Ferrocenylnaphthalene Diimide-Based Electrochemical Detection of Aberrant Methylation in hTERT Gene

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Ferrocenylnaphthalene diimide-based electrochemical detection of aberrant methylation in *hTERT* gene

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Abstract: Since aberrant methylation at CpG sites is linked to the silencing of tumor suppressor genes, DNA methylation analysis is important for cancer diagnosis. We developed ferrocenylnaphthalene diimide (FND), which has two ferrocenyl moieties at the substituent termini, as an electrochemical indicator for hybridized DNA duplexes. In this study, we attempted to detect aberrant methylation of human telomerase reverse transcriptase gene (*hTERT*), an efficient cancer marker, using FND-based hybridization coupled with electrochemical detection via a multi-electrode chip.

Key words: methylation; electrochemical detection; ferrocene; hybridization; *hTERT* gene

Introduction

Since aberrant methylation of CpG sites in the genome is known to be related to silencing of tumor suppressor genes, such methylation signals can be used as markers for cancer diagnosis or therapy [1]. Properties that are specific to methylated cytosine are useful for its detection. For example, cytosine is converted to uracil after bisulfite treatment, whereas methylated cytosine is not converted to uracil. This methylation-specific behavior of cytosine has been exploited in the development of bisulfite sequencing [2] and methylation-specific polymerase chain reaction (PCR) methods [3]. Combined bisulfite restriction analysis [4] has also been reported for this purpose, in which methylation is screened through digestion with restriction enzymes, because methylated cytosine is not digested. Although this method is effective in practice, it may be more difficult for actual diagnoses, where methylated cytosine must be detected from a limited cancer cell population among a number of normal cells. An electrochemical technique that enables highly sensitive detection has been reported for this purpose. Although nucleic bases, including methylated cytosine, are electrochemically active under high redox potential [5], it is difficult to detect their electrochemical behaviors using conventional electrodes such as gold and glassy carbon electrodes. Kato *et al*. [6] successfully monitored the electrochemical signals of methylated cytosine using an electron cyclotron resonance nanocarbon film electrode carrying an extensive chemical window. Wang and colleagues [7] also successfully monitored methylated cytosine signals using a glassy carbon electrode coated with an overoxidized polypyrrole/nanocarbon film. Bartosik *et al*. [8] detected methylated cytosine from a bisulfite-treatment sample using a hanging mercury drop electrode. Since uracil converted by bisulfate treatment is redox-inactive under this condition, an electrochemical signal that is specific to methylated cytosine can be obtained. Although this method provides highly sensitive detection of methylated cytosine and the ability to quantify the amount of cytosine in a sample, it is difficult to determine the specific site of the gene that is methylated.

On the other hand, we developed ferrocenylnaphthalene diimide (FND, Fig. 1A), which possesses two ferrocenyl moieties at the substituent termini, as an electrochemical indicator of hybridized DNA duplexes [9-15]. FND binds to double-stranded DNA in the threading intercalation mode and is difficult to dissociate from a complex with double-stranded DNA [9, 12, 14]. We succeeded in detecting single-base mismatches of single nucleotide polymorphisms

using an FND-based hybridization assay for *p53* [10] and lipoprotein lipase genes (*LPL*) [11]. Recently, we applied this system to the detection of aberrantly methylated genes, p16 [13], CDH4 [14, 15]. Such methylation was observed in the CpG sites on the promoter region of specific genes and is known to be associated with colon cancer [16]. Hence, methylation of cytosines will serve as a suitable cancer marker. Precise and quick detection of specific methylation on a given gene is required for the diagnosis and treatment of cancer. In this paper, the gene studied here is human telomerase reverse transcriptase (*hTERT*), an efficient any-cancer marker [17], and its specific aberrant methylation sites were analyzed in PCR products by using FND-based hybridization (Fig. 1B) coupled with electrochemical detection via a multi-electrode chip (Fig. 2A). FND intercalates between every two base pairs in a fully matched DNA duplex, and a large current based on the ferrocene of FND concentrated on the electrode is observed. On the other hand, FND does not intercalate with mismatched base pairs in a DNA duplex [11]; thus, the electrochemical responses of FND are vastly different between fully matched and mismatched DNA duplexes.

In this paper, 24–meric DNA probes carrying various methylation patterns were designed to represent 10 different kinds of CpG sites. They were immobilized on a multi-electrode chip and used for the analysis of aberrant CpG methylation sites with three different types of 24-meric DNA as a model sample or 121-meric PCR products obtained from model samples.

Experimental

Materials

FND (Fig. 1A) was the same as that used previously [12]. Oligonucleotides were custom synthesized by Genenet (Fukuoka, Japan). RNase-free water, 2.0 M potassium acetate (AcOH-AcOK, pH 5.5), 3.0 M KCl, and 5.0 M NaCl were purchased from Life Technologies (Carlsbad, CA). TBE buffer (1×, 89 mM Tris base, 89 mM borate, and 1.0 mM ethylenediamintetraacetic acid, pH 8.0). The forward and reverse primers were as follows: M-F primer; 5′-GAG GTA TTT CGG GAC CTT TCG C-3′, M-R primer; 5′-ACT CCG AAC ACC ACG AAT ACC G-3′, U-F primer; 5′-GGG AGG TAT TTT GGG AGG TTT TGT-3′, U-R primer; 5′-CAA ACT CCA AAC ACC ACA AAT ACC A-3′.

The sequences of probe DNA, HS-M0(+), HS-M1(+), HS-M2(+), HS-M3(+), HS-M4(+), HS-M5(+), HS-M4'(+), HS-M3'(+), HS-M2'(+), and HS-M1'(+), which are disulfide derivatives, and target template DNAs [M5(-), M3(−) and M0(−)] are shown in Table 1 and Table 2. The PCR products M5 and M3, each 121-meric, were amplified by using the 131-bp synthetic oligonucleotides M5(−) and M3(−) as templates by employing the M-F and M-R primers. The PCR product M0 (126-meric) was amplified with the 136-bp synthetic oligonucleotide M0(−) as template by using the U-F and U-R primers. The study target was the promoter region of the *hTERT* [18]. ZymoTaq PreMix was purchased from Zymo Research (Orange, CA). Genomic DNA with its cytosines randomly methylated (CpGenomeTM Universal Methylated DNA) or unmethylated (CpGenomeTM Universal Unmethylated DNA) was obtained from Millipore (Billerica, MA).

Clinical Samples

This research was approved by the Ethics Committee of Kyushu Dental College (No. 10-19) and the specimens were collected from patients and healthy volunteers after obtaining their informed consent. The specimens were collected from June 2011 from 1 patient with oral squamous cell carcinoma (SCC) diagnosed at the Diagnostic and Surgical Science Surgery in Kyushu Dental College, and 1 healthy volunteer. Both clinical specimens were tissue and exfoliated oral cells (EOCs). The EOCs were collected by scratching the tongue and buccal mucosa in the right and left sides 10 times each using a sponge-type brush. The collected cells were suspended in 20 mL of saline and then centrifuged at 20,000 × *g* for 5 min at 4 °C. The pellets obtained were homogenized in 500 μL of lysis buffer (10 mM Tris-HCl [pH 7.5], 1.0 mM $MgCl_2$, 1.0 mM benzamidine, 5.0 mM 2-mercaptoethanol, 0.5% 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate, and 10% glycerol) and stored at −80 °C. The tissue samples were obtained by surgical resection. Tissue sections were approximately 1.5 mm × 1.5 mm × 1.5 mm, were homogenized in 500 μL of lysis buffer, and stored at −80 ° C. Prior to assay, the lysate was centrifuged at 20,000 × *g* for 30 min at 4 °C, the supernatants were collected, and the genome DNA was extracted by using the DNeasy Blood & Tissue Kit (Qiagen).

Bisulfite treatment

CpGenomeTM Universal Methylated DNA, as the methylated genome sample, and CpGenome^{TM} Universal Unmethylated DNA, as the unmethylated genome sample, were obtained from Millipore (Billerica, MA). One microgram of genomic DNA was treated with bisulfite by using EpiTect (Qiagen). The products were used as templates for PCR, and the sequence of the PCR products was determined by custom DNA sequencing (Hokkaido System Science, Sapporo, Japan).

Methylation-specific PCR (MSP) sample preparation

PCR products were generated from a 100-μL PCR mixture consisting of 1× ZymoTaq PreMix, 0.4 μM F Primer, 0.4 μM R Primer, and 40 pM synthetic oligonucleotide [M5(−), M3(−) or M0(−)] or bisulfite-treated genomic DNA with its cytosines randomly methylated (see above) or unmethylated (see above). The PCR products were obtained by using the following program: 94 °C for 1 min, 40 cycles at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min, and 72 °C for 10 min for final elongation. After PCR, the products (50 μL) were recovered by using a PCR Purification kit (Qiagen). The sequence of the PCR products from the genomic DNA was checked by custom DNA sequencing (Hokkaido system science. Co., Ltd., Hokkaido, Japan). Before electrochemical measurement, the PCR products in 2× SSC (30 mM Sodium Citrate, 0.30 M NaCl, pH 7.0) were incubated at 95 °C for 10 min for thermal denaturation.

Combined Bisulfite Restriction Analysis (COBRA)

Ten microliter (50 ng) of MSP product was treated with 2.0 U AccII (Takara bio, Shiga, Japan) in 10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM DTT, and 50 mM NaCl at 37 °C for 1 h. The samples were checked by native PAGE on 12.5 % polyacrylamide (19:1 acrylamide/bisacrylamide) gels at 120 V for 120 min in 0.7× TBE buffer (1× TBE; 89 mM Tris base, 89 mM borate, and 1.0 mM ethylenediaminetetraacetic acid, pH 8.0). Gels were stained with GelStar (Cambrex, Baltimore, MD) in 1× TBE buffer for 30 min.

Apparatus

PCR was carried out using the Program temp control system PC-320 (Astec Co., Ltd, Fukuoka, Japan). Electrochemical measurements were carried out with an ALS 650C electrochemical analyzer (CH Instrument, Austin, TX). The multi-electrode chip carrying 10 working electrodes and its electroanalyzer, IM-AN100, were developed in our laboratory.

Preparation of DNA probe-immobilized electrodes

A gold electrode with an area of 2 mm^2 was polished with 6 and 1 μm of diamond slurry and 0.05 μm of alumina slurry in this order and then washed with Milli-Q water. Subsequently, the electrode was electrochemically polished by scanning 10 times from −0.2 to 1.5 V at a scan rate of 100 mV/s in 1 M H_2SO_4 aqueous solution and washed with Milli-Q water [19]. This electrode was dipped in 500 μL of 0.50 M NaCl solution containing 0.10 pmol/μL DNA probe (Table 1) for 16 h at 25 °C. After washing with Milli-Q water, the electrode was dipped in 1.0 μL of 1 mM 6-mercaptohexanol for 1 h at room temperature.

Hybridization and SWV measurement

Hybridization with the PCR products (Table 2) was carried out by soaking a probe DNA-immobilized electrode in 95 μL of 2.0 ng/μL target DNA in 2× SSC for 2 h at 20 °C. Square wave voltammetry was measured in 0.10 M AcOH-AcOK (pH 5.5), 0.10 M KCl, and 5.0 μM FND at 20 °C. The electrode current was measured after incubation for 2 min in that electrolyte. All data were standardized using Δi values, which are defined as $(i/i_0 - 1) \times 100\%$, where i_0 and i refer to the current of SWV before and after hybridization, respectively.

Preparation of DNA probe-immobilized a multi-electrode chip

A multi-electrode chip (Fig. 2A, Tanaka Kikinzoku Kogyo K.K.) contains 10 Au working electrodes, one Au counter electrode, and one Au reference electrode. The Au reference electrode was covered by Ag/AgCl ink (BAS, Japan). A multi-electrode chip was subjected to O_2 plasma treatment (Cute-MP, Femto Science, Gyeonggi, South Korea) at 0.5 Torr for 30 s. This electrode was dipped in 2.0 μL of 1.0 M NaCl solution containing 0.05 pmol/μL DNA probe (Table 1) for 3 h at 25 °C. After washing with Milli-Q water, the electrode was dipped in 2.0 μL of 1 mM 6-mercaptohexanol for 1 h at 45 °C.

Hybridization and differential pulse voltammetry (DPV) measurement of probe DNA immobilized multi-electrode chip

Hybridization with the PCR products from clinical samples was carried out by soaking a probe DNA-immobilized electrode in 95 μL of 2.0 ng/μL target DNA in 2× SSC for 2 h at 20 °C. DPV was measured in 0.10 M AcOH-AcOK (pH 5.5), 0.10 M KCl, 5.0 μM FND by IM-AN100 (Fig. 2B, I'm Co., Ltd., Fukuoka, Japan). The electrode current based on the ferrocene parts of FND was measured after incubation for 2 min in that electrolyte. All data were standardized using Δi values, which are defined as $(i/i_0 - 1) \times 100\%$, where i_0 and i refer to the current of DPV before and after hybridization, respectively. The following parameters were employed: DPV, Init E = 0 V; Final E = 0.6 V; Incr E = 10 mV; Amplitude = 0.05 V; Pulse Width = 0.025 s; Sample Width = 0.005 s; Pulse Period = 0.01 s; Quiet Time = 2 s; and Resistance = 510 kΩ.

Results and Discussion

A 24-meric region of the *hTERT* gene containing up to five methylation sites was selected as the probe (Table 1). The anti-sense strand of the PCR products was allowed to hybridize with both sense strands of the DNA probes and the electrochemical response was taken (Fig. 1). In principle, a methylated sample DNA can hybridize best with a methylated DNA probe. Since the cytosines of unmethylated DNA were converted to thymines by bisulfite treatment,

the unmethylated sites did not match with the DNA probe for the methylated target to give hybrid DNA carrying mismatched sites, whose number depends on the number of unmethylated sites.

Concentration dependence of PCR product

The DNA probe-immobilized electrode for M5(+) was prepared to detect methylated target DNA. This electrode allowed to form a fully matched DNA duplex with M5(−) DNA and a five bases-mismatched DNA duplex with M0(−) DNA. Figure 3 shows the result of the SWV measurement of the electrode in 0.10 M AcOK-AcOH (pH 5.5) containing 0.10 M KCl and 5.0 μM FND after hybridization of 0.1–4.0 ng/μL PCR product carrying M5(−) or M0(−). A quantitative current increase was observed in the range of 0.1–2.0 ng/μL M5(−) PCR product as a fully matched combination, whereas no current increase was observed in the case of M0(−) PCR product as a mismatched one. Figure 3 also shows that the discrimination limit between the two sequences is 0.6 ng/μL (1.2 pmol).

Detection of the PCR mixture

Since clinical samples are always a mixture of methylated and unmethylated DNA, we tried to detect the mixture of M5(−) and M0(−) DNA. Figure 4 shows the current increase percentage for the M(5)-immobilized electrode after hybridization with 2.0 ng/μL of the M0(−)/M5(−) mixture at ratios of 100:0, 75:25, 50:50, 25:75, and 0:100. A quantitative current increase was observed over 50% M5(−): 60 and 80% current increases were observed in the case of 50/50 and 25/75 [i.e., 1.0 and 1.5 ng/μL M5(−)], respectively. The current increases for these concentrations of M5(+) are in agreement with the results in Fig, 2, suggesting that the M5(+) probe-immobilized electrode can discriminate the M5(−) DNA.

Detection of hybridization between PCR products of M0, M3, or M5 and the DNA probes

Figure 5 shows the results for M5, M3, and M0 PCR products having five, three, and no methylation sites, respectively, after hybridization with the six DNA probes shown in Table 1. As expected, the M5 PCR product gave the largest current increase (96%) with the M5 probe, which decreased with increasing number of mismatched sites (Fig. 5A). Likewise, the M3(−) PCR product gave the largest (88%) current increase with the M3(+) probe (Fig. 5B). The current increase decreased to 57% with M2(+) or M4(+), which both have one mismatch, and to 30% with M1(+) or M5(+) probe, which have two mismatches. The unmethylated M0 PCR product gave the largest increase with M0(−) probe and the value decreased with the number of mismatched bases (Fig. 5C). These results reveal that the current increase varies from 95 to 50% with 0 to 2 mismatches.

Detection of the PCR product from commercially available genomic DNA

Then, methylated and unmethylated human genomic DNAs were tested after bisulfite treatment followed by MSP, Combined Bisulfite Restriction Analysis (COBRA, Fig. 6A), and electrochemical measurements (Fig. 6B, C).

Figure 6A shows the results of the MSP and COBRA methods. Methylated genomic DNA was treated with bisulfite and amplified with a 121-bp target region as the MSP. Since the MSP product has a CGCG sequence in three sites digestible with AccII, the methylation of these sites was detected with the COBRA method. When all the CGCG sites were digested with AccII, bands of 21-, 46-, 8-, and 46-mer were obtained (Fig. 6D). the MSP product from the commercially available genomic DNA produced three bands at 21, 46, and 54 bp, but the band at 121 bp was missing after the COBRA method. Differently from the 46-bp and 54-bp bands, the 21-bp band is not contained in the target region of the M5(+) DNA probe. The existence of the 54-bp band indicates that the third or fourth CG sites are not methylated. On the other hand, the 126-bp band was only observed in the unmethylated genomic DNA in the gel electrophorogram after bisulfite treatment and subsequent PCR amplification with unmethylated specific DNA primer. Four bands at 121, 77, 68, and 49 bp were observed after COBRA of the PCR product of the unmethylated genomic DNA. This result shows that this DNA was a mixture of unmethylated DNA and M5(+) DNA methylated at the third or fourth GC site.

Table 3 shows the methylated cytosine content obtained from the sequencing of these DNAs. Part of the fourth or fifth GC sites was unmethylated in the M5(+) sequence of the methylated genomic DNA, which was in good agreement with the results from COBRA. In the unmethylated genomic DNA, all the methylated GC sites were a mixture of methylated or unmethylated state and the methylation frequency was higher at the second, third, and fourth GC sites (70%), which was also is good agreement with the COBRA results.

Figure 6B and C show the results of the electrochemical detection with the six DNA-probe electrodes after hybridization with the MSP sample from the methylated genomic DNA. The current increase was 96, 61, 52, 34, 28, or 29% for M5(+), M4(+), M3(+), M2(+), M1(+), or M0(+) DNA probe, respectively. These current increases are similar to that of M5(−), as shown in Fig. 4A, and indicate that the target MSP product has almost completely methylated cytosines in the GC sites. Similarly, the current increase in the case of M5(+), M4(+), M3(+), M2(+), M1(+), or M0(+) probe was 17, 38, 36, 59, or 16%, respectively, after hybridization with the unmethylated genomic DNA. By comparison with the current-based increasing ratio obtained in Fig. 4, these current increases show that the expected number of mismatched bases is 3, 2, 2, 2, 1, or 3, respectively,. Although, according to the above observation, the sequence of this sample is probably M1(+), this sample was concluded to be a mixture from 59% of the current increase.

Detection of the PCR product from clinical samples using the multi-electrode chip

The multi-electrode chip used in this study, which carries 10 working electrodes, was developed with a dedicated electroanalyzer, M-AN100. Ten DNA probes were immobilized on this chip and treated with the PCR products derived from the clinical samples, which are genomic DNA from tissue slices from the cancer patient and exfoliated cells from the healthy volunteer treated with bisulfite and subsequently subjected to MSP. Figure 7A or B shows the result for the cancer patient or healthy volunteer, respectively, after hybridization with 2.0 ng/μL of each PCR product in 2× SSC for 2 h at 20 °C. In the case of cancer patient, the highest current increase measured was 85% for the M4′(+) probe, with 60% for M5(+), and below 40% for other DNA probes. Since full methylation would give 100%

current increase, as shown in Fig. 4, this sample was expected to contain mainly the M4´ (+) sequence. In the case of the healthy volunteer, the highest current increase observed was 118% for the M1(+) probe, while 40–60% current increases was observed for the other probes. This result indicates that the sample from the healthy volunteer mainly contains singly methylated DNA and small amounts of other differently methylated DNAs. This result is reasonable and the procedure based on the multi-electrode chip system could become a convenient screening method.

Conclusion

DNA probes carrying up to five methylation sites were designed and used for the detection of methylation on PCR products and genomic DNA. Up to 90% current increase was observed for the fully matched combinations of the probe and sample. The current increase remained approximately 50% for singly mismatched combinations and was 20–30% for doubly mismatched combinations. Therefore, we conclude that the number of methylated sites on genomic DNA can be deduced from the magnitude of the current increase.

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Table 1 Probe DNA sequences

Probe DNA	Sequence
M0 (+)	5′-HO-(CH2)6-S-S-(CH2)6-TAG TTG TGT TTA TGT GTT TTT GTT-3′
M1 (+)	5′-HO-(CH2)6-S-S-(CH2)6-TAG TCG TGT TTA TGT GTT TTT GTT-3′
M2 (+)	5′-HO-(CH2)6-S-S-(CH2)6-TAG TCG CGT TTA TGT GTT TTT GTT-3′
M3 (+)	5′-HO-(CH2)6-S-S-(CH2)6-TAG TCG CGT TTA CGT GTT TTT GTT-3′
M4 (+)	5′-HO-(CH2)6-S-S-(CH2)6-TAG TCG CGT TTA CGC GTT TTT GTT-3′
M5 (+)	5′-HO-(CH2)6-S-S-(CH2)6-TAG TCG CGT TTA CGC GTT TTC GTT-3′
M4′ (+)	5′-HO-(CH2)6-S-S-(CH2)6-TAG TTG CGT TTA CGC GTT TTC GTT-3′
M3′ (+)	5′-HO-(CH2)6-S-S-(CH2)6-TAG TTG TGT TTA CGC GTT TTC GTT-3′
M2′ (+)	5′-HO-(CH2)6-S-S-(CH2)6-TAG TTG TGT TTA TGC GTT TTC GTT-3′
M1′ (+)	5′-HO-(CH2)6-S-S-(CH2)6-TAG TTG TGT TTA TGT GTT TTC GTT-3′

Table 2 Sequences of the PCR products carrying no, two, and five methylated sites

Sample	Sequence
M5(−)	3′-36 mer - ATCAGCGCAAATGCGCAAAAGCAA - 61 mer-5′
M3(−)	3′-36 mer - ATCAGCGCAAATGCACAAAAACAA -61 mer-5′
M0(−)	3′-39 mer - ATCAACACAAATACACAAAAACAA -63 mer-5′

Table 3. Methylated cytosine content from sequencing

No. of CG*	1	2	3	4	5
MG	100%	100%	100%	80%	70%
UG	57%	71%	70%	70%	62%

* M5(+); 3′-TAGTC1GC2GTTTAC3GC4GTTTTC5GTT-5′

Figure Legends

Fig. 1. (A) Chemical structure of ferrocenylnaphthalene diimide (FND) and (B) electrochemical discrimination between methylated and unmethylated DNA by the FND-based hybridization assay.

Fig. 2. (A) Multi-electrode chip before (a) and after screen masking of the electrode (b). (c) The reference electrode was covered by Ag/AgCl ink. (B) Chip holder (a) and electroanalyzer (IM-AN100).

Fig. 3. Current increases of the M5(+) DNA probe-immobilized electrode after hybridization with 0.1–4.0 ng/μL of M5(−) or M0(−) DNA sample.

Fig. 4. Current increases of M5(+) DNA probe-immobilized electrode after hybridization with 0.1−4.0 ng/μL of the mixture between M5(−) and M0(−). MG% is the percentage of M5(-) present in the total samples: MG% = M5(-)/[M5(-)+M0(-)]

Fig. 5. Current increases for the DNA probe-immobilized electrodes carrying M0–M5(+) after hybridization of the 2.0 ng/μL PCR probes carrying M5(−) (A), M3(−) (B), or M0(−) (C). The electrochemical measurements were conducted in 0.10 M AcOH-AcOK (pH 5.5) containing 5.0 μM FND and 0.10 M KCl at 20 °C.

Fig. 6. (A) Results of COBRA, (B) current increases for the DNA probe-immobilized electrodes carrying M0–M5(+) after hybridization of 2.0 ng/μL methylated human genomic DNA (MG)s. (C) Current increases for the DNA probe-immobilized electrodes carrying M0–M5(+) after hybridization of 2.0 ng/μL unmethylated human genomic DNA (UG)s. The electrochemical measurements were conducted in 0.10 M AcOH-AcOK (pH 5.5) containing 5.0 μM FND and 0.10 M KCl at 20 °C. (D) Cleaving patterns of MG and UG by COBRA.

Fig. 7. Current increases of the multi-electrode chip after hybridization with the genomic DNAs from the tissue slice from cancer patient (A) and exfoliated cell from the healthy volunteer (B) treated with bisulfite and subsequently subjected to the MSP method.

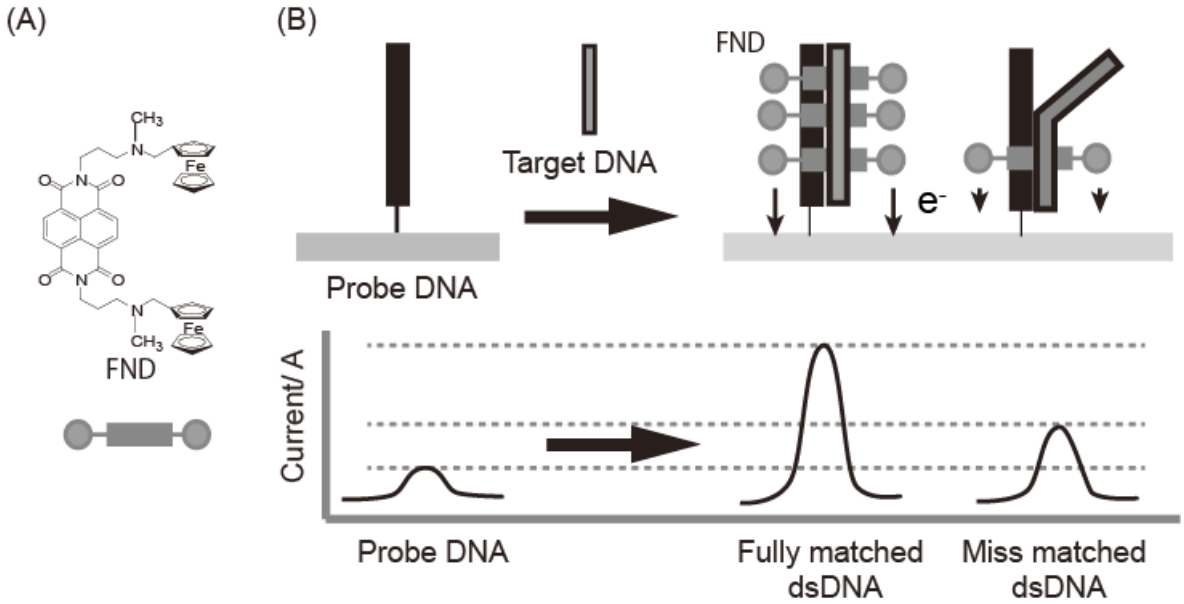

Fig. 1. (A) Chemical structure of ferrocenylnaphthalene diimide (FND) and (B) electrochemical descrimination between methylated and unmethylated DNA by the FND-based hybridization assay.

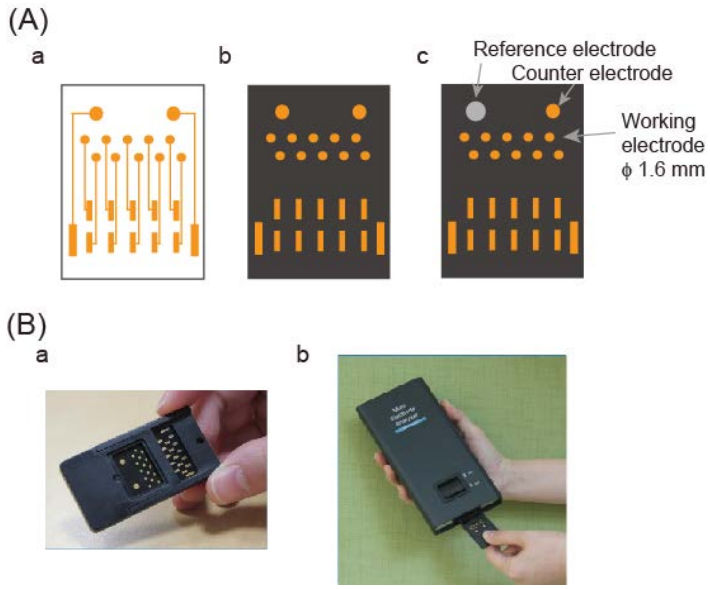

Fig. 2. (A) Multi-electrode chip developed by the Authors before (a) and after screen masked electrode (b). (c) Reference electrode was covered by Ag/AgCl ink. (B) Chip holder (a) and electro analyzer, IM-AN100.

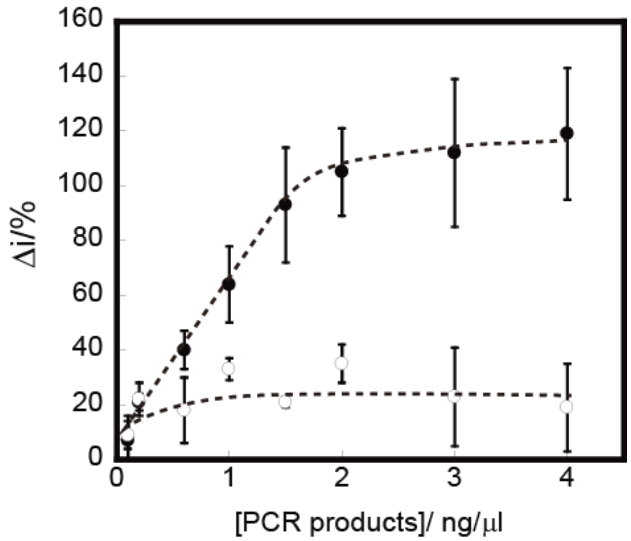

Fig. 3. Current increases of M5(+)DNA probe-immobilized electrode after hybridization with 0.1-4.0 ng/μL of M5(-) or M0(-) DNA sample.

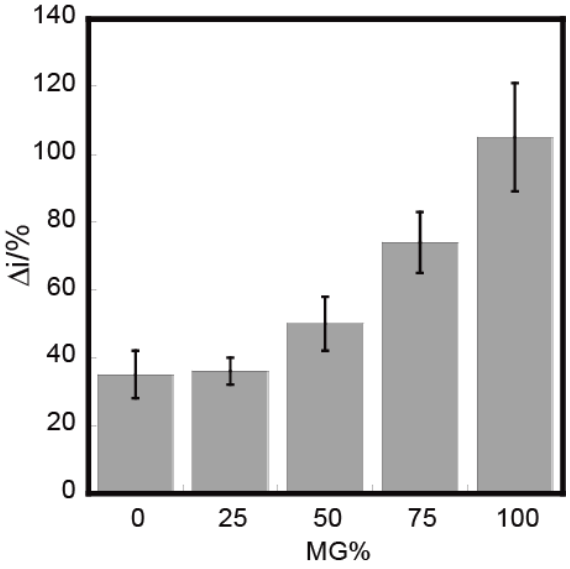

Fig. 4. Current increases of M5(+)DNA probe-immobilized electrode after hybridization with 0.1-4.0 ng/μL of the mixture between M5(-) and M0(-). MG% means the percentage of M5(-) is contained in the total samples. MG% = M5(-)/[M5(-)+M0(-)]

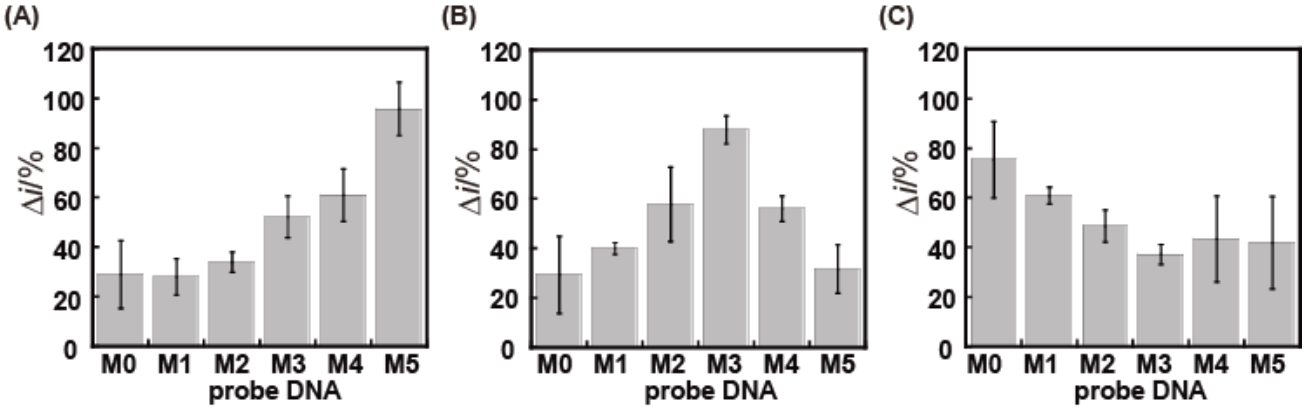

Fig. 5. Current increases for DNA probe-immobilized electrodes carrying M0 - M5(+) after hybridization of 2.0 ng/μL PCR probes carrying M5(-) (A), M3(-) (B), or M0(-) (C) Electrochemical measurements were conducted in 0.10 M AcOH-AcOK (pH 5.5) containing 5.0 μM FND and 0.10 M KCl at 20 °C.

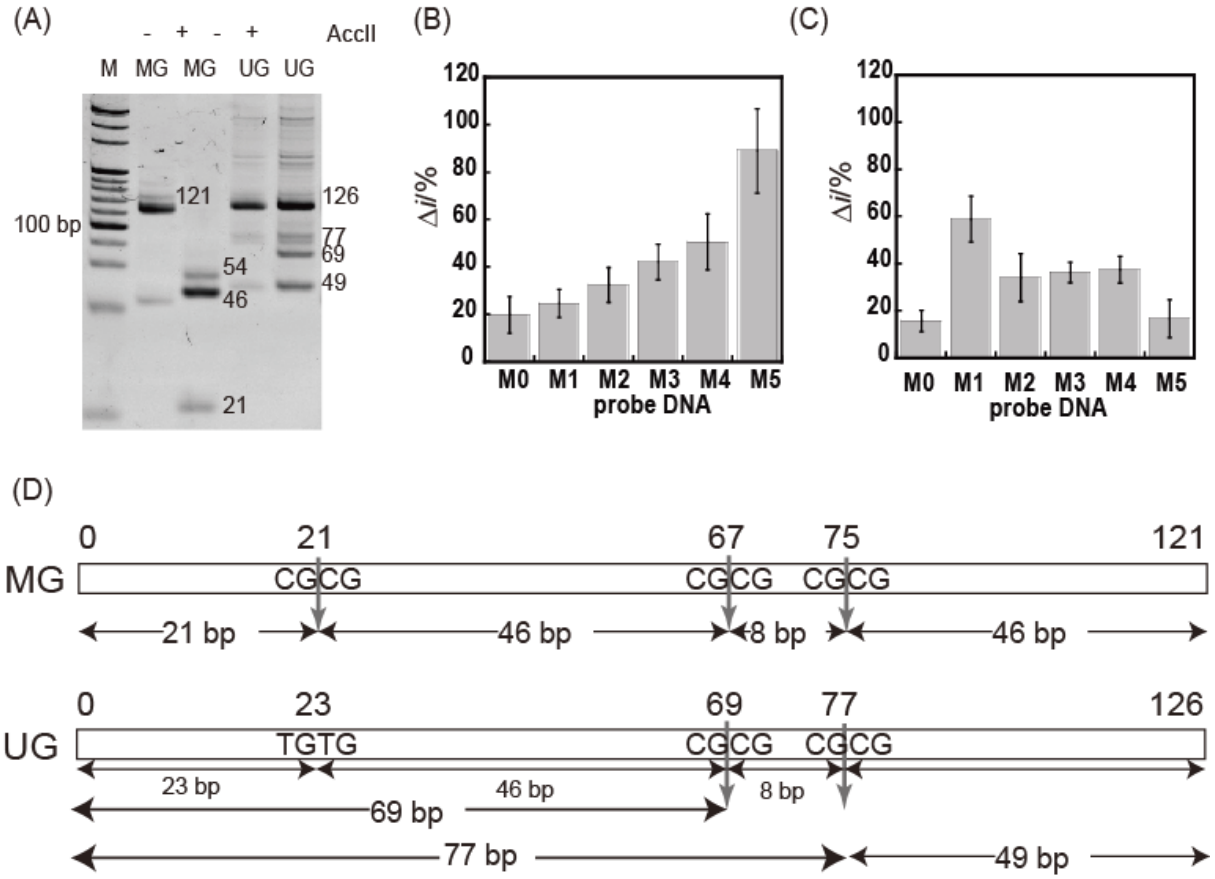

Fig. 6. (A) Results of COBRA, (B) Current increases for DNA probe-immobilized electrodes carrying M0–M5(+) after hybridization of 2.0 ng/μL methylated human genomic DNA (MG)s. (C) Current increases for DNA probe-immobilized electrodes carrying M0– M5(+) after hybridization of 2.0 ng/μL unmethylated human genomic DNA (UG)s. Electrochemical measurements were conducted in 0.10 M AcOH-AcOK (pH 5.5) containing 5.0 μM FND and 0.10 M KCl at 20 °C. (D) Cleaving patterns of MG and UG by COBRA.

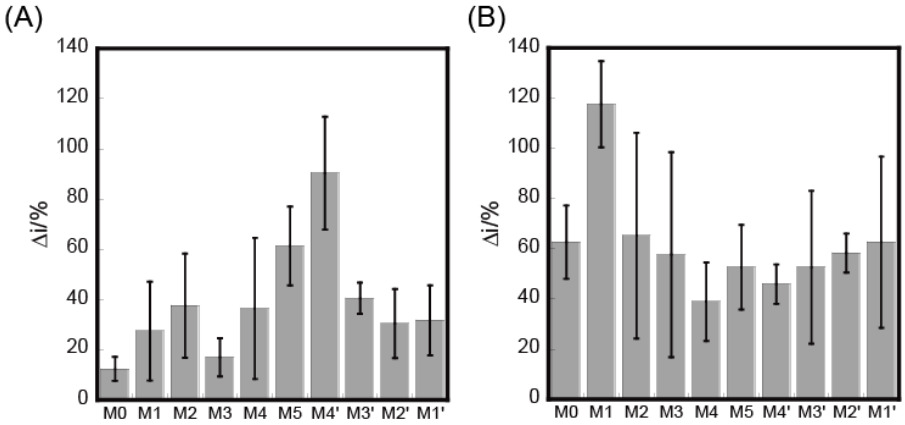

Fig. 7. Current increasese of multi electrode chip after hybridization with genome DNAs from tissue slice from cancer patient and exfoliated cell (A) and healthy volunteer (B) and treated with bisulfite and subsequently carried out by MSP method.