Multiprobe Assay for Clinical SEPT9 Methylation Based on the Carbon Dot-Modified Liquid-Exfoliated Graphene Field Effect Transistor with a Potential to Present a Methylation Panorama

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ABSTRACT: The hypermethylation in the promoter region of the SEPT9 gene is associated with the development of colorectal cancer (CRC). Although its clinical significance for early diagnosis and screening of CRC has been demonstrated, the tedious operations in the conventional DNA methylation (DNAm) detection hinder its wide application. Herein, an electronic method for determining SEPT9 methylation in CRC patients is proposed by using the carbon dot-modified liquid exfoliated graphene field effect transistor (CDs-LEG-FET) as the DNAm sensor, the specifically designed probes to capture the SEPT9 gene and the immunologic recognition to recognize 5-methylcytosine (5mC) positions on the anchored sequences. The identification and nanomorphology of the as-prepared materials and devices are executed first by the characterizations of UV−vis, Raman, atomic force microscopy, Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy, and electronic measurements. Then, the role of CDs in enhancing DNAm sensitivity of CD-LEG-FET is manifested by comparing it with that of CD-free LEG-FET. Third, the captured SEPT9 genes on CD-LEG-FETs by different probes are evaluated, and the optimized temperature for hybridizing the target ssDNA sequences is determined to be 48 °C. Furthermore, the detection sensitivity for the low-quantity of DNA samples is demonstrated to be as low as 2 ng. Finally, the methylation degree of the tumor and corresponding noncancerous tissue DNA samples were examined by the proposed electrical assay and methylight assay in parallel. The diagnostic value of the electrical assay is confirmed by using the receiver operating characteristic curves; meanwhile, the superiority of the CD-LEG-FET platform is found to present a methylation panorama of the target gene.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors and causes huge burdens worldwide. Surgical treatment is relatively effective for CRC patients in early stages, and a favorable prognosis can be expected for them. However, it is completely different for advanced CRC patients, and treatment often involves a combination of multiple therapies. Despite the implementation of multimodal treatment regimes, including surgical resection, preoperative and postoperative chemotherapy, as well as the molecular-targeted therapy, relapses are still common, which obviously deteriorate the survival of those patients. This prompts people to attach great importance to the early diagnosis of disease, determine the risk factors related to CRC, and consider more accurate screening methods. Although considerable efforts in the development of noninvasive diagnostic testing, colonoscopy and sigmoidoscopy remain to be utilized as golden standard for the detection of colorectal tumors. Epigenetic changes in genomic tumor DNA are biologically stable and usually cancer-specific, or even patient-specific. Therefore, its role in tumor diagnosis has received increasing attention. In recent years, growing evidence has shown that the SEPT9 gene is associated with malignant tumors, especially for CRC. The SEPT9 gene is located on the human chromosome 17q25.3 contains 17 exons, and spans $240 \times 10^3$ bp. The $5'$-end regulatory region of the SEPT9 gene has a CpG-rich area (CpG island), which is the main site of DNA methylation (DNAm). In mammals, 60−90% of CpG sites are methylated, and most of the remaining unmethylated residues are clustered in CpG islands within functional gene promoters. A study found that the hypermethylation of the CpG island in the promoter region of the SEPT9 gene, which acts as a tumor suppressor gene, inhibited the normal expression of the gene.

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and consequent loss of its tumor suppressor function, thereby promoting the development of CRC. In addition, the SEPT9 gene methylation detection according to previous report, named as Epi proColon assay, is the first FDA-approved blood-based assay for CRC screening, and permitted to be used as a CRC screening test for average-risk population over 50 years old. The applications of Epi proColon assay demonstrate the sensitive detection of the methylation degree of SEPT9 gene is of great significance for early diagnosis and screening of CRC, prognosis evaluation, treatment monitoring, and so forth. Even though this FDA-approved method has been proven to be an accurate and reliable molecular test, it still involves multiple steps, such as bisulfite conversion (BC) and triplicated taqman probe-dependent polymerase chain reaction (PCR) reactions for each sample, which results in high cost and hard-to-guarantee quality control.

In our previous work, we developed a DNAm evaluation method, which is based on the well-known sensing platform of the field effect transistor (FET) and by using the liquid exfoliated graphene (LEG) as the conducting channel, abbreviated as LEG-FET. To streamline the tedious operation in traditional SEPT9 detection procedure, we would like to apply this LEG-FET DNAm evaluation method for the clinical SEPT9 assay. As opposed to the commonly studied DNAm detection technologies which depend on bisulfite sequencing, mass spectroscopy, high performance capillary electrophoresis, the value of the proposed LEG-FET protocol is in its promising potential in directly evaluating the methylated degree in the real DNA samples, without the operation of BC and PCR, which is also the current trend in developing the next generation of DNAm detection tools. In addition, carbon dots (CDs) are utilized in this work to immobilize more probes on the LEG-FET, to capture more single-stranded DNA (ssDNA) filaments, and result in the improved sensitivity for DNAm detection.

Herein, the experiments are executed from material synthesis, characterization, device fabrication to the SEPT9 methylation detection, as well as the evaluation for its application value. The scheme is shown in Figure 1, in which CDs are synthesized by the one-step hydrothermal method (Figure 1A), then they are drop-coated on the channel surface of LEG-FETs (Figure 1B) to form the CD-modified LEG-FETs (CDs-LEG-FETs). In subsequence, the devices are exploited for the purpose of evaluating the SEPT9 methylation degree of the clinical CRC patients, the main considerations in this procedure are outlined at here, in a nut shell, which are: (1) the target SEPT9 sequences are fixed onto the CDs-LEG-FETs by the oligonucleotide hybridization with the specifically designed probes; (2) the 5-methylcytosine (5mC) positions on them can be recognized by the antibody of 5mC and transduced as the output current between the electrodes of FET’s source and drain (I_{DS}), according to the immunologic recognition and FET’s sensing mechanism.

Although the graphene and its derivate-based FETs have been widely developed and approved to be a kind of the versatile and reliable biochemical sensors, there are few works about their applications of DNAm determination, especially the CRC-related SEPT9 gene. Herein, the SEPT9 assays for the DNA samples from CRC patients are conducted in parallel by the CDs-LEG-FETs and the methylight assay, for the purpose of assessing the feasibility of the proposed method. The comparisons between two methods could provide a fundamental evidence for developing FET-based DNAm sensors, give impetus to a new generation of testing tools for epigenetic research, and provide a potential alternative for the clinical detection of CRC.

**RESULTS AND DISCUSSION**

**UV−Vis and AFM.** UV−vis spectra illustrated the optical properties of CDs and LEG (Figure 2A). The peak at 344 nm is the characteristic absorption of the CDs synthesized from citric acid and ethylenediamine, which is attributable to n−π* transition of C==O bonds. The strong UV−vis absorption of LEG (the blue line) at 268 nm is consistent with the graphite’s typical UV−vis feature (270 nm). In addition, two predominant peaks of D band (1346 cm\(^{-1}\)) and G band (1570 cm\(^{-1}\)) can be clearly identified, which can demonstrate the graphite state of the as-prepared LEG. The D band is induced by the hybridized vibrational mode (A_{1g}) associated with graphene edges and the intensity ratio of D peak to G peak is about 0.92, which indicates the presence of some disordered graphene structure. The G band corresponds to the in-plane vibration mode E_{2g} of the graphite. In addition, the 2D band (2683 cm\(^{-1}\)) is typical indicator of few-layer-graphene, which is caused by two phonon double resonance process. Furthermore, it is found that the Raman features of LEG and CDs are combined in the contour of the CD-decorated LEG sample (the blue line of “CDs&LEG” in Figure 2B), which indicates CDs are decorated on LEG.

The atomic force microscopy (AFM) examinations are conducted on the layers of CDs, LEG, and CD-coated LEG. The dispersed particles of CDs and laminar LEG nanosheets could be seen in Figure 2C,D. Their thicknesses are calculated according to the method in our previous work, which are about 1−7 nm for CDs and 3 nm for LEG (more evidences about the morphology and size distribution of CDs are provided in Figure S1). The CDs on the LEG layer could be identified by the bright-white dots on the light-yellow bottom layer (Figure 3E). Meanwhile, their 3D AFM images (Figure 2F−H) indicate the surface roughness of LEG (1.11, obtained

![Figure 1. Protocol of the CD-modified LEG-FETs and experimental design for the SEPT9 assay. (A) CDs’ synthesis process based on the one-step hydrothermal method. (B) Main experimental procedures for the modification and functionalization of the LEG-FETs.](https://dx.doi.org/10.1021/acsomega.0c02022)
by Nanoscope) is increased by CDs decoration to 1.34, which could confirm the particles of CDs were coated on the LEG layer.

**Analysis of Surface Components.** The Fourier transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) analyses are conducted and shown in Figure 3. CDs’ FTIR analysis is briefly described below according to Figure 3A:27,28 the peak at around 3293 cm$^{-1}$ indicates the existence of $\text{--N--H}$ stretching in amide bond; the peak at 2925 cm$^{-1}$ is assigned to the stretching sp$^3$ --C--H bond, and two sharp peaks at 1645 cm$^{-1}$ (assigned to $\nu _{\text{C}═\text{O}}$) and 1545 cm$^{-1}$ (assigned to $\nu _{\text{C}―\text{N}}, \delta _{\text{N}―\text{H}}$) also illustrate the existence of amide linkage in CDs. That means, the CDs are formed through the amide linkage between CA and EDA molecules, as shown in Figure 1A. Furthermore, the small peaks at about 1396 and 1290 cm$^{-1}$ are deduced to the bending vibrations of C–H, C―N and C―O bonds.

The XPS analyses are performed to gain an insight into the surface groups and elemental states on the films of pure CDs, LEG, CDs&LEG. First, in the XPS wide spectra (Figure 3B), three peaks at 283.5, 398.5, and 531.1 eV demonstrate the main elements are C 1s, N 1s, and O 1s, and the existence of Si in “LEG” (blue line) is from the APTES treatment on the substrate, while its disappearance after being modified by CDs (green line) indicates the substrate is covered by CDs.

The CDs’ core spectra of C 1s and N 1s are shown in Figure 3C. The peak at 284.4 eV corresponds to the sp$^2$-hybridized graphitic carbon, the peaks at 285.6, 287.6, and 288.8 eV are attributed to C―N, C═O, and O―C═O configurations, respectively.29,30 In N 1s core spectra of CDs, the peaks at 399.6, 400.5, and 401.5 eV are attributed to the C―N―C, N―(C)$_3$, and N―H.31 More analyses about O 1s core spectra are shown in Figure S2, which indicate that oxygen exists mostly in the form of C═O, C―OH, and O―C═O, respectively.26,30 These surface components based on XPS analyses are consistent with the above mentioned FTIR discussion.

The elemental compositions of LEG can be proofed by core spectra of C 1s and N 1s in Figure 3D. LEG’s carbon elements in the states of sp$^2$ and sp$^3$ are identified by the fitted peaks at 284.5 and 285.1 eV in the C 1s core spectrum, and the peaks at 286.3 and 288.6 eV are assigned to C―OH and O―C═O, respectively. For its N 1s spectra, the N 1s-fitted peak at 400.3 eV corresponds to N―C, which is induced by the reaction between APTES and GA; the peak at 401.7 eV corresponds to graphitic nitrogen, which may be induced from N atom’s incorporation in the ultrasonic exfoliation process.32 The O 1s spectrum (Figure S2B) is deconvoluted into two peaks at 531.4 and 532.7 corresponding to C―OH and O―C═O, respectively.

The decoration of CDs on LEG can be verified by the core spectra of C 1s and N 1s in Figure 3E. In comparison with the spectra of LEG (Figure 3D), the appearance of the peaks at 287.9 eV (C 1s) and 399.6 eV (N 1s) in Figure 3E indicates the successful decoration of CDs on LEG because they are in accordance with the CDs’ XPS features in Figure 3C.

**Effects of CDs’ Decoration on LEG-FET and the Methylation Evaluation.** Figure 4A shows the normalized transferring curves of CD-modified LEG-FET.
that the typical bipolar transfer characteristics of LEG-FET are maintained by the CDs decorated ones. The voltage of charge neutral points (the black stars) under different values of $V_{DS}$ is 0 V, which may be caused by the carboxylate and hydroxyl groups which are identified by the XPS and FTIR examinations (as illustrated in Analysis of Surface Components). Because these groups are negatively charged and in large number, the change of $V_{DS}$ is not enough to change the voltage of charge neutral point for the CD-decorated LEG-FET. Moreover, the volt–ampere characteristics of the CD-LEG-FETs are also examined and shown in Figure S3, they agree with our previous work.15

The effect of CD decoration on LEG-FETs performance in the methylation detection is evaluated by using the testing

![Figure 3](image-url)

Figure 3. FTIR of the synthesized CDs (A). XPS wide spectra (B); C 1s and N 1s XPS core spectra of CDs (C), LEG (D), CDs&LEG (E).
ssDNA chains as proof of concept. The testing ssDNA chains have the similar sequences, which are 5′- TTGCGCGGCGTCCGTCCTGTT GACTTC-3′ and with −NH₂ groups at the 5′ terminal, different in the amount of 5mC sites (NmC) on them. The currents (I_DS) changing ratios of CD-decorated and CD-free LEG-FETs are compared in Figure 4B, it is found there are positive and linear changing curves for both, when NmC is from 0 to 4. This phenomenon is in accordance with the basic working principle of an immune-based FET sensor, which is outlined as: when there are more 5mC sites on the testing chains, more 5mC antibodies can be immobilized by the immune recognition, which lead to the increased surface impedance at the interface of the solution and the channel film. However, there is also difference between them, that is the slope of red line (1.1573) is almost twice of the black line (0.6583), that means, on the CD-decorated LEG-FETs more ssDNA chains can be fixed than the CDs-free ones, which is in line with our expectation.

Captured SEPT9 Genes by Different Probes under Different Temperature. As presented in Figure 5A, probe 251, 1031, and 1649 correspond to the 5′ end, middle, and 3′ end of CpG island, respectively. The sequences and Tm of the three probes are shown in Figure 5B. The 5′ ends of these probes are all modified by amino, and with similar Tm values (about 50 °C). The results of the methylight assay (green line area) and pyrosequencing (purple line area) in the middle of the CpG island are used as the contrast target for the electrical detection to evaluate the feasibility and desirability of the scheme. Universal methylated and unmethylated human DNA were used as positive and negative control samples, and the initial DNA detection amount was set to 40 ng. To optimize the reaction temperature, we compared the test results of control samples incubated at 37 and 48 °C, respectively. As is shown in Figure 5C and Table S2, the data of −ΔI_DS/I_DS0 are the changing ratios of FETs’ output currents (I_DS) before and after the incubation with 5mCab solution. It can be found that along with the temperature increases, the current change rate increases. Significant different PC/NC (positive control/negative control) ratios were observed in the electrical assay with probe 1031 (2.64 for 37 °C vs 8.32 for 48 °C) and 1649 (3.03 for 37 °C vs 16.70 for 48 °C). While for probe 251, little differences were obtained (10.93 for 37 °C vs 8.10 for 48 °C). We speculate that 48 °C is close to Tm values of the three probes, and control DNA samples can hybridize with higher specificity with probes at this temperature, thence it will eventually produce a greater current response. However, why different probes respond differently to temperature changes remains to be explained. Therefore, the optimized incubation
the methylation level of SEPT9 reports. Then, taken into the consideration of the similar tumor vs 0.47 for normal), which is consistent with previous tissues were investigated by the electrical assay system with thirty-one pairs of CRC and corresponding noncancerous CD-LEG-FET-based DNAm sensor. First, the methylation of cancerous tissue DNA samples were evaluated by the proposed methylation degree of the tumor and corresponding non-

cancerous tissue DNA samples. (D–F), ROC analysis of the probe 1031 assay system (D), three probes assay system (E) and methylight assay (F).

Figure 6. Results of the tumor and corresponding noncancerous tissue DNA samples from patients with CRC. (A) Results of electrical assay system with probe 1031 and three probes. (B) Results of methylight assay. (C) Results of pyrosequencing in one pair of randomly selected CRC samples. (D–F), ROC analysis of the probe 1031 assay system (D), three probes assay system (E) and methylight assay (F).

temperature of DNA samples is selected as 48 °C for subsequent experiments.

To determine the detection sensitivity of this electrical assay, its performances for different quantity of DNA samples were also evaluated. As is shown in Figure 5D and Table S2, the electrical assay system with each of the three probes come up with consistent results. The amounts of DNA samples were changed by using their diluted solutions (40 μL) in different concentrations (0.05, 0.1, and 1 ng/μL). The current changing rates decreased with the decreasing of the DNA amount. This indicated: as the amount of DNA sample decreases, fewer methylated SEPT9 gene fragments will be captured by the probes, and then fewer antibodies will attach to the surface of the LEG-FET, so the measured current response will be reduced accordingly. However, there were always significant differences between the positive and negative control samples. Even with the DNA amount as low as 2 ng, the PC/NC ratio remained 3.61 (probe 251), 3.09 (probe 1031), and 2.76 (probe 1649). This detection limit is lower than that of the recently reported electrochemical sensing strategy.20 Besides, according to Figure 5D, the highest changing ratios are obtained by using 40 ng (1 ng/μL) DNA samples, so it is used in the following tests for CRC tissue samples.

Performance of the CD-LEG-FET-Based DNAm Sensor in Detecting CRC Tissue Samples. In this part, the methylation degree of the tumor and corresponding noncancerous tissue DNA samples were evaluated by the proposed CD-LEG-FET-based DNAm sensor. First, the methylation of thirty-one pairs of CRC and corresponding noncancerous tissues were investigated by the electrical assay system with probe 1031. As the results shown in Figure 6A and Table S3, the methylation level of SEPT9 in tumor tissues was significantly higher than that in normal tissues (4.28 for tumor vs 0.47 for normal), which is consistent with previous reports.22,23 Then, taken into the consideration of the similar $T_m$ values and the scattered locations of the three probes, we can’t help but wonder, if combined application of multiple probes could further improve the performance of the CD-LEG-FET-based DNAm sensor? It turned out that this conjecture was reasonable. For the results with “Three probes”, the disparity of the SEPT9 methylation level between the tumor and normal tissues (15.42 for tumor vs 0.79 for normal) was much higher than that with single probe 1031 (T/N ratio of 9.11 for single probe vs 19.52 for three probes), as results shown in Figure 6A and Table S3. Therefore, the combined application of multiple probes scattered on the CpG island of SEPT9 gene could be another satisfactory strategy to further improve the specificity and sensitivity of the CD-LEG-FET-based DNAm sensor.

To further evaluate the application value of our proposed analytical method, clinical DNA samples were also assessed by methylight, an extensive and mature applied methylation detection method.34 The result of methylight was in coincidence with that obtained by the electrical assay (Figure 6B). The results from CDs-LEG-FET based DNAm sensor with both probe 1031 and three probes were significantly correlated with that from methylight (Figure S4). Those results illustrated that, CDs-LEG-FET based DNAm sensor was comparable with, if not better than, the methylight assay in detecting the SEPT9 methylation of CRC tissue DNA samples. Moreover, pyrosequencing, the recent gold standard for DNAm detection,35 was also employed to verify the different methylation level between the tumor and normal tissues. One pair of CRC DNA samples were randomly selected (patient 20), and the methylation level of SEPT9 in tumor tissues was indeed higher than that in normal tissues (33.0% for tumor vs 8.7% for normal).

Receiver operating characteristic (ROC) curves indicate the sensitivity–specificity profile for evaluating the diagnostic value of the electrical assay and methylight assay, as shown in Figure 6D–F. The areas under the curve (AUC) of this electrical assay with probe 1031 and three probes were 1, and for the methylight assay, the AUC was 0.98. Once again, the above data demonstrated the electrical assay we have established
provided comparable value in distinguishing CRC tissues from normal tissues. The CD-LEG-FET-based DNAm sensor may be expected to be an alternative to the methylight assay. As a matter of fact, the CD-LEG-FET-based DNAm sensor is superior to the methylight assay in some ways. On the one hand, as is well known that methylight is a PCR-based assay, and the value obtained at last depends on the methylation status of limited CpG sites covered by the primers and probes. By taking into the consideration of the high heterogeneity among the tumor cells, false negative results may be produced when these CpG sites are hypomethylated and vice versa. While, for the CD-LEG-FET-based DNAm sensor, the methylation value is determined by the methylation status of all CpG sites on the DNA fragments captured by the probes, presenting a methylation panorama of the target gene. On the other hand, the CD-LEG-FET-based DNAm sensor is more cost-effective and high-speed than the methylight assay because BC and triplicated tagman probe-dependent PCR is no longer needed. Those superiority may make the CD-LEG-FET-based DNAm sensor a promising alternative to the methylight assay.

The present study was limited by its relatively small sample size. Even though the methylation levels of CRC samples were significantly higher than that of normal samples, methylation differences among various stages of CRC patient were unable to be concluded. In the future study, a large cohort will be used to verify the distinguish capability of the CD-LEG-FET-based DNAm sensor for patients with CRC. In addition, we will focus on the diagnostic value of our proposed assay by the detection of blood-based specimens in the subsequent research. For the detection of blood samples, the core focus is whether the detection system is sufficiently sensitive. Because unlike the tissue samples, there is only trace amount of circulating DNA in the plasma of CRC patients. According to our data, the CD-LEG-FET-based DNAm sensor is able to effectively detect the methylation differences in as low as 2 ng DNA. Combined application of multiple probes and optimized reaction conditions, such as temperature and salt concentration will guarantee the high sensitivity of this assay, which is of potentially significant value for blood samples detection.

### CONCLUSIONS

The proposed CD-LEG-FET-based DNAm determination method is described and characterized first, the improved DNAm evaluation by CDs decoration is also demonstrated. In sequence, to realize its application in determining the methylation level in the clinical CRC patients’ SEPT9 genes, three kinds of probes are utilized to capture the target DNA sequence. It could be found that the results by these probes are mutually verified and in good consistency with each other. Moreover, the optimized experimental conditions for DNA sample’s dilution and the incubation temperature are decided through sufficient examinations by using the negative and positive controls. The ultimate experimental system based on the proposed CD-LEG-FET sensor is exploited to realize the clinical assay for CRC patients’ DNA samples. It is found there is a good agreement between the results obtained by the FETs’ method and the methylight assay, which demonstrates that the FET sensing platform could be developed as an alternative tool for clinical methylation evaluation. What’s more, the probes’ effects for determining the methylation level on SEPT9 genes are also examined by using each of three probes and their combinations. It is found an improved diagnostic value could be achieved by using the combination of three probes, which are even higher than the traditional PCR-based methylation method. In general, the current experimental works provided a positive answer for the comparability of the FET-based system for evaluating DNAm in the target genes, its potential in discovering new biomarkers can be anticipated.

### EXPERIMENTAL SECTION

#### Materials

Crystalline flake graphite powder 99% (Beijing HWRK Chem Co. Ltd., China), dimethyl-formamide (J&K Scientific, USA), glutaraldehyde (GA) and 3-amino-propyl-triethoxysilane (APTES, Sigma-Aldrich, USA), citric acid (CA), C₆H₈O₇·H₂O and ethylenediamine (EDA, C₂H₄N₂) (Tianjin chemical reagent Co., Tianjin, China) were the main chemicals for fabricating CDs-LEG-FET. The antibody of 5mC (clone number 33D3, ab10805) was purchased from Abcam (UK), named as 5mCab in this work. Bovine serum protein (Glenview Scientific Inc., USA) was used as a blocking reagent. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and N-hydroxysulfo-succinimide (NHS) from Thermo. Other chemicals including Tris—HCl, H₂SO₄ (98.0%), H₂O₂ (30.0%), HCl (30.0%), phosphate-buffered saline (PBS), NaOH, and so on, were analytical reagents from Tianjin Chemical Reagent wholesale company (China). Oligonucleotide primers and probes used in this work were synthesized by Saigon Biotech Co. Ltd., Shanghai (China).

**DNA Sample Preparation.** Universal methylated and unmethylated human DNA (Zymo Research, Orange, CA, USA) were used as positive and negative control. CRC and corresponding noncancerous tissues (n = 31) were obtained from Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). All of the specimens were pathologically confirmed and all CRC patients gave written informed consent on the use of clinical specimens for medical research. All procedures undertaken in studies involving human participants were in accordance with the 1964 Helsinki Declaration ethical standards and approved by the Research Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Genomic DNA was extracted from tissue samples by using a commercial DNA extraction kit (Promega, Madison, WA, USA). To prepare ssDNA fragment suspension for CDs-LEG-FET’s assay, 0.5 μg of the DNA samples were diluted to 0.05, 0.1, and 1 ng/μl in Eppendorf tubes with TE (Tris—EDTA) buffer, and subjected to 18 ultrasonic rounds on ice (a total of 3.5 min, with 5 inverts per round) using an ultrasonic crusher (JY92-II, Scientz Biotechnology Co., Ningbo, China); after being incubated for 30 min on heater at 98 °C, the tubes were immediately put on ice and incubated for another 30 min. All samples were stored at 4 °C and detected within one week. For the methylight assay, Genomic DNA (200 ng) was bisulfite converted following EZ DNA-Methylation Gold kit instructions (Zymo Research, Irvine, CA, USA). PCR for methylight assays and the calculation for the percentage of methylated reference was undertaken as described previously. The pyrosequencing assay were performed by Saigon Biotech Co. Ltd., Shanghai (China). Sequence information for the primers and probes used is listed in Table S1 and Figure SB.

**CDs Preparation.** CDs were prepared by the one-step hydrothermal reaction, as schematically shown in Figure 1A. In this bottom-up synthesis process, there are abundant branching sites (the carboxylate groups) on the molecule of CA, which is the carbon source of CDs. The reaction can be...
outlined as: by the condensation reaction between the carboxylate groups in CA and the amino groups in EDA, the monomers of CD can be formed, then they are dehydrated and polymerized in the so-called carbonization procedure, which results in the linked carbon nano-scaffold.

The main experimental procedures were: (1) the mixture of CA (1.0507 g) and EDA (335 μL) were dissolved in 10 mL of deionized water (DIW) and transferred into a 50 mL autoclave; (2) they were reacted in the autoclave at 200 °C for 5 h; (3) after being naturally cooled, the reaction products were dialyzed with for 48 h to obtain brown-black CDs suspension; (4) then, the lyophilization was conducted on the CDs suspension to obtain the powder of CDs.

**Modification and Functionalization of LEG-FET.** LEG-FETs were fabricated following the procedures in our previous work, the main operations for the modification and functionalization of the sensing surface of LEG-FET (i.e., the LEG channel) were outlined here: (1) the LEG surface was activated by aldehyde groups, 100 μL of GA solution (2.5%) was pipetted onto the surface of the LEG channel and incubated at room temperature for 1 h. (2) CDs were drop-coated on the aldehyde activated LEG, that is: 100 μL of CDs solution (0.034 mg/mL) was pipetted into the testing pool and incubated at the thermostatic oscillator (60 rpm, 37 °C, 4 h). (3) The carboxyl groups in the CDs layer were activated by 2 mM EDC and 5 mM NHS. (4) the probe sequences were anchored on the activated CDs layer by the covalent bond between the amino groups and the activated carboxylate groups on CDs, that is, the probe solutions (80 μL, in PBS) were pipetted to the surface of the devices, after the incubation in the thermostatic oscillator (60 rpm, 37 °C, 4 h), they were gently rinsed out by DIW. So far, the devices were functionalized and ready to capture the detected gene sequences.

**Determination of DNAm by LEG-FET.** First, on the probe-functionalized LEG-FETs, the ssDNA fragment suspension (40 μL, in TE buffer) was pipetted and incubated in the thermostatic oscillator (60 rpm, 48 °C, 2 h). After being gently rinsed by DIW to remove the residue samples, the currents of the ssDNA-anchored LEG-FET were measured and named as $I_{PSO}$. Second, the SmCab solutions (80 μL) were added on the ssDNA-anchored LEG-FET and incubated in the thermostatic oscillator (60 rpm, 37 °C, 1 h). After rinsing with DIW to remove unspecific absorbed SmCab, the currents of the devices were measured and named $I_{PS}$. Finally, the DNAm degree on the anchored ssDNA sequences were evaluated by the changing rate of currents $I_{PSO}$ that was $I_{PS}/I_{PSO}$.

**Apparatus.** Characterizations for the as-prepared LEG, CDs are executed by atomic force microscopy (AFM), Raman spectroscopy, XPS, FTIR, and UV–vis spectrum. The instruments employed in these examinations are listed here: (1) Dimension Icon (Bruck, USA) for AFM; (2) Axis Ultra DLD (Kratos Analytical Ltd., UK) for XPS; (3) Microconfocal Raman spectrometer RTS-HiR-AM (Titian Electro-Optics Co. Ltd., China); (4) UV754 N (Shanghai Precision Science Instrument Co. Ltd., China) for UV–vis; and (5) FTIR-650 (Tianjin Gangdong Technology Development Co. Ltd., China). Meanwhile, Digital source meter B2900A (Agilent Technology Co. Ltd., USA) is utilized to perform the electronic measurements.

**Statistical Analysis.** Quantitative data were expressed as data plots or histogram with mean ± SD. Differences between the mean values of two groups were analyzed by the Student t-test. ROC curves were generated to assess diagnostic efficiency. The Pearson correlation coefficient method was used to evaluate the association between the result of methylight and the CD-LEG-FET-based DNAm sensor. All statistical tests were performed using SPSS program (version 13.0; Chicago, Illinois, USA). Value of $P < 0.05$ was taken as statistical significance.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02022.

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**Author Contributions**

D.D. and J.Z. contributed equally to this manuscript. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

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