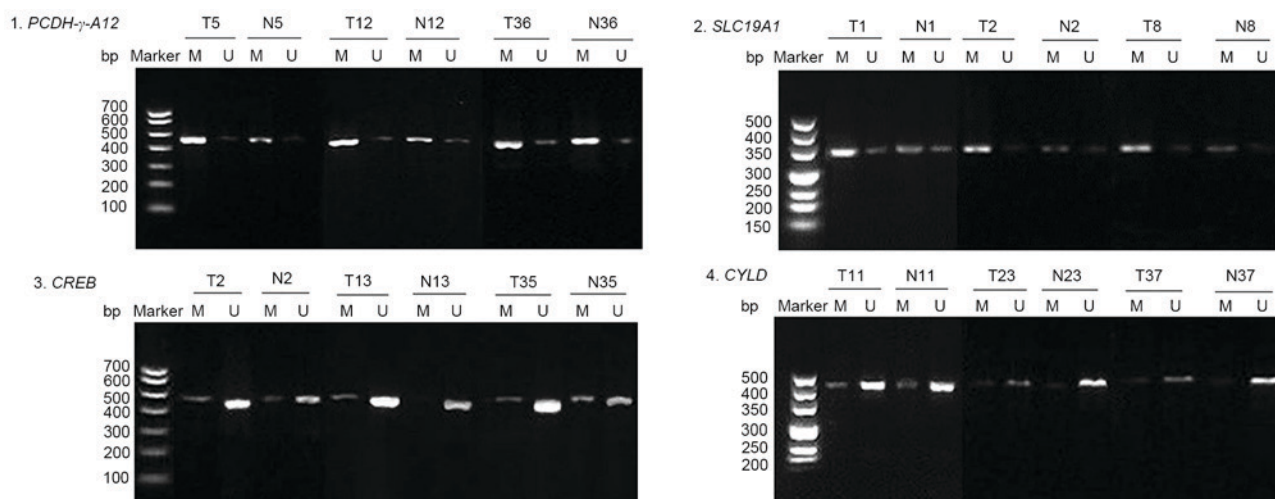


Figure 1. Representative results for methylation status of protocadherin γ subfamily A12 (*PCDH-γ-A12*), solute carrier family 19 A 1 (*SLC19A1*), cAMP responsive element binding protein (*CREB*) and cylindromatosis (*CYLD*) genes in colorectal cancer tissues (T) and adjacent normal tissues (N). M, methylated; U, unmethylated.

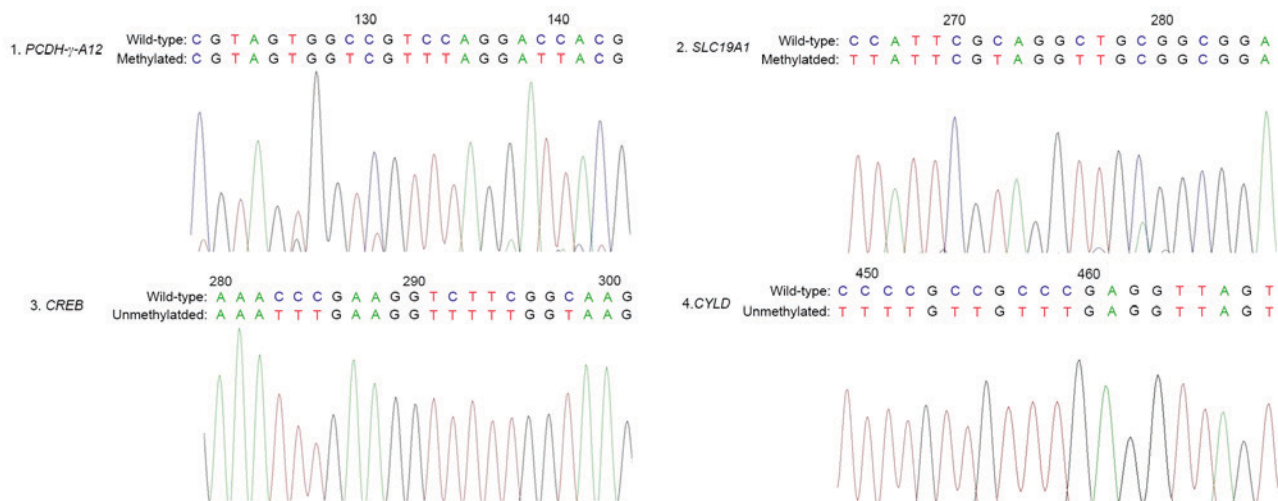
methylation pathways may be involved in the tumorigenesis of CRC and associated with the aggressiveness of clinical disease (37). In the present study, the correlation between the

methylation of *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* and colorectal cancer clinical features was examined. However, no significant correlation was identified between *PCDH-γ-A12*,

Table IV. Methylation status of *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* genes in lymph metastasis and non-lymph metastasis colorectal cancer tissues.

Gene	Subgroup	Total	M	U	M%	χ^2	P-value
<i>PCDH-γ-A12</i>	Cases	21	21	0	100.00	6.171	0.013
	Controls	21	14	7	66.67		
<i>SLC19A1</i>	Cases	21	20	1	95.24	5.091	0.024
	Controls	21	13	8	61.90		
<i>CREB</i>	Cases	21	7	14	33.33	1.109	0.292
	Controls	21	4	17	19.05		
<i>CYLD</i>	Cases	21	4	17	19.05	0.194	0.659
	Controls	21	2	19	9.52		

PCDH-γ-A12, protocadherin γ subfamily A12; *SLC19A1*, solute carrier family 19 A 1; *CREB*, cAMP responsive element binding protein; *CYLD*, cylindromatosis; M, methylated; U, unmethylated.

Figure 2. Bisulphite sequencing of protocadherin γ subfamily A12 (*PCDH-γ-A12*), solute carrier family 19 A 1 (*SLC19A1*), cAMP responsive element binding protein (*CREB*) and cylindromatosis (*CYLD*) genes.

SLC19A1, *CREB* and *CYLD* methylation and the clinical features, which may be due to the lack of power in the samples used.

CEA is a member of a family of cell surface glycoproteins that are excessively produced in the majority of human colorectal carcinomas (51). CEA measurement is mainly used as a tumor marker to monitor colorectal carcinoma treatment, to identify recurrences following surgical resection and to localize cancer spread through measurement of biological fluids (52,53). CA19-9 is a useful tumor-associated antigen for the serological detection of colorectal carcinomas, and may be used to monitor patients with advanced colorectal carcinomas (54). One aim of the present study was to observe whether the status of *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* promoter methylation had a correlation with the serum level of CEA and CA19-9. However, no significant correlation was observed between *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* promoter methylation and the serum level of CEA and CA19-9. This may imply that aberrant methylation of *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* combined with conventional tumor markers could serve as complementary

markers in the diagnosis of CRC. However, further study is necessary to confirm this hypothesis.

In conclusion, *PCDH-γ-A12* and *SLC19A1* promoters, but not *CREB* and *CYLD* promoters, are hypermethylated and contribute to the occurrence and metastasis of colorectal cancer. These findings may provide a new direction in the detection and treatment of CRC. Future research is required to determine the detailed mechanisms of how the *PCDH-γ-A12* and *SLC19A1* genes contribute to the risk of CRC.

Acknowledgements

Not applicable.

Funding

This study was supported by grants from the Natural Science Foundation of Zhejiang Province (LY15H160015 and LS14H26001), the K. C. Wong Magna Fund in Ningbo University, the Science and Technology Innovation Team of Ningbo (2011B82014), the Specialized Research Fund for

Table V. Association between *PCDH-γ-A12*, *SLC19A1*, *CREB* and *CYLD* methylation in CRC serum and clinicopathological features.

Characteristics	Subgroup	Patient, n	<i>PCDH-γ-A12</i>			<i>SLC19A1</i>			<i>CREB</i>			<i>CYLD</i>		
			M	U	P-value	M	U	P-value	M	U	P-value	M	U	P-value
Gender	Male	28	25	3	0.306	21	7	0.690	7	21	1	4	24	1.000
	Female	14	10	4		12	2		4	10		2	12	
Age (years)	≤60	16	14	2	0.887	10	6	0.109	4	12	1	3	13	0.846
	>60	26	21	5		23	3		7	19		3	23	
TNM stage	1, 2	21	18	3	1.000	17	4	1.000	6	15	0.547	2	19	0.659
	3, 4	21	17	4		16	5		5	16		4	17	
Lymph metastasis	Yes	21	21	0	0.013	20	1	0.024	7	14	0.292	4	17	0.756
	No	21	14	7		13	8		4	17		2	19	
Distant metastasis	Yes	8	7	1	1.000	8	0	0.245	4	4	0.209	3	5	0.128
	No	34	28	6		25	9		7	27		3	31	
CEA	≥5.0 ng/ml	15	12	3	1.000	10	5	0.313	3	12	0.754	3	12	0.742
	<5.0 ng/ml	27	23	4		23	4		8	19		3	24	
CA19-9	≥37 U/ml	9	7	2	1.000	6	3	0.600	3	6	0.903	2	7	0.818
	<37 U/ml	33	28	5		27	6		8	25		4	29	
Tumor location	Colon	26	21	5	0.887	23	3	0.109	8	18	0.618	3	23	0.846
	Rectum	16	14	2		10	6		3	13		3	13	
Differentiation	Poor	10	9	1	0.871	9	1	0.570	4	6	0.468	2	8	0.941
	Moderate	32	26	6		24	8		7	25		4	28	
	Good	0	0	0		0	0		0	0		0	0	
Tumor size	<5 cm	28	23	5	1.000	22	6	1.000	7	21	1	3	25	0.640
	≥5 cm	14	12	2		11	3		4	10		3	11	
Histological classification	Adenocarcinoma	40	33	7	1.000	31	9	1.000	10	30	1	4	36	0.558
	Mucinous	2	2	0		2	0		1	1		1	1	
	adenocarcinoma													
	Undifferentiated	0	0	0		0	0		0	0		0	0	
	carcinoma													

PCDH-γ-A12, protocadherin γ subfamily A12; *SLC19A1*, solute carrier family 19 A 1; *CREB*, cAMP responsive element binding protein; *CYLD*, cylindromatosis; M, methylated; U, unmethylated; TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

the Social Development of Hangzhou (20160533B21) and the Scientific Innovation Fund of the Affiliated Hospital of Hangzhou Normal University.

Availability of data and materials

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

Author's contributions

MY and SD designed the research. CZ, JL, TH and CC conducted the experiments. CZ, QH and HJ analyzed the data. The manuscript was drafted by CZ and SD. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Human Research Ethics Committee of Ningbo University and written informed consent was obtained from all participants.

Consent for publication

All participants provided written informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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