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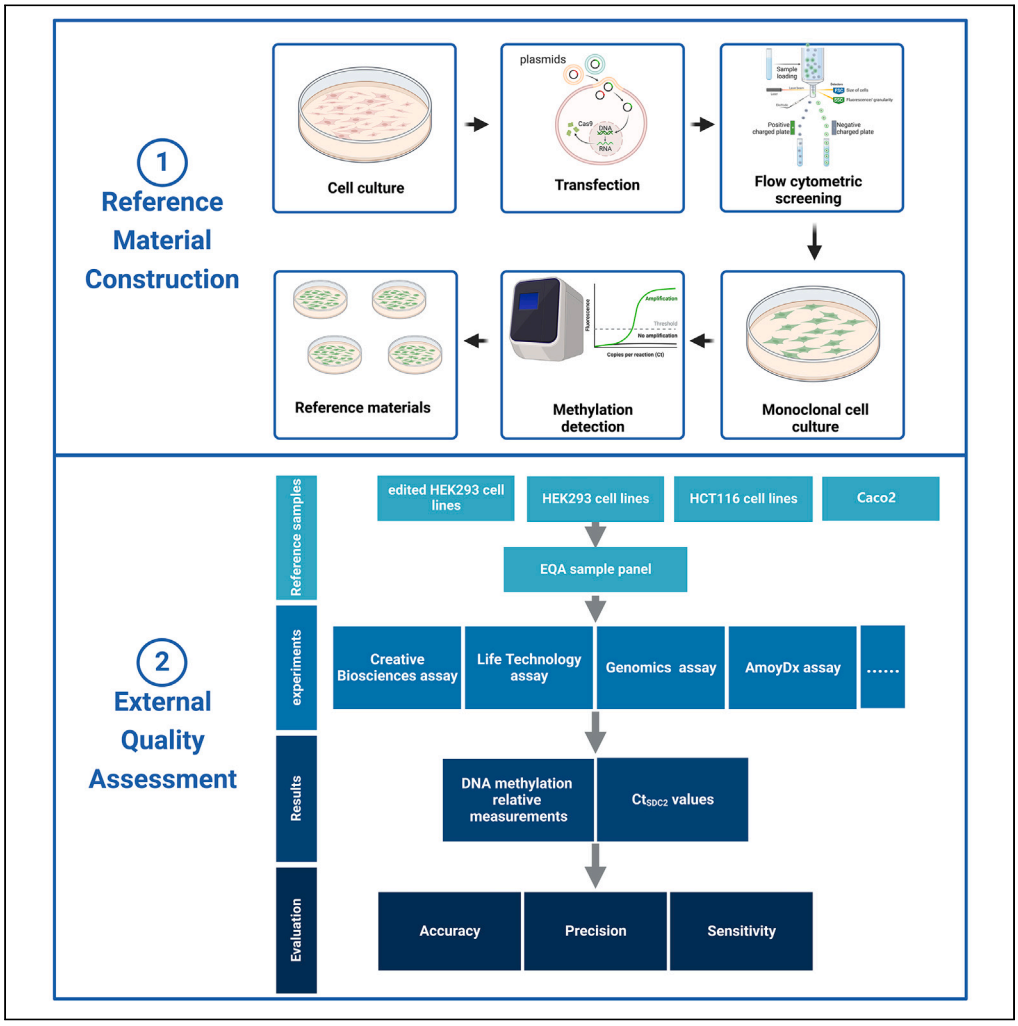

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Highlights

Early colorectal cancer can be screened by SDC2 methylation marker

New methylation SDC2 EQA materials reflect actual patients with tumor

EQA studies are essential for precise and reliable SDC2 methylation testing

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Interlaboratory consistency of *SDC2* promoter methylation detection in colorectal cancer using the post-optimized materials

Lijing Zhang,[1,2,3] Duo Wang,[1,2,3] Ziqiang Li,[1,2,3] Guigao Lin,[1,2,3] Jinming Li,[1,2,3,4,*] and Rui Zhang[1,2,3,*]

SUMMARY

Fecal DNA-based Syndecan 2 (*SDC2*) methylation detection is a promising non-invasive strategy for early colorectal cancer (CRC) screening. In China, commercial assays for *SDC2* methylation detection vary in sensitivity and specificity, yet there is no standardized external quality assessment (EQA) to ensure accuracy. This study utilized CRISPR-Cas9 and homology-directed repair (HDR) technologies to edit the *SDC2* promoter in 293T cells, creating hypermethylated and heterogeneous cell lines. These cell lines were used to develop an EQA panel for *SDC2* methylation. We established a 10-sample panel, encompassing a range of methylation levels, and conducted an EQA across 140 laboratories. Among 1,400 results, 0.57% were incorrect. The optimized EQA materials effectively monitor the accuracy of *SDC2* methylation detection in CRC, supporting reliable and consistent clinical testing and contributing to early CRC screening and diagnosis in China.

INTRODUCTION

Cytosine–> methylation of CpG sites is crucial for gene expression regulation and cellular function maintenance in vertebrates.[1,2] Dysregulation of DNA methylation can lead to diseases like cancer. Colorectal cancer (CRC) is a prevalent malignant tumor worldwide, ranking third in incidence and second in mortality.[3] In 2020, there were over 1.88 million new cases of CRC globally, resulting in approximately 915,000 deaths.[4] CRC development involves genetic and epigenetic abnormalities, including aberrant DNA methylation.[5] Fecal DNA testing, particularly fecal DNA methylation testing, has shown promise in improving the early detection rate of CRC.[6–8]

The Syndecan 2 (*SDC2*) gene encodes the *SDC2* protein, which plays a role in cell adhesion, migration, and proliferation.[9] Abnormal *SDC2* methylation occurs early in tumor development and is maintained in advanced CRC.[10] *SDC2* methylation is considered an epigenetic biomarker for early fecal DNA testing in CRC.[11] A fecal DNA-based *SDC2* methylation assay has been used for screening CRC and late-stage intestinal adenomas, with high sensitivity and specificity.[12–14] This non-invasive screening method using fecal samples has the potential to increase patient participation in early CRC detection.[8,15] In China, various commercial kits have been used for detecting methylated *SDC2* DNA in feces in clinical settings. However, the detection processes involved are complex, leading to potential variations in laboratory test results.[16] External quality assessment (EQA) programs for *SDC2* methylation assay are necessary to ensure satisfactory analytical performance and maintain test result accuracy. Thus, the ideal EQA reference material is crucial for methylation detection standardization. Current reference materials are mainly tumor patient clinical samples or M.SssI-modified DNA with fully methylated CpG sites.[17,18] Collecting clinical samples involves challenges such as ethical approval, patient consent, and accurate record-keeping. M.SssI-modified DNA, though artificially methylated, does not replicate the diverse methylation patterns of actual tumor samples,[19–21] which are critical for evaluating detection methods.[19–22] It also fails to simulate real sample handling procedures like DNA extraction, impacting the assessment of laboratory workflows.

Thus, a post-optimized reference material is needed, and it should be easily obtainable and exhibit heterogeneous methylation patterns, simulating *SDC2* methylation in patients with CRC. From the point of view of molecular specificity, both methylated cell samples and clinical stool samples contain DNA molecules, and DNA methylation is a modification that occurs at the level of DNA molecules. Whether it is methylated cell samples or clinical fecal samples, both contain DNA molecules, and DNA methylation occurs at the molecular level of DNA. Therefore, both types of samples can be used as DNA sources for methylation detection. Besides, for the methylation detection method, such as direct bisulfite sequencing, methylation-specific PCR (MSP), methylation-sensitive restriction enzyme digestion, and other methods, the specificity of these methods is mainly based on the sequencing reads of methylation-specific primers, methylation-sensitive enzymes, or methylation sites, not relation to the sample type itself.[23,24] Thus, cell samples and clinical stool samples can be considered to be relatively consistent in terms of molecular specificity and specificity of the methylation test methods.

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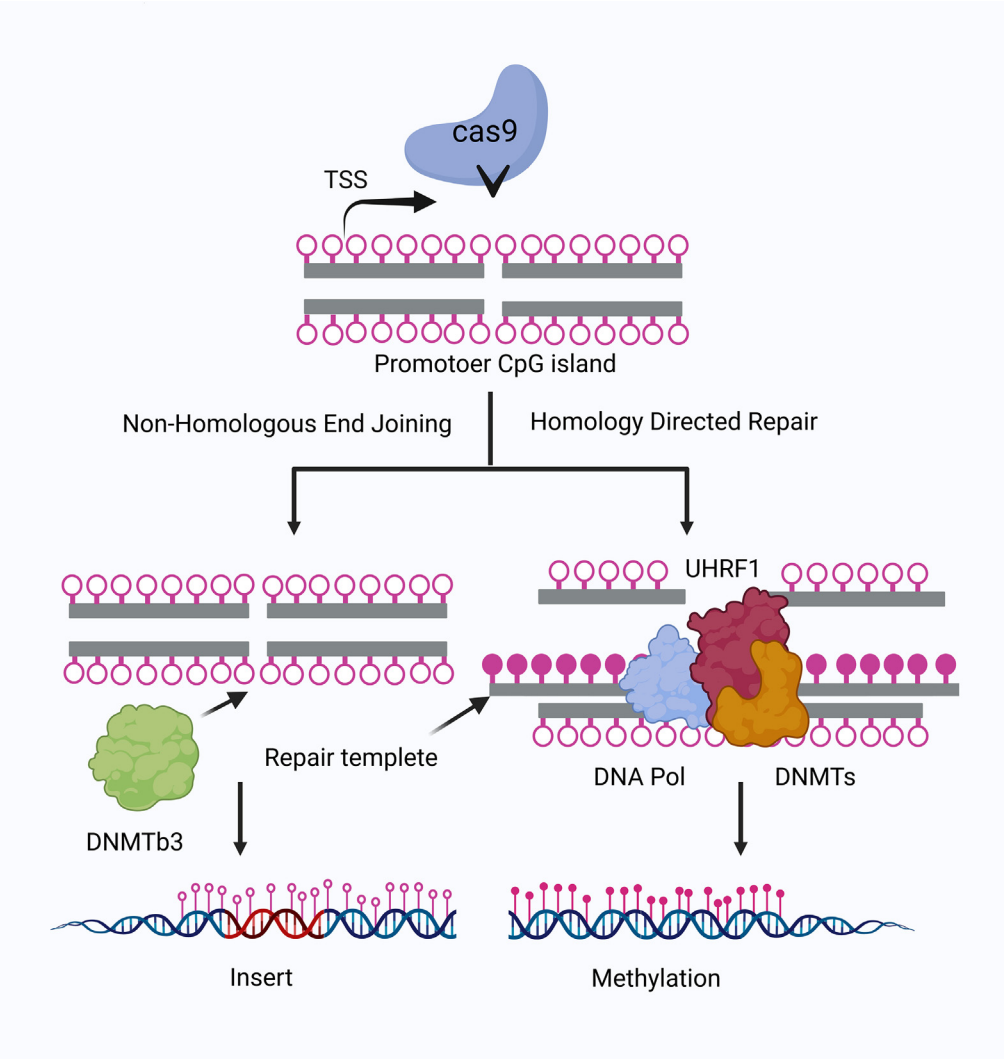

Figure 1. Proposed mechanism of targeted methylation through endogenous DNA repair pathways

HDR repairs CpG island double-strand breaks using NHEJ or HDR. Non-homologous end joining (NHEJ) induces error-prone repair and unstable methylation, potentially involving DNMT3b. Exogenous methylated repair templates enable HDR repair, recruiting UHRF1 and DNMTs to copy methylation into endogenous DNA.

Recent studies have utilized CRISPR-Cas9 combined with double-stranded DNA (dsDNA) templates for homology-directed repair (HDR) to target DNA methylation.[25–28] Based on this principle, our laboratory successfully used CRISPR-Cas9 combing HDR to target MGMT methylation and constructed an MGMT hypermethylated and heterogeneous cell line[25](Figure 1), which makes it possible to construct hypermethylated and heterogeneous *SDC2* cell lines as post-optimized EQA materials that can mimic the epigenetic changes in patients with CRC. In addition, the heterogeneous *SDC2* cell lines can participate in the preprocessing procedures of clinical samples, providing a good way to evaluate the integrity and accuracy of the *SDC2* methylation assay process.

By utilizing CRISPR-Cas9 and HDR technology to construct heterogeneous methylation *SDC2* cell lines, this study aims to overcome the limitations of current reference materials and use the innovative EQA material to conduct a nationwide EQA to assess the reliability of *SDC2* methylation analyses in various laboratories and standardize the *SDC2* testing.

RESULTS

Generation of *SDC2* promoter-methylated monoclonal cell lines using CRISPR-Cas9-targeted editing and dsDNA HDR

Transfection status in each group

After CRISPR-Cas9 combined with HDR-targeted HEK293T cell editing, the fluorescence expression of cells in each group after transfection is shown in Figure 2B.

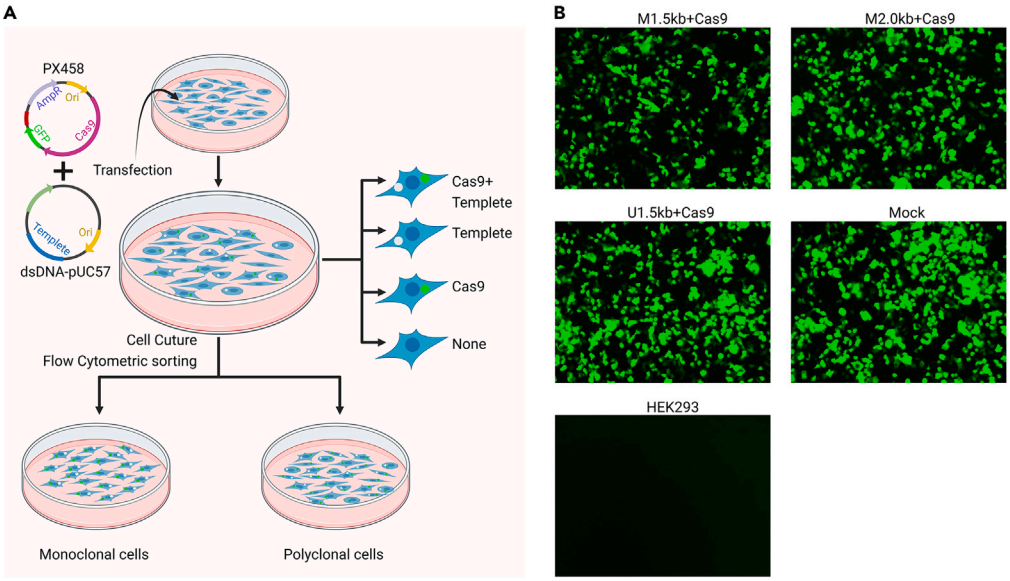

Figure 2. Flow cytometry analysis of transfected HEK293T cells

(A) Cells were co-transfected with plasmids and screened after two days.

(B) Five groups were transfected with different components: M-1.5kb (methylated 1.5kb dsDNA template), M-2.0kb (methylated 2.0kb dsDNA templates), U-1.5kb (unmethylated 1.5kb dsDNA templates), mock (PX458 plasmids only), and HEK293T (no transfection).

Targeted DNA methylation induced by dsDNA repair templates

To monitor homologous repair efficiency, a 2 bp mutation was introduced in the repair templates to create a non-native NcoI restriction enzyme site in the *SDC2* promoter region (Figures 3A and 3B). This mutation prevented repeat cutting of edited loci by CRISPR and enabled monitoring of HDR efficiency by PCR and restriction enzyme digestion. After transfection of the templates into the cells, we observed comparable HDR rates between the same length of unmethylated and methylated homologous repair template groups (Figure 3C). In contrast, the HDR rates were significantly different between the non-homologous and homologous template groups (Figure 3C). We measured the level of *SDC2* promoter methylation by MSP and the Human *SDC2* Gene Methylation Detection Kit. The MSP results showed that CpG sites 45, 46, 62, and 63 of the *SDC2* promoter were methylated in cells transfected with the SpCAS9 plasmid and methylated templates (M-1.5kb/M-2.0kb) (Figure 3D). The qPCR assay indicated that in the absence of a homologous repair template (U-1.5kb/Mock), the cycle threshold (Ct) was greater than 38, suggesting that DNA methylation rarely occurs after dsDNA fractures without HDR. In contrast, the methylation level of the *SDC2* promoter was significantly higher (Ct < 38) in the methylated homologous template transfection group than in the unmethylated template group (Figure 3E). These results suggest that exogenous methylated repair templates can promote DNA methylation of endogenous *SDC2* promoters.

Targeted DNA methylation stability

To investigate the persistence of DNA methylation obtained from exogenous templates during cell differentiation, we analyzed changes in promoter methylation levels of *SDC2* genes in edited HEK293T cells at various time points after transfection (5, 10, 15, 30, and 40 days). Positive monoclonal cells (60 days after transfection) were tested by PCR and NcoI restriction enzyme digestion, revealing that the 2 bp mutation inherited from the homologous repair template was stably maintained with cell passage (Figure 4A). qPCR results showed that the methylation levels generated by the methylated dsDNA templates fluctuated during cell differentiation, but ultimately stabilized and did not disappear with cell passage (Figures 4B and 4C).

Methylation heterogeneity in monoclonal cell lines

Finally, pyrosequencing was used to examine methylation patterns in 2 methylation-positive monoclonal cells, revealing extensive methylation alterations in the *SDC2* promoter characterized by heterogeneous methylation patterns. The average methylation level at the CpG site of Mono-*SDC2*-1 was 80.75%, the average methylation level at the CpG site of Mono-*SDC2*-2 was 30.67%, and the average methylation level at the CpG site of wild-type HEK293T was 1.33% (Figure 5). The methylation level of *SDC2* gene promoter region of these two monoclonal cell lines was significantly different from that of wild-type HEK293T cells. Furthermore, the successfully constructed *SDC2* methylated monoclonal cell lines exhibit a heterogeneous methylation pattern, which can simulate epigenetic changes observed in clinical samples.

The 140 *SDC2* methylation EQA report from 140 laboratories

This study invited a total of 140 clinical laboratories, including 81 hospital laboratories and 59 commercial diagnostic laboratories, to participate, using 11 commercial assay kits. The information summary of each assay method is presented in Table S1, and the compliance rate of

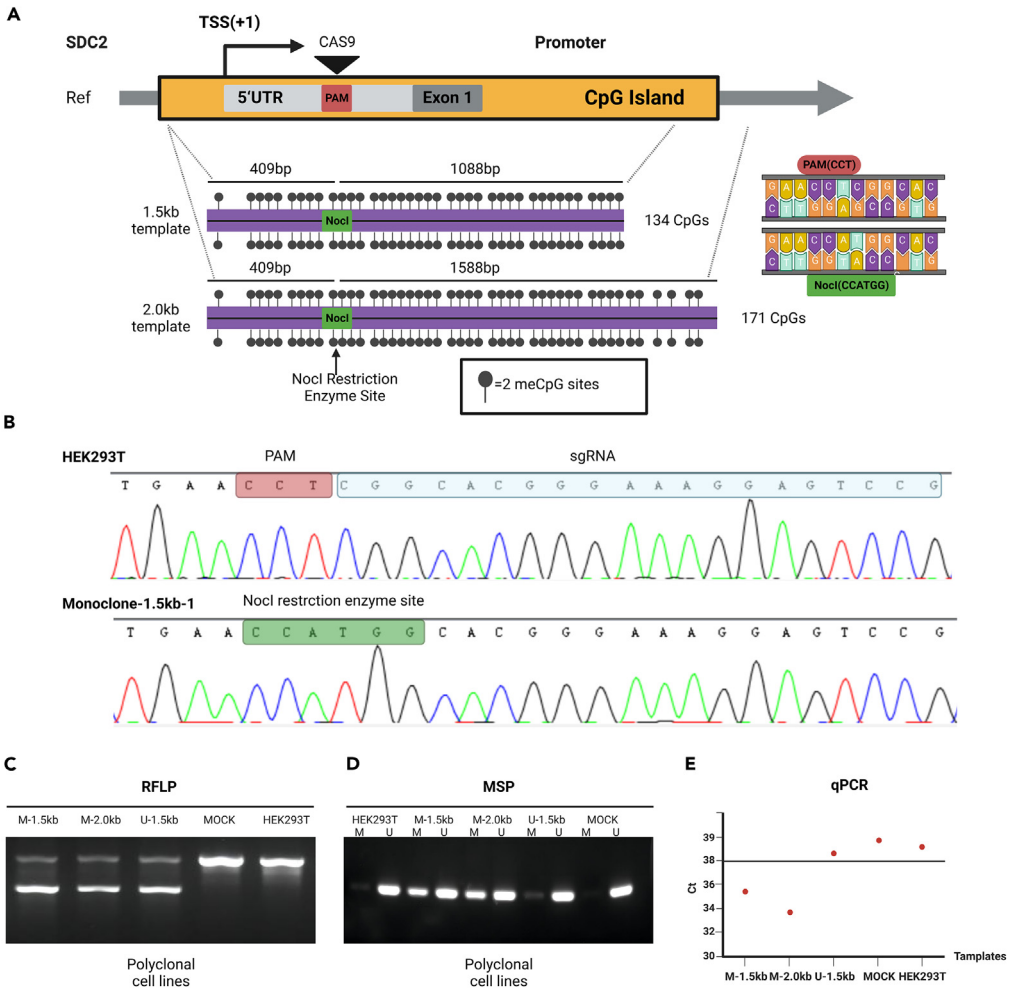

Figure 3. Editing and analysis of SDC2 DNA methylation using dsDNA templates

(A) 1.5kb and 2.0kb dsDNA templates for HDR.

(B) 2 bp mutation in the templates (CCATGG).

(C) HDR assay: NcoI digestion of amplicons targeting the mutation site, with restriction fragment length polymorphism (RFLP) primers outside the repair templates.

(D) Comparison of targeted methylation levels at CpG sites 45, 46, 62, and 63 by MSP.

(E) Comparison of *SDC2* promoter methylation levels at CpG sites 54–62.

different kits for each EQA sample is shown in Table S2. All laboratories used commercial analysis methods, with the most commonly used assay kit being the Methylation Detection Kit for Human *SDC2* Gene (Creative Biosciences Co., Ltd.) (109/140, 77.86%), followed by the *SDC2* and TFPI2 gene methylation combined detection kit (Life Technology Co., Ltd.) (15/140, 10.71%). All results were submitted within the designated 1-week deadline.

In this quality evaluation, the valid results of 140 laboratories were received, as shown in Table 1. Among them, there were 139 laboratories (99.29%, 139/140) with qualified EQA scores, and 1 unqualified laboratory (0.71%, 1/140). Of the 140 valid results reports, 134 (95.71%, 134/140) fully matched the evaluation results, and 6 had at least one evaluation result inconsistent (4.28%, 6/140). Of the 1,400 samples reported from 140 laboratories, 8 samples (0.71%) from 6 laboratories were qualitatively incorrect, with 5 false negative (FN) results and 3 false positive (FP) results. FN results were generated in EQA sample S2, S7, and S8 (Table S2). In these EQA samples, there were three replicate samples (S4, S5, and S6). To evaluate the precision of the laboratory *SDC2* methylation assay, the SD and coefficient of variation (CV) of the Ct_{SDC2} values were calculated for the three replicate samples from each laboratory. Among them, CV>5% had 3 (3/140, 2.14%) participating laboratories (see Table S3). Two hypomethylated samples (S7 and S8) were used as limit of detection samples to evaluate the sensitivity of *SDC2* methylation detection in the laboratory, and the positive result indicates that the laboratory had good detection sensitivity. The results showed that 3 laboratories failed to correctly detect hypomethylated samples, indicating that they failed to more actively detect hypomethylated *SDC2* samples.

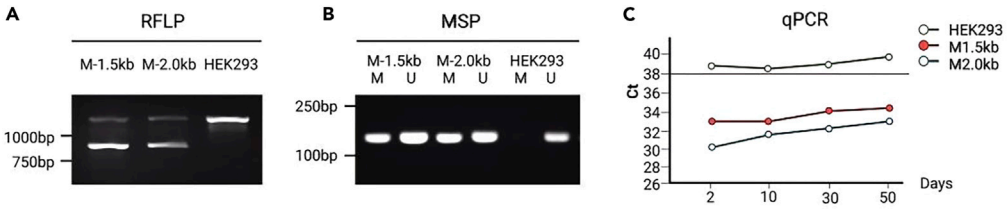

Figure 4. Validation of methylation and mutation stability in screened clonal cell lines

(A) 2 bp mutation stability in monoclonal cells confirmed by RFLP assay.

(B) MSP detected methylation levels in monoclonal cells and HEK292T cells.

(C) Comparison of *SDC2* promoter methylation levels at CpG sites 54–62 between monoclonal cells and HEK292T cells.

Detection of CpG sites of commercial kits

The 11 commercial kits, 5 of which were approved by the National Medical Products Administration (NMPA), and 6 kits are applying for NMPA approval, and the 11 kits cover the CpG islands of the CpG sites 1–10 and 43–116 (Table S1). The 2 monoclonal cells used for EQA were examined by pyrosequencing that showed they had heterogeneous methylation patterns (Figure 5). Since our EQA materials were *SDC2* methylation heterogeneous, inconsistent methylation levels at each CpG site can cause differences in Ct values between kits.

DNA extraction methods of laboratory

The EQA samples used in this study are cell mixture, consistent with clinical samples, and can evaluate the entire process of DNA extraction from the experiment to the results report. Among the laboratories participating in the EQA program, a total of 57 laboratories used manual DNA extraction, and 82 experiments used automated methods to extract DNA. Ct values of laboratory S4, S5, and S6 for artificial and automated DNA extraction are shown in Table 2. The results showed that CV of artificial DNA extraction was significantly higher than that of automatic DNA extraction ($p > 0.05$).

DISCUSSION

A summary of the main findings

In this study, we edited 3 monoclonal cell lines with heterogeneous methylation patterns in the *SDC2* promoter region. Methylation heterogeneity is observed in embryonic development, epigenetic reprogramming, and cancer, impacting gene regulation. In patients, DNA methylation is metastable. Modifications of methylase and demethylase lead to dynamic changes in single CpG site in intracellular.[29] Additionally, aging induces significant changes in methylation patterns, resulting in heterogeneity.[30] In patients with CRC, heterogeneous methylation patterns may arise from distinct cell populations or reflect variations in methylation status within cells. Our constructed monoclonal cells exhibited a heterogeneous pattern, closely resembling the disease situation in clinical patients with CRC after methylation stabilization.

Fecal DNA-based *SDC2* methylation detection has emerged as a promising non-invasive strategy for early CRC screening. Currently, *SDC2* gene methylation testing is not yet Food and Drug Administration-approved for CRC screening, and most fecal *SDC2* kits in China are used in conjunction with other genes or confirmed by colonoscopy. Further large-scale, multicenter studies are needed to validate the effectiveness of *SDC2* testing and to determine whether differences in sensitivity and specificity arise from the *SDC2* gene itself or other genes, as well as to explore optimal approaches for clinical application.

However, it poses technical challenges due to low DNA content and complex procedures. DNA extraction from stool is difficult, and the sulfite conversion process is tedious, leading to potential issues such as underconversion, DNA degradation, and low recovery.[31] In China, various commercial assays for *SDC2* methylation detection are available, each with different sensitivity and specificity rates, making it crucial to ensure reliable and consistent results.

To address these challenges, we conducted an EQA study to evaluate the performance of Chinese laboratories in detecting *SDC2* methylation in fecal DNA. Our EQA panel consists of CRISPR-CasS-targeted methylation-edited tumor cell lines and simulated clinical samples from multiple unedited tumor cell lines, including positive and negative samples. Our experimental results demonstrate the stability of *SDC2* methylation level after storage, validating the suitability of our panel as an EQA material. Negative samples composed of unedited HEK293T and Caco2 cell lines confirmed the specificity of our panel for CRC detection. These EQA samples possess the following key characteristics: direct availability, stability during storage, and specificity for CRC screening, making them suitable and valid as EQA materials. EQA panels made from cell lines are traceable, allowing regular EQA testing and facilitating comparison of *SDC2* methylation results between laboratories.

The implications of the results of the study

In our EQA study, we comprehensively evaluated laboratory results, including Ct values and qualitative outcomes. The majority of laboratories (95.71%, 134/140) accurately identified EQA samples, indicating satisfactory performance of *SDC2* methylation detection in China. However, six laboratories reported FN or FP results, which were not related to the panel samples. Among laboratories whose qualitative results were consistent, disparities still existed in Ct values. The variability between laboratories may arise from differences in technology, staff capabilities,

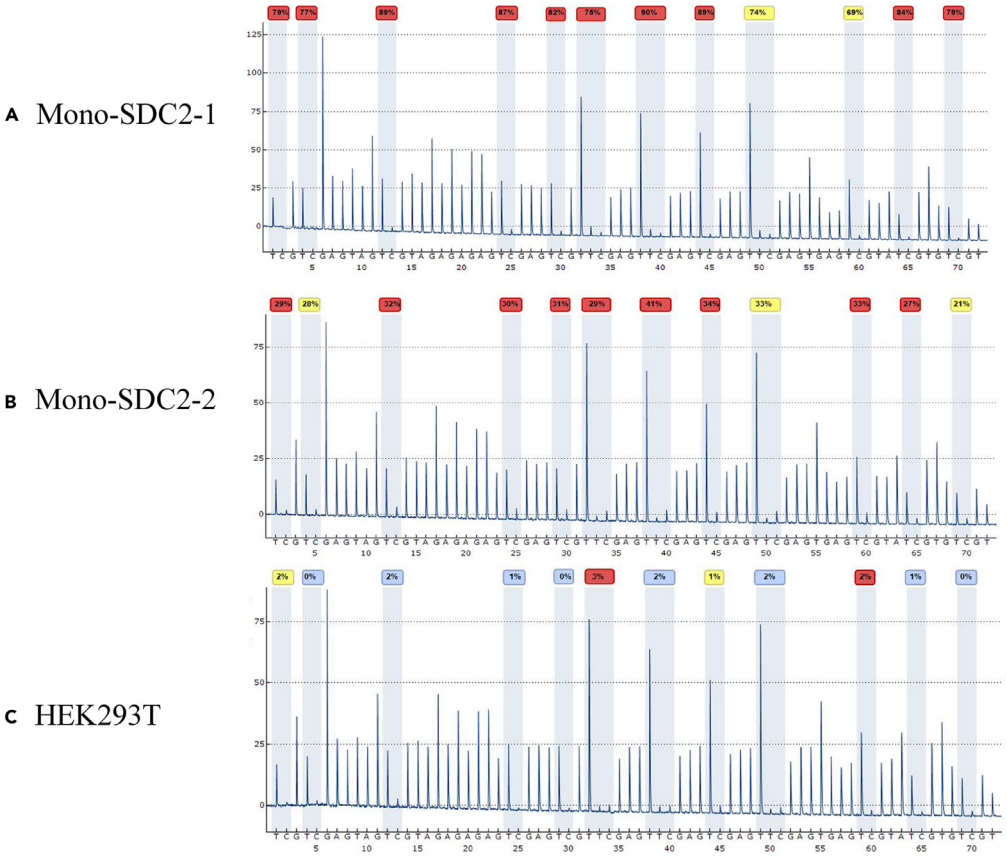

Figure 5. Pyrosequencing reveals distinct methylation levels at the CpG site across different clonal cell lines

(A) The average methylation level at the CpG site of Mono-SDC2-1 was 80.75%.

(B) The average methylation level at the CpG site of Mono-SDC2-2 was 30.67%.

(C) The average methylation level at the CpG site of wild-type HEK293T was 1.33%.

and the various commercial *SDC2* methylation kits. In our study, manual nucleic acid extraction, common among participating laboratories, may introduce variability and errors due to technical variations or uneven sample processing. The detection of SDC2 methylation in fecal DNA has some technical challenges, such as the low DNA content in feces, which increases the difficulty of DNA extraction; The process of sulfite transformation is complicated, which may lead to incomplete transformation. There are also problems with DNA degradation and low recovery rates. Implementing automated systems for specimen handling and processing can minimize the variability and operational errors in nucleic acid extraction.[32]This aligns with the previous study on *SDC2* methylation.[33] Thus, automated instrumentation is recommended to clinical use for minimizing operational errors. Ct value analysis revealed operator submission errors as the cause of FN and FP results from two laboratories. Accurate reporting of results is crucial, and ongoing staff training is necessary to ensure correct information processing and communication, aligning with the ISO15189 standard.[34] Besides, the ability of the kits to detect methylation heterogeneity at each CpG sites may lead to differences in Ct values. Ct is a parameter used in qPCR to determine the number of PCR cycles required for an amplified signal to reach a certain threshold. It is commonly used to measure the initial amount of target DNA in a sample. This variability may be due to sample biological factors, technical differences in the kits, or both. In patients with CRC, DNA methylation levels are heterogeneous, i.e., inconsistent at each CpG site, whereas our EQA samples conform to clinical patient methylation heterogeneity. It is important to note that different kits detect different CpG sites using different primers or probes, which may affect the amplification and detection of target DNA regions. Differences in Ct values between these kits may have implications for interpreting the methylation status of specific CpG sites within the *SDC2* gene promoter region.

The *SDC2* hypermethylated cell lines constructed in our laboratory can be used as EQA materials for fecal methylation detection, as well as daily clinical testing work for indoor quality control material monitoring, and the accuracy of blood cfDNA *SDC2* methylation detection can be assessed in the future. By introducing these cell lines as quality control samples in each experiment, the laboratory can promptly detect and correct any technical or experimental operational issues that may lead to result deviations. This helps ensure the stability and reliability of the laboratory's methylation analysis process, improving the consistency and comparability of experimental results and providing reliable data support for the application of fecal methylation analysis in clinical practice.

Table 1. Performance scores for EQA results

Assays	Number of labs	EQA score	
		100	<100
Creative Biosciences	109	107	2
Life Technology	15	15	0
Genomics	3	3	0
RealBio Technology	2	2	0
AmoyDx	3	3	0
Anhui Targene Medical Technology	2	0	2
Nanjing Vazyme Biotech	1	1	0
Hangzhou Heyi Gene Technology	1	0	1
Wuhan YZY Medical Science and Technology	1	1	0
CEYUANPUHUI	1	0	1
Yunying Medicine	1	1	0
Renhe Future Biotechnology	1	1	0
Total	140	134	6

Limitations of the study

Single EQA limitation: The EQA conducted in this study was performed only once, which may not fully reflect all potential issues with detection accuracy. To gain a comprehensive understanding of detection accuracy and stability, multiple rounds of EQA are necessary to capture performance variations over time and under different conditions.

Sample representativeness and stability: Although the EQA samples used in this study have been validated, it is essential to confirm their stability over extended storage and usage periods. Further research should focus on assessing sample performance under various conditions to ensure their effectiveness as EQA materials.

Need for prospective studies: While EQA provides feedback on laboratory detection accuracy, assessing the real-world effectiveness and clinical applicability still requires prospective studies. These studies should include large-scale clinical trials to compare the performance of different testing methods and validate their effectiveness in practical applications.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jinming Li (jmli@nccl.org.cn).

Materials availability

The cell lines generated in this study have been deposited (the SDC2 Project, Mono-SDC2-1 and Mono-SDC2-2); plasmids generated in this study have been deposited to the SDC2 Project, Px458-sgRNA-SDC2. This study did not generate new unique reagents.

Data and code availability

- This paper does not report original data.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported will be shared by the lead contact upon request.

ACKNOWLEDGMENTS

We would like to thank the 140 laboratories who actively participated in our study and reported their test results to NCCL in a timely manner. This study was supported by National Key Research and Development Program of China (2023YFF0613303).

Table 2. Ct values for duplicated EQA samples by manual and automatic nucleic acid extraction

Method of extraction	Number of labs	Ct_{SDC2} (S4) Mean ± SD	Ct_{SDC2} (S5) Mean ± SD	Ct_{SDC2} (S6) Mean ± SD
Manual	57	34.99 ± 0.20	34.88 ± 0.21	34.94 ± 022
Automatic	82	35.29 ± 0.08	35.43 ± 0.09	35.33 ± 0.09

AUTHOR CONTRIBUTIONS

L.Z.: writing – original draft; J.L.: writing – review and editing and funding acquisition; D.W.: reviewing original draft; Z.L.: reviewing original draft; G.L.: reviewing original draft.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Real-time PCR methylation detection
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- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Lipofectamine™ 3000	Thermo Fisher Scientific	
Opti-MEM™	Thermo Fisher Scientifie	
EZ DNA Methylation-Gold™ Kit	ZYMO RESEARCH	
ZymoTaq™ PreMix	ZYMO RESEARCH	
Human Methylated & Non-methylated DNA Set	ZYMO RESEARCH	
Changan ® Human SDC2 gene methylation detection kit (Real-time PCR method)	Creative Biosciences	
Fecal storage solution	Creative Biosciences	
NcoI restriction enzyme	New England Biolabs Company	
Experimental models: Cell lines		
HEK293T	Basic Medical Cell Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences	N/A
Caco2	Basic Medical Cell Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences	N/A
HCT116	Basic Medical Cell Center, Institute of Basic Medicine, Chinese Academy of Medical Sciences	N/A
Oligonucleotides		
sgRNA-2 targeting sequence: CACCGCGGACTCCTTTCCCGTGCCG	This paper	N/A
RFLP-F: GGGAGCCAGAGGAAAAGAAG	This paper	N/A
RFLP-R: ACACTCGCGGGTCTCTTAAA	This paper	N/A
MSP-M F: GAAATTAATAAGTGAGAGGGCGTC	This paper	N/A
MSP-M R: GAATCCGAAACAAAATACCG	This paper	N/A
MSP-U F: TAGAAATTAATAAGTGAGAGGGTGTTG	This paper	N/A
MSP-U R: AAATCCAAAACAAAATACCACA	This paper	N/A
QSP (51-62CpG)F: TGTYGGGAGTGTAGAAATTAATAAG	This paper	N/A
QSP (51-62CpG)R: ACACRAATCCRAAACAAAATAC 5′Biotin labeling	This paper	N/A
Sequening primer 1: TTTTAGGGGAGTAGTTGAGGG	This paper	N/A
QSP (144-155CpG)F: GAYGGGAGGATATTTTTATAGGAGT	This paper	N/A
QSP (144–155 CpG)R: AAACRAAACTCCTCCCRACC 5′Biotin labeling	This paper	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sequening primer 2: GGGAGTGTAGTAAGTAGGG	This paper	N/A
Px458-sgRNA DNA sequences: see Table S4		
pUC57-1.5kb homologous dsDNA template DNA sequences: see Table S5		
pUC57-2.0kb homologous dsDNA template DNA sequences: see Table S6		

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines used in the experiments

The following three cell lines were used in this study.

HEK293T, this cell line consists of human colorectal cancer cells, characterized by epithelioid adherent growth. It is derived from a woman and was sourced from the Basic Medical Cell Center at the Institute of Basic Medicine, Chinese Academy of Medical Sciences.

The Caco2 cell line is composed of human colorectal cancer cells with epithelioid adherent growth. The sex is noted as human. These cells were also sourced from the Basic Medical Cell Center at the Institute of Basic Medicine, Chinese Academy of Medical Sciences.

HCT116 is a cell line made up of human colorectal cancer cells, also characterized by epithelioid adherent growth. The sex is listed as human, and this cell line was obtained from the Basic Medical Cell Center at the Institute of Basic Medicine, Chinese Academy of Medical Sciences.

Cell culture

HEK293T, HCT116 and Caco2 cells were cultured in DMEM high glucose+ L-glutamine medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) and 1×Penicillin/Streptomycin (Invitrogen). The bottles were then incubated horizontally in a 37°C humidified air ambient incubator containing 5% CO2.

METHOD DETAILS

Cell transfection

Six hours before transfection, the complete medium in the 6-well dishes was replaced with Opti-MEMTM medium containing 10uM SCR7.

(1) Take five 15 mL EP tubes, add 125μL Opti-MEM and 7.5μL Lipofectamine 3000, and mix with Pap straw;
(2) Five 1.5 mL EP tubes were added with 125μL Opti-MEM, 1.5μL P3000 and 2.5 μg plasmid. The 2.5μg plasmid component in each group was:1.25μg (PX458-sgRNA2) + 1.25μg(ds1500-pUC57-CpG+); 1.25μg (PX458-sgRNA2) + 1.25μg(ds2000-pUC57-CpG+); 1.25μg (PX458-sgRNA2) + 1.25μg (ds1500-pUC57-CpG-); PX458-sgRNA2 transfection with 2.5μg and blank control without plasmid; mix each pasteurized straw;

Then the corresponding mixture of A and B was evenly mixed, incubated at room temperature for 10-15min, and then rotated into 6-well plate. Two days after transfection, monoclonal cells were screened by flow cytometry (Figure 2A). The cells were then diluted to 1 cell/well in 96-well plates, and the remaining polyclonal cells were enriched in 24-well plates. After the monoclonal and polyclonal cells were subcultured to 24-well plates, DNA was extracted using a DNA extraction kit for subsequent experiments.

Plasmid and repair template generation

To create *SDC2* promoter methylated monoclonal cell lines, CRISPR sgRNAs were designed using http://crispr.mit.edu/and cloned into the pSpCas9(BB)-2A-GFP (PX458) plasmid to target the *SDC2* promoter. The 1.5kb and 2.0kb homologous dsDNA template and the non-homologous dsDNA repair template were synthesized by Sangon Biotech (Shanghai) Co., Ltd. and cloned into the PUC57 plasmid for HDR. These templates were hypermethylated using M.SssI CpG Methyltransferase before transfection. HEK293T cells were transfected with a plasmid encoding SpCas9 nuclease and a sgRNA specific to the *SDC2* promoter, along with the methylated repair templates. To test whether DNA double-strand breaks in the absence of HDR could lead to *SDC2* methylation via NHEJ, cells were also transfected with just the SpCas9 plasmid. To prevent the sgRNA from cutting the homologous repair template, a 2bp PAM mutation was introduced to create the NcoI restriction recognition site (CCATGG) on the repair template. For methylation of plasmid repair templates, 20ug of plasmid DNA was methylated in a 100ul reaction containing 50U of M.SssI CpG Methyltransferase (New England Biolabs), 640uM SAM, 50 mM NaCl, 10mM Tris–HCl (pH 7.9), and 10mM EDTA for 4h at 37°C. The enzyme was inactivated for 20 min at 65°C, and the plasmid DNA was purified using a DNA Purification Kit.

Promoter-targeting site selection of the SDC2 gene

Enter the UCSC website (http://genome.ucsc.edu/) and select the Human GRCh38/hg38 database to retrieve the basic information of the CpG island of the SDC2 gene promoter. According to the CpG island assessment criteria, the sequence length is required to be greater than 200 bp, the GC content is greater than 50%, and the ratio of the observed CpG to the expected value is greater than 0.60. The UCSC website retrieved the CpG island information of SDC2 gene promoter, and the CpG island of SDC2 gene promoter is 1860 bp long and located in chromosome 8 (chr 8:96493520-96495379).

Design and synthesis of the sgRNA

Log in to http://crispr.mit.edu/, select CRISPOR online design tool for sgRNA design; use CRISPOR online design tool for sgRNA design. The previously retrieved CpG island sequence of the SDC2 gene promoter was entered into the search box to generate the corresponding sgRNA sequence; Main principle of sgRNA screening: select sgRNA with a comprehensive score higher than 90. To imize matching with non-target sites and reduce off-target effects. Avoid sgRNA sequences ending with more than four T, and avoid problems associated with the template of multimeric T. The optimal GC% content was 40%–60%, which contributes to the stability of sgRNA. G or GG was chosen as the 5′ base to improve the transcription efficiency of sgRNA. Before designing the sgRNA-targeted binding sites, check the genomic sequence of this region for the presence of SNPs or Indels to ensure the sgRNA specificity.

CRISPR/Cas9 combined with HDR to target DNA methylation

This technique uses CRISPR/CAS9 technology to trigger DNA double-strand breaks at target gene loci, and then uses heterologous methylated DNA sequences as DNA templates for homologous recombination repair, so as to achieve targeted methylation modification of target gene loci.[28] HDR is a complex intracellular biological process, including end removal of broken chains, template chain invasion, Holliday junction formation, and DNA synthesis.[35] In this process, endogenous methylation regulatory proteins such as DNMT1, DNMT3A, and Np95 are recruited to the cleavage site and are responsible for selective methylation. Therefore, this epigenetic gene-editing technology, which combines CRISPR/CAS9 and HDR technologies, simulates the biological processes that naturally occur in cells for HDR and methylation maintenance in DNA replication.

HDR assay via polymerase chain reaction (PCR) and RFLP assay

40μg of genomic DNA from CRISPR edited cells was amplified with 2U ZymoTaq PreMix hotstart polymerase (Zymo Research) in a reaction containing 0.25 mM dNTPs, 1× Reaction Buffer, and 0.25μM each primer. Copying of the 2 bp PAM mutation via HDR from the repair template creates a NcoI restriction enzyme site (CCATGG) in the amplicon. 1μg of PCR product was digested with 5U NcoI-HF (New England Biolabs) in 1× CutSmart buffer for 30 min at 37°C. The digested products were then analyzed on 2% agarose gels for HDR assay and stained with ethidium bromide (Invitrogen).

Pyrophosphate methylation sequencing (QSP)

This experiment also employed QSP to measure the *SDC2* methylation levels in monoclonal cell lines and EQA samples. Genomic DNA was extracted and bisulfite converted. PCR amplification was performed using specific primers, and the products were visualized using gel electrophoresis. Biotin-labeled products were mixed with beads and subjected to pyrosequencing to detect fluorescence signals during DNA synthesis.

Real-time PCR methylation detection

Real-time PCR kit for DNA extraction and transformation

3.0mL of cell suspension, negative and positive controls were added to the labeled new 10mL centrifuge tube; Add 2.0mL lysate and 50μL magnetic bead M1 (mix bead magnetic M1), shake 1900rpm for 5s, incubate 95°C on the dry thermostat for 15min, remove on the tube rack, avoid air conditioning and vent, and sit at room temperature for 1h; Turn off the dry thermostat after use; Instantaneous separation, 10mL magnetic frame adsorption for 5min, 4.2mL of liquid, the remaining liquid to the marked new 2mL centrifuge tube with the magnetic beads, instantaneous separation, 2mL magnetic frame adsorption for 1min, discard waste liquid; Add 800μL wash fluid W1, shaking at 1 min at room temperature at 1300rpm; Instantaneous separation, magnetic frame adsorption for 1min, discard waste liquid, instantaneous separation for another 15s, magnetic frame adsorption for 1min, discard waste liquid again; Add 50μL of solution A to each tube, vortex mix evenly, instantaneous separation, constant temperature mixing machine at 1300rpm for 20min; Instantaneous separation, magnetic frame adsorption for 1min, transfer the solution to a marked new 2mL centrifuge tube;

100μL of solution B was added, vortexed, and incubated with 65°C on the dry thermostat for 70min; Instantaneous separation, each tube with 450μL binding liquid, 50μL magnetic beads M2 (magnetic beads M2 before mixing), instantaneous separation, constant temperature mixing machine at 1300rpm for 15min; Instantaneous separation, magnetic frame adsorption for 1min, discard waste liquid, instantaneous separation for another 15s, magnetic frame adsorption for 1min, discard waste liquid again; Add 800μL of wash liquid W2 to each tube and transfer it to the marked new 2mL centrifuge tube, instantaneous separation, constant temperature mixing instrument on 1300 rpm at room temperature concussion for 1min, instantaneous separation, magnetic frame adsorption for 1min, discard waste liquid; Each tube with 200μL of solution C supernatant, instantaneous separation, constant temperature mixing machine at 1300rpm for 15min; Instantaneous

separation, magnetic frame adsorption for 1min, discard waste liquid, instantaneous separation for another 15s, magnetic frame adsorption for 1min, discard waste liquid again; Add 200μL of washing liquid W2, instantaneous separation, constant temperature mixing machine at 1300rpm for room temperature shock for 1min, instantaneous separation, magnetic frame adsorption for 1min, discard waste liquid; Add 200μL of lotion W2 to each tube and repeat the above operation; Add 100μL of lotion W2 to each tube, instantaneous separation, room temperature shock at 1 min at 1300rpm; Instantaneous separation, magnetic frame adsorption for 1min, discard waste liquid, instantaneous separation for another 15s, magnetic frame adsorption for 1min, discard waste liquid again; Open the lid and dry at room temperature for 10min, open the constant temperature mixing instrument for 65°C preheating; After confirming drying, 60μL of eluate was added to each tube, vortex mixed and instantaneous, constant temperature mixing instrument at 1300rpm and incubated at 65°C for 10min; Instantaneous separation, magnetic frame adsorption for 1min, transfer the liquid to a labeled 1.5mL centrifuge tube, and store at-20°C.

The Real-time PCR reaction

Combine 10μL of DNA supernatant, 10μL of PCR reaction solution-1, and 10μL of PCR reaction solution-2 to achieve a total volume of 30μL. The PCR reaction protocol includes the following steps: denaturation at 95°C for 5 min; followed by 48 thermal cycles, with each cycle consisting of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C; finally, cooling at 40°C for 30 s. The terminal detection uses FAM and Texas Red.

The quality control criteria are as follows: a Ct value of <35 in the Texas Red channel and <35 in the FAM channel indicates a positive control; a Ct value of ≥40 or no Ct in both the Texas Red and FAM channels indicates a blank control; a Ct value of <35 in the Texas Red channel and ≥40 or no Ct in the FAM channel indicates a negative control.

The criteria for sample detection results are as follows: a Ct value of ≤36 in the Texas Red channel and ≤38 in the FAM channel indicates a positive result; a Ct value of >36 or no Ct in the Texas Red channel and >38 or no Ct in the FAM channel indicates a negative result; if the Texas Red channel Ct value is > 36 or no Ct, and the FAM channel Ct value is any value, the result is considered invalid.

MSP

MSP was conducted using two pairs of primers designed for methylated and non-methylated DNA. The CpG sites45, 46, 62, and 63 in the *SDC2* gene promoter were analyzed, and the methylation degree was determined.

Preparation of EQA samples

An EQA panel comprising 8 positive samples (S1-S8) and 2 negative samples (S9 and S10) was prepared. *Samples with SDC2 gene promoter methylation levels as hypermethylation, intermediate and low methylation were included, respectively.* Each sample is 4mL, first, 10 samples are mixed in proportion. After mixing, the methylation level and sample homogeneity and stability verification are verified; Methylation levels were analyzed using bisulfite sequencing, and Ct values of methylated *SDC2* were detected using the *SDC2* DNA Methylation PCR Fluorescence Kit. To meet the EQA material requirements, 6 cell lines (3 monoclonal HEK293T cells, wild HEK293T cells, HCT116 and Caco2 cells) were prepared and proportionally mixed with the preservation solution to form EQA samples (10^5 cells/ml). Traceability, reproducibility, and interchangeability were ensured. Positive samples with varying methylation levels were created, including duplicate samples for inter-laboratory reproducibility assessment. Two samples with low methylated DNA content were prepared as limit of detection samples. Negative samples simulating *SDC2* methylation-negative samples were also included. After sample preparation, bisulfite sequencing and qPCR kit were used to verify the methylation levels. Identical aliquots were provided to 140 participating laboratories. EQA sample panels were distributed through dry ice delivery. Laboratories were required to analyze the panel and report results, including sample details, extraction methods, detection methods, instrument/reagent information, raw data, and qualitative results within one week via an online system.

QUANTIFICATION AND STATISTICAL ANALYSIS

EQA sample methylation reported as binary qualitative ("positive" or "negative") and Ct values. Outliers (for each laboratory and each sample) are defined and identified as errors in the qualitative results. Results categorized as qualified (100% correct) or improvable (≥1 incorrect). Exploratory data analysis conducted on study samples, including mean, standard deviation (SD), and coefficient of variation (CV) of cyclic threshold (Ct) values by each lab. Statistical comparisons performed using means, SDs, CV, t-tests, chi-square tests, one-way ANOVA, Student's t-tests, and linear regression analysis with SPSS 27.0 software (IBM SPSS 27.0, Chicago, IL, USA). Significance level was set at $p < 0.05$, two-tailed.