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Rapid and Sensitive Assay of *K-ras* Mutations in Pancreatic Cancer by Electrochemical Detection with Ferrocenyl-naphthalene-diimide

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Abstract. *The DNA chip is a very powerful tool for genetic analysis. Conventional DNA chips that utilize fluorescence detection systems are very complicated, expensive and impractical, but the electrochemical array (ECA) chip is gaining popularity. To investigate the validity of the ECA chip, which utilizes ferrocenyl-naphthalene-diimide (FND), k-ras mutations in 20 pancreatic cancer tissues were examined. DNA was isolated from 20 pancreatic cancer tissues and subjected to a 2-stage polymerase chain reaction (PCR). The k-ras mutations were detected with the ECA chip. To verify the reliability of the ECA chip, the DNA was also analyzed by direct sequencing and the PCR-dependent preferential homoduplex formation assay (PCR-PHFA). The ECA chip could detect one mutation in a background of 1000 wild-type DNAs. K-ras mutations were identified in 17 out of 20 (85%) pancreatic cancer samples. Three mutations of codon 12 of k-ras, GTT, GAT and AGT, were detected. K-ras mutations were detected in 13 out of 20 (65%) samples by sequencing and in 17 out of 20 (85%) samples by PCR-PHFA. These findings were concordant with the ECA chip result. The FND-ECA chip is a sensitive, rapid and reliable method for screening point mutations in a variety of clinical samples.*

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Key Words: Electrochemical array (ECA) chip, ferrocenyl-naphthalene-diimide (FND), *k-ras* mutation, restriction enzyme *Bst*NI, assymmetric PCR (A-PCR).

DNA chips have been in use since the late 1980s and this technology has been a very powerful and valuable tool for genetic analysis because it is sensitive, specific, accurate, quick and versatile. Fluorescence labeling is typically used to visualize hybridization on chips (1). Despite the high accuracy and sensitivity of fluorescence detection systems, fluorescence readers are expensive and difficult to handle. Therefore, DNA chips cannot be commonly used as a routine diagnostic tool at present.

Electrochemical detection systems have gained popularity since the early 1990s, and several electrochemical DNA sensors that transduce DNA information into electrochemical signals have been developed (2-4). The detection of mutations by most of these sensors is based on the difference in thermal stability between mismatched and fully matched DNA duplexes. However, assessments of thermal stability require precise temperature control, which is expensive and difficult to accomplish, as well as significant amounts of time. We developed ferrocenyl-naphthalene-diimide (FND) as a hybridization indicator specific for double-stranded DNA (5, 6). FND intercalates into double-stranded DNA but cannot interact with single-stranded DNA (7, 8). When target DNA hybridizes to a probe immobilized on the electrodes of the ECA chip, the FND concentrated on the electrodes gives rise to an electrochemical signal proportional to the amount of hybridized target DNA (9, 10).

Pancreatic cancer is the fourth leading cause of cancer-related death in the world (11). Because pancreatic cancers tend to grow rapidly, invade adjacent organs and metastasize, they are difficult to diagnose and treat at an early stage. Many techniques for detecting tumors of the pancreas at an early stage have been developed and improved over the past decades; however, the results to date have not been

Table IA. *Primer design.*

PCR	Primer name	Primer sequence	Expected product size (bp)	Specific notes
For the first PCR				
First diagnostic	Kras12MvaI	GAATATAAACTTGTGGTAGTTGGAcCT	159	Amplify a fragment of *k-ras* exon 1. Presence of amplicon indicates that the template has mutant *k-ras*.
	1213SeqR	CTGTATCAAAGAATGGTCCTGC		
First control	PCRcontF2	GGTGTAGTGGAAACTAGGAATTAC	291	Control of PCR
	PCRcontR	CTTACCTGTCTTGTCTTTGCTG		
First *BstN*I control	MvaIcontF3	AATTACTCTTACCAATGCAACAGAC	279	Amplify a fragment of *k-ras* exon 4a that contains *BstN*I site. Presence of amplicon indicates *BstN*I failure.
	MvaIcontR4	CTACACCTAAGTAGTTCTAAAGTGG		
For the second PCR				
A-PCR diagnostic	Kras12MvaI	GAATATAAACTTGTGGTAGTTGGAcCT	100	Amplify a fragment of *k-ras* exon 1. Presence of amplicon indicates that the template has mutant *k-ras*.
	1213R2	TTGTTGGATCATATTCGTCCAC		
A-PCR control	PCRcontF	TTATGACAAAAGTTGTGGACAGG	197	Control for PCR
	PCRcontR2	CTTCTTGCTAAGTCCTGAGCC		
A-PCR *BstN*I control	MvaIcontF4	GCTTTTTATACATTGGTGAGGGAG	183	Amplify a fragment of *k-ras* exon 4a that contains *BstN*I site. Presence of amplicon indicates *BstN*I failure.
	MvaIcontR2	TGGTTGCCACCTTGTTACC		
Special diagnostic	Kras12-1LP	P-GTGGCGTAGGCAATGATTCTGAA TTAGCTGTATCGTCAAGGCACTC	204	Amplify the single-strand target for variant of the first letter at *k-ras* codon 12 of exon 1.
	Kras12-2LP	P-TGGCGTAGGCAAGTGATCTGAAT TAGCTGTATCGTCAAGGCACTC	204	Amplify the single-strand target for variant of the second letter at *k-ras* codon 12 of exon 1.
Special control	PCRcontLP	P-TGTTACTAATGACTGTGTTTGTCTCT GGGAAAGAAAAAAAAGTTATAGCAC	151	Control for PCR
Special *BstN*I control	MvaIcontLP	P-CAAAGAAGAAAAGACTCTGCATTTT TTAATTTTCACACAGCCAGGAG	114	Amplify a single-stranded fragment of *k-ras* exon 4a that contain s *BstN*I site. Presence of amplicon indicates *BstN*I failure.

Table IB. *Probe design.*

Probe	Target	Probe sequence
Control PCR (PC)		HS-TATGACAAAAGTTGTGGACAGGTTTTGAAAGATATTTG
Control *BstN*I (RC)		HS-GTGAGGGAGATCCGACAATACAGATTGAAAAAAATCAG
Mutant probe (1C)	CGT	HS-GAAAATGACTGAATATAAACTTGTGGTAGTTGGAcCac
Mutant probe (1A)	AGT	HS-GAAAATGACTGAATATAAACTTGTGGTAGTTGGAcCaa
Mutant probe (1T)	TGT	HS-GAAAATGACTGAATATAAACTTGTGGTAGTTGGAcCat
Mutant probe (2C)	GCT	HS-AAAATGACTGAATATAAACTTGTGGTAGTTGGAcCgGc
Mutant probe (2A)	GAT	HS-AAAATGACTGAATATAAACTTGTGGTAGTTGGAcCIIa
Mutant probe (2T)	GTT	HS-AAAATGACTGAATATAAACTTGTGGTAGTTGGAcCgGt

satisfactory. Therefore, it is necessary to develop more effective systems to screen for pancreatic cancer (12, 13).

The mutation of codon 12 of exon 1 of *k-ras* is one of the most common alterations in pancreatic cancer with a frequency of 75-90% (14). *K-ras* mutations in various cancers have been analyzed, however, *k-ras* mutations in pancreatic cancers have never been analyzed with an FND-ECA chip. The aim of the present study was to investigate the validity and practicality of the FND-ECA chip as a diagnostic test tool, by examining *k-ras* mutations in pancreatic cancer cell lines and tissues and comparing the results with those of conventional techniques.

Materials and Methods

Tumor cell lines. Four pancreatic cancer cell lines, A549, MIA Paca2, LS174T and SW480, each containing a mutation in codon 12 of *k-ras,* were used. The GGT sequence of codon 12 is mutated to AGT in A549 cells, TGT in MIA Paca2 cells, GAT in LS174T cells and

GTT in SW480 cells. The human umbilical vein endothelial cell line (HuVEC), which has no *k-ras* mutations, was used as the control. The HuVEC cell line was purchased from Kurabo (Tokyo, Japan), A549 and MIAPaca2 were obtained from the Japanese Cancer Resource Bank (Tokyo, Japan), LS174T was obtained from the European Collection of Animal Cell Culture (Wiltshire, UK) and SW480 was purchased from the American Type Culture Collection (Rockville, MD, USA).

Tissue samples. Pancreatic cancer tissues were obtained from 20 patients who were diagnosed and treated by surgical resection during the period April 2000 to March 2003 at the Department of Surgery and Oncology, Graduate School of Medical Science, Kyushu University (Fukuoka, Japan). The study was conducted according to the recommendations of the World Medical Association Declaration of Helsinki. Informed consent was obtained from all patients and the study was approved by the Institutional Review Board of Kyushu University. The pancreatic tissues were frozen at –80˚C immediately after resection.

DNA extraction. Genomic DNA was extracted by means of a standard phenol and chloroform method (15) from the cell lines and pancreatic cancer tissues. The tissue specimens were digested in sodium dodecylsulfate (SDS) and proteinase K at 56˚C overnight and genomic DNA was extracted with phenol-chloroform and precipitated with ethanol.

Primer and probe design. The sequences of primers and probes used for this experiment are shown in Table I. All oligonucleotides used in this research were custom-synthesized by Operon Biotechnologies K.K. (Tokyo, Japan).

Polymerase chain reaction (PCR). Two-stage PCR reactions are needed to detect single nucleotide mutations with FND-ECA chips. In the first PCR, a 159-bp region of *k-ras* codon 12 of exon 1 is amplified from genomic DNA in the presence of the restriction enzyme *Bst*NI, while in the second PCR, a specific single-stranded target DNA is made from the product of the first PCR. The second PCR is an asymmetric PCR (A-PCR).

For the first PCR, 3 sets of primers were used (Table IA). The diagnostic primers Kras-12 MvaI and 1213 SeqR were used to amplify a 159-bp fragment of exon 1 of the *k-ras* gene that contains a *Bst*NI site (CCTGG) in wild-type *k-ras* amplicons. Because *Bst*NI is included in the first PCR, wild-type *k-ras* cannot be amplified, whereas the mutant DNA that lacks the *Bst*NI cleavage site is selectively amplified by these primers. The PCR control primers PCRcontF2 and PCRcontR amplify a 291-bp fragment of exon 4a of *k-ras* as a control for the PCR conditions. Restriction enzyme control primers MvalcontF3 and MvalcontR4 amplify a 279-bp fragment of exon 3 that contains a *Bst*NI cleavage site. The presence of this amplicon indicates that *Bst*NI digestion failed. For the PCR, 50 ng of genomic DNA template were added to a 20-μl reaction solution containing 0.1 μM of each diagnostic primer (Kras-12 MvaI and 1213 SeqR), 0.05 μM of each PCR control primer (PCRcontF2 and PCRcontR), 0.1 μM of each enzyme control primer (MvalcontF3 and MvalcontR4), 10 U *Bst*NI, 1 U Taq polymerase-Hot Start version (Takara Bio, Shiga, Japan), 2 mM dNTP and 10 x PCR buffer. After predenaturation at 94˚C for 2 min, 15 cycles of PCR amplification (denaturation at 94˚C for 30 sec, annealing at 60˚C for 60 sec and elongation at 72˚C for 10 sec), were performed followed by a final elongation at 72˚C for 30 sec. The products were analyzed by electrophoresis on 10% acrylamide gels.

In the second PCR (A-PCR), 2 μl of PCR product were added to 20 μl of the second PCR solution that contained 1 μM special diagnostic primer (Kras12-1LP or Kras12-2LP, Table IA), which amplify the single-strand target DNAs, with mutations of the first or second position of codon 12, 0.25 μM special PCR control primer (PCRcontLP, Table IA), 1 μM *Bst*NI control primer (MvaIcontLP, Table IA), 0.1 μM of each diagnostic primer (Kras-12 MvaI and 1213R2, Table IA), 0.05 μM of each PCR control primer (PCRcontF and PCRcontR2, Table IA), 0.1 μM of each enzyme control primer (MvalcontF4 and MvalcontR2, Table IA), 1 U Taq polymerase-Hot Start version, 0.25 mM dNTP and 10x PCR buffer. Two primer sets, set 1 and set 2, which contained Kras12-1LP and Kras12-2LP, respectively, were prepared to amplify 3 SNP targets with one set simultaneously. Set 1 and set 2 corresponded to variants of the first and second letter at *k-ras* codon 12, respectively. To amplify single-strand-specific targets that were hybridized to probes on the ECA chip, only the special primer was applied, making the second PCR an asymmetric PCR (A-PCR). The A-PCR consisted of an initial activation of polymerase at 94˚C for 2 min, followed by 40 cycles of PCR amplification (denaturation at 94˚C for 30 sec, annealing at 60˚C for 60 sec and elongation at 72˚C for 10 sec), and final elongation at 72˚C for 30 sec and denaturation at 95˚C for 1 min. After confirmation of the quality of the PCR products by electrophoresis on a 10% acrylamide gel, the A-PCR products were subjected to hybridization and electrochemical measurement with the ECA chip.

Preparation of ECA chip-immobilized DNA probe. Mutant probes for 6 *k-ras* gene mutations and 2 control probes (M probes and C probes listed in Table IB) were immobilized on individual gold electrodes of an ECA chip, as described previously (6).

Hybridization, ligation and electrochemical measurement. Prior to hybridization of the A-PCR products to the C or M probes immobilized on the gold electrodes of an ECA chip, the ECA chip was denatured and the baseline current (I_0) was measured, as described previously (16). The A-PCR products (10 μl) were hybridized to the probes at room temperature for 10 min. The ligation reaction between the A-PCR products and the probes was then carried out at room temperature for 5 min. The ECA chip was subsequently denatured, washed and used for the electrochemical measurement. Measurement of electric current (I_1) was performed at room temperature with an electrochemical analyzer, STR3000 (TUM Gene, Inc. Kimitsu, Japan). The results were evaluated by the score calculated from the following equation. ΔI is the hybridization/ligation efficiency of a probe and is calculated as $\Delta I=(I_1 - I_0)/ I_0$ x 100. The procedure from hybridization to electrochemical measurement (I_0, I_1) takes less than 1 h.

Direct DNA sequencing analysis and PCR-preferential homoduplex formation assay (PCR-PHFA). To verify the reliability of the ECA chip data, DNAs extracted from all samples were analyzed by direct DNA sequencing and PCR-PHFA. Direct sequencing was done with the dideoxy chain termination method with a BigDye Terminator V1.1 Cycle Sequencing Kit (PE Applied Biosystems) and an automated DNA sequencer (ABI PRISM 3100, PE Applied Biosystems). PCR-PHFA was carried out with a commercially available kit (Wakunaga Pharmaceutical, Hiroshima, Japan).

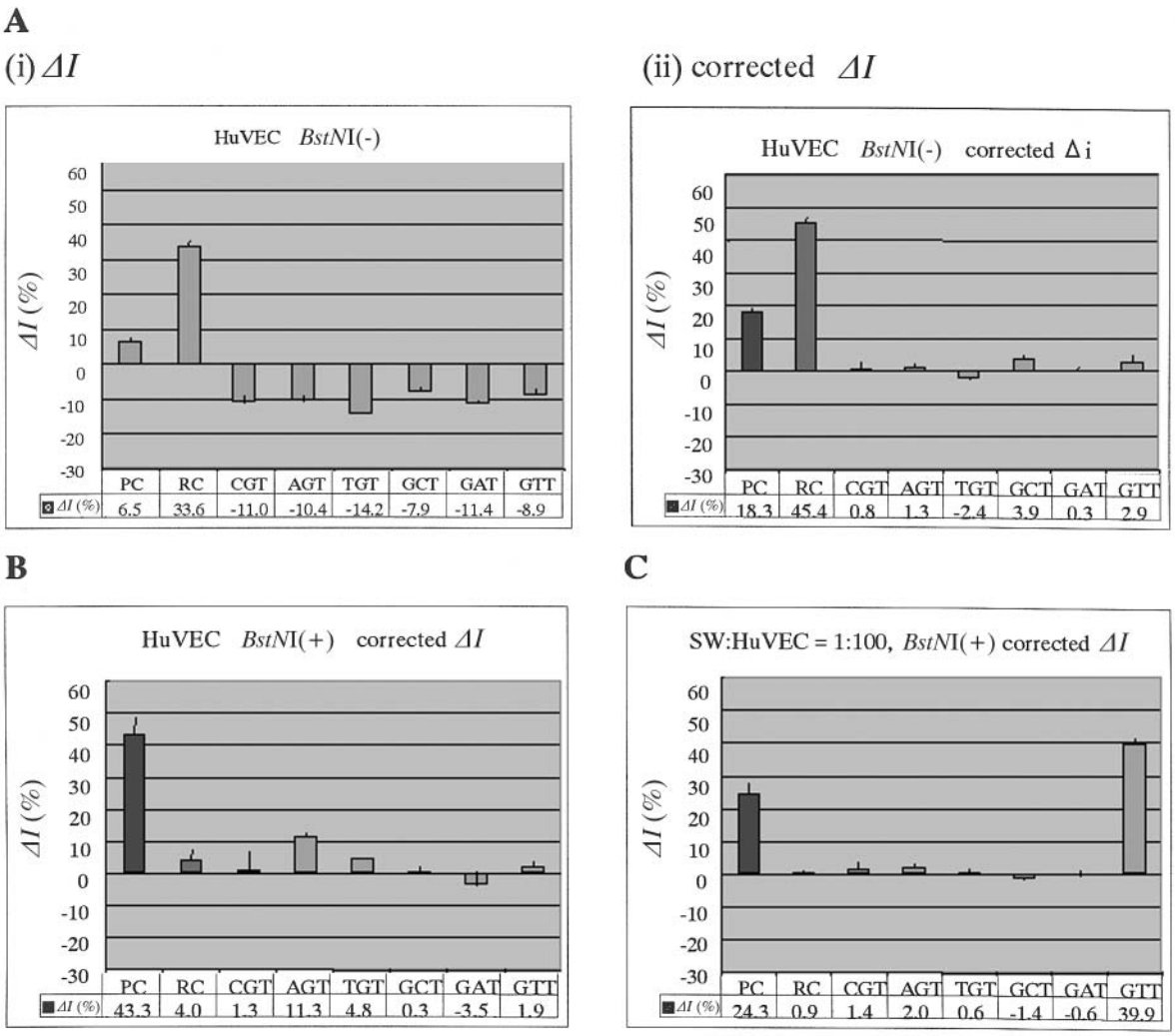

Figure 1. *ECA chip analysis of cell lines. A. HuVECs (wild-type) amplified without restriction enzyme BstNI. (i) $\Delta I = (I_1 - I_0)/I_0$ x 100. (ii) Corrected ΔI is the remainder after the average of the 4 lowest value data among 6 probes are substracted from each ΔI. B. HuVEC DNA amplified in reactions containing BstNI. C. SW480 cells carrying the GTT mutation in codon 12 of k-ras and 100-fold wild-type cells amplified in reactions containing BstNI.*

Results

Analysis of the ECA chip data. Two control probes and 6 mutant *k-ras* probes (Table IB) were immobilized on the ECA chip. One of the 2 control probes was the PCR C probe (PC) and the other the *Bst*NI C probe (RC). The 6 mutant-specific probes were 1C/CGT, 1A/AGT, 1T/TGT, 2C/GCT, 2A/GAT, 2T/GTT and were designed to detect a mutational transition from GGT to CGT, AGT, TGT, GCT, GAT or GTT, respectively. The ECA chip analyses of the PCR products of the cell lines amplified with or without restriction enzyme are shown in Figure 1. In some cases, *ΔI* ($(I_1 - I_0)/ I_0$ x 100) yielded a negative value (Figure1A- (i)). When this happened, the average of the 4 lowest value data among the 6 probes was substracted from each *ΔI* and defined the value as a corrected *ΔI* (Figure 1A- (ii)). The collected *ΔI* was defined as positive when the value was more than 20%. When the first PCR was carried out without *Bst*NI, the PCR product of wild-type DNA from HuVEC cells yielded a positive *ΔI* for both the PC and RC probes and a negative *ΔI* for the 6 mutant probes (Figure 1A- (ii)), indicating that the 2-stage PCR was carried out correctly and that the HuVEC DNA did not carry any *k-ras* mutations. When *Bst*NI was included, the ECA chip yielded a positive *ΔI* for the PC probe. *ΔI* was negative for the RC probe, indicating that *Bst*NI functioned appropriately in this system (Figure 1B). As shown in Figure 1C, the SW480 cells, which carry the GTT mutation of *k-ras* codon 12,

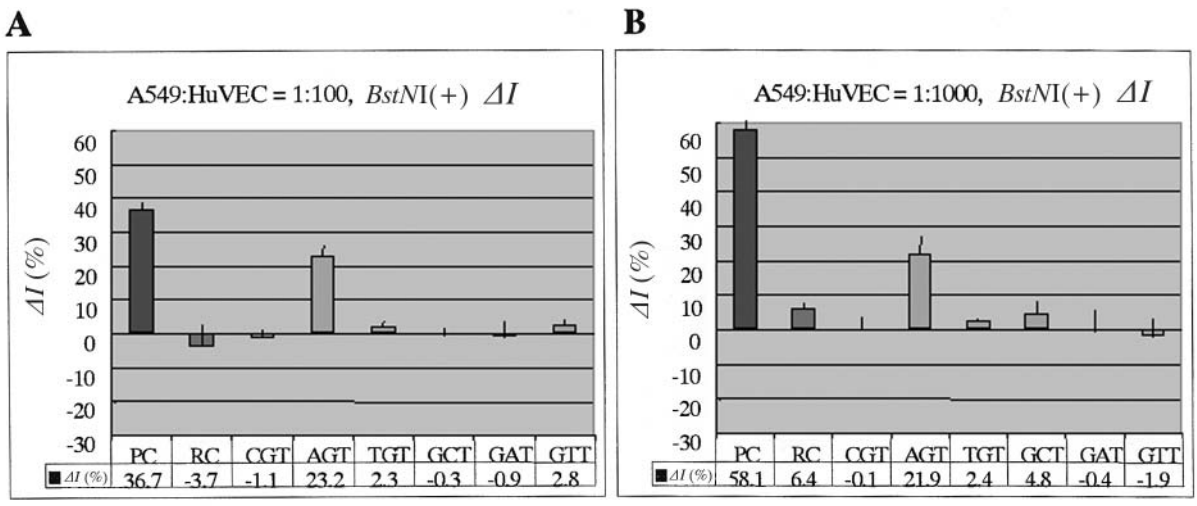

Figure 2. *Sensitivity of the ECA chip. The ECA chip detected the AGT mutation in codon 12 of k-ras in A549 cells mixed with 100-fold wild-type cells (A) and with 1000-fold wild-type cells (B).*

Table II. *Clinical data.*

Patient No.	Sex	Age (years)	Tumor site	TNM classification	Tumor stage[a]	Surgery	Outcome (months survival)
1	M	69	Phb	T4N1M0	IVa	TP+SP	Dead (9)
2	F	63	Pt	T3N1M0	III	DP+SP	Dead (17)
3	M	63	Ph	T4N1M0	IVa	PpPD	Dead (24)
4	F	69	Phb	T3N2M0	IVa	PD+IOR	Dead (9)
5	M	75	Ph	T3N1M0	III	PpPD	Dead (19)
6	M	36	Ph	T4N3M1	IVb	PD	Dead (7)
7	F	59	Ph	T4N1M0	IVa	PD	Dead (9)
8	M	75	Ph	T3N1M0	III	PD	n.d.
9	M	72	Pb	T3N0M0	III	DP+SP	Dead (34)
10	M	60	Pt	T4N1M0	IVa	TP+SP	Dead (5)
11	M	56	Ph	T3N1M0	III	PpPD	Dead (3)
12	M	62	Pb	T4N1M0	IVa	DP+SP	n.d.
13	M	58	Pb	T4N1M0	IVa	DP+SP	Alive (27)
14	F	57	Ph	T3N1M0	III	PpPD	Alive (32)
15	M	64	Ph	T3N0M0	III	PpPD	Alive (31)
16	F	61	Ph	T3N0M0	III	PpPD	Alive (30)
17	F	53	Ph	T4N3M1	IVb	PpPD	Dead (16)
18	M	72	Ph	T4N2M0	IVb	PpPD	Alive (28)
19	M	67	Pbt	T4N1M0	IVa	DP+SP	Dead (13)
20	F	64	Pbt	T3N1M0	III	DP+SP	Dead (5)

[a]Classification by Japan Pancreas Society.

showed a positive *ΔI* for the PC and GTT probes. The other 3 cell lines, A549 (GGT→AGT), MIA Paca2 (GGT→TGT) and LS174T (GGT→GAT), showed a positive *ΔI* for the PC probe and corresponding mutant probes (data not shown). These results showed that *k-ras* mutations can be identified accurately with the ECA chip.

Determination of the sensitivity of the ECA chip. To determine the sensitivity of the ECA chip, 4 tumor cell lines carrying *k-ras* mutations were mixed with 100- to 1000-fold wild-type HuVEC cells. When the A549 cell line was tested in a background of 100-fold wild-type HuVEC cells, more than 20% of *ΔI* was observed with the PC probe and mutant

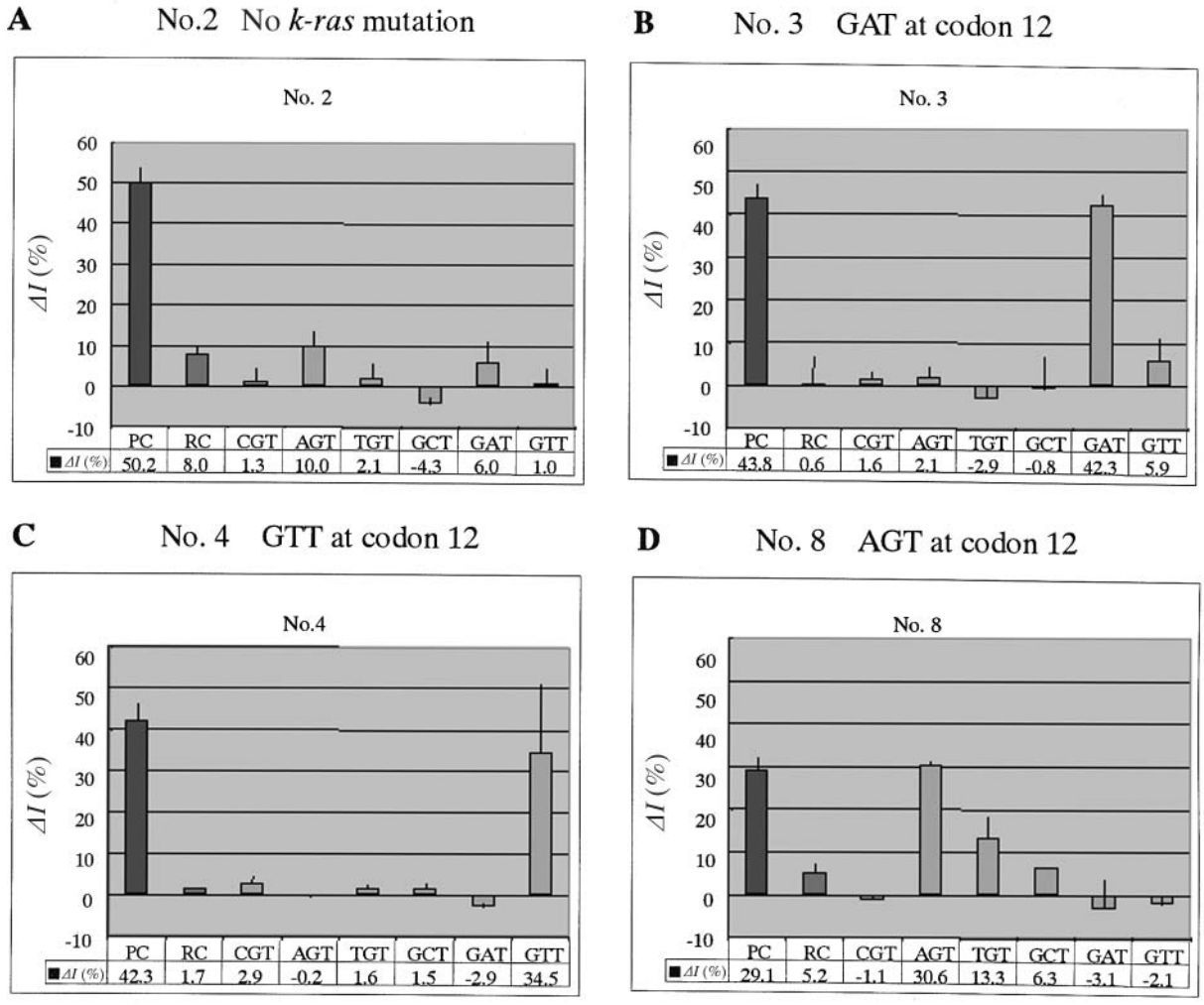

Figure 3. *ECA chip analysis of pancreatic cancer tissues. Three mutations in codon 12 of k-ras, GAT, GTT, and AGT were found on the 20 tissue samples investigated (B-D). ΔI over 20% was not obtained with any mutant probe in samples without k-ras mutations (A).*

probe for AGT (Figure 2A). A similar result was obtained when mutant DNA was mixed with 1000-fold excess wild-type DNA (Figure 2B). At a 10,000-fold excess of wild-type DNA, the mutation could not be detected (data not shown). In the other 3 cell lines, a positive *ΔI* was also detected for the mutant probe corresponding to each mutation and in the 1000-fold excess wild-type DNA (data not shown).

ECA chip analysis of tissue samples. DNAs extracted from 20 pancreatic cancer specimens were analyzed with the FND-ECA chip. The clinical features of the 20 pancreatic cancer patients are shown in Table II. Three mutations of codon 12 of *k-ras*, GAT, GTT and AGT, were identified (Figure 3) and mutations were present in DNAs from 17 out of 20 (85%) of the pancreatic cancer specimens. The GGT to GTT, GAT, or AGT mutations of codon 12 of *k-ras* were recognized in 12, 4, or 1 sample, respectively (Table III). Direct sequence analysis detected *k-ras* mutations (8 GTT, 4 GAT and 1 AGT) in 13 out of 20 samples (65%). PCR-PHFA identified mutations in 17 (85%) samples. These results were the same as the FND-ECA chip data, suggesting that the detection ability of the ECA chip data was superior to that of direct sequencing and similar to that of PCR-PHFA (Table IV).

Discussion

The present results indicate that the FND-ECA chip is a useful tool for the accurate, rapid and sensitive detection of *k-ras* mutations in human pancreatic cancer tissues. The mutation detection rate of the FND-ECA chip, 85%, was comparable to that of the highly sensitive PCR-PHFA

Table III. *K-ras mutations.*

	Direct sequence	ECA chip	PCR-PHFA
Total no. of positive cases	13	17	17
Type of *K-ras* mutation			
AGT	1	1	1
CGT	0	0	0
TGT	0	0	0
GAT	4	4	4
GCT	0	0	0
GTT	8	12	12

Table IV. *K-ras mutations in exon 1.*

Patient No.	Direct sequence	ECA chip	PCR-PHFA
1	(-)	GTT	GTT
2	(-)	(-)	(-)
3	GAT	GAT	GAT
4	GTT	GTT	GTT
5	GAT	GAT	GAT
6	GTT	GTT	GTT
7	GTT	GTT	GTT
8	AGT	AGT	AGT
9	GTT	GTT	GTT
10	GTT	GTT	GTT
11	GTT	GTT	GTT
12	(-)	GTT	GTT
13	(-)	(-)	(-)
14	GAT	GAT	GAT
15	GTT	GTT	GTT
16	(-)	GTT	GTT
17	GAT	GAT	GAT
18	(-)	GTT	GTT
19	(-)	(-)	(-)
20	GTT	GTT	GTT

method. Furthermore, the FND-ECA chip could detect different mutations and the measurement procedure takes less than 1 h. Pancreatic cancer tissues contain both cancer cells and non-cancerous cells, including normal duct, acinar, islet, inflammatory and fibroblast cells. Therefore, the FND-ECA chip must possess sufficient sensitivity to detect point mutations of tumor cells, at least, in a background of 100- to 1000-fold non-cancerous cells.

To increase the hybridization efficiency, 2-stage PCR was used. The first PCR containing *Bst*NI allowed for the simultaneous amplification of mutant *k-ras* and the inhibition of the amplification of wild-type *k-ras*. The second PCR generated the single-stranded and self-looped target that allows high affinity binding to probes immobilized on the ECA chip. Ligation between a fixed probe and target increases the contrast between positive and negative signals. FND allows the ECA chip to detect double-stranded DNA efficiently (8). An FND-ECA chip can detect one mutant in a background of 1000 wild-type (Figure 2).

In the present study, the detection rate of mutations in codon 12 of *k-ras* was 85%, consistent with the frequency of 75-90% reported previously (14). We detected the GTT, GAT and AGT mutations, corroborating previous reports that these mutations are common in pancreatic cancers (17, 18).

Theoretically, an FND-ECA chip can simultaneously detect multiple mutations of different types, including SNPs, deletions, insertions, translocations and short tandem repeats. For example, alterations in the *p53* tumor suppressor genes have been detected in 40-76% of pancreatic cancer tissues and mutational hot-spots are mainly located in exons 5 to 8 (19). Detection of *p53* in pancreatic cancer specimens by FND-ECA chip is now in progress in our laboratory. Our goal is to establish parallel analyses of multiple DNA targets in pancreatic cancer with the ECA chip to develop an automated analytic system for clinical use.

In conclusion, the FND-ECA chip provides a sensitive, rapid and reliable screening assay for the detection of point mutations in a variety of clinical samples and is, therefore, suitable for use as a screening method.

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