Table 1

(1) PCR Primers and amplification conditions

1. Target DNA preparation

SNP site	Sequence		Amplification condition							Target length
	F	R	Primer concentration	dNTP	Enzyme	Human genome	Reaction volume	Anneal (X)	Step ★	
C481T	ctaTTTTTGATCACATTGTAAGAAGAA	GCTCTCTCCTGATTTGGTCC	30pmol	0.3mM	1.25U	30ng	50μl	53°C	3step	342bp
G590A G857A	ATACTTATTTACGCTTGAACCTC	gttCCTTATTCTAAATAGTAAGGGAT	30pmol							317bp

2. PCR-RFLP analysis

SNP site	Sequence		Amplification condition							Target length
	F	R	primer concentration	dNTP	Enzyme	Human genome	Reaction volume	Anneal (X)	Step ★	
C481T G590A G857A	AGATGTGGCAGCCTCTAGAA	ATTAGTGAGTTGGGTGATAC	20pmol	0.4mM	1.25U	30ng	50μl	59°C	3step	534bp

★Step
95℃ 5min
98℃ 10sec
X℃ 30sec
72℃ 30sec
×40cycle
72℃ 1min

(2) PCR-RFLP analysis

SNP site	C481T		G590A		G857A	
Restriction enzyme	*KpnI*		*TaqI*		*BamHI*	
Reaction temperature	37℃		65℃		30℃	
Before treatment fragment length	534bp		534bp		534bp	
Genotype	C	T	G	A	G	A
After treatment fragment length	444bp 90bp	534bp	200bp 170bp 164bp	370bp 164bp	467bp 67bp	534bp

(3) Probes

Name	Sequence
3'SH control	ATGCTTTCCGTGGCA-SH
5'SH control	HS-GTTTCTGCTCCCGGA
481-C	HS-TGGTCCAGGTACCAGA
481-T	HS-TTTGGTCCAAGTACCAGA
590-G	HS-CCTCGAACAATTGA
590-A	HS-GAACCTCAAACAATTGA
857-G	TGGTGATGGATCCCTTAC-SH
857-A	TGGTGATGAATCCCTTACTATT-SH

(1) Primer sequences and PCR conditions for target DNA preparation and PCR-RFLP analysis: The primers were obtained as custom synthesis products from SIGMA Genosys. Small letters are S-oligo. PCR was carried out using GeneAmp PCR System Model 9700 (Applied Biosystems, Foster City, CA, USA). Pyrobest DNA Polymerase (TAKARA Bio, Shiga, Japan), and attached buffer and dNTP were used for this reaction.
(2) Enzyme used for PCR-RFLP analysis, and the digested pattern after treatment: The restriction enzymes were purchased from Daiichi Pure Chemicals (Tokyo, Japan).
(3) Sequences of probe DNA: HS- means thiol modification on 5' or 3' end of probe DNA. Sequences of control probes are irrelevant to NAT2 sequence.

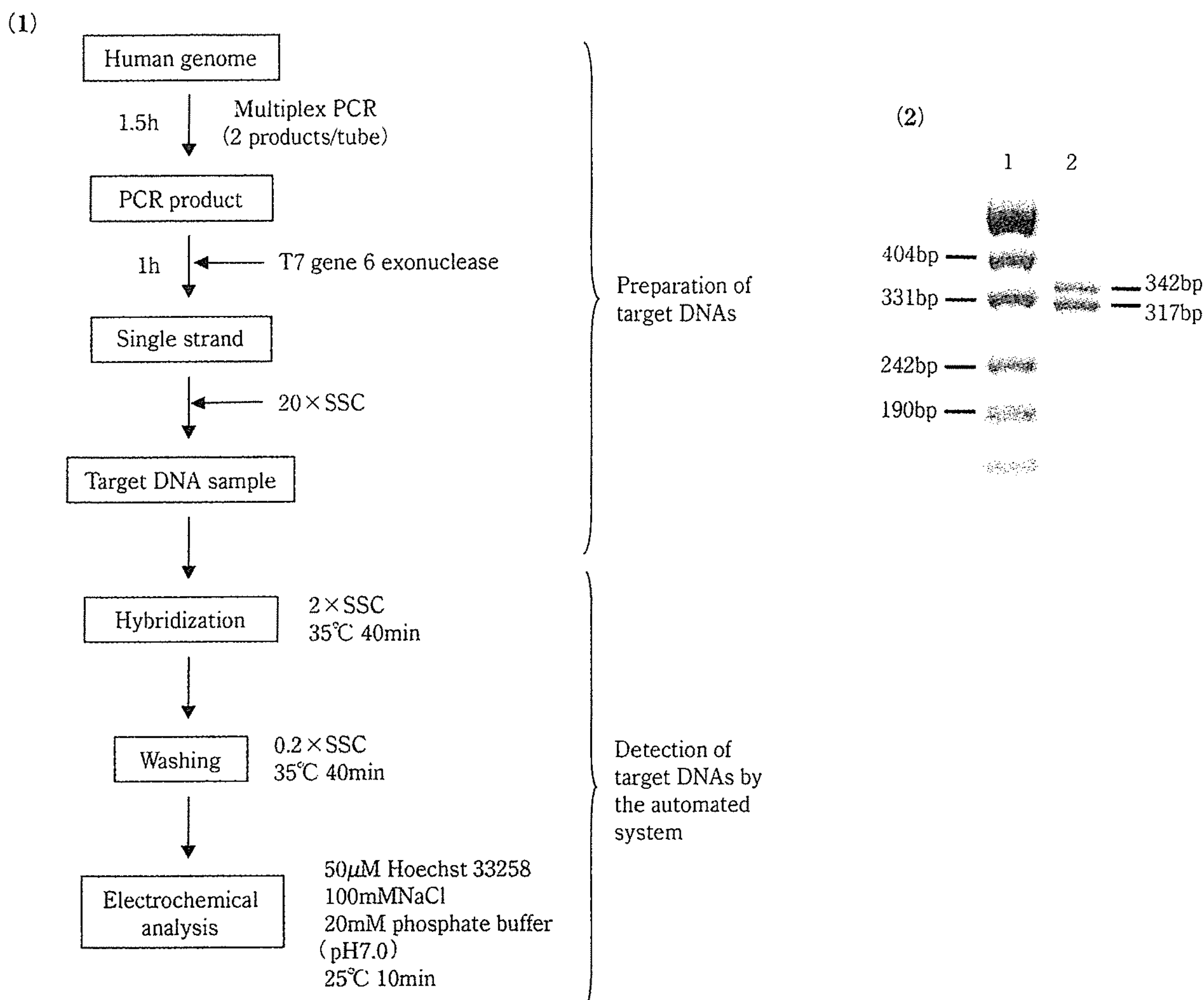

Figure 2

(1) Protocol for detection of NAT2 3SNPs by electrochemical DNA chip.
(2) PCR products obtained by the multiplex PCR procedure.
PCR products were confirmed by 4% agarose gel electrophoresis.
Lane 1: Maker pUC19/MspI (Nippon Gene, Toyama, Japan), Lane 2: PCR products
342bp: C481T target, 317bp: G590A and G857A target

(S/N) ratio was calculated from these voltammetric experiments. The signal means the I_{pa} value from hybridization on wild or mutant probes, whereas the noise means the I_{pa} value on control probe. The S/N ratios on 590-G probe and 590-A probe for target 590 G/G were 2.1 and 1.1, respectively. Similarly, S/N ratios on 590-G probe and 590-A probe for target 590A/A were 1.2 and 2.5, and for target 590 G/A were 2.0 and 2.2, respectively. Comparable results were obtained for the other SNPs. S/N ratios of perfect-match-combinations between probes and target DNAs were in the range from 2.0 to 2.5, and S/N ratios of single-base-mismatch combinations between probes and target DNAs were in the range from 1.0 to 1.2, respectively. The 5'SH control probe was used as control for T481C and G590A polymorphisms and the 3'SH control probe was used as control for G857A polymorphism.

The typing results of 38 samples with the automated system are shown in **Fig. 5 (B)** as a scatter chart. For each probe, four electrodes were assigned for validation and an average of I_{pa} from the four electrodes was adopted for genotyping analysis. An average of CV values of four electrodes on the control probes (5'SH and 3'SH) was 4.4%. Similarly, an average of CV values of four electrodes on the perfect-match-combinations between probes and target DNAs was 2.7%, and on the single-base-mismatch combinations

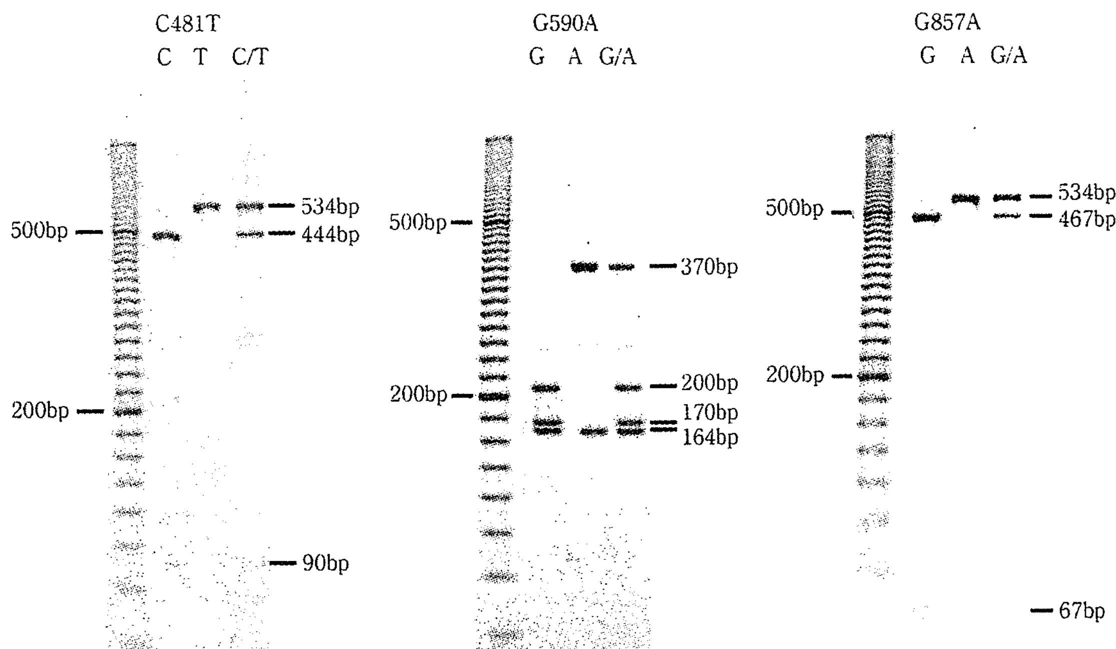

Figure 3 Results of PCR-RFLP analysis for NAT2 3SNPs.
A marker is a 20bp DNA ladder (TAKARA Bio, Shiga, Japan).

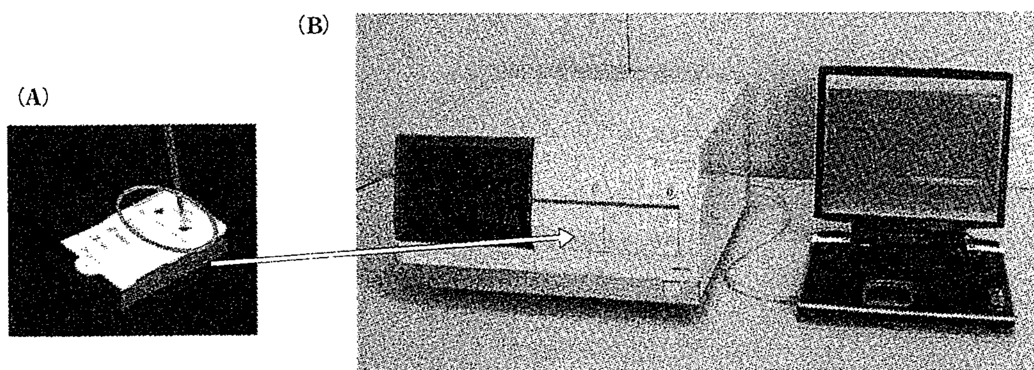

Figure 4 DNA chip cassette and automated DNA detection system.

(**A**) DNA chip cassette: DNA chip cassette includes the probe-immobilized-DNA chip substrate. The size is 30mm×50mm×6mm.

(**B**) Automated DNA detection system: The automated system has a space that stores reagents, such as 0.2×SSC washing buffer and Hoechst33258 solution. Using two cassettes, simultaneous measurement is possible. The size is 450mm×450mm×200mm.

between probes and target DNAs was 4.2%. The distribution of each genotype of 3SNPs was clearly distinguished. The genotyping results by the detection system were all consistent with those by PCR-RFLP analysis. The turnaround time for genotyping with this system was 90 min.

IV. Discussion

In this study, NAT2 3SNPs genotyping was performed using the detection system that automatically performs the process after hybridization. As a result of genotyping of 38 samples, the feasibility of the automated system and that of the SNP genotyping algorithm were verified.

In **Fig. 5 (B)**, there is wide variation among samples with an identical genotype. A difference of amplifi-

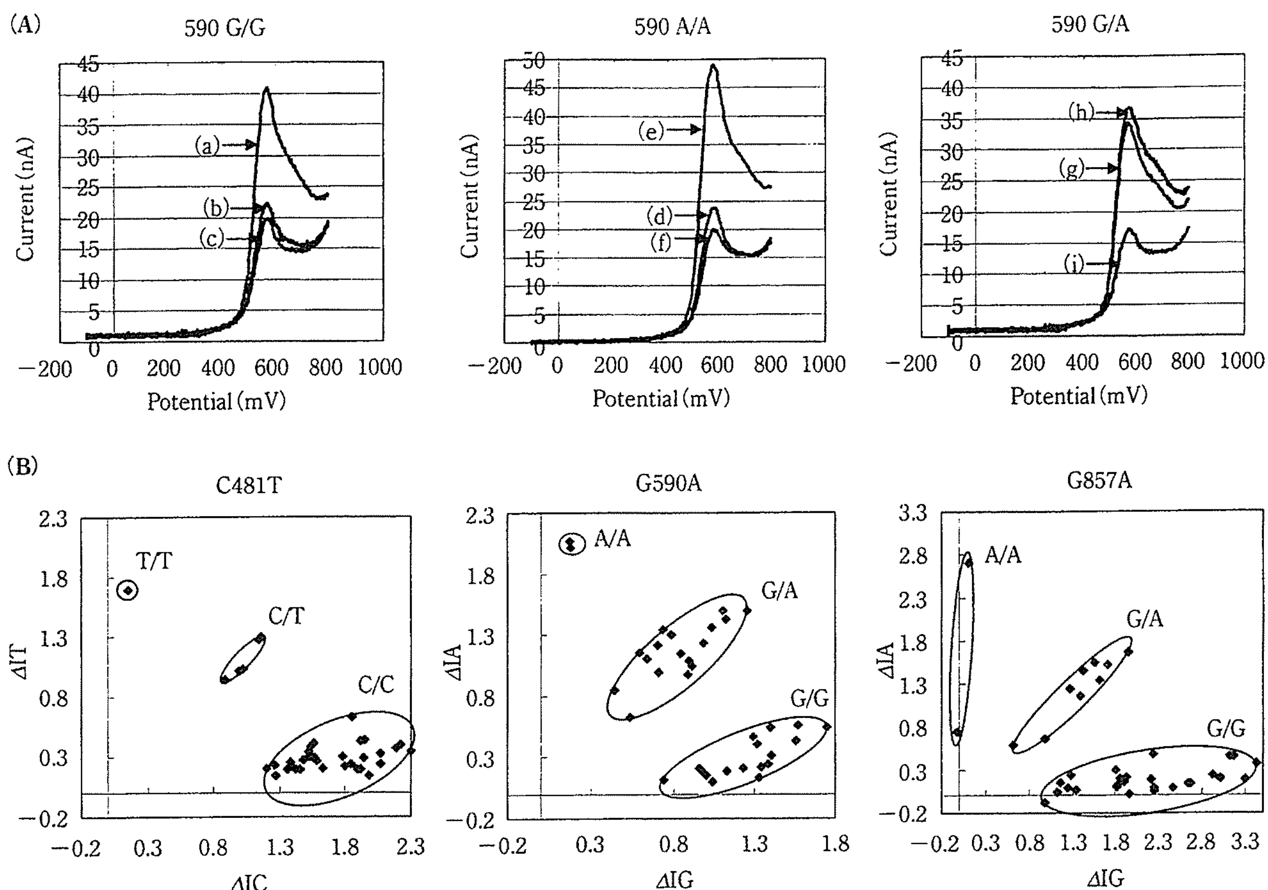

Figure 5 The electrochemical detection results of NAT2 3SNPs analyzed with the automated system.
(**A**) Voltammograms for G590A polymorphisms
(**B**) Typing results of 38 samples: IW: A average of *Ipa* values on wild probe, IM: A average of *Ipa* values on mutant probe, IB: A average of *Ipa* values on control probe
It is referred to as Δ IW=IW−IB, Δ IM=IM−IB. X-axis: Δ IW/IB, Y-axis: Δ IM/IB

cation efficiency by each human genome and a difference of treatment efficiency of double-stranded DNAs by T7 gene6 exonuclease are speculated as reasons. In particular, the treatment by the T7 gene6 exonuclease might be unstable and excessive digestion of DNA might occur. To solve such problem, we are now examining the combination of the LAMP[16] method, which is a novel technique for the amplification of specific DNA sequences under isothermal condition and contains the single-stranded DNAs in the amplification products.

Some studies on developments in electrochemical-based DNA or SNP detection technologies have been published. However, most of these studies can be categorized as fundamental verification steps because they use synthesized short oligonucleotides as the targets. There are only a few studies involving simultaneous genotyping of patient blood samples by use of the electrochemical DNA chip strategy. In these studies, the sandwich hybridization method using a DNA capture probe, a signaling probe, and a single-stranded target DNA has been reported[4]. This is similar to our method, but it is disadvantageous that the ferrocene modification to the signaling probe is essential. On the other hand, the modification step is unnecessary and the procedures are very simple in the case of our method. Moreover, the Hoechst 33258 is available commercially and is cheap.

The DNA chip used in this study had 40 working electrodes. And the diameter of the electrode was 200μm. The number of electrodes can be increased and the diameter can be changed according to the applications.

Moreover, we are now developing a fully automated system containing the DNA extraction and amplification. The fully automated system can detect DNAs from samples such as blood, mucous, and hair roots. Therefore, use of the system will not be restricted to technicians, but anyone will be able to use the system anywhere. We expect that the electrochemical DNA chip and the automated system will contribute to personalized medicine in the future.

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References

1) Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP, Fodor SP. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. Proc Natl Acad Sci U S A 1994; 91: 5022-6.
2) Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995; 270: 467-70.
3) Marshall A, Hodgson J. DNA chips: an array of possibilities. Nat Biotechnol 1998; 16: 27-31.
4) Umek RM, Lin SW, Vielmetter J, Terbrueggen RH, Irvine B, Yu CJ, et al. Electronic detection of nucleic acids: a versatile platform for molecular diagnostics. J Mol Diagn 2001; 374-84.
5) Palecek E. Adsorptive transfer stripping voltammetry: determination of nanogram quantities of DNA immobilized at the electrode surface. Anal Biochem 1988; 170: 421-31
6) Yang IV, Thorp HH. Modification of indium tin oxide electrodes with repeat polynucleotides: electrochemical detection of trinucleotide repeat expansion. Anal Chem 2001; 73: 5316-22.
7) Patolsky F, Lichtenstein A, Willner I. Highly sensitive amplified electronic detection of DNA by biocatalyzed precipitation of an insoluble product onto electrodes. Chemistry 2003; 9: 1137-45.
8) Boon EM, Ceres DM, Drummond TG, Hill MG, Barton JK. Mutation detection by electrocatalysis at DNA-modified electrodes. Nat Biotechnol 2000; 18: 1096-100.
9) Hashimoto K, Ito K, Ishimori Y. Sequence-specific gene detection with a gold electrode modified with DNA probes and an electrochemically active dye. Anal Chem 1994; 66: 3830-3.
10) Hashimoto K, Ishimori Y. Preliminary evaluation of electrochemical PNA array for detection of single base mismatch mutations. Lab Chip 2001; 1: 61-3.
11) Takahashi M, Okada J, Ito K, Hashimoto M, Hashimoto K, Yoshida Y, et al. Construction of an electrochemical DNA chip for simultaneous genotyping of single nucleotide polymorphisms. Analyst 2005; 130: 687-93.
12) Nakamura T, Sakaeda T, Takahashi M, Hashimoto K, Gemma N, Morita Y, et al. Simultaneous determination of single nucleotide polymorphisms of MDR1 genes by electrochemical DNA chip. Drug Metab Pharmacokinet 2005; 20: 219-25.
13) Ohno M, Yamaguchi I, Yamamoto I, Fukuda T, Yokota S, Maekura R, et al. Slow N-acetyltransferase 2 genotype affects the incidence of isoniazid and rifampicin-induced hepatotoxicity. Int J Tuberc Lung Dis 2000; 4: 256-61.
14) Tanaka E, Taniguchi A, Urano W, Nakajima H, Matsuda Y, Kitamura Y, et al. Adverse effects of sulfasalazine in patients with rheumatoid arthritis are associated with diplotype configuration at the N-acetyltransferase 2 gene. J Rheumatol 2002; 29: 2492-9.
15) Martinez C, Agundez JA, Olivera M, Martin R, Ladero JM, Benitez J, et al. Lung cancer and mutations at the polymorphic NAT2 gene locus. Pharmacogenetics 1995; 5: 207-14.
16) Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000; 28: E63.