Chapter 6

DNA Electrochemistry shows DNMT1 Methyltransferase Hyperactivity in Colorectal Tumors

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Introduction

Colorectal cancer is the third most prevalent cancer worldwide, causing approximately 700,000 mortalities annually.[1] The investigation into the causes of this disease is especially important, as its diagnosis is on the rise among people under the age of 50, and one of the major causes of mortality from colorectal cancer is metastasis due to its late detection.[2] Many molecular factors have been found to contribute to the onset of this disease including a host of genetic mutations[3-6] and epigenetic modifications,[7-10] as well as the inactivation of DNA repair pathways.[11-13]

While many factors likely contribute to the initiation and development of colorectal cancer, epigenetic modifications are of special interest, as they are connected to the progression of a variety of cancers.[14, 15] DNA methylation in particular has garnered significant interest, as aberrant DNA methylation has been found to be a hallmark of many cancers,[16, 17] including colorectal cancer.[18] Both hypermethylation and hypomethylation are linked to tumorogenesis. However, genomic hypermethylation in particular is often found in colorectal cancer and has been linked to the methylation of tumor suppressor genes and genes that control the translation of DNA repair proteins, leading to their silencing and therefore tumorigenesis.[3, 19, 20]

In humans, there are two classes of methyltransferases: *de novo* methyltransferases (DNMT3a, DNMT3b, and DNMT3L) and maintenance methyltransferases (DNMT1). *De novo* methyltransferases are in relatively low copy number and are responsible for establishing methylation patterns on the genome, meaning that they have a large preference for unmodified DNA.[21] In contrast, DNMT1, the most abundant mammalian methyltransferase, is a maintenance methyltransferase responsible

for transferring the genomic methylation pattern from the parent DNA strand to the daughter strand during DNA replication.[22] Because of its vital role in maintaining genomic methylation patterns during DNA replication, DNMT1 may be important in these molecular transformations within the cell that lead to the development of colorectal cancer.

Despite the potential importance of DNMT1 activity in disease initiation and progression, there is currently no clinical test for its activity. Generally, quantitative PCR (qPCR), which can be used to quantify gene expression of this protein, is used as a correlative measurement for the total amount of DNMT1 present.[23] Other methods, such as bisulfite sequencing,[24, 25] are used to detect specific, disease-relevant methylation patterns for early clinical diagnosis. However, such techniques are very costly and have limited efficacy.[26] In order to obtain a direct measure of methyltransferase activity, the current laboratory gold standard involves radiolabeling DNA with a tritium-labeled methyl group.[27] This assay not only produces relatively high variability but also requires the use of radioactivity and specialized instrumentation for measurement, making it impractical for clinical use.

We have previously developed an electrochemical method for the assessment of DNMT1 activity from crude cultured cell and tissue lysate.[28] This assay is conducted on a multiplexed, two working electrode platform (Figure 6.1) that enables electrochemical readout from disperse DNA monolayers with signal amplification and no necessary background correction. Using this platform, low-density DNA monolayers are formed through electrochemical activation of an inert copper precatalyst into an active catalyst.[29]

Electrochemical readout is accomplished through the measurement of current generated from a catalytic cycle.

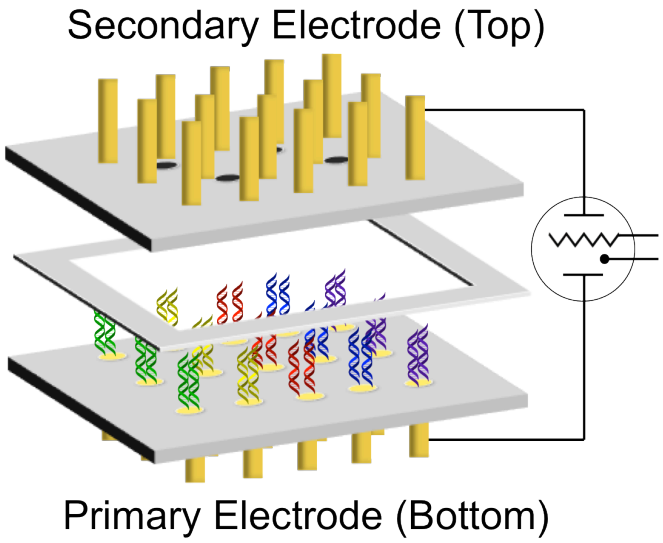

Figure 6.1 Electrochemical array for DNMT1 activity detection. The array contains two sets of fifteen gold electrodes, each embedded in a Teflon plate. Each electrode has a 1 mm diameter. The two complementary Teflon arrays are assembled with a 150 μm spacer between them, which was previously determined to be the optimal distance such that signals are not diffusion-limited.[28] The electrodes of the primary (bottom) array are modified with DNA of the desired sequences such that DNA-mediated charge transport is detectable. The electrodes of the secondary (top) array are bare for electrochemical detection.

In this cycle, DNA-mediated charge transport reduces an intercalative redox probe, methylene blue, to leucomethylene blue. This form of the probe has a reduced affinity for DNA, destacking from the helix and entering solution. In solution, leucomethylene blue reduces the ferricyanide electron sink to ferrocyanide, in turn becoming reoxidized to methylene blue. Amplified DNA-mediated electrochemical signals are generated without necessitating background correction through the detection of the current generated from the reoxidation of ferrocyanide at the secondary electrode array, which is proportional to the amount of ferrocyanide present (Figure 6.2). This cycling enables significant signal amplification, leading to very sensitive detection. This array combined with an electrochemical methyltransferase assay enables rapid detection, and requires no background correction or complex instrumentation. This assay requires only 500 μg of tissue per electrode, which is a significantly smaller sample than is currently removed with a biopsy.[28]

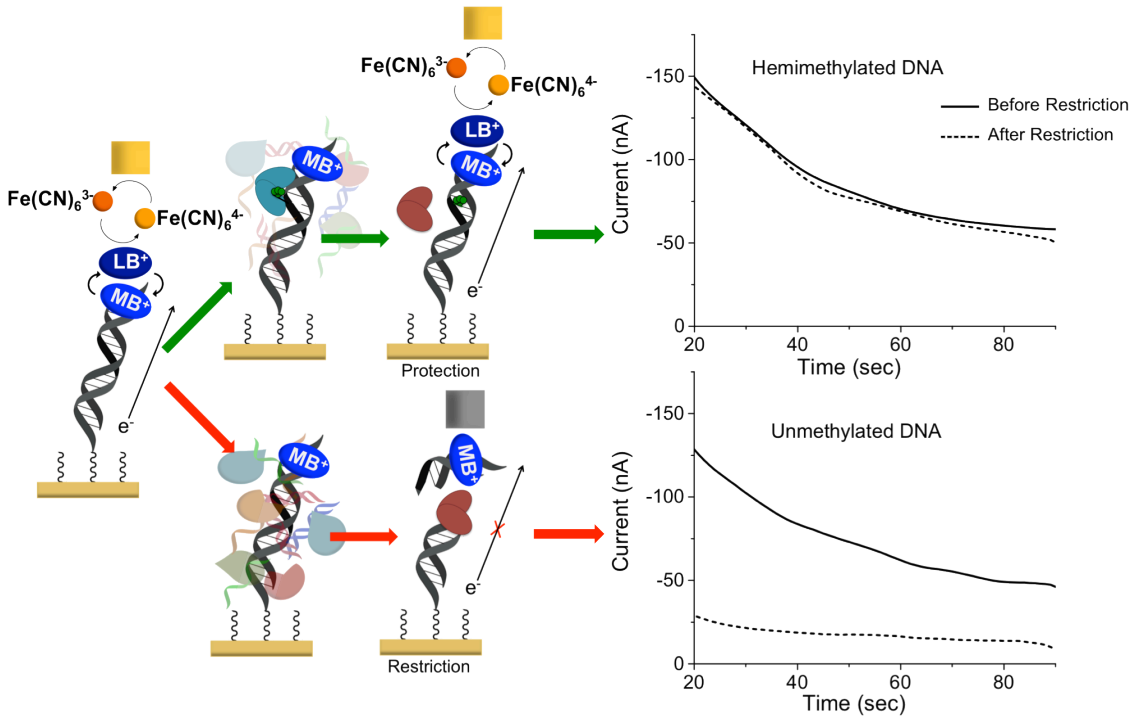

Figure 6.2 Signal-on electrochemical assay for DNMT1 detection. Left: The bottom (primary) electrode modified with a dilute DNA monolayer is responsible for generating electrochemical signals through DNA-mediated charge transport (CT) amplified by electrocatalysis. Methylene blue, a DNA intercalating redox probe, is reduced by DNA CT and enters solution as leucomethylene blue, where it can interact with an electron sink, ferricyanide. Upon interaction with leucomethylene blue, ferricyanide is reduced to ferrocyanide, reoxidizing the leucomethylene blue to methylene blue in the process. Current is generated and detected at the secondary electrode from the reoxidation of ferrocyanide. The current generated is proportional to the amount of ferrocyanide oxidized. To detect DNMT1, crude lysate is added to the electrode. If DNMT1 (blue) is capable of methylating DNA (green arrow), the DNA on the electrode becomes fully methylated. If the protein is not active, the DNA remains hemimethylated or unmethylated (red arrow). A methylation-specific restriction enzyme (*BssH*II) is then added that cuts the unmethylated or hemimethylated DNA (red arrow), significantly attenuating the electrochemical signal, while leaving the fully methylated DNA (green arrow) untouched. Constant potential amperometry (right) is used to measure the percent change before and after restriction enzyme treatment. If the restriction enzyme does not affect the DNA (top), the signals overlay. If, however, the restriction enzyme cuts the DNA, the signal is significantly attenuated (bottom). Constant potential amperometry is run for 90 s with a 320 mV potential applied to the secondary electrode and a -400 mV potential applied to the primary electrode relative to an AgCl/Ag reference. All scans are in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) with 4 μM methylene blue and 300 μM potassium ferricyanide.

Using this platform, we evaluate DNMT1 activity in ten sets of tumor tissue and healthy adjacent tissue, as well as in cultured colorectal carcinoma and normal colon cells (Figure 6.3). Significantly more DNMT1 activity is observed electrochemically in the majority of the tumor samples as compared to their healthy tissue counterparts, making this assay promising as an early clinical diagnostic for cancerous transformations.

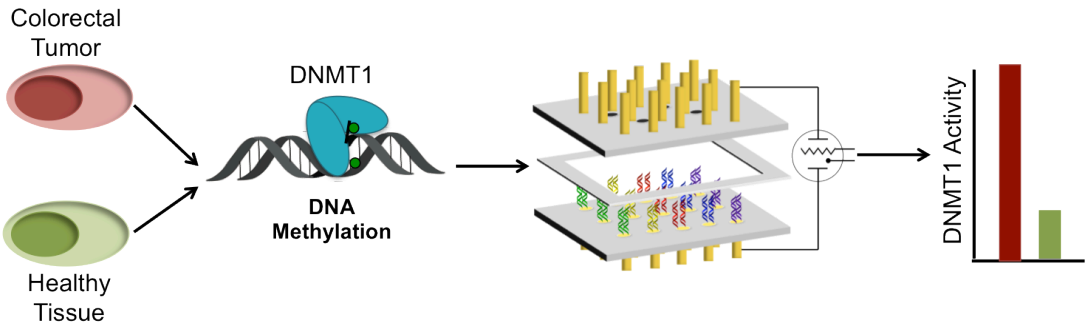

Figure 6.3 Overview of electrochemical DNMT1 analysis from tumors. Tumor and healthy tissues are lysed, and nuclear lysate is used to detect DNMT1 methyltransferase activity. The lysate is applied to a multiplexed, two working electrode platform that enables the conversion of methylation events into an electrochemical signal. Generally, we find hyperactivity of DNMT1 in tumor samples as compared to the healthy adjacent tissue.

Materials and Methods

DNA Synthesis and Purification

Hexynyl-modified oligonucleotides were synthesized on an Applied Biosystems 3400 DNA synthesizer, and unmodified complementary DNA was purchased from IDT. The terminal hexynyl moiety that was incorporated into the 5′ end of one of the strands was purchased from Glen Research. DNA was deprotected and removed from solid support with ammonium hydroxide (60° C for 16 h). Following a preliminary round of HPLC, oligonucleotides were treated with 80% acetic acid in water for 20 minutes. Each oligonucleotide was purified by high-performance liquid chromatography (HPLC) on a PLRP-S column (Agilent) with a gradient of acetonitrile and 50 mM ammonium acetate. Oligonucleotides were desalted by ethanol precipitation and quantified by ultraviolet-visible spectrophotometry based on their extinction coefficients at 260 nm (IDT Oligo Analyzer). Masses were verified by matrix-assisted laser desorption (MALDI) mass spectrometry. Based on the UV-Vis quantification of the DNA, DNA duplexes were formed by thermally annealing equimolar amounts of single-stranded oligonucleotides in deoxygenated phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) at 90° C for 5 minutes followed by slowly cooling to 25° C.

The following sequences were prepared:

Alkyne: 5′-C_2-$(CH_2)_6$-GA CTG AGT ACT GCG CGC ACT GAT AGC-3′

Unmodified Complement: 5′-GCT ATC AGT GCG CGC AGT ACT CAG TC-3′

Methylated Complement: 5′-GCT ATC AGT GCG C^mGC AGT ACT CAG TC-3′

The *BssHII* restriction site is shown in red.

RT-qPCR

Total RNA was extracted from 30 mg of tissue and 3,000,000 cultured cells of each cell type. Tissue samples were homogenized in a total RNA extraction lysis buffer from a RNEasy kit (Qiagen). The total RNA extracted using this kit was eluted into a 300 μL of RNAse-free water.

Following RNA isolation, RT-PCR was performed to make a complete cDNA library using a Transcription First Strand cDNA Synthesis kit (Roche) and standard RT-PCR thermocycler conditions (C1000 Touch^{TM} Thermal Cycler, Bio-Rad). Total cDNA concentrations were determined by nanodrop. For these samples, 250 ng/μL of total DNA was used.

Three *DNMT1* primer sets were evaluated for consistency and reproducibility. *β-Actin* expression was evaluated to normalize the *DNMT1* results with primers from RealTime Primers; *GAPDH* was also evaluated as a housekeeping gene but was observed to be extremely variable between qPCR trials for the same samples. *DNMT1* primers were obtained from RealTime Primers, qSTAR and Genocopeiea. *DNMT1* primers from RealTime primers were found to have the most consistent results over multiple trials, and were therefore used for all tissue sample evaluation. Each sample was run in quadruplicate for RT-qPCR measurements. RT-qPCR was performed on a CFX 96 Real-Time PCR Detection system (Bio-Rad). RT-qPCR results were calculated using a relative quantification method, with the $\Delta\Delta C_t$ to determine fold excess of the mRNA between the adjacent normal and tumor tissue. Error was propagated through the experiment for four samples of each primer and sample set.

Cell and Tumor Preparation for ^{3}H and Electrochemistry

100 mg of each tissue sample was prepared as a crude cell lysate. Tissue samples were homogenized before nuclear isolation using a 3 mL homogenizer with 100 strokes of the pestle. Cultured cells were harvested upon confluence (approximately 7,000,000 cells) and taken directly to rinsing. Both tissue and cultured cells were rinsed with 1 mL phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) and centrifuged. The tissue and cells were then prepared with a commercial NE-PER™ Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). Following nuclear lysis, the lysate was buffer exchanged with a 10 kDa spin column into DNMT1 reaction buffer (50 mM Tris HCl, 1 mM EDTA, 5% glycerol, pH 7.8), flash frozen and stored at -80° C.

Once the nuclear lysate was aliquotted and frozen, total protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Pierce) for protein concentration. Concentrations of lysate were found to range from 1000-6000 μg/mL of protein.

^{3}H Assay

Tritium assays were performed using the protocol previously established in our lab.[30] The DNA used as a substrate for electrochemical measurements, with sequences provided in a previous section, including the hexynyl terminal modification, was used in this radioactive methyltransferase activity assay. 0.5 μCi ^{3}H-SAM with 20 μM DNA, 100 μg/mL of BSA, and lysate (final concentration of 500 μg/mL total protein) were combined to a total volume of 20 μL in DNMT1 reaction buffer (50 mM Tris HCl, 1 mM

EDTA, 5% glycerol, pH 7.8). Samples with purified DNMT1 (BPS Biosciences) were used as a positive standard, along with a negative standard with no methyltransferase. Reactions were incubated for 2 h at 37° C, followed by quenching with 30 μL of 10% TCA in water. The solutions were then spotted onto DE81 filter paper (Whatman) and air-dried for 15 minutes. Each filter paper was then washed by individually soaking it in 10 mL of 50 mM Na_2HPO_4 for 15 minutes, followed by rinsing with 50 mM Na_2HPO_4 and 95% ethanol. Filter papers were heated to 37° C and allowed to dry for 15 minutes before liquid scintillation counting. Fold excess for radioactive measurements of a given tumor set was determined by taking the ratio of the counts for the tumor lysate on the hemimethylated substrate to the tumor lysate on the unmethylated substrate divided by that same ratio for the normal tissue lysate.

Electrochemistry

Electrochemistry was performed on a bipotentiostat (CHInstruments 760E) with two working electrodes, a platinum counter electrode, and an AgCl/Ag reference electrode. Constant potential amperometry electrochemical measurements were recorded for 90 seconds with an applied potential of 320 mV to the secondary electrode and -400 mV to the primary electrode. Constant potential amperometry measurements were performed in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) with 4 μM methylene blue and 300 μM potassium ferricyanide. Scans were taken individually at each of the 15 secondary pin electrode sets. The percent signal remaining data are reported as compared to pure DNMT1, with variation in the data representing the standard error across three measurements of three electrodes, all at a given condition.

To incubate electrodes with desired proteins, a 1.25 mm deep Teflon spacer was attached to the primary electrode surface with clips to form an isolated 4 μL volume well around each electrode. Generally, three electrodes on the device were incubated with 65 nM DNMT1, 160 μM SAM, and 100 μg/mL BSA as a positive control. For electrodes incubated with lysate, the lysate was diluted to a final concentration of ~200 μg/mL and directly combined with SAM to a final SAM concentration of 160 μM with 50 μg/mL BSA. Three electrodes modified with hemimethylated DNA were treated with tumor lysate and three treated with adjacent normal tissue lysate. Similarly, three electrodes modified with unmethylated DNA were incubated with tumor lysate and three with normal tissue lysate. Each electrode had 4 μL of the desired solution added to the well. The platform was incubated at 37° C for 1.5 h in a humidified container. The DNA monolayers were then treated with 1 μM protease solution in phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) for 1 h, followed by thorough rinsing with phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) and scanning by constant potential amperometry. The restriction enzyme *BssH*II was then added at a concentration of 1500 units/mL for 1.5 h at 37° C. *BssH*II was prepared by buffer exchange into DNMT1 reaction buffer (50 mM Tris HCl, 1 mM EDTA, 5% glycerol, pH 7.8) using a size exclusion column (10 kDa, Amicon). The electrodes were again rinsed with phosphate buffer and scanned. Results from three trials per tumor sample were aggregated and averaged. Fold excess for electrochemical measurements of a given tumor set was determined by taking the ratio of the percent signal remaining for the tumor lysate on the hemimethylated substrate to the tumor lysate on the unmethylated substrate divided by that same ratio for the normal tissue lysate.

Western Blot Analysis of Lysate for DNMT1

The relative amount of DNMT1 protein in each tumor set was established by Western blot. Samples were diluted to a final loading of 60 μg of protein per lane with DNMT1 reaction buffer (50 mM Tris HCl, 1 mM EDTA, 5% glycerol, pH 7.8) and Lammli reagent with betamercaptoethanol. Samples were probe sonicated for 10 seconds at 20% power, followed by boiling at 90° C for 5 minutes. Samples were loaded onto 4-12% polyacrylamide gels in MOPS SDS buffer (50 mM MOPS, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.7) and run at 175 mV for 2.5 hours at 4° C. Gels were subsequently transferred to membranes with a dry transfer procedure for 1.5 h. Membranes were blocked with 5% milk in TBST (20 mM Tris base, 150 mM NaCl, 0.1% Tween-20, pH 7.6) at room temperature for 1 hour, followed by overnight incubation with a 1^{o} antibody in 5% milk in TBST (1:1000 for DNMT1 (R & D) and 1:1000 for Lamin A (Santa Cruz)). The membranes were then rinsed with TBST buffer. Lamin A membranes were incubated with goat anti-rabbit 2^{o} antibody (Abcam) (1:5000 in 5% milk with 0.02% SDS in TBST) or Donkey Anti Sheep for DNMT1 (Santa Cruz) (1:5000) for 1 hour and then rinsed with TBST. Membranes were scanned on a Li-Cor Odyssey CLx infrared gel scanner, and bands were quantified using Image Studio software.

Results

DNMT1 Activity Measured Electrochemically

The electrochemical assay for DNMT1 activity involves the signal-on detection of methylation events on synthetic substrate DNA tethered to an electrode surface.[30] The process involves two steps: first, the pure methyltransferase or crude lysate is added to the surface. Subsequently, a methylation-specific restriction enzyme is used to cut any DNA that was not methylated during the previous treatment step. Successful methylation, resulting in protection from a methylation-specific restriction enzyme, maintains an electrochemical signal (Figure 6.2), while inactivity of the methyltransferase on the DNA-modified surface results in a significantly diminished electrochemical signal following restriction enzyme treatment.

When pure DNMT1 is titrated onto a DNA-modified electrode modified with hemimethylated DNA, a binding curve is generated, and a binding constant can be extracted based on the percent of the electrochemical signal that remains following restriction of the substrate DNA. A similar binding curve is generated from the addition of pure DNMT1 to the lysate of 4000 HCT116 *DNMT1*$^{-/-}$ cultured cells (the previously determined optimal lysate concentration for DNMT1 activity detection).[28] The titration data, along with the curve fit to the Hill binding model, are shown in Figure 6.4. Based on the inflection point of this curve, a K_D of pure DNMT1 on this DNA-modified electrode is found to be 31±1.3 nM, and 32±1.8 nM for pure DNMT1 in HCT116 *DNMT1*$^{-/-}$ lysate. These values are in good agreement with previously determined values for the K_D of DNMT1 in solution (26 nM).[31] A surface K_D on the same order as solution values indicates that not only is our platform especially sensitive for the detection of this

protein but also that the morphology of the DNA on our surface mimics the native substrate for the DNMT1 protein, allowing full access of the protein to the DNA bound to the surface.

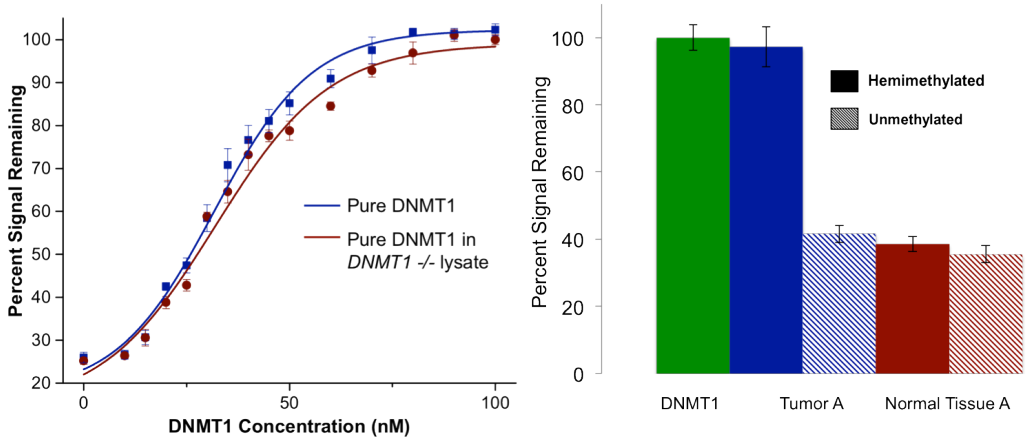

Figure 6.4 Detection of DNMT1 in pure form and from crude lysate. A titration of pure DNMT1 protein (*left*) demonstrates the sensitivity of this method of detection. In blue is shown the titration of pure DNMT1 on our electrodes, while in red is shown pure DNMT1 added to HCT116 *DNMT1*$^{-/-}$ cultured cell lysate. When the data are fit to a Hill binding model (fits shown as solid traces in plot), a K_D of 31±1.3 nM protein is extracted for pure DNMT1 and 32 nM±1.8 nM for DNMT1 added to lysate. The data from an array used to measure the DNMT1 activity from tumor A (*right*) show the differential between active lysate on electrodes and inactive lysate. The green bar shows electrodes treated with 65 nM pure DNMT1 as a positive control. The blue bars show electrodes treated with tumor A lysate on hemimethylated substrate (solid) and unmethylated substrate (dashed). The red bars show electrodes treated with adjacent normal tissue A lysate on hemimethylated DNA (solid) and unmethylated DNA (dashed). As can be seen, a significantly higher amount of signal protection is observed for the tumor tissue on the hemimethylated substrate than for the adjacent normal tissue on that substrate. The error bars show standard error across three electrodes.

Similarly, the differential signal protection from tumor and adjacent healthy tissue sets can be measured electrochemically. Ten tumor sets (A-J) were evaluated, and an example (tissue set A) is shown in Figure 6.4. Electrochemical data for this set show significant DNMT1 hyperactivity in the tumor sample as compared to the normal adjacent tissue over several replicates. With the data shown, an electrochemical assay using pure, isolated DNMT1 (green bar in Figure 6.4) is always included as a positive control. Also, it should be noted that the tumor lysate as well as the adjacent healthy tissue lysate are tested for methyltransferase activity using both a hemimethylated and an unmethylated DNA substrate. It is clear, even without normalization to the healthy adjacent tissue, that tumor A has significantly more DNMT1 activity than the healthy tissue. The incorporation of a comparison between the hemimethylated and unmethylated substrates, though, ensures that the activity we monitor is specific to DNMT1, and not to any *de novo* methyltransferases, given the noted preference of DNMT1 for a hemimethylated substrate.[32]

The data for each tissue set tested (A-J) have been aggregated into a single graph indicating the fold excess of DNMT1 activity in the tumor tissue as compared to the normal adjacent tissue (Figure 6.5). To calculate fold excess, each tumor and adjacent normal tissue has been normalized for the signal protection for the unmethylated DNA substrate to account for methylation that is not DNMT1-specific. Subsequently, the tumor tissue value is normalized to the adjacent healthy tissue, producing a ratio. If the fold excess is >1, then the DNMT1 activity in the tumor is higher than in the adjacent tissue. A fold excess of <1 indicates lower activity in the tumor relative to the healthy tissue, while a value of 1 indicates equivalent expression. As can be seen in Figure 6.5,

none of the tumors exhibits lower DNMT1 activity than their healthy tissue counterparts. In fact, the vast majority of the samples show significantly higher activity of DNMT1 when compared to the healthy tissue.

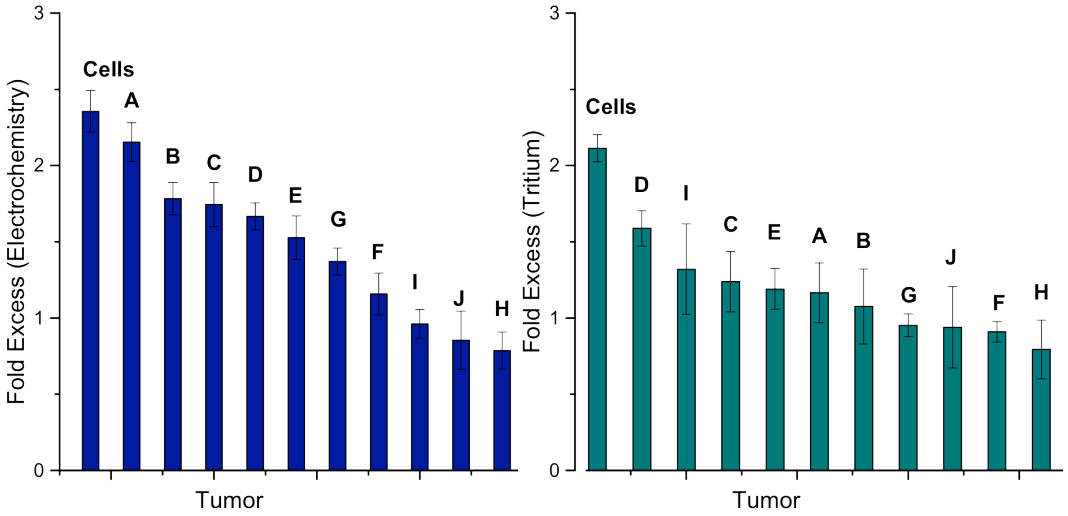

Figure 6.5 DNMT1 activity measured electrochemically and radioactively. The fold excess activity measured electrochemically (left) shows hyperactivity (fold excess > 1) in all but two of the tissue samples. Those that do not show hyperactivity show equivalent DNMT1 activity between tumor and normal tissue (fold excess ~1). When DNMT1 activity is measured with radioactive labeling (right), the same hyperactivity is not observed, likely because the measurement is convoluted by genomic DNA in the lysate samples. In both cases, the data for both the tumor and normal tissue on the hemimethylated substrate are first normalized to that of the unmethylated substrate, and the data for the tumor tissue are then normalized to the normal adjacent tissue. Each of the letters represents one of the tumor and healthy adjacent tissue sets, and the bar denoted 'cells' represents the result from the comparison between HCT116 colorectal carcinoma and healthy CCD-18Co colorectal cultured cells.

Radiometric Assay for DNMT1 Activity

In addition to measuring DNMT1 activity with our electrochemical assay, the current generally used radiochemical assay for DNMT1 activity[27] was used to assess the ten tumor samples. This assay involves the addition of substrate DNA and tritium-labeled S-Adenosyl methionine (SAM) cofactor, the source of methyl groups, to crude lysate or pure DNMT1. Protein activity is extrapolated from the amount of radioactive methyl groups added to the DNA following incubation, determined through scintillation counting of the samples in triplicate. Just as with the electrochemical assay, both hemimethylated and unmethylated substrate DNA were tested with the crude lysate for methylation. Analogously to the electrochemical assay, the resulting fold excess determination is calculated based on the ratio of hemimethylated substrate to unmethylated substrate counts for the tumor sample normalized to the hemimethylated divided by the unmethylated counts for the normal tissue. As can be seen in Figure 6.5, although the trend in fold excess activity is similar for tritium labeling as for electrochemistry, the extent of hyperactivity is diminished across all samples. Additionally, the majority of the samples have essentially statistically equivalent activity measured in this manner. This is, in part, due to the correction for the activity on unmethylated DNA. In this assay, genomic DNA from the lysate remains in the reaction mixture and is therefore capable of being methylated and contributing to the overall activity, even though it is not the target substrate. This seems to have led to higher activity in the unmethylated substrate samples for the tumors in many cases, which, when corrected, may lead to overall lower fold excess activity. The values that simply compare the hemimethylated tumor counts to their normal tissue counterparts are shown in Figures

6.6 and 6.7. Because of this significant difference in fold excess of DNMT1 activity, tritium labeling of DNA appears to be a less precise measurement of specific DNMT1 activity from lysate than electrochemical measurements.

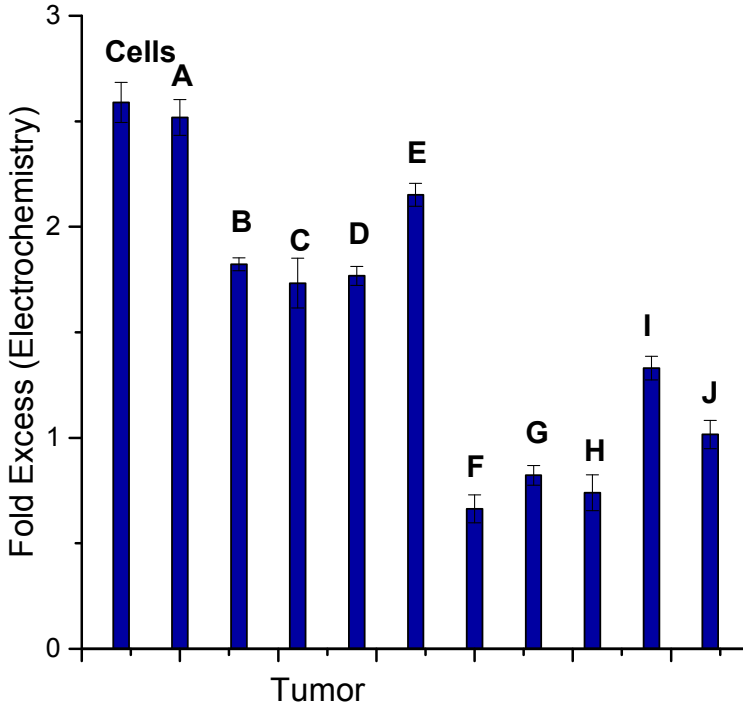

Figure 6.6 DNMT1 activity measured electrochemically without normalization to the unmethylated substrate. The fold excess activity measured electrochemically shows hyperactivity in all but two of the tissue samples. Those that do not show hyperactivity have equivalent DNMT1 activity between tumor and normal tissue. The data for activity on the hemimethylated substrate for the tumor tissue are normalized to that of the normal adjacent tissue.

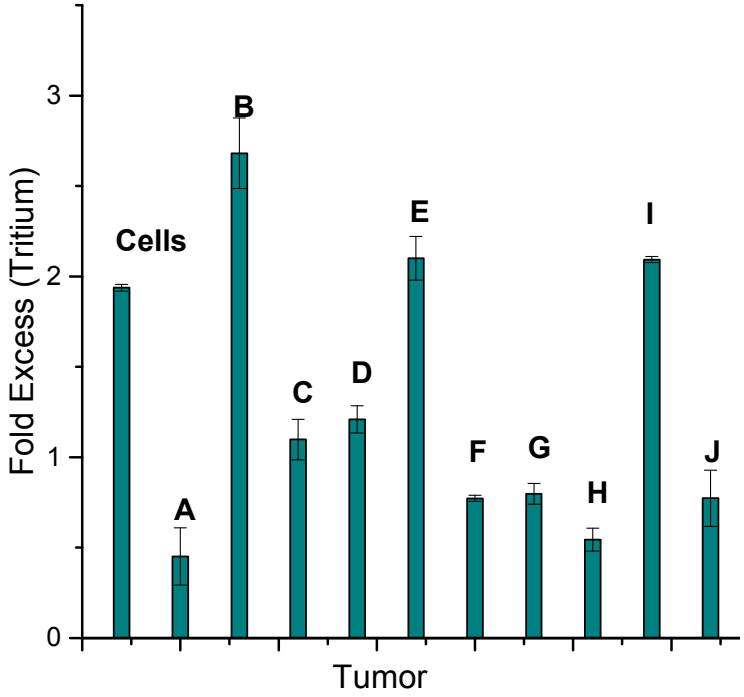

Figure 6.7 DNMT1 activity measured with radioactivity. The fold excess activity measured with radioactive labeling does not show similar hyperactivity in the tumor sample as when measured electrochemically. The data for activity on the hemimethylated substrate for the tumor tissue are normalized to that of the normal adjacent tissue.

DNMT1 Expression Measured by RT-qPCR

Currently, the most common method to analyze methyltransferases from lysate is through evaluation of genomic expression of the methyltransferase of interest. Thus, as a baseline with which to compare our electrochemical data, the expression of the *DNMT1* gene in each tumor and adjacent healthy tissue sample was evaluated by reverse transcription-quantitative PCR (RT-qPCR). A relative quantification method was used to determine the fold excess of *DNMT1* gene expression in the tumor sample as compared to the adjacent normal tissue sample. For each set of samples, the relative abundance of *DNMT1* is first normalized to the expression of *β-Actin* in each sample, followed by comparison between the normal tissue and tumor tissue. A fold excess of *DNMT1* from each tumor and normal tissue pair is calculated, and a value of >1 indicates higher expression in the tumor as compared to the normal tissue, <1 indicates lower expression in the tumor as compared to the healthy tissue, and a value of 1 indicates equivalent expression.

Multiple primer sets were evaluated for consistency over multiple trials, and the most consistent results were obtained with RealTime Primers, which were used for both *DNMT1* and *β-Actin* quantification by RT-qPCR. As can be seen in Figure 6.8, there is a large degree of variability among the ten tumor sets regarding the overexpression of *DNMT1* in the tumors as compared to the normal adjacent tissues. Sample B has the most significant upregulation, with a 22±5 fold excess of *DNMT1* expression, while sample G has significant downregulation of *DNMT1* in the tumor sample, with a fold excess of 0.3±0.1. Overall, no trends were observed with regards to the expression of *DNMT1* among these samples; tumors A, B, E, and F, as well as the HCT116 cells

compared to the CCD-18Co cells, all have significant upregulation of *DNMT1* in the tumor tissue. Samples C, D, and I have equivalent *DNMT1* expression compared to adjacent tissue, while samples G, H, and J have significant downregulation of *DNMT1* in the tumors.

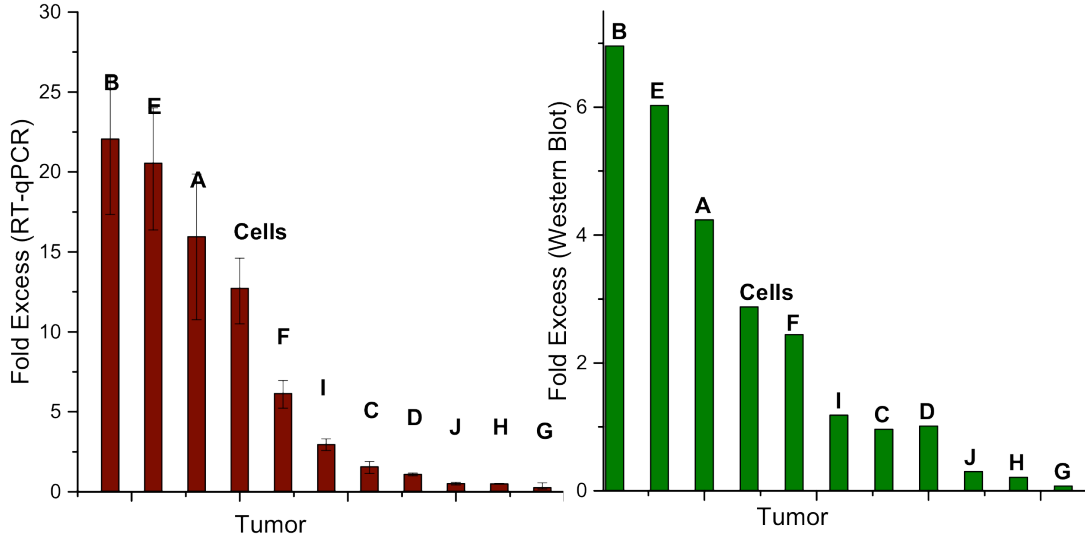

Figure 6.8 *DNMT1* expression and DNMT1 protein quantification. The fold excess *DNMT1* expression is determined with RT-qPCR (left), which shows just as many samples with overexpression of *DNMT1* in the tumor (fold excess > 1) as with equivalent expression (fold excess ~1) and underexpression (fold excess < 1). The RT-qPCR expression data for *DNMT1* expression in the tumor tissue are normalized to that of the normal adjacent tissue. The error represents the standard error across four replicates. The DNMT1 protein content (right), determined by Western blot, follows the same trend as the fold excess *DNMT1* expression; overexpression in the tumor sample correlates to more protein in that sample as compared to the normal adjacent tissue. The same trends are observed for those samples with equivalent expression and underexpression. For DNMT1 protein quantification, the measured intensity of the DNMT1 band is normalized to the Lamin A loading control, and subsequently, data for the tumor tissue are normalized to the normal adjacent tissue. Each of the letters represents one of the tumor and healthy adjacent tissue sets, and the bar denoted 'cells' represents the result from the comparison between HCT116 colorectal carcinoma and healthy CCD-18Co cultured cells.

Protein Content Measured by Western Blot

In addition to *DNMT1* expression evaluated by RT-qPCR, the total DNMT1 protein content of each tissue sample was evaluated by Western blotting. Total nuclear protein (60 μg) was added to each lane, and the amount of DNMT1 protein in the tumor tissue as compared to the healthy adjacent tissue was determined through quantification of the bands resulting from the Western blot (Figure 6.8). Again, a large amount of variability is observed in the total amount of DNMT1 within each lysate sample. The bands for DNMT1 protein as well as the nuclear protein Lamin A, used as a loading control, are given in Figure 6.9. Importantly, the ratio of DNMT1 in the tumor as compared to the normal adjacent tissue correlates directly to the fold excess of the *DNMT1* gene expression quantified by RT-qPCR.

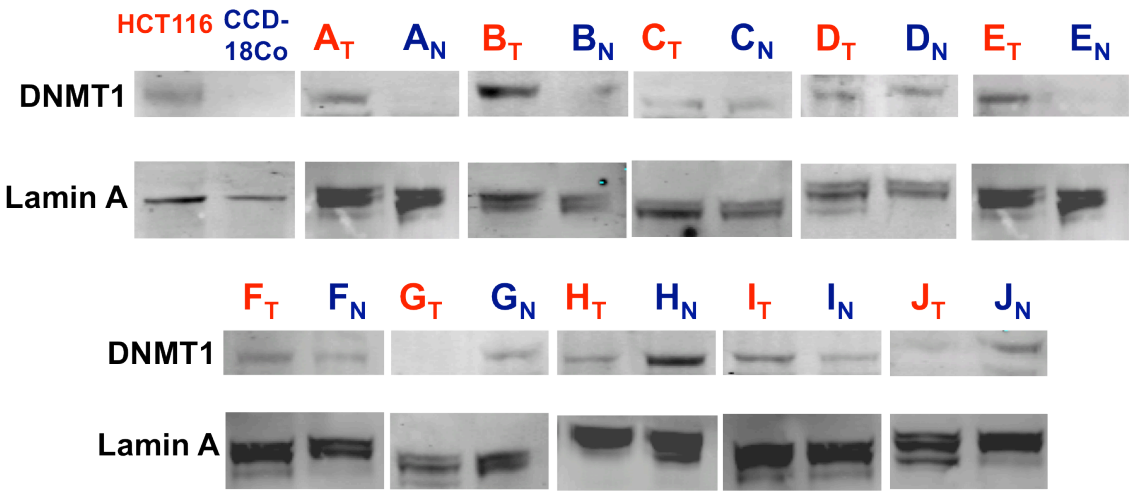

Figure 6.9 Western blots used for quantification. Shown are the bands used for quantification of differential DNMT1 protein content in the tumor as compared to normal tissue. The top bars show the DNMT1 in each tissue set as well as cell lysate. The bottom bars show the loading control, Lamin A, to which each DNMT1 concentration is normalized. 60 µg of protein per lane were loaded onto the gels. 1^o antibody for DNMT1 (R & D) and Lamin A (Santa Cruz) is incubated overnight. Goat anti-rabbit 2^o antibody (Abcam) or Donkey Anti Sheep for DNMT1 (Santa Cruz) is subsequently incubated, followed by imaging. Quantification is performed with Li-COR Odyssey Image Studio software.

Figure 6.10 then compares our electrochemical measurement of activity for the various tumor samples normalized to the adjacent healthy tissue, with *DNMT1* expression also normalized. Remarkably, as is evident in the Figure, there is no correlation between expression levels and resultant activity. While, for example, sample D has comparable expression levels in the tumor and adjacent tissue, the protein is found to be hyperactive electrochemically. In the case of sample B, the high activity seen electrochemically appears instead to be a function of the very high expression levels. Thus the electrochemical measurements allow a clear determination of methyltransferase activity associated with a given sample, and comparisons with expression and/or Western blotting permit the evaluation of whether the high activity in a given tumor sample results from high protein content or protein hyperactivity. The electrochemical assay is seen to provide the most direct measure of DNMT1 activity, and not simply its cellular abundance.

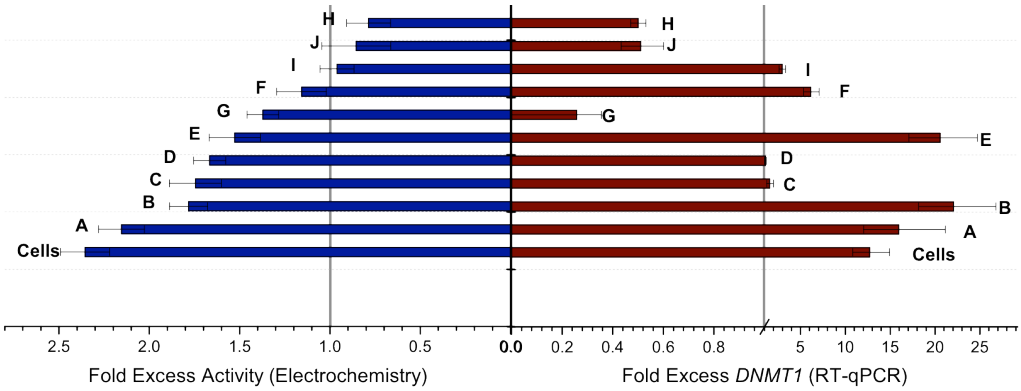

Figure 6.10 Direct comparison between DNMT1 activity measured electrochemically and *DNMT1* expression. The two bar graphs directly compare the fold excess protein activity (blue) measured electrochemically and the fold excess gene expression (red). There is no correlation evident between the amount of expression of *DNMT1* and the eventual activity of DNMT1 found in the tissue.

Discussion

The electrochemical platform developed for the measurement of DNMT1 activity allows the detection of this protein from crude cultured cell and colorectal tissue lysates. However, the utility of this platform for potential clinical diagnostic applications rests in the analysis of many samples. Here, ten tumor samples and adjacent healthy tissue have been evaluated. Our electrochemical platform facilitates extremely sensitive protein detection, with femtomole detection limits for the DNMT1 protein.[28] Furthermore, the electrochemical assay is a signal-on method of detection, ensuring specific DNMT1 discrimination from a host of proteins, DNA and RNA fragments found in crude lysates. Importantly, measurements of methyltransferase activity using the hemimethylated versus unmethylated substrate distinguishes the methyltransferase activity as associated with DNMT1 versus other methyltransferases, since only DNMT1 is preferentially active on the hemimethylated substrate.[32]

From the electrochemical analysis of the ten samples tested, in addition to lysate from cultured colorectal carcinoma cells, a pattern was found to emerge. Hyperactivity in the tumor samples as compared to the normal tissue is clearly found in the majority of tissue specimens analyzed. Furthermore, the few samples that did not show hyperactivity in the tumor tissue have equal activity between the tumor and healthy tissue. We therefore observe hyperactivity of DNMT1 in the majority of the measured tumor samples. In fact, a one-way ANOVA analysis of variance between the tumor and normal adjacent tissue DNMT1 activity was performed. We found a confidence level of $p < 0.05$, indicating a 95% confidence that the DNMT1 activity in the tumor tissues is different from the healthy adjacent tissues.

Currently, the most prevalent method of methyltransferase activity measurement is a radioactivity assay involving the application of tritium-labeled SAM cofactor to the methyltransferase of interest in pure form or in a mixture from crude lysate.[28] Substrate DNA is added to the reaction, and activity is measured based on scintillation counts resulting from tritium-labeled methyl groups added to DNA. This assay is also used here as a comparison to our electrochemical assay. The results from this activity assay, however, show less hyperactivity in the tumor samples as compared to the electrochemical assay. Indeed, the radioactivity assay does not show for most samples a statistically clear hyperactivity of DNMT1 in tumorous tissue. This lack of a clean correlation is likely due to methylation of genomic DNA present in the lysate, which is inseparable from measurements of the methylation of the synthetic, target DNA added to the reaction. Tritium labeling, therefore, does not provide a pure measurement of DNMT1 activity on a target substrate when measured from lysate, as methylation of native DNA can affect the levels of methylation measured, and therefore, the ratio of methylation on hemimethylated versus unmethylated substrates. It is especially important to note that this is not a problem with our electrochemical assay, as the only DNA methylation events that contribute to the electrochemical signal are those that occur on the target DNA in the electrochemical device. Noteworthy also is that the tritium-based assay is not suitable for clinical applications due to the large amounts of reagents required and the necessity of radioactivity for detection.

Perhaps more important in the context of clinical relevance is the comparison of DNMT1 activity measurements to the primary method currently applied in analysis of clinical samples: gene expression of the *DNMT1* gene by RT-qPCR. Many studies have

focused on correlations between the expression of this methyltransferase gene and tumorigenesis.[23, 33-35] However, upon analysis of the ten sample sets involved in this study, no such correlation is observed. There are just as many samples that have upregulation of *DNMT1* in tumors as equivalent expression and underexpression. We also observe no correlation between the activity of DNMT1 in a sample and its gene expression level as measured by RT-qPCR. Thus while RT-qPCR has potential applications for general research regarding methyltransferases, in and of itself it is insufficient as a diagnostic to identify potentially tumorous transformations.

Western blotting for total DNMT1 protein content in the lysate samples yields similar results as RT-qPCR. The DNMT1 protein levels are found to correlate to the expression of *DNMT1*, but not to the activity of the protein measured electrochemically. This finding indicates that it is not simply the amount of protein present that is responsible for the hyperactivity in the tumor samples observed electrochemically. Other post-translational factors must influence the activity of DNMT1.[36, 37]

If one considers the various sources of changes in protein activity, it is understandable that the electrochemical assay, which directly measures the methyltransferase activity on a hemimethylated target, would yield the clearest diagnostic for DNMT1 as a source of epigenetic change within a given tissue. Measurement of protein activity is closest in time and space to the epigenetic changes responsible for cancerous transformation, certainly as compared to the measurement of mRNA for the methyltransferase, which reflects effects on transcription, and even measurements of the abundance of protein, reflecting the status of DNMT1 following translation. What is key for cancerous transformation is the activity of the methyltransferase itself, turning on and

off particular genes as a result of methylation. The fact that only some of the tumorigenic changes in a sample depend on the amount of DNMT1, measured by Western blot or expression levels, underscores that point; tumorigenic changes are also seen to depend upon enzymatic hyperactivity of a given DNMT1. In developing assays for epigenetic changes as a source of tumorigenic change, this point needs to be kept in mind. In sum, then, using our electrochemical data, it is clear that tumorigenesis does indeed correlate with DNMT1 hyperactivity, and hence the electrochemistry provides a useful early and sensitive diagnostic for cancerous transformation.

Significance

We have developed an electrochemical platform based upon DNA charge transport for the measurement of DNMT1 activity from crude lysate samples. Using this platform to analyze ten colorectal carcinoma samples, as well as cultured colorectal carcinoma cells, we find a direct correlation between hyperactivity of DNMT1 and tumorous tissue. Significant hyperactivity of the protein is found in the majority of samples. This hyperactivity does not correlate with either overexpression or total amount of DNMT1 within the sample. Instead, significant enzyme hyperactivity is frequently evident. Furthermore, DNMT1 hyperactivity as an indicator of cancerous transformation, measured electrochemically, is not cleanly observed with the current standard techniques of DNMT1 analysis, including tritium labeling, RT-qPCR, and Western blotting. Thus our electrochemical platform has the potential to provide a sensitive method of detecting DNMT1-related cancerous transformations and with greater reliability than current DNMT1 analysis techniques.

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