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Quantification of Gene-specific DNA Methylation in Oesophageal Cancer via Electrochemistry

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ABSTRACT

Development of simple and inexpensive method for the analysis of gene-specific DNA methylation is important for the diagnosis and prognosis of patients in cancer. Herein, we report a relatively simple and inexpensive electrochemical method for the sensitive and selective detection of gene-specific DNA methylation in oesophageal cancer. The underlying principle of the method relies on the affinity interaction between DNA bases and unmodified gold electrode. Since the affinity trend of DNA bases towards the gold surface follows as adenine; (A) > cytosine (C) > guanine (G) > thymine (T), a relatively larger amount of bisulfite-treated adenine-enriched unmethylated DNA adsorbs on the screen-printed gold electrodes (SPE-Au) in comparison to the guanine-enriched methylated sample. The methylation levels were (*i.e.*, different level of surface attached DNA molecules due to the base dependent differential adsorption pattern) quantified by measuring saturated amount of charge-compensating $[\text{Ru}(\text{NH}_3)_6]^{3+}$ molecules in the surface-attached DNAs by chronocoulometry as redox charge of the $[\text{Ru}(\text{NH}_3)_6]^{3+}$ molecules quantitatively reflects the amount of the adsorbed DNA confined at the electrode surface. The assay could successfully distinguish methylated and unmethylated DNA sequences at single CpG resolution and as low as 10% differences in DNA methylation. In addition, the assay showed fairly good reproducibility (% RSD = <5%) with better sensitivity and specificity by analysing various levels of methylation in two cell lines and eight fresh tissues samples from patients with oesophageal squamous cell carcinoma. Finally, the method was validated with methylation specific-high resolution melting curve analysis and Sanger sequencing methods to study the methylation in oesophageal cancer samples.

Keywords: DNA methylation, gene-specific methylation, Electrochemical detection, Chronocoulometry, Oesophageal squamous cell carcinoma

1. Introduction

DNA methylation is one of the clinically relevant epigenetic biomarkers that regulate gene expression via controlling transcriptional alteration, genomic stability, X chromosome inactivation, genomic imprinting and mammalian cell development[1]. Recent studies on epigenetic research demonstrate that aberrant DNA methylation plays a critical role in the pathophysiology of human cancers including oesophageal squamous cell carcinoma (ESCC)[2,3]. For example, gene-specific promoter hypermethylation is an important driver in the development and progression of many human cancers *via* transcriptional inactivation and suppressing of gene function[4-8]. More recently, it has also been demonstrated that DNA methylation can be used as tumour-specific therapeutic targets in ESCC [2]. Therefore, sensitive and specific profiling of gene specific DNA methylation in ESCC could have potential implication for prediction of prognosis as well as to therapy response in clinical settings.

Until recently, gene specific DNA methylation in ESCC is generally detected *via* methylation specific PCR approaches along with bisulfite sequencing[9,10]. Over the past several decades, a variety of molecular biological approaches including methylation-sensitive single nucleotide-primer extension, methylight, methylation-sensitive high resolution melting, enzyme-linked immunosorbent assay (ELISA) based methylation assays, mass spectroscopy and fluorescence readout based methods have been conspicuously exploited to quantify the level of the DNA methylation in many human cancers [11-15]. However, most of these approaches are relatively simpler and robust but typically require large sample volumes, sophisticated instruments, multi-step procedures, hazardous radiolabeling, complex fabrication, expensive antibodies, etc. Furthermore, these assays are affected by multiple

external controls for quantitative analysis, background fluorescence interference, high labour and bioinformatics costs which limit their use in routine clinical applications.

In recent years, much attention has been focused on the development of sensitive, specific, relatively simple and inexpensive method for the analysis of DNA methylation using electrochemistry, colorimetry, surface plasmon resonance and Raman scattering readouts [17-22]. While most of these readout methods have their own merits and demerits, electrochemical readout offers additional advantages in clinical diagnostics applications due to their relatively higher sensitivity and specificity, cost-effectiveness and compatibility with the miniaturization [23-25]. In these assays, sensor requires a surface-attached capture probe to hybridize the complementary target sequence, and form duplex DNA that intercalatively bind with a redox-active transition-metal cations (e.g., $[\text{Ru}(\text{NH}_3)_6]^{3+}$) for the generation of electrochemical signals [26-29]. As described in many conventional electrochemical assays [30-33], the saturated amount of charge-compensation $[\text{Ru}(\text{NH}_3)_6]^{3+}$ complex (RuHex) on the electrode surface is electrochemically determined, which is directly proportional to the number of negatively charged phosphate residues and thereby the surface density of the target DNA.

Previously, we demonstrated the use of direct adsorption of bisulfite treated and asymmetric PCR-amplified DNA sequences on to an unmodified gold electrode (without the use of complementary capture probe and hybridization step) to quantify the level of DNA methylation present in the sequence via measuring the total adsorbed DNA on to the electrode surface [34,35]. Since the adsorption (*i.e.*, physisorption) trend of the DNA bases to gold surfaces follows as adenine (A) > cytosine (C) > guanine (G) > thymine (T) [36,37], two DNA sequences with different methylation patterns (*i.e.*, bisulfite treated adenine-enriched unmethylated and guanine-enriched methylated DNA sequences) should have different adsorption affinity towards gold surface. Indeed, a relatively large amount of unmethylated

DNA was adsorbed on the gold electrode in comparison to the methylated DNA. In this system, we showed that monitoring the Faradaic current generated by the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ system alone could be used for the interrogation of DNA methylation level present in the bisulfite treated samples[34,35]. While this assay is relatively simple, it follows an electron transfer kinetic-based mechanism, where density of the DNA sequences at the electrode surface should be sufficiently low[31]. Additionally, the risk of false-positive responses at low concentration of target is well known when using a detection technique based on attenuation of the interfacial electron transfer reaction of a redox process (i.e., “signal-off” approach).

In order to avoid this complexity, in the current study, we explored whether simply monitoring the total charge generated by the electrostatically-attached RuHex onto the adsorbed DNA could report on the level of DNA methylation present in samples, where generated total redox charge is the function of adsorbed DNA sequences on the electrode surface [26-29]. Since in this “signal-on” approach, the charge of the RuHex complex is quantitatively reflecting the amount of the adsorbed DNA at the electrode surface[30], the electrochemical signal generated by the chronocoulometric (CC) interrogation of DNA-bound RuHex will give the level of methylation present in the amplified samples. It is also important to note that unlike RuHex based conventional methods [30], the current method detects DNA methylation by simply monitoring the adsorbed target DNA on an unmodified SPE-Au electrode. Since we use direct adsorption of target DNA on an unmodified electrode rather than the conventional biosensing approach of using recognition and transduction layers, this method substantially simplifies the detection system by avoiding the complicated chemistries underlying each step of the sensor fabrication.

In this method, we first optimized the adsorption parameters (i.e., adsorption time, pH of the solution, and concentration of DNA) for the direct adsorption of target DNA onto the

unmodified SPE-Au surface. Then, we detected the level of promoter methylation present in *FAM134B* gene in a panel of oesophageal squamous cell carcinoma (ESCC) cell lines and tissue samples derived from patients with ESCC. We also validated the results with methylation specific-high resolution melting (MS-HRM) curve analysis and Sanger sequencing.

2. Experimental

2.1. Genomic DNA preparation

All reagents and chemicals were analytical grade and purchased from Sigma Aldrich (St Louis, MO, USA) unless otherwise noted. UltraPure DNase/RNase-free distilled water was obtained from Invitrogen (Carlsbad, CA, USA). Whole genome amplification DNA was prepared according to the manufacturer's protocol from REPLI-g whole genome amplification kit (Qiagen, Hilden, Germany). Two ESCC cell lines (HKESC-1 and HKESC-4) were kindly provided from our research group [38-39]. Another ESCC cell line, KYSE-510, purchased from Leibniz Institute DSMZ (German collection of microorganisms and cell cultures). 100% methylated Jurkat genomic DNA was obtained from New England Biolabs (Ipswich, MA, USA). Eight fresh frozen tissue samples from patients with ESCC and two non-neoplastic oesophageal tissues (as controls) were recruited for this study. Ethical approval was taken from the Griffith University human research ethics committee for the use of these samples (GU Ref Nos: MED/19/08/HREC and MSC/17/10/HREC). After histopathological confirmation, genomic DNA was purified from all ESCC tissue samples with all prep DNA/RNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocols. Blood and cell culture DNA mini kit (Qiagen, Hilden, Germany) was used for the purification of DNA from ESCC cell lines. All patients had not received pre-operative were free from radio/chemotherapy, matched with gender (all male) and clinical staging (stage III & IV).

Mean age group of the patients were 65 ± 14 ranging from 45 to 74 years. Screen-printed gold electrodes were acquired from Dropsens (Spain).

2.2. Bisulfite modification

Bisulfite conversion and purification of the genomic DNA was performed with MethylEasyXceed kit (Human Genetic Signatures Pty. Ltd., NSW, Australia) as recommended by the manufacturer. DNA quantification and purity was checked *via* Nanodrop Spectrophotometer (BioLab, Ipswich, MA, USA). Concentration of bisulfite treated DNA was noted in ng/ μ L and then stored at -20°C until use. Approximately 500 ng genomic DNA from each samples were the starting amount for the bisulfite treatment.

2.3. DNA quantification

The DNA copy number normalization of *FAM134B (JK1)* genes in bisulfite treated cell and WGA DNA samples were analysed by the Rotor-Gene Q PCR detection system (Qiagen, Hilden, Germany). qRT-PCR was performed in a total volume of 10 μ L reaction mixture containing 5 μ L of 2XSensiMix SYBR No-ROX master mix (Bioline, London, UK), 1 μ L of each 250 nM primer, and 1 μ L of equal concentrated target cell and WGA DNA samples with 2 μ L of nuclease-free water. Thermal cycling programs encompassed initial denaturation and activate the hot start DNA polymerase in one cycle of 7 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C (denaturation), 30 seconds at 60°C (annealing) and 20 seconds at 72°C (extension).

2.4. Asymmetric PCR

Asymmetric PCR of the bisulfite treated DNA was carried out using AmpliTaq Gold 360 master mix (ThermFisher scientific, Waltham, MA USA) to generate ss-DNA amplicons.

Asymmetric PCR was performed by using 60 μL reaction mixtures comprising 30 μL of AmpliTaq Gold 360 master mix, 1 μL of 125 nM forward primer and 375 nM reverse primer, 1 μL of 30 ng bisulfite treated DNA and 28 μL of nuclease-free water. PCR cycling programs was performed under the following conditions: 95°C for 10 minutes followed by 49 cycles of 30 seconds at 95°C (denaturation), 30 seconds at 61°C (annealing) and 20 seconds at 72°C (extension).

2.5. Determination of the surface area of the electrodes

Screen-printed electrode with the three-electrode system printed on a ceramic substrate (length 33 \times width 10 \times height 0.5 mm) were purchased from Dropsens (Spain). In the three-electrode system, working (SPE-Au, diameter = 4 mm), counter and reference electrodes were gold, platinum, and silver-modified electrodes. The effective working area of the electrodes were determined under cyclic voltammetric conditions for the one-electron reduction of $\text{K}_3[\text{Fe}(\text{CN})_6]$ [2.0 mM in water (0.5 M KCl)] and use of the Randles-Sevcik equation (1),

$$i_p = (269 \times 10^5) n^{3/2} A D^{1/2} C \nu^{1/2} \dots \dots \dots (1)$$

where i_p is the peak current (A), n is the number of electrons transferred ($\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$, $n = 1$), A is the effective area of the electrode (cm^2), D is the diffusion coefficient of $[\text{Fe}(\text{CN})_6]^{3-}$ (taken to be $7.60 \times 10^{-5} \text{cm}^2 \text{s}^{-1}$), C is the concentration (mol cm^{-3}), ν is the scan rate (Vs^{-1}).

2.6. Electrochemical measurements of DNA methylation

All electrochemical measurements were performed on a CH1040C potentiostat (CH Instruments, TX, USA). Cyclic voltammetric (CV) experiments were performed in 10 mM PBS solution containing 2mM $[\text{K}_3\text{Fe}(\text{CN})_6]$ electrolyte solution. Chronocoulometric readouts

were obtained in 10mM tris buffer (pH 7.4) in the presence and absence of 50 μ M RuHex with a potential step of 5 mV and pulse width of 250 ms, and sample interval of 2 ms. For synthetic DNA samples, 5 μ L (diluted in SSC5X buffer to get 100 nM of DNA) sample was adsorbed on SPE-Au surface. For clinical samples analysis, 5 μ L (diluted in SSC5X buffer to get 50 ng of DNA) were used for adsorption experiments. The electrodes were then washed three times with PBS prior to perform CC readouts. The total redox charge (Q_{total}) corresponding to RuHex electrostatically bound to the surface localized DNA was calculated using Eq. 2.

$$Q_{total} = Q_{target} - Q_{dl} \dots \dots \dots (2)$$

where Q_{target} is the charge flowing through the target DNA-attached (methylated or unmethylated) electrode containing both Faradaic (redox) and non-Faradaic (capacitive) charges, and Q_{dl} is the double-layer charge (capacitive charge) from the intercept at $t = 0$, respectively. The redox charge difference (ΔQ) in CC signals between unmethylated and methylated was estimated using Eq. 3.

$$\text{Chargedifference}(\Delta Q) = Q_{total, \text{methylated}} - Q_{total, \text{unmethylated}} \dots \dots (3)$$

where $Q_{total, \text{methylated}}$ and $Q_{total, \text{unmethylated}}$ are the CC signals estimated for the unmethylated and methylated samples, respectively.

2.7. Methylation specific-high resolution melting (MS-HRM) curve analysis

MS-HRM was carried out based on the modified versions of the previously published procedure [13]. Briefly, HRM curve analysis was demonstrated on the Rotor-Gene Q detection system (Qiagen) using the Rotor-Gene ScreenClust Software. PCR was performed in a 10 μ L total volume containing 5 μ L of 2Xsensimix HRM master mix, 1 μ L of 20 ng/ μ L

bisulfite modified genomic DNA, 2 μ L RNase free water and 1 μ L of each primer. The thermal profile comprised 15 min at 95°C, followed by 50 cycles of 30 seconds at 95°C, 30 seconds at 61°C and 20 second at 72°C. HRM analyses were carried out at temperature ramping from 70-95°C. The normalization of melting curve was performed as previously reported[40].

2.8. Sanger sequencing

To further confirm the methylation status of FAM134B promoter region, we employed Sanger sequencing analysis. The purified DNA was mixed with the primer (12 ng of DNA + 1 μ L of 10 pmol primer in 12 μ L of H₂O) sequence using the Big Dye Terminator (BDT) chemistry Version 3.1 (Applied Biosystems). Sanger sequencing was performed and analyzed using a 3730xl Capillary sequencer (Applied Biosystems) under standardised cycling PCR conditions in the Australian Genome Research Facility (AGRF, Brisbane).

2.9. Statistical analysis

Statistical analyses were performed *via* pairwise comparisons between two conditions using student's t-test. Significance level of the tests was taken at $p < 0.05$.

3. Results and discussion

3.1 Principle of the quantification of gene specific DNA methylation assay

We first extracted double stranded (ds)-DNA from the cancer cell lines and clinical tissue samples from ESCC patients to demonstrate the working principle of the method. We performed a bisulfite conversion step for converting unmethylated cytosines in ds-DNA into uracils while methylated cytosines remain unchanged. Then, an asymmetric PCR amplification step was performed to convert all ds-DNA into ss-DNA amplicon. In this step,

cytosines in the complementary strand would be copied into guanines and uracils into adenines ensuing guanine-enriched methylated and adenine-enriched unmethylated samples. The samples were then directly adsorbed on a SPE-Au electrode surface. The adsorbed ss-DNA samples were detected by CC interrogation in presence of an electroactive complex RuHex. Here, RuHex cations act as the signaling molecule that binds to the anionic phosphate of DNA strands in a stoichiometric manner [41]. Previous studies have clearly showed that redox charge of RuHex is quantitatively indicating the amount of DNA strands localized at the electrode surface [13, 41-42]. In the present method, since the adsorption strength of DNA bases towards gold differ as A > C > G > T, adenine-enriched unmethylated DNA leads to a higher level of adsorbed DNA on the gold electrode surface in comparison to guanine-enriched methylated DNA, resulting in a significant difference in CC signals for unmethylated and methylated targets. As schematically presented in Fig. 1, methylated DNA results in a relatively low level of CC charges (*i.e.*, a significant charge density/ μCcm^{-2}) in comparison to that of the unmethylated DNA.

3.2. Synthetic sample design

Recent studies suggested that alterations in *FAM134B* gene have a significant impact in gastrointestinal carcinomas and neurological diseases via regulating its expression patterns and cellular autophagy [43-46]. It has also been reported that *FAM134B* is mutated in metastatic lymph node tissues and its DNA copy number significantly altered in oesophageal squamous cell carcinoma tissues [43]. In this proof-of-concept study, we have used gold-DNA affinity interaction for detecting gene-specific DNA methylation in *FAM134B* promoter region containing designated CpG sites located within a length of 48 bases. In order to execute our approach, we have designed synthetic samples containing 0, 1, 5 and 11 CpG sites

within the promoter region of *FAM134B* gene which mimic the bisulfite treated and asymmetric PCR processed methylated and unmethylated DNA regions.

3.3. Assay Optimization

The extent of the adsorption of the target DNA on unmodified SPE-Au depends on the adsorption condition such as adsorption time, solution pH and amount of DNA. We first optimized the adsorption time (5-40 mins) of target DNA samples by measuring the redox charge differences between the 10 ng/ μ L synthetic methylated (11 CpG) and unmethylated (0 CpG) DNA in a solution of pH 7. As depicted in Fig. 2A, the maximum level of difference in charge densities between methylated and unmethylated samples was achieved at 5 min of adsorption time and gradually decreases with increasing time. At >20 min of the adsorption time, the difference in charge densities is minimum. This can be explained by the fact that longer adsorption time led to the saturation of the electrode surface with the methylated and unmethylated samples, causing a similar level of the surface confined redox process (*i.e.*, RuHex localized at electrode) in CC for both the methylated and unmethylated cases providing two CC signals with almost identical magnitudes. Therefore, 5 min of the adsorption time was chosen for all subsequent experiments.

We further investigated the effect of DNA concentration (2.5-40 ng/ μ L) on the change of the charge densities between the methylated and unmethylated DNA samples onto a SPE-Au in a solution of pH 7 for 5 min of adsorption. As seen in Fig. 2B, a significant change in charge densities (80.24) between methylated and unmethylated samples was found at the DNA concentration of 5 ng/ μ L. The maximum redox charge density difference *i.e.*, 95.54 was achieved using 10 ng/ μ L of DNA between these two samples. This is related to the increasing level of adsorbed DNA (*i.e.*, larger RuHex redox probes) on the electrode surface with increasing concentrations. At >20 ng/ μ L DNA concentration, a sharp decrease in the charge

densities changes was observed. For example, 40 ng/ μ L of DNA concentration resulted the charge densities changes of 40.34 between methylated and unmethylated samples. These findings clearly indicate that the amount of DNA at >10 ng/ μ L offers almost similar level of CC signals for both the methylated and unmethylated sequences. This can be explained by the fact that saturation of both sequences on the electrode surface was achieved within 5 min of adsorption at higher DNA concentrations which eventually leads to a similar level of redox charge densities. Thus, 10 ng/ μ L of DNA concentration was selected as an optimal concentration for all subsequent experiments. We then estimated the effect of the pH of the solution on the adsorption of target DNA by varying the pH of the solution from 3.0 to 9.5. Fig. 2C clearly showed that the redox charge density changes between methylated and unmethylated DNA samples were found to be 35.25 at pH 3.0. The optimal charge density changes 95.55 was achieved at neutral pH (*i.e.*, 7), whereas at $>pH = 7.0$, a gradual decrease in charge density changes was recorded. These results clearly showed that pH of the buffer solution influence the competition between DNA and gold electrostatic forces (*i.e.*, inherent interaction between DNA bases and gold electrodes). At neutral pH, negative charge of the phosphate backbone of DNA is optimal to hinder the adsorption of methylated samples while still allowing the unmethylated DNA with higher adenine contents to be adsorbed strongly. At basic pH, the gold surface would be more negatively charged and electrostatic repulsion with the negatively-charged phosphate backbone of DNA could reduce overall DNA adsorption. On the other hand, at the lower pH (3), cytosines and adenine in the target sequences would be protonated which could facilitate faster adsorption for methylated and unmethylated samples resulting saturation of both targets on the gold surface within a very short time leading to a reduced level of charge density changes. Therefore, we selected pH 7 as an optimal pH for our assay.

3.4. Synthetic sample analysis

To evaluate the applicability of our approach for the detection of various level of CpG methylation within the promoter region of *FAM134B* gene, four synthetic DNA samples containing 0, 1, 5 and 11 CpG sites were examined. Fig. 3 shows that the decrease of the redox charge densities is a function of the number of CpG sites. This is due to the decrease of the adenine contents with increasing methylated CpG sites in the target sequence (i.e., low level of adsorbed DNA leading to the lowering of the charge densities). The linear regression equation was estimated to be y (charge, μCcm^{-2}) = -8.2926 (number of CpG sites) + 103.9 with a correlation coefficient (R^2) of 0.9999. The level of redox charge responses showed in Fig. 2 clearly indicates that our assay can effectively detect DNA methylation at a single CpG level of resolution. A similar result has also been reported previously based on gold-DNA [34] and graphene-DNA [40] affinity interaction based approaches.

3.6. Heterogeneous sample analysis

Heterogeneous methylation can arise as a mixture of fully methylated and unmethylated DNA in varying proportions in tissue samples from cancer patients [47]. A heterogeneous mixture of cancer cells may contain both fully unmethylated and methylated DNA like imprinted gene *H19* [48]. Accurate quantification of heterogeneous DNA methylation pattern plays critical role for the detection and prediction of clinical prognosis in human cancers [47]. It is therefore important to screen the degree of methylation pattern in a high background of unmethylated DNA samples. To evaluate the assay performance for detecting heterogeneous DNA methylation pattern, we analysed the dependence of the CC responses on various degree of methylation. The samples were made by mixing synthetic standards of methylated and unmethylated DNA sequences to get 0%, 10%, 25%, 50%, 75%, 90% and 100% methylation, Fig. 4. The total charge densities decrease with increasing levels of methylation, probably due

to the increasing adenine contents in the target DNA sequences. The linear regression equation was found to be y (charge, μCcm^{-2}) = -0.8653 (% methylation) + 102.6 with a correlation coefficient (R^2) of 0.9998 . A methylation change as low as 10% could be detected from $10\text{ng}/\mu\text{L}$ of DNA. This data clearly demonstrate that our approach is sensitive enough in detecting methylated DNA in the nanogram regime. It is important to note that this level of data was much better than the findings of our previous gold-DNA based approach [34,35], and was also comparable to recent approaches [18-22].

3.7. Gene specific methylation detection and validation in cell line and clinical sample

To demonstrate a complex biological application, we applied our assay to detect the methylation status at the eleven CpG sites of the targeted *FAM134B* promoter which have been reported to be methylated in ESCC [40]. Purified DNA amplicons obtained from whole genome amplification and Jurkat DNA was used as fully unmethylated DNA and 100% methylated control, respectively. For avoiding any PCR bias, we quantified the gene copy number prior to PCR amplification [34,35]. Purified genomic DNA samples generated from three ESCC cell lines were then amplified asymmetrically and analyzed using our approach under the optimized conditions, Fig. 5A. As indicated in Fig. 5B and C, significant redox charge responses were observed in three cancer cell lines, unmethylated WGA, and 100% methylated Jurkat DNA samples signifying the presence of different percentage of methylation. When compared to that of the fully unmethylated WGA and 100% methylated Jurkat DNA samples, the level of the total redox charges obtained for the DNA sequences derived from HKESC-4, KYSE-510 and HKESC-1 cell lines indicated that HKESC-4 is partially and other two could be highly methylated (i.e., hypermethylated) at *FAM134B* promoter gene. The relative standard deviation (%RSD) over three independent experiments in quantifying DNA methylation from these cell line samples analysis was found to be $<5\%$.

These data were validated with MS-HRM curve analysis and Sanger sequencing. As can be seen in Fig S1, MS-HRM curve analysis showed that DNA samples derived from HKESC-4 is partially methylated while KYSE-510 and HKESC-1 DNA samples are hypermethylated. Moreover, Sanger sequencing also confirmed the different degree methylation in WGA, Jurkat DNA and different ESCC cell lines sample (Fig. S2 and 3). These data clearly indicate that the proposed assay could be a useful alternative for detecting *FAM134B* promoter gene methylation in cell-derived samples.

To further demonstrate the potential utility of our method in analysing clinical samples, we extended our assay to analyse eight tissue DNA samples derived from patients with primary ESCC. Two oesophageal non-cancerous tissue DNA samples were also used as a control (see Experimental for details). As indicated in Fig. 5D, all samples showed different degree of methylation. The level of total redox charge of two normal samples clearly showed that these two samples are unmethylated in comparison to that of the WGA and Jurkat DNA samples. Similarly, by comparing the level of total redox charges found for WGA and Jurkat DNA samples (Fig. 5C), we can estimate that four DNA samples derived from P5, P6, P7 and P8 cancer patients were relatively highly methylated, while P1, P2, P3 and P4 samples were partially methylated (i.e., low methylation) at *FAM134B* promoter gene. We then validated our assay performance with well-known MS-HRM curve analysis and Sanger sequencing. As can be seen in Fig S1B and C, MS-HRM curve analysis identified almost similar methylation level in WGA, N1 and N2 samples. Also, P5, P6, P7 and P8 cancer patients were highly methylated with respect to that of P1, P2, P3 and P4 samples. Sanger sequencing also confirmed that P5, P6, P7 and P8 samples were relatively highly methylated (see typical sequencing data in Fig.S2-S4). Moreover, %RSD over three independent experiments in quantifying DNA methylation from clinical samples analysis were found to be <5%. These data clearly indicated that CC signals generated by our assay were able to quantify different

degree of DNA methylation in ESCC tissue samples. Also, our assay is highly reproducible with greater sensitivity and specificity without costly fluorescence labels used in many of current methylation detection techniques[49-51]. In addition, validation studies with MS-HRM curve analysis and Sanger sequencing further suggested that our assay could detect DNA methylation in easy and inexpensive way from cancer patients.

Our method offers several advantages over current methodologies. First, the method involves the direct adsorption of target DNA onto an unmodified electrode rather than the conventional biosensing approach of using recognition and transduction layers, and hence it substantially simplifies the detection method by avoiding the use of complicated chemistries underlying each step of the sensor fabrication. It also avoids the use of capture probe as well as hybridization step. Second, it circumvents the need for the use of radioactive labels, methylation-sensitive restriction enzymes, antibodies, and sequencing analysis. Third, the use of commercially available and disposable SPE-Au (containing a three-electrode system) successfully eliminates the utilization of typical electrochemical cells, counter and reference electrodes thereby offering a relatively inexpensive (~USD \$5 per SPE-Au) platform for DNA methylation detection. Moreover, the use of SPE-Au potentially avoids the usual time-consuming cleaning steps associated with conventional electrodes making the analysis much faster. Fourth, the detection step of our proposed assay can take only ten min in total (excluding bisulfite treatment and asymmetric PCR steps) to achieve electrochemical readout, which is considerably faster than many recent electrochemical DNA methylation assays [13,29,40].

4. Conclusion

We have reported a simple and new method for the quantification of targeted *FAM134B* gene-associated DNA methylation *via* the different adsorption affinity interaction of DNA

bases with gold. The detection was achieved by simply monitoring their direct adsorption of bisulfite-treated and PCR amplified sequences onto a SPE-Au. The adsorption of the DNA sequence representing methylated and unmethylated was then quantified *via* CC interrogation of the DNA-bound RuHex complexes. Most importantly, our developed assay can successfully quantify *FAM134B* promoter methylation at varying level in a panel of ESCC cell lines and clinical samples from ESCC patients. The analytical performance of our method has been shown a good agreement with the data obtained using MS-HRM analysis and Sanger sequencing. We anticipated that our approach could be potentially useful for the detection of epigenetic biomarker in both clinical diagnostics and research.

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Appendix A. Supplementarydata

Supplementary data associated with this article can be found in the online version at

<http://dx.doi.org/.....>

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Figure Legends

Fig. 1. Principle of the quantification of gene specific DNA methylation assay. The adenine-enriched unmethylated ss-DNA adsorbs relatively larger amount on SPE-Au electrode in compare to that of the guanine-enriched methylated ss-DNA. A significant electrochemical signals were generated by the CC interrogation of DNA-bound $\text{Ru}(\text{NH}_3)_6^{3+}$ complexes. Inset, typical CC signals showing the adenine-enriched unmethylated DNA that produces higher CC charge in comparison to guanine-enriched methylated DNA.

Fig. 2. The redox charge difference obtained for the adsorption of unmethylated (0 CpG) and methylated (11 CpG) DNA sequences at (A) various time (B) concentration and (C) pH of the solution. Each data point (A-C) represents the average of three repeat trails, and error bars represent the standard deviation of measurements (%RSD = <5% for $n = 3$).

Fig. 3. (A) Typical CC curves for the sample containing 0, 1, 5, 11 CpG site and its corresponding background signal. (B) CC readout represent redox charges of RuHex bound to DNA with respect to the designated CpG sites. Each data point represents the average of three repeat trails, and error bars represent the standard deviation of measurements (%RSD = <5% for $n = 3$).

Fig. 4. (A) Typical CC curves for the sample containing 0%, 10%, 25%, 50%, 75%, 90% and 100% and their corresponding background signals. (B) CC readout represents redox charges of RuHex bound to DNA with respect to the designated methylation level. Each data point represents the average of three repeat trails, and error bars represent the standard deviation of measurements (%RSD = <5% for $n = 3$).

Fig. 5.(A) Representative amplified PCR products of FAM134B promoter region in 1.5% agarose gel. FAM134B were present in all the samples (2-7) except non template control (8). Hundred basepairs DNA ladder is used for comparison. (B) Total redox charges for detecting *FAM134B* promoter region in three oesophageal cancer cell lines and unmethylated WGA and Jurkat DNA samples. (C) Typical CC curves for detecting methylation of *FAM134B* promoter region in three oesophageal cancer cell lines, unmethylated WGA and Jurkat DNA and its corresponding background signal, and (D) Total redox charges for detecting *FAM134B* promoter region two normal (N1 and N2) and eight (P1-P8) oesophageal cancer tissue samples. Each data point represents the average of three repeat trails, and error bars represent the standard deviation of measurements (%RSD = <5% for $n = 3$). Statistical significance was determined by pairwise comparison between 2 conditions using student t-test. *, $p = 0.005$ to 0.05 and **, $p = 0.0005$ to 0.005 .

μCcm^{-2}

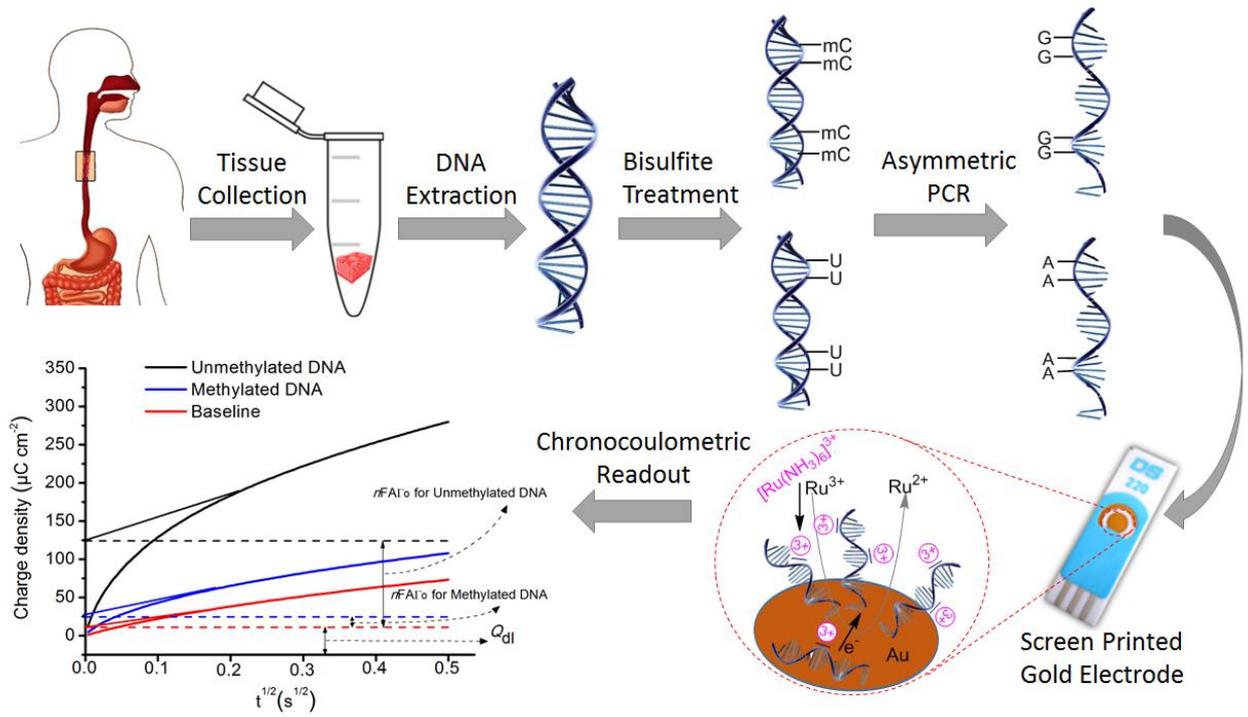


Fig. 1

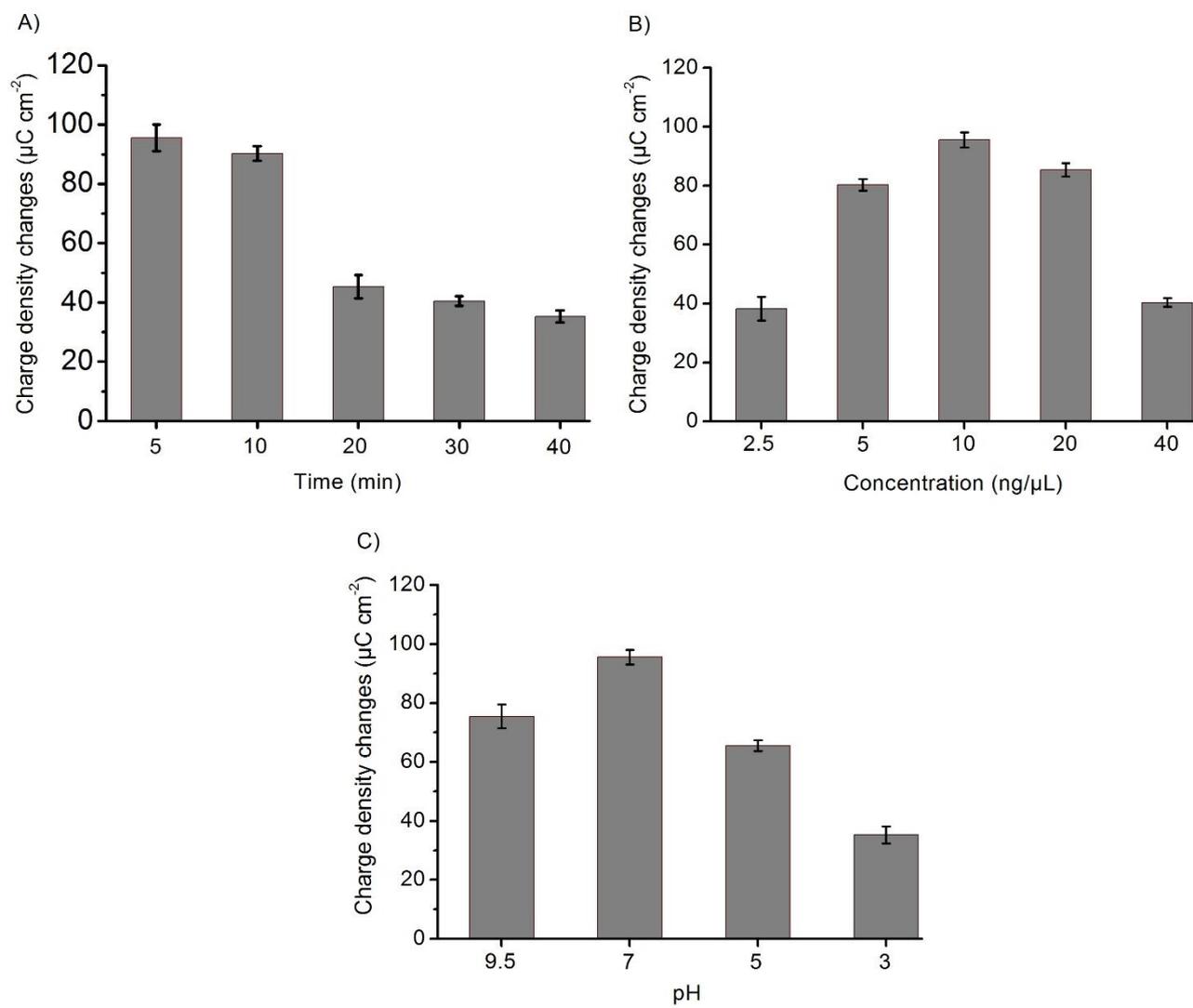


Fig. 2

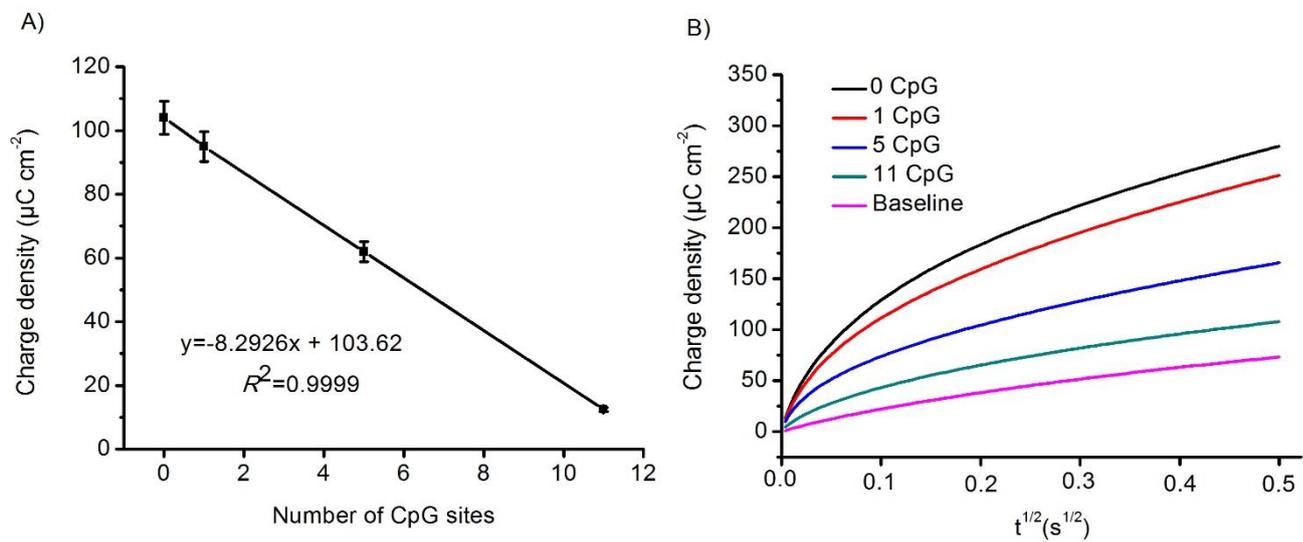


Fig. 3

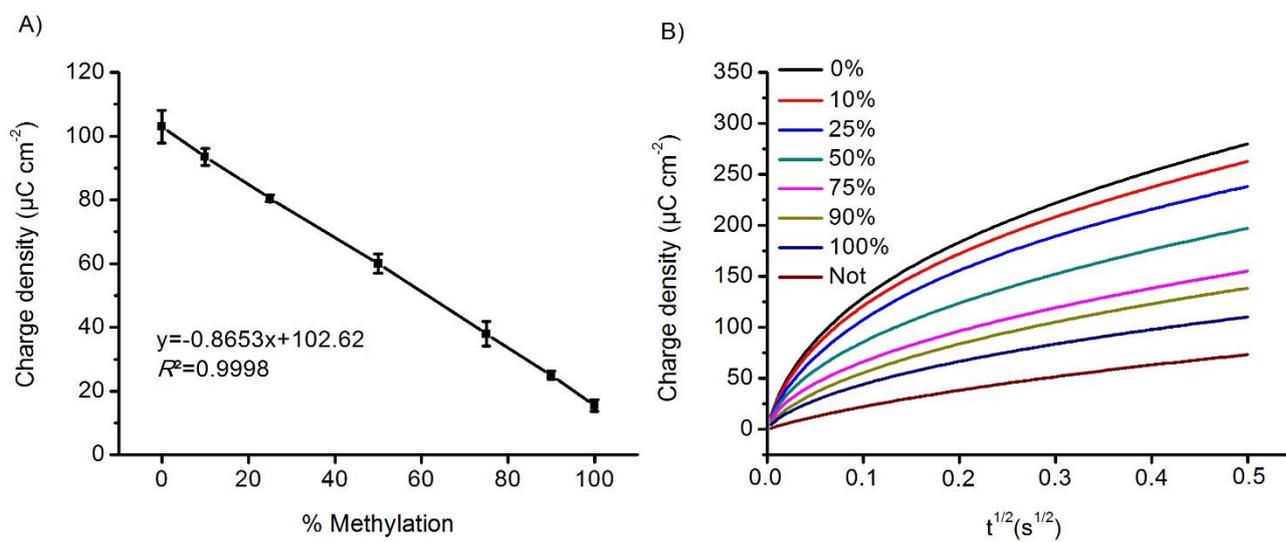
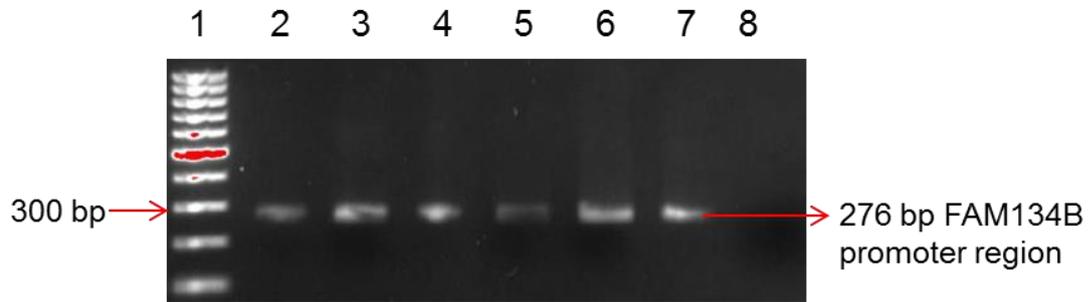
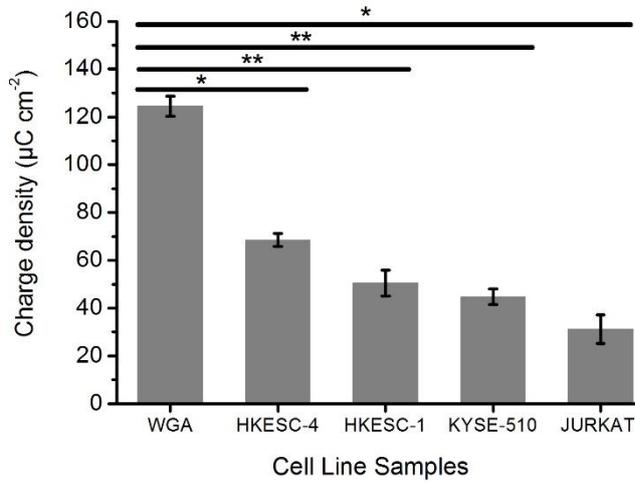


Fig. 4

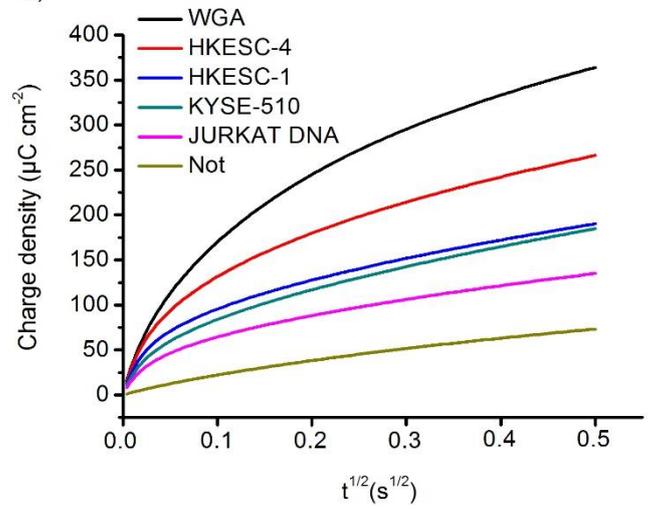
A)



B)



C)



D)

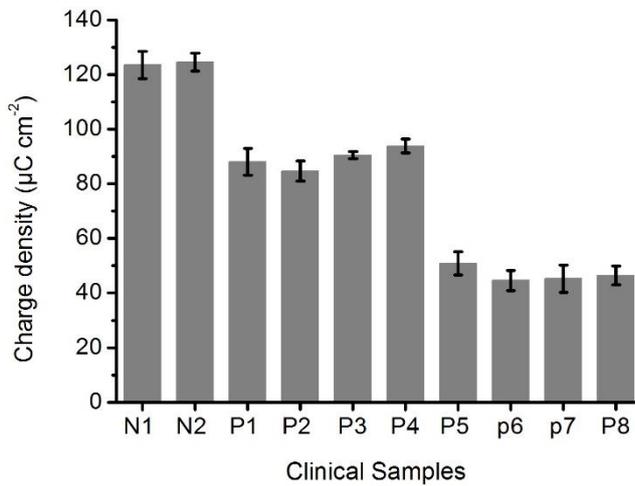


Fig. 5