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A novel method of identifying genetic mutations using an electrochemical DNA array

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ABSTRACT

We describe the development of a new type of DNA array chip that utilizes electrochemical reactions and a novel method of simultaneously identifying multiple genetic mutations on an array chip. The electrochemical array (ECA) uses a threading intercalator specific to double-stranded nucleotides, ferrocenylnaphthalene diimide (FND), as the indicator. ECA does not require target labeling, and the equipment is simple, durable and less expensive. The simultaneous multiple mutation detection (SMMD) system using an ECA chip and FND utilizes an enzyme to simultaneously distinguish several genetic mutations such as single nucleotide polymorphism (SNP), insertion, deletion, translocation and short tandem repeat. We examined this SMMD system using an ECA chip, by detecting seven different mutations on the lipoprotein lipase (LPL) gene for 50 patients in a blind test. It turned out that all the results obtained were concordant with the sequencing results, demonstrating that this system is a powerful tool for clinical applications.

INTRODUCTION

The DNA chip is a very powerful tool for genetic analysis, and it is applied for gene expression analysis and gene mutation detection. Genomic mutation analysis, such as single nucleotide polymorphism (SNP) detection, is particularly essential in the fields of drug response prediction, drug side-effects prediction and preventive medicine. Several SNP analysis reports were performed in a chip format, and most of these utilize the difference in the melting temperature (T_m) resulting from a single nucleotide mismatch in double-stranded nucleotides. This T_m difference caused by a single nucleotide mismatch is very small, and in previous reports multiple probes (1), temperature control (2) or electrical fields (3) have been utilized to detect such minute differences. Instead of applying such types of physical force, an enzymatic approach to distinguish a single nucleotide difference (4,5) was also reported.

Irrespective of the approach taken to distinguish a single nucleotide difference, fluorescence emission detection is the most commonly used technology for visualizing such differences. Although fluorescent dyes are readily available and easy to handle, the detection system for fluorescence emission requires optical components, which tend to be expensive and fragile. Additionally, the dye itself is cost consuming. Instead of such optical detection methods, electrochemical detection methods are gaining popularity and a wide variety of systems are available [for review, see (6–8)]. As an example, catalytic currents generated due to the oxidation of immobilized guanines on electrodes were measured for triplet repeat detection (9). Reporter molecules with oxidation–reduction properties (10–12) or π stacks of DNA (13) were used to detect double-stranded nucleotides. These methods convert the nucleotide property to an electrical signal, and the system itself is composed of a relatively simple electrical amplifier, that is less expensive and more compact than the fluorescence detection system. Recent advancement makes electrochemical detection methods further attractive. For instance, incorporation of new material such as carbon nanotubes is improving its sensitivity (14), and spatial separation of hybridization and electrochemical detection is enhancing target specificity (15).

We have been studying a DNA sensor that uses an oligonucleotide probe-immobilized gold electrode and ferrocenylnaphthalene diimide (FND) as the electrochemically active indicator (16,17). Recently, we reported the application of this system for SNP detection (18,19). In these systems, detection of a mutation depends on the T_m difference between the completely matched and the mismatched probe–target pair. This DNA sensor was evaluated by detecting two types of human lipoprotein lipase (LPL, EC 3.1.1.34) gene mutations (18).

Human LPL is a glycoprotein enzyme with a molecular mass of 61 kDa (20), and it is mainly synthesized in adipocytes (21). The human LPL cDNA predicts a translated molecular weight of 50 394 with 448 amino acid residues in the mature form of the protein and in the absence of any sugar moiety (22). The human LPL gene, 30 kb in length, is located on chromosome 8p22 (23) and consists of 10 exons

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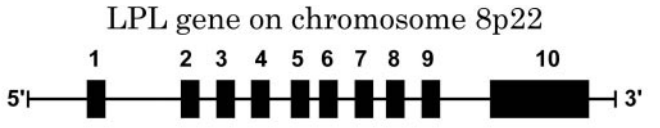

LPL mutation	Exon		Mutation site	Enzyme for the detection of the mutation
Y61X	3	Nonsense	TA<u>T</u>(438) -> A	EcoT22I
V200A	5	Missense	G<u>T</u>(854)T -> C	PvuII
A221-del	5	Deletion	<u>G</u>(916) -> deletion	AluI
R243C	6	Missense	<u>C</u>(982)GC -> T	Eco47III
A261T	6	Missense	<u>G</u>(1036)CC -> A	StuI
A334T	7	Missense	<u>G</u>(1255)CC -> A	StuI
W382X	8	Nonsense	TG<u>G</u>(1401) -> A	MboII

Figure 1. LPL gene and properties of the seven LPL mutations used in this study. The substituted or deleted nucleotides are underlined.

(24–26): Exons 1 through 9 contain coding regions, whereas exon 10 contains a 3′ non-coding region (Figure 1). The LPL protein is anchored on the cell surface by a proteoglycan chain (27). It plays a key role in regulating triglyceride levels in circulation by hydrolyzing the triglycerides in chylomicrons and very low-density lipoproteins (VLDL) at the first step in their metabolism (28). The free fatty acids released from the triglycerides are either used as energy or re-esterified for endogenous triglyceride storage in adipocytes.

Homozygous or compound heterozygous LPL deficiency is a rare autosomal recessive genetic disorder and its frequency is estimated to be one per million population (29). The disease causes severe fasting hypertriglyceridemia (type I hyperlipoproteinemia) due to massive accumulation of chylomicrons and normal or marginally increased VLDL. This disease is often present in infancy or early childhood with episodes of abdominal pain, pancreatitis, xanthomatosis, lipemia retinalis and hepatosplenomegaly (29). Acute pancreatitis is occasionally a fatal complication of the disease. Heterozygous LPL deficiency usually reveals a normolipidemic state. However, this may cause mild hypertriglyceridemia (type IV hyperlipoproteinemia), if heterozygotes are exposed to factors that lead to overproduction of VLDL in the liver, such as high alcohol intake (30,31) and/or a hyperinsulinemic state (31). Recent prospective epidemiological studies have shown that hypertriglyceridemia elevates the risk of coronary heart disease (32).

Clinically, a first screening for primary LPL deficiency is performed by analyzing the LPL activity (29) and the immunoreactive LPL mass (29,33) in plasma obtained after injection of heparin (post-heparin plasma; PHP). Abnormalities of the LPL gene in subjects with LPL deficiency have been detected by analysis of single-strand conformation polymorphism (SSCP) (34), and mutation sites in the LPL gene have subsequently been identified by DNA sequencing. To date, over 60 distinct mutations in the LPL gene have been reported in various ethnic groups, including Japanese people (29). Most mutations are characteristic of specific ethnic groups (29), but the G188E mutation resulting in a non-functional LPL has been identified in various ethnic groups (35–37). This ethnic specificity of the LPL gene impedes an easy scan for LPL gene aberrations in patients with type I or type IV hyperlipoproteinemia. Therefore, developing a simple and reliable screening method for several specific mutations is important for rapid genetic diagnosis of LPL deficiency.

In this study, we report a novel genetic mutation analysis system—the simultaneous multiple mutation detection (SMMD) system, which uses an electrochemical array (ECA) chip and FND. This system utilizes enzymatic recognition instead of T_m difference for single nucleotide mismatch detection. Hence, the system does not require precise temperature control, which is difficult to achieve and expensive; therefore, the system can be built in a very compact and inexpensive form. We demonstrate the validity of the SMMD system using an ECA chip, by examining 50 patient samples and using the LPL gene as a model system. Four missense mutations, V200A (38), R243C (38), A261T (38) and A334T (unpublished data)], one deletion mutation, A221-del (Arita) (30), and two nonsense mutations, Y61X (38) and W382X (37), were studied.

MATERIALS AND METHODS

Genomic DNA extraction

Genomic DNA was isolated from the peripheral whole blood of subjects with LPL mutation of Y61X, V200A, A221-del, R243C, A261T, A334T and W382X, and of 42 subjects in whom LPL genes were confirmed to be normal by PCR–RFLP analyses using restriction enzyme specific to the seven individual LPL mutations.

Primer and probe design

Table 1 shows primers and probes used for this experiment. All oligonucleotides used in this research were custom-synthesized by Qiagen K.K. (Tokyo, Japan). The sequences of these synthesized oligonucleotides are listed in Table 1.

LPL gene and properties of the seven LPL mutations used in this study as a model

LPL gene consists of 10 exons (Figure 1), and exons 1 through 9 are coding regions, whereas exon 10 is a non-coding region. The seven LPL mutations used in this study are Y61X (exon 3), V200A (exon 5), A221-del (exon 5), R243C (exon 6), A261T (exon 6), A334T (exon 7) and W382X (exon 8). Their mutation sites are described in Figure 1, confirmed by both sequencing and PCR–RFLP method using the restriction enzymes listed in Figure 1.

In vitro preparation of plasmid DNAs containing wild or mutant DNA fragments of the LPL gene and their application in developing criteria for the judgment of zygosis

Exons 3, 5, 6, 7 and 8, including their flanking regions of the LPL gene, were amplified by the PCR using the primer pairs (Table 1a) from the genomic DNA of a subject in whom LPL gene was confirmed to be normal by sequencing or in subjects carrying the seven mutations listed in Figure 1. The PCR products containing wild DNA fragments of the exons 3 (340 bp), 5 (337 bp), 6 (353 bp), 7 (202 bp) or 8 (205 bp), and those containing each mutation of Y61X (exon 3), V200A (exon 5), A221-del (exon 5), R243C (exon 6), A261T (exon 6), A334T (exon 7) or W382X (exon 8) were purified with QIAquick spin columns. The purified PCR products were ligated into pCR2.1 vectors. The recombinant DNAs were

Table 1. Primers and probe sequences

(a) First PCR primer	
Ex3-FP	CTGTGCCAATGGGTTTCCA
Ex3-RP	CACTGTTTTGGACACATAAGTCTC
Ex5-FP	GAAATTTACAAATCTGTGTTCCTGCT
Ex5-RP	CATTGGGTCAATAAGGGTTAAGGA
Ex6-FP	AGACATGCCAAATGAAACACTCT
Ex6-RP	ACTCCTTGGTTTCCTTATTTACAACA
Ex7-FP	TTCATAAAGATTGATCAACATGTTCGA
Ex7-RP	ACTGGTGCCATGATGACCG
Ex8-FP	GAGAGCTGATCTCTATAACTAACCA
Ex8-RP	CTCTGATCTTCTGAATGGCGA
(b) Second PCR primer	
Y61X-CP	TAGGTGGGTATTTTAAGAAAGCTTGT
Y61X-SP	GAGAGTTGGGTGCCTCTCTCTTGTACAGGGCGGCC-ACAAG
V200A-CP	GTAGACGTCTTACACACATTCACCAGAGGG
V200A-SP	TGGGCATGTTGACATCCTGGCTGAAAAGTACCTCC-ATTCGGG
A221-del-CP	CAGTTGGGCATGTTGACATTTACCCG
A221-del-SP	CTATCCGCGTGATTCTTCTAAATAATATTTACCTCC-AAGTCCTCTCTCTGC
R243C-CP	GCACCTGTAGGCCTTACTTGGATTTTCT
R243C-SP	CTCGTGGGAGCACACCCAGATGTGGACCAGCTAG-TGAA
A261T-CP	CCCACGAGCGCTCCATTCATCTCT
A261T-SP	CCTACAGGTGCAGTAGAGCCCTTTCTCAAAGGCT-TCCTTGG
A334T-CP	CCTCCCCAACAGTCTTCCATTACCAAGTAAAG
A334T-SP	CCTTTGAGATTTCTCTGGGATGTTCTCACTCTCGG-CCACGGTGCCAT
W382X-CP	AGACCTACTCCTTCCTAATTTACACAGAGGTAG
W382X-SP	AAGAGTGATTCATACTTTCTGCTCCACCAGTCTG-ACCAGC
(c) Probe	
Y61X-W probe	AAAGCTTGTGTCATCATCTTCAGGTAACAGGAAT-GTA**T**
Y61X-M probe	AAAGCTTGTGTCATCATCTTCAGGTAACAGGAAT-GTA**A**
V200A-W probe	GGGTCCCCTGGTCGAAGCATTGGAATCCAGAAAC-CAG**T**
V200A-M probe	GGGTCCCCTGGTCGAAGCATTGGAATCCAGAAAC-CAG**C**
A221-del-W probe	TGGAGGTACTTTTCAGCCAGGATGTAACATTGGA-GAA**G**
A221-del-M probe	ATGGAGGTACTTTTCAGCCAGGATGTAACATTGG-AGA**A**
R243C-W probe	TCATTCAACAGAGAGTCGATGAAGAGATGAATGG-AGC**G**
R243C-M probe	TCATTCAACAGAGAGTCGATGAAGAGATGAATG-GAGC**A**
A261T-W probe	CATCGACTCTCTGTTGAATGAAGAAAATCCAAGT-AAG**G**
A261T-W probe	CATCGACTCTCTGTTGAATGAAGAAAATCCAAGT-AAG**A**
A334T-W probe	TTTTTCTGGGACTGAGAGTGAAACCCATACCAAT-CAG**G**
A334T-M probe	TTTTTCTGGGACTGAGAGTGAAACCCATACCAAT-CAG**A**
W382X-W probe	TAGATATTGGAGAACTACTCATGTTGAAGCTCAA-ATG**G**
W382X-W probe	TAGATATTGGAGAACTACTCATGTTGAAGCTCAA-ATG**A**

In (a), FP denotes 'forward primer' and RP denotes 'reverse primer'. In (b), CP and SP indicate 'counter primer' and 'special primer', respectively. The underlined nucleotides in the SP are the tag sequences essential to make a stem structure in a self-loop formation (cf. Figure 3D). In (c), W probe and M probe indicate 'wild-type probe' and 'mutant-type probe', respectively.

transformed into transfection-competent *Escherichia coli* cells using an Original TA Cloning Kit (Invitrogen, Carlsbad, CA). The presence or absence of the individual mutations was verified by DNA sequencing. The plasmid DNAs with or without the mutations were amplified by PCR using the primer pairs (Table 1a), and purified with QIAquick spin columns. The PCR products amplified from the plasmid DNAs without the mutations were used for hybridization experiments as wild homozygotes, whereas those from the plasmid DNAs with the mutations were used as mutant homozygotes. In the preparation of wild/mutant heterozygote, plasmid DNAs containing each mutation and the corresponding wild DNA were mixed in an equal ratio, amplified by PCR using the primer pairs (Table 1a), and used as heterozygotes for the hybridization experiments.

PCR amplification and purification of PCR products

The two-stage PCR reactions are essential for detection of SNP by an SMMD system using ECA chip. The first PCR was performed to amplify the regions with SNPs from plasmid and genomic DNA; the second was to make a specific target for the SMMD reaction from the first PCR products. In the first PCR, 25 ng of genomic DNA or 0.05 ng of plasmid DNA was added as template to 50 µl reaction solution that contained 0.2 µM of each primer (Table 1a), 2.5 U Taq polymerase-Hot Start version (Takara Bio, Shiga, Japan), 0.25 mM dNTP and 1× enzyme buffer. PCR consisted of an initial activation of polymerase at 95°C for 5 min, followed by 40 cycles of PCR amplification (annealing at 60°C for 90 s, elongation at 72°C for 10 s and denaturation at 95°C for 30 s), and final elongation at 72°C for 10 min. Five microliters of that PCR product (not purified) was added to 50 µl of the second PCR solution that contained 2.5 µM of each special primer (SP) (Table 1b), 0.625 µM of each counter primer (CP) (Table 1b), 2.5 U Taq polymerase-Hot Start version, 0.25 mM dNTP and 1× enzyme buffer. Two primer sets were prepared in order to amplify three SNPs target with one set. Each set was combined with the SP and CP primers of Y61X/V200A/W382X or A221-del/R243C/A334T. The target to detect A261T was amplified by itself without a mix of primers. In order to amplify single-strand-specific targets that were hybridized to probe on ECA tips, the concentration of the SP was 4-fold higher than that of the CP, and the second PCR was designated as asymmetric PCR (A-PCR). The A-PCR consisted of an initial activation of polymerase at 95°C for 5 min, followed by 40 cycles of PCR amplification (annealing at 65°C for 90 s, elongation at 72°C for 10 s and denaturation at 95°C for 30 s), and final elongation at 72°C for 10 min. The A-PCR products were subjected to hybridization and electrochemical measurement by the SMMD system using an ECA chip without purification.

Preparation of ECA chip immobilized DNA probe

Mutant probes (M probes listed in Table 1c) for the seven LPL gene mutations and the corresponding wild probes (W probe listed in Table 1c) were immobilized on individual gold electrodes of an ECA chip (Figure 2A) as described earlier (17).

Hybridization, ligation and electrochemical measurement process

Prior to hybridization of the A-PCR products to the W probe or M probe immobilized on the gold electrodes of an ECA chip, the ECA chip was denatured with 0.5 M NaOH for 5 min and

washed with MiliQ water. Following this, the base line measurement (I_0) was carried out. The measurement solution was prepared with the following components: 1 ml of 0.2 M acetate–acetic buffer (pH = 5.6), 200 µl of 1 M KCl, 700 µl of MiliQ water and 100 µl of 1 mM FND (synthesized at Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). The electrical potential was applied from 0 to 600 mV versus Ag/AgCl, and I_0 was recorded by subtracting the base line current from the peak current at 440 mV automatically by the equipment. The A-PCR products (35 µl) (non-purified) and 15 µl of 20× SSC were mixed to final 50 µl of hybridization solution. The mixture was applied to the cellulose acetate membrane on the electrodes of ECA chip placed in a wet box containing 6× SSC that allowed hybridization of the A-PCR products to the probes after incubation at room temperature for 10 min. Then the cellulose acetate membrane was removed, and another new membrane was put on the ECA chip. The ECA chip with the membrane was placed in a wet box containing MiliQ water, and 50 µl ligase solution containing 2.8 Weiss Unit (39) T4 DNA ligase (Takara Bio Inc., Shiga, Japan) and 1× ligase buffer was applied to the membrane. Ligation reaction between the A-PCR products and the probes was carried out at room temperature for 5 min. Following this, the ECA chip was denatured with 0.5 M NaOH for 5 min, washed with MiliQ water and immersed into the measurement solution described above. Measurement of electric current (I_1) was performed at room temperature with an electrochemical analyzer (Figure 2B). The results were evaluated by the score calculated from the following equation.

Score = $\alpha/\delta - \beta/\delta$, where α is ΔI of W probe; β is ΔI of M probe; and δ is the average of α and β. Here, ΔI is the hybridization/ligation efficiency of particular probe and calculated as $\Delta I = (I_1 - I_0)/I_0 \times 100$.

RESULTS

Principle of SMMD system using an ECA chip and FND

Previously, we reported a DNA sensor for detecting SNP, in which the gold electrode had an immobilized oligonucleotide probe, and FND was used as the indicator (18,19). In this system, the T_m difference between a completely matched probe–target pair and a single nucleotide mismatched probe–target pair was utilized to detect SNP. This DNA sensor was applied to detect mutation in two types of human LPL gene (18). Consequently, the DNA sensor was fabricated in an array format (Figure 2A) and this ECA had 25 gold electrodes on a ceramic base. An electrochemical reaction measuring device was also manufactured (Figure 2B). This ECA chip reader could measure a maximum of 10 µA at a 1 nA resolution, and approximately 3 min was required to read a 25 electrode chip.

The principle of the SMMD method is shown in Figure 3. Initially, the first PCR product from the genomic DNA is used as the template for A-PCR (Figure 3A). The A-PCR is carried out using CP and SP in the ratio of 1:4. The SP contains the sequence (tag sequence) that is complementary to the sequences (yellow-colored sequences) between the mutation point and the special primer (Figure 3B). The A-PCR product forms a self-loop and stem structure between the tag sequence and the yellow-colored sequence (Figure 3D). The electrical current, designated as I_0, is measured in a buffer containing FND, prior to hybridization between the self-looped A-PCR product and the probe immobilized on the gold electrode (Figure 3E). After the measurement of I_0, the self-looped A-PCR product is hybridized with probes fixed on the gold electrodes of an ECA chip (Figure 3F, right and left). The ligation reaction follows the hybridization reaction (Figure 3F). The probes on the gold electrodes are prepared so that the 3′ end nucleotide is complementary to either the wild-type or the mutant-type A-PCR product. The wild-type probe (W probe) has the same nucleotide as the wild-type genome at the 3′ end (Figure 3E, right), and the mutant-type probe (M probe) has the same nucleotide as the mutant-type genome (Figure 3E, left). When the A-PCR product is hybridized with the W probe or the M probe, the ligase enzyme recognizes the matched or unmatched nucleotide pair at the 3′ end of the probe. If the 3′ end nucleotide is complementary (or matched), the enzyme repairs the nick between the 3′ end of the probe and the 5′ end of the A-PCR product. As a result, the probe and the A-PCR product become one molecule. However, if the last nucleotide is not complementary (or is mismatched), the enzyme does not repair the nick, and the probe and the A-PCR products remain as two different molecules. After the ligase reaction, the ECA chip is denatured, and the hydrogen bonds between the probe and the

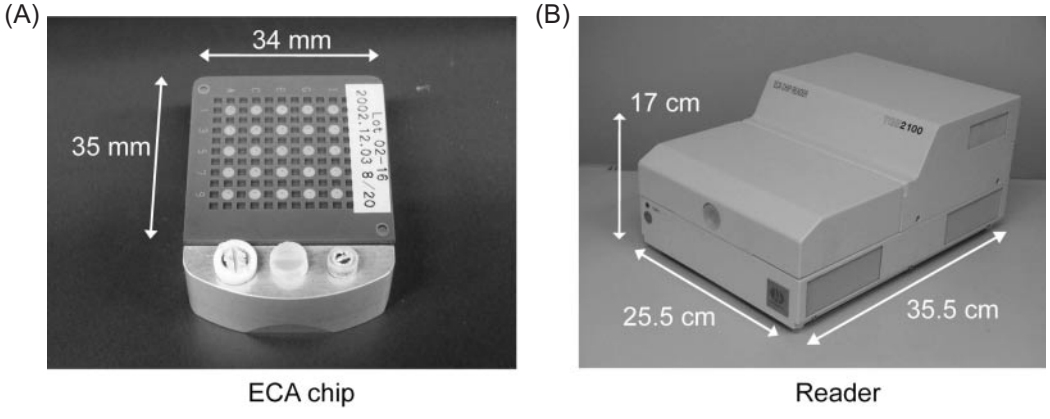

Figure 2. An ECA chip and an ECA chip reader. (**A**) The ECA chip has 25 gold electrodes with a ceramic base. The left electrode in the front is the reference electrode, and the one to the right is the counter electrode. (**B**) The ECA chip reader is compact and durable. It weighs only 7 kg.

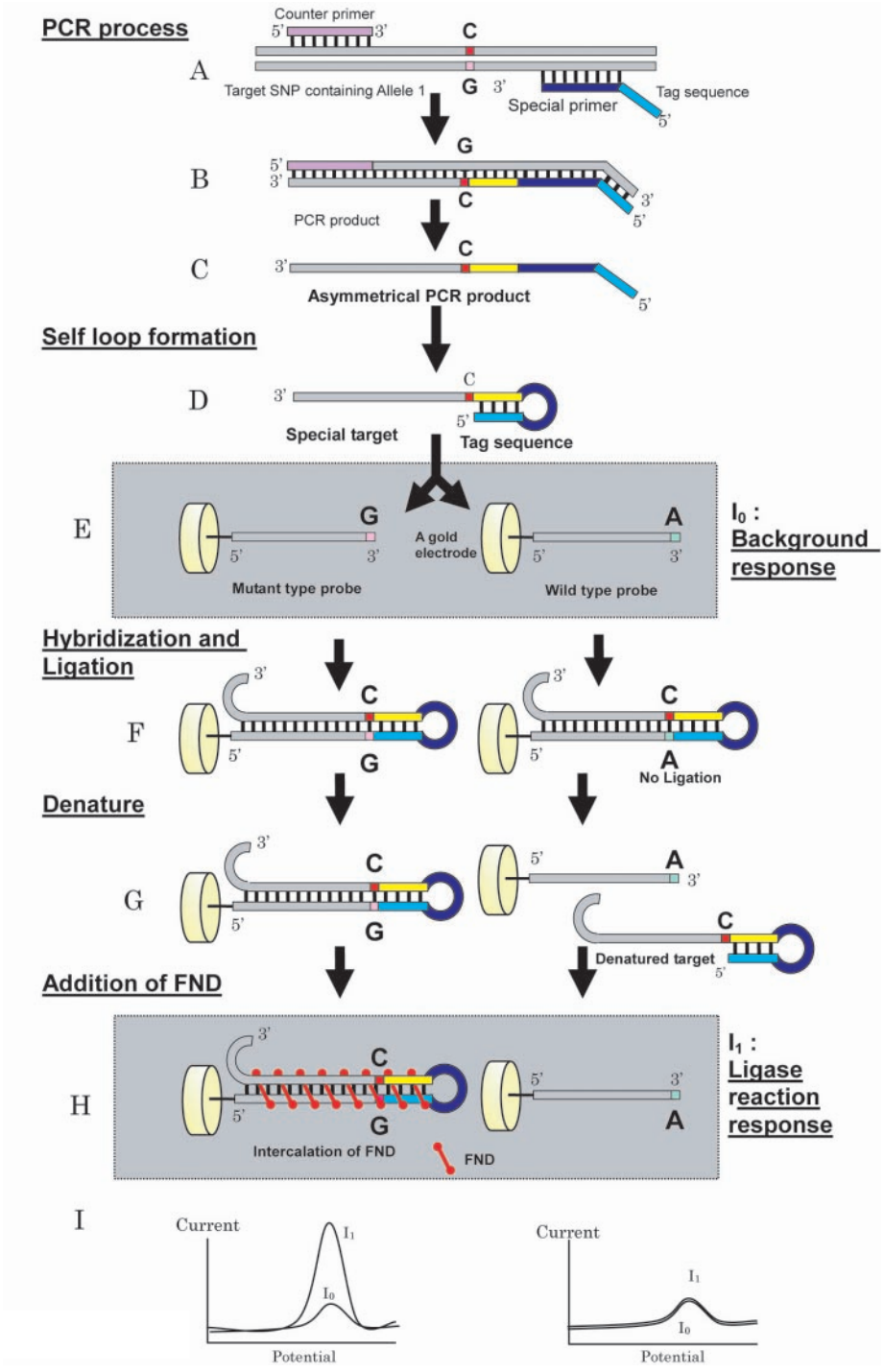

Figure 3. The process for the determination of mutations by the SMMD system using an ECA chip and FND. As the first step, asymmetric PCR (A-PCR) was performed with counter primer and special primer in the ratio of 1:4 and the first PCR product, as a template, from genomic DNA (**A**). The special primer contains the Tag sequence (cyan) at its 5′ end, which is a complement to the genomic region [yellow-colored region in (**B**)] between the mutation point (red) and the special primer. Since the A-PCR product contains self-complemental sequences [cyan- and yellow-colored sequences in (**C**)], it forms a self-loop (**D**). Before the hybridization and ligation reactions of the self-looped A-PCR product and a probe immobilized on the ECA chip, electric current (I_0, base line current response) was measured in a buffer containing FND (**E**). After the measurement of I_0, the self-looped A-PCR product was hybridized with two types of probes, a wild type and a mutant type, fixed on individual electrodes of the ECA chip. If the 3′ end nucleotide of the probe is completely matched with the self-looped A-PCR product, indicated as a bar, denoting hydrogen bond between red (c, cytosine) and pink (g, guanine) nucleotides, the enzyme ligase glues the 3′ end nucleotide on the probe with the 5′ end of the self-looped A-PCR product [left panel of (**F**)]. However, if the 3′ end nucleotide of the probe is mismatched with the 5′ end of the self-looped A-PCR product, the enzyme recognizes the gap, and it does not glue the probe and the A-PCR product [right panel of (F)]. After the ligation reaction, the ECA chip was denatured. When the A-PCR product is glued to the probe fixed on the electrode, the A-PCR product is not released from the probe [left panel of (**G**)]. When the A-PCR product is not glued, it is released from the probe [right panel of (G)]. After washing out excess nucleotides, individual electrode was measured for its electrochemical current of FND molecule by an ECA chip reader. The electrode containing double-stranded DNA gives higher current [left panels of (**H**) and (**I**)] than the one containing only a single-stranded short DNA [(right panels of (H) and (I)].

A-PCR product are disrupted. If the probe and the A-PCR product are joined by the ligase, the denaturation treatment only affects the double-stranded region, and it does not separate the probe and the product. When the ECA chip is returned to mild conditions, a self-looped product is again formed (Figure 3G, left). If the probe and the A-PCR product are not joined by the ligase, the denaturation treatment separates the A-PCR product from the probe (Figure 3G, right). Subsequently, FND is added and the electrochemical measurement is carried out. The measured electrical current is designated as I_1. Since FND is an electrochemically active threading intercalator for double-stranded nucleotides, the measurement gives a higher signal for an electrode containing the double-stranded products (Figure 3H and I, left), and a smaller signal for an electrode containing only the single-stranded probe (Figure 3H and I, right).

In this electrochemical measurement, the FND and ECA chip system (Figure 2) developed by our group were used. The ECA chip has 25 gold electrodes, and the measurement system can monitor the oxidation current of FND at a potential of +440 mV versus Ag/AgCl, by differential pulse voltammetry (DPV). Since FND is attracted to single-stranded oligonucleotides by static force, the current in the electrodes were measured prior to the SMMD reaction. This current value (I_0) and the current value after the SMMD reaction (I_1) were used in the evaluation of the formation of double-stranded DNA on individual electrodes by the following equation: $\Delta I = (I_1 - I_0)/I_0 \times 100$. One set of probes, the M probe and the W probe, was used for the detection of a single nucleotide mutation. If the ΔI of the M probe is higher than that of the W probe, the A-PCR product contains only the mutant-type nucleotide, and hence the sample is judged as being a mutant homozygote. If the ΔI of the W probe is higher than that of the M probe, the A-PCR product contains only the wild-type nucleotide, and the sample is judged as being a wild-type homozygote. If the ΔI of the W probe and the M probe is approximately the same, the sample is judged as being a heterozygote.

Establishment of SMMD system using an ECA chip and FND

The LPL gene SNPs were used for the evaluation of the SMMD system using an ECA chip. Among the Japanese people, there are 22 known SNPs in the LPL gene, the mutations of which all result in a non-functional LPL molecule. Seven SNPs (Y61X, V200A, A221-del, R243C, A261T, A334T and W382X) were selected as models for this evaluation. Special primers and probes were designed as shown in Table 1.

As the first step of the evaluation, plasmids containing the wild type as well as the mutant type of individual mutations were produced and tested with the ECA chip system. The mutations were judged by the score calculated from the equation described in Materials and Methods.

Occasionally, I_1 is smaller than I_0, and this phenomenon was observed more frequently with a mismatched probe. With a mismatched probe, the target is not joined by the ligase, and the probe may be detached from the electrode surface during the washing and denaturing process, between the I_0 and I_1 measurement. When I_1 was smaller than I_0, the score was calculated as $\Delta I = 0$. Therefore, the maximum and minimum of the score is 2.0 and −2.0, respectively.

Figure 4A shows the result of the SMMD system using an ECA chip for plasmid samples. In this figure, three experiments were performed for each plasmid sample, and the individual results are plotted next to each other. As shown in the figure, when the sample was a wild-type homozygote, the score was between 1.0 and 2.0, and when the sample was a mutant homozygote, the score was between −1.0 and −2.0. If the sample was a heterozygote, the score was between −0.5 and 0.5. Although some variation was observed, all measurement results were within the above criteria.

As the next step, known patient samples were tested and the result is shown in Figure 4B. The Y61X, V200A, A261T and A334T mutant homozygote samples could not be obtained; however, all other samples showed the same results as the plasmid samples. Based on these results, the SMMD system using an ECA chip was considered as an effective genomic mutation detection system.

Validation of the SMMD system using an ECA chip in a blind format

The genomic DNA was extracted from the blood of 50 patients including homozygotes of A221-del and R243C, and heterozygotes of Y61X, V200A, A221-del, A261T, A334T and W382X. The extracted DNA was partially used for the SMMD system using an ECA chip, and the remainder was used for sequencing in order to determine mutation type. The results of the SMMD system using an ECA chip are shown in Figure 5. All the samples were within one of three criteria and were judged for their mutation type. The results of all 50 samples for six single nucleotide substitutions and one single nucleotide deletion, obtained by the SMMD system using an ECA chip, were concordant with the sequencing results.

DISCUSSION

Several methods have been reported for SNPs analysis, and some of them can detect repeating nucleotides, which is one of the most difficult nucleic mutations to detect (40). Among them, DNA microarray technology provides a high throughput, simple and accurate typing method because of its solid surface hybridization reaction, and some of these methods have already been commercialized. Currently, the most popular microarray SNP detection method utilizes the T_m difference of a single nucleotide mismatch and visualizes it with fluorescent probes. However, in order to detect a small difference in the T_m of a single nucleotide mismatch, several improvements have been achieved in the system, such as precise temperature control, electrical field control and so forth (1–3).

Several methods utilizing enzymatic recognition have been reported. For instance, polymerase was used for single nucleotide extension method to distinguish specific nucleotide at mutation point (4,41). Reverse transcriptase was used for nucleotide extension method in which the enzyme extend DNA probe when the probe has perfectly matched with target RNA (42). Ligase was used for oligonucleotide ligation assays (OLA) (43,44) and ligase chain reaction (LCR) (45) or ligase detection reaction (LDR) (46). MutS enzyme that recognize single base mismatch was used either immobilized on solid phase (47,48) or added to probe–target hybridization product

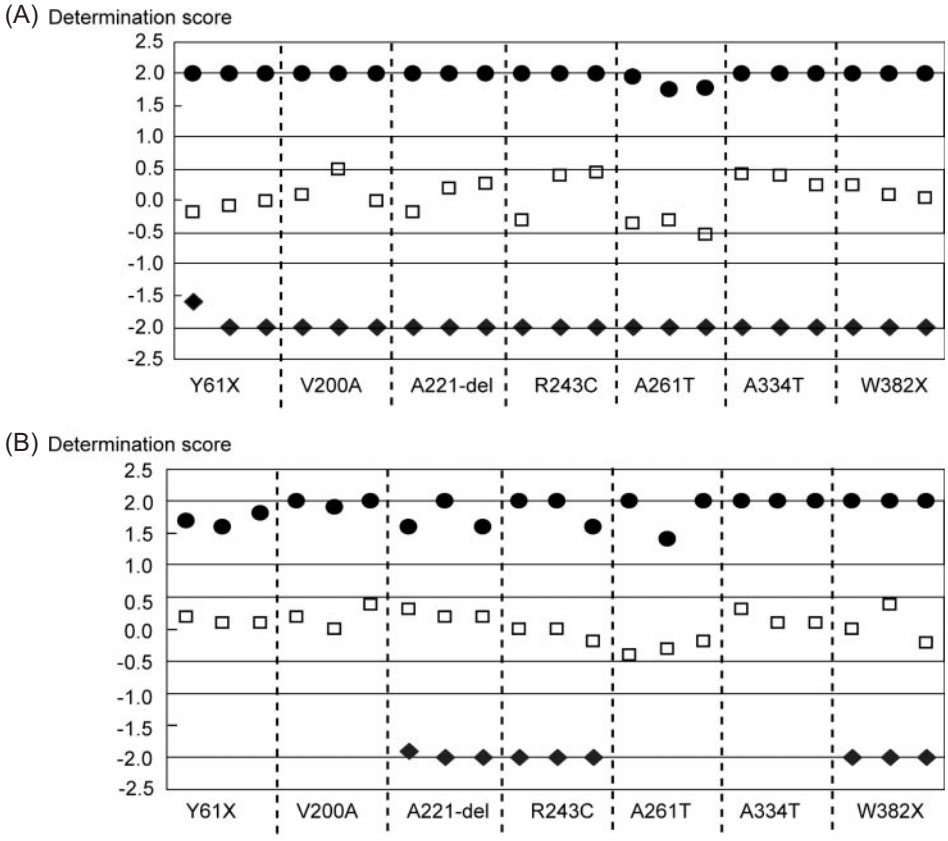

Figure 4. Establishment of criteria for the determination of zygosis by the SMMD system using an ECA chip. (**A**) Samples of wild homozygote (solid black circles), heterozygote (white squares) and mutant homozygote (solid black diamonds) were prepared from the plasmid DNA containing the seven mutations of the LPL gene or the corresponding wild DNA as described in Materials and Methods. Three experiments were carried out for individual samples, and the results have been plotted as the score values calculated from the equation $\alpha/\delta - \beta/\delta$, where α is $\Delta I\ [(I_1 - I_0)/I_0 \times 100]$ of W probe; β is ΔI of M probe; δ is average of α and β. (**B**) Samples of wild homozygote (solid black circles), heterozygote (white squares) and mutant homozygote (solid black diamonds) were prepared from genomic DNA samples of patients. The samples which are homozygotes of Y61X, V200A, A261T and A334T could not be obtained. Three experiments were performed for individual samples, and the results have been plotted as in (A).

(49). Although such enzymatic recognition method has its popularity, the novel SNP detection method introduced in this report utilizes a combination of enzymatic recognition and electrochemical detection; therefore, the system does not require temperature control and is very simple, compact and inexpensive. The detection can be carried out at room temperature and the measurement process takes <1 h. Based on the results of the blind test, the SMMD system using an ECA chip is shown to be a simple, accurate and reliable genetic mutation detection method.

There are several reasons for this highly reliable result and one of these is the ligation reaction between the probe and the target DNA by the ligase enzyme. There was a report that utilized a ligation reaction on a microarray (50); however, this method did not take advantage of the ligase to join the probe fixed on the solid surface and the target nucleotide. The ligation reaction between a fixed probe and a target, as in SMMD, allowed for the use of highly stringent washing conditions, such as 0.5 M NaOH, and this resulted in a high contrast between the positive and negative signals. If the method is utilized for only hybridization, it cannot use such stringent washing conditions, and as a result, the contrast between the positive and negative signals is rather ambiguous. In addition, the target prepared for the SMMD system using the ECA chip contains a self-loop, and this self-loop may contribute to higher hybridization efficiency. Although both the single strand and the self-looped target are of the same length, the latter showed higher hybridization efficiency to the same probe (data not shown). Furthermore, the ECA system utilizes an intercalator for its measurement, and the results are evaluated based on two electrical current measurements (I_0 and I_1). The first current measurement (I_0) indicates the probe fixation efficiency of an individual electrode. Although reproducible DNA immobilization method on gold electrode was employed as described before (17), I_0 value of 25 pins showed 5–10% of coefficient of variation (data not shown). Since ECA chip's electrode surface is solid gold of 1 mm diameter and the surface crystal structure is not uniform, the amount of immobilized DNA has certain variation. However, I_0 measurement works as an internal control, and its incorporation to Score calculation cancel out DNA immobilization variation for end result. In the fluorescent dye system, it is very difficult to estimate the amount of probe fixed at a single spot. Due to this ambiguity, the interpretation of the result is quite difficult, and multiple control probes are used to improve the signal to noise ratio (1). In an ECA system, internal control is provided

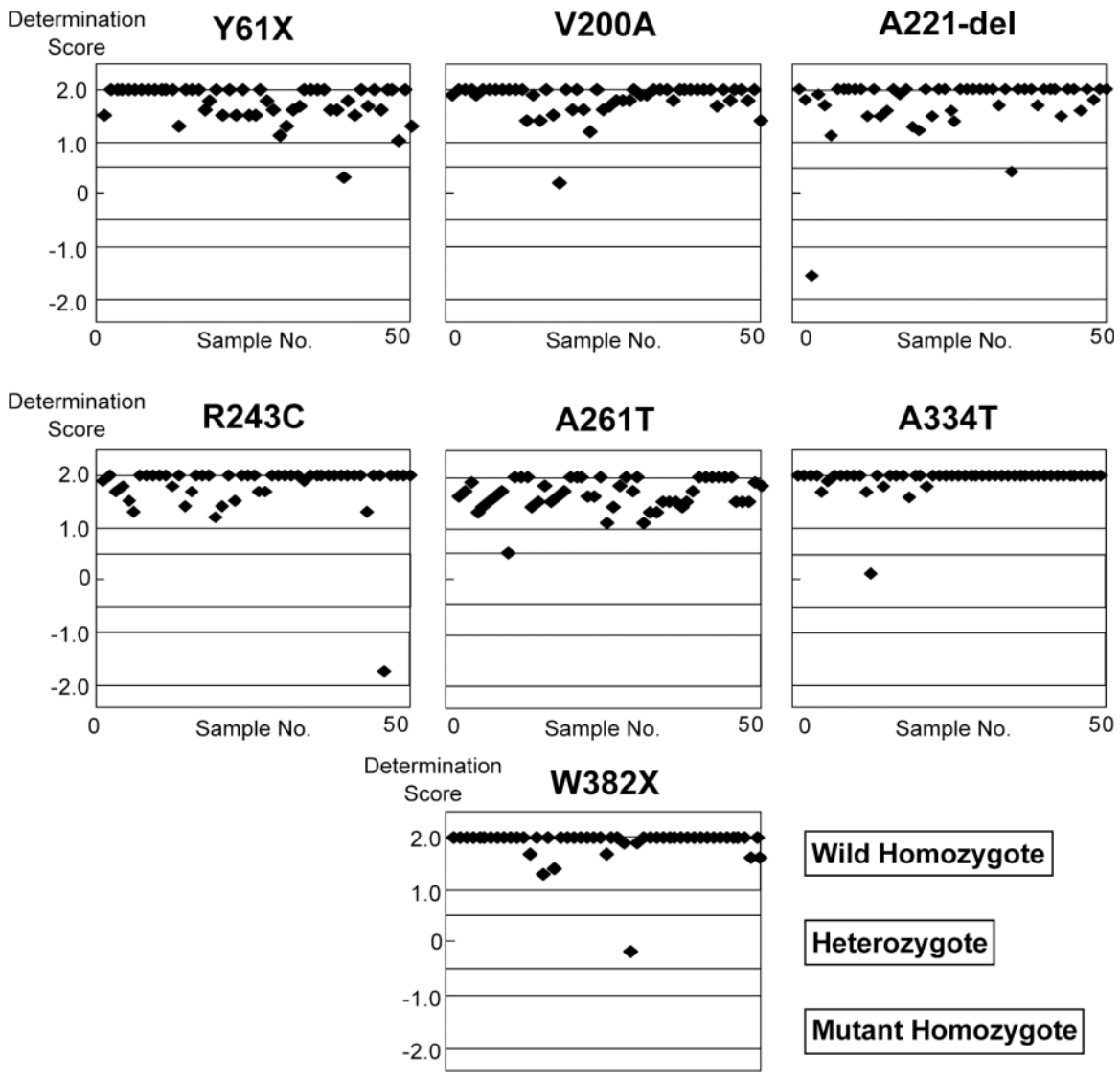

Figure 5. Blind test for determination of LPL mutations by the SMMD system using an ECA chip. Samples from 50 patients, including homozygotes of A221-del and R243C, and heterozygotes of Y61X, V200A, A221-del, A261T, A334T and W382X were examined for the determination of genotype for the above seven mutations. The electrical currents measured were converted to the score (−2.0∼2.0) by using the equation described in Figure 4. The results of the 50 patients have been plotted for the seven mutations. The sample in which the score is between 1.0 and 2.0 is a wild homozygote, the sample in which the score is between −0.5 and 0.5 is a heterozygote, and the sample in which the score is between −2.0 and −1.0 is a mutant homozygote.

by the I_0 measurement; therefore, additional probes for accurate typing are not necessary.

In this report, several SNPs and single nucleotide deletions were examined; however, it is expected that single nucleotide insertion, translocation and short tandem repeat can also be detected by the SMMD system using an ECA chip. Previously, mutation detection methods for other than SNP were reported; however, design of the probe appeared to be quite difficult and there has been no report of a system that simultaneously detected multiple types of mutations. There are some limitations for the SMMD system using an ECA chip. For instance, long repeat mutation detection cannot be detected, because loop region of the A-PCR product becomes too long for efficient hybridization, and quite frequently long repeat contain self-complemental sequence, which disturb loop formation of A-PCR product. Also, it is very difficult to detect multiple nucleotide mutations adjacent each other. In spite of such limitations, the SMMD system using an ECA chip can simultaneously detect multiple mutations of different types in a simple, efficient, reliable and inexpensive manner, and it would be a very powerful tool for clinical applications.

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