Detection of single nucleotide polymorphisms by minisequencing on a polypyrrole DNA chip designed for medical diagnosis

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With the increasing availability of genetic information and its relationship to human diseases, there is a growing need in the medical diagnostic field for technologies that can proceed to the parallel genotyping of multiple markers. In this paper, we report the development of a new flexible microarray-based method that aims to be inexpensive, accurate, and adapted to routine analysis. The construction of the MICAM[®] (MICrosystem for Analysis in Medicine) DNA chip is based on the controlled electrosynthesis of a conducting polymer film bearing oligonucleotide probes on gold electrodes. First, accessible 3'OH-ends of grafted probes are directly used to conduct single template-dependent nucleotide extension reactions with fluorescence-labeled chain terminators. Then, the fluorescence of incorporated dideoxynucleotides on controls and probes of interest are recorded to assess base calling. Here, we present the development of the methodology to assign the genotype of *TP53* (tumor protein p53) codon 72 polymorphism and its application to analysis of genomic DNA from cell lines and from human colorectal samples. The genotyping results obtained by minisequencing on the polypyrrole DNA chip were 100% concordant with data obtained by polymerase chain reaction-restriction fragment length polymorphism and direct sequencing. Moreover, the developed probe array assay has been successfully applied to the detection of *TP53* loss of heterozygosity.

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The DNA of the human genome has numerous sequence variations, among which base substitutions are the most common. Depending on the location of the single-nucleotide variation, including single nucleotide polymorphisms (SNPs) and point mutations, different consequences may be brought about at the phenotypic level. SNPs in gene coding regions may alter the function or the structure of the encoded proteins and be associated with inherited monogenic disorders. On the other hand, mutations may be associated with complex multigenic pathologies such as diabetes,¹ cardiovascular diseases,² or cancer.³ Particularly in the field of oncology, mutations or polymorphisms in some cancer-related genes may be potential markers and

contribute to the diagnosis of the pathology, determination of prognosis⁴ or management of the therapy.⁵ In this context, it is interesting for medical diagnostic purposes to proceed to the parallel genotyping of multiple SNPs/mutations.

In recent years, a multitude of genotyping methodologies for the detection of single nucleotide variations have been described.^{6–8} Classic genotyping technologies such as direct sequencing are complex, expensive, and time-consuming when large numbers of markers are required for a patient. To avoid these limitations, a promising approach would be to use arrays of oligonucleotides (DNA chips) on a solid support in miniaturized assays. The concept of oligonucleotide arrays was originally introduced for *de novo* nucleotide sequence-based hybridization.⁹ High-density oligonucleotide arrays such as variation detection arrays manufactured by Affymetrix have been developed to analyze single nucleotide variations using the principle of allele-specific oligonucleotide (ASO) hybridization.¹⁰ Hybridization chips contain thousands of oligonucleotides, matching normal or mutant sequences. Under

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controlled hybridization conditions, probe–target heteroduplexes could be discriminated from perfectly matched probe–target duplexes, thus allowing the analysis of several polymorphisms or mutations. Nevertheless, this method presents limitations when multiple markers are simultaneously analyzed.¹¹ Indeed, annealing efficiency and thermal stability depend strongly on both the nucleotide sequence composition and the stringency of the reaction conditions. It is then difficult to design a single set of experimental conditions that would provide optimal signal intensities and allow a simultaneous discrimination of a large number of sequence variants. To circumvent limitations of hybridization-based approaches, enzyme-mediated reactions have been developed.

Particularly, minisequencing is becoming the preferred allelic discrimination technology for the detection of known polymorphisms. Minisequencing is based on the extension of the 3′OH-end of a probe by a single labeled dideoxynucleotide (ddNTP). Unlabeled PCR-amplified test DNA is hybridized to the probes, and extension by a DNA polymerase occurs only when the labeled ddNTP is complementary to the nucleotide of the target DNA adjacent to the 3′OH-end of the probe. This method is more accurate than ASO because the distinction between sequence variants depends on polymerase-catalyzed incorporation. The vast majority of array assays designed for genotyping by minisequencing such as tag arrays developed by Orchid Biosciences¹² and Affymetrix¹³ relies on a two-step process. It includes a liquid-phase enzymatic reaction followed by a detection step based on the capture of extension products on an array containing complementary hybridization tags.¹⁴ Recently, microarray-based minisequencing using solid-phase immobilized extension primers has been proposed for the analysis of the complete sequence of selected genes.¹¹

Based on the availability of the human genome,^{15,16} high-density genotyping arrays have been designed to conduct large-scale linkage analysis or association studies, combining high-throughput with low data point cost. Unfortunately, such formats are not well adapted to medical diagnostic tests, where only a limited number of markers is needed.

Here, we describe a new flexible microarray-based method, especially designed for diagnostic purposes. This technology was designed to be inexpensive, accurate, and rapid, with a throughput adapted to routine analysis. The process is named MICAM[®] (MICrosystem for Analysis in Medicine). The construction of the MICAM[®] DNA chip, based on the fixation of 5′ pyrrole-labeled oligonucleotides on gold electrodes, has been described previously.^{17,18} The application of the MICAM[®] DNA chip to the detection of *K-ras* mutations in tumor samples by using ASO hybridization has been published recently.¹⁹ Here, we report the ability to perform

minisequencing reactions on the developed polypyrrole DNA chip using the free 3′OH-ends of grafted probes. The assay was applied to the *TP53* (tumor protein p53) codon 72 SNP genotyping²⁰ of DNA from cell lines and human colorectal samples. Recently, it has been demonstrated that this SNP could be an important determining factor for the prediction of treatment response and survival in clinical oncology.²¹

Materials and methods

Cell Lines

Breast and pancreatic carcinoma cell lines with a known *TP53* codon 72 genotype, SK-BR-3 (homozygous, CGC (arginine (Arg))) and MIA PaCa-2 (homozygous, CCC (proline (Pro))), respectively, were purchased from the American Type Culture Collection and cultured under standard conditions.

Colorectal Samples

Surgical samples from colorectal carcinomas and the corresponding normal mucosa were resected from colorectal cancer patients (CRLC Val d'Aurelle, Montpellier, France) and stored in liquid nitrogen until extraction.

DNA Extraction

High-molecular-weight DNA from cell lines and patient colorectal samples was extracted using a classic phenol–chloroform protocol as described previously.²² All DNA samples were stored at –80°C. The determination of *TP53* codon 72 polymorphism was achieved through direct sequencing or polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) as described previously.^{23,24}

Preparation of Chips

MICAM[®] biochips are the result of microelectronic research and are made on a silicon support bearing 128 gold electrodes.¹⁷ Probes to be grafted (Table 1) are synthesized *ex situ* and purified. They are labeled at their 5′-end with a pyrrole residue using pyrrole-phosphoramidite building blocks and tailed with a poly(T)₁₀ spacer arm. The pyrrole group enables probe fixation on the gold electrodes using an electrocopolymerization process as described previously.¹⁸ Briefly, each of the 128 electrodes (50 μm) was successively functionalized by electrochemical polymerization (1 V/saturated calomel electrode) in an aqueous 0.1 M LiClO₄ solution containing 20 mM pyrrole and 1 μM of the pyrrole oligonucleotide to be addressed.

Table 1 Characteristics of the grafted probes

Probe	5' → 3' sequence ^a	Function
P72S	GCTCCCAGAATGCCAGAGGCTGCTCCCC	<i>TP53</i> codon 72 genotyping probe
PC	CCTACCGTACCCCTCCCGT	Positive control
SEPA	^b TTTAGCCTTAACGCCTATGACGTCA	Extension control
SEPC	TTTAGCCTTAACGCCTCTGACGTCA	Extension control
SEPG	TTTAGCCTTAACGCCTGTGACGTCA	Extension control
SEPT	TTTAGCCTTAACGCCTTTGACGTCA	Extension control
DCbio	Bio-GCCTTGACGATACAGCTA	Detection control

Bio = biotin.

^aEach probe was 5'-labeled with a pyrrole residue and a d(T)₁₀ oligonucleotide linker.^bFor each SEP, the nucleotide in bold enabled template-independent extension with the complementary chain terminator.

Target Preparation

PCR

A region of 80 bp containing the polymorphic site of interest was amplified from cell lines and patient DNA samples by PCR. Amplification was performed in a T_{GRADIENT} Thermocycler (Whatman-Biometra, Goettingen, Germany) with a final volume of 25 μ l. The reaction mix contained 1 $\times$ Qiagen PCR buffer, 200 μ M dNTP, 0.48 μ M of each forward (5'-GAA GACCCAGGTCCAGATGA-3') and reverse (5'-GGTGTAGGAGCTGCTGGTG-3') primer, 2.5 U Hot-StarTaq[®] DNA Polymerase (Qiagen, Courtaboeuf, France), and 100 ng of genomic DNA. The forward primer was 5'-phosphorylated to allow the enzymatic digestion of the sense strand. The enzyme was first activated during an initial denaturation step for 15 min at 95°C, followed by 17 cycles at 94°C for 30 s, 66°C for 30 s, and 72°C for 90 s, with a touch down of -0.5°C for the annealing temperature between each cycle. Then, 14 normal cycles were carried out at 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s. A final extension step of 10 min at 72°C completed the reaction. Qualitative and quantitative analysis of PCR products was performed on a DNA1000 LabChip[®] (Agilent Biotechnologies, Massy, France) and analyzed with the 2100 Bio-analyzer (Agilent Biotechnologies).

Generation of ssDNA

The primer excess was first eliminated during an exonuclease I (Amersham Biosciences, Orsay, France) digestion, then the 5'-phosphorylated sense strand was digested with lambda exonuclease (Amersham Biosciences). The reactions were driven at 37°C for 30 and 45 min, respectively. After each digestion, the enzyme was inactivated at 80°C for 15 min. The resulting purified antisense strand was kept at 4°C until subsequent reactions.

Genotyping of *TP53* Codon 72 on the Chip

Minisequencing reactions

All minisequencing reactions were carried out in a hermetic reaction chamber placed in a temperature-

controlled incubator. Before use, the chips were hydrated for 5 min with 20 μ l of ultrapure water DNase/RNase free (Sigma, Saint Quentin Fallavier, France). A prehybridization step was first conducted for 15 min at 45°C in a buffer containing 9 mM phosphate, 2.5 mM KCl, 0.5 M NaCl, 0.05% Tween-20, 1 $\times$ Denhardt, 10 μ g/ml salmon sperm DNA, and 0.2 μ M polyA. During this time, the targets (1 pmol of the single-stranded PCR product and 500 fmol of a positive control (PC) oligonucleotide) were heated at 95°C for 5 min. The PCR product contained the *TP53* codon 72 polymorphic site of interest, which was the template for the single extension of the P72S probe (Table 1). The PC target (PCT: 5'-GTAGTGACTTCATTANACGGGAGG GGGTACGGTAGGTACGGCTTGTTCTT-3'), with no homology with the human genome, was used to check the global quality of the various steps (hybridization, extension, and detection). Nucleotide N (A, C, G, or T) was the template for the single extension of the PC probe (Table 1). It was complementary to the biotinylated ddNTP used in the minisequencing reaction. Target hybridization was performed in a final volume of 20 μ l buffer (9 mM phosphate, 2.5 mM KCl, 0.5 M NaCl, 0.05% Tween-20, 1 $\times$ Denhardt, 10 μ g/ml salmon sperm DNA) for 1 h at 45°C. The chips were then washed for 5 min with the washing buffer. Next, 20 μ l of the extension mix (20 pmol of the appropriate biotin-labeled ddNTP, 20 pmol of each of the other unlabeled ddNTPs, and 8 U of the Thermo Sequenase[™] (Amersham Biosciences) were added. The extension reaction was carried out at 45°C for 5 min and then at 60°C for 15 min.

Denaturation and detection

To stop the reaction and melt the complexes formed by the target and the extended probe, the chip was washed successively with distilled water for 10 s, then with 50% formamide at 60°C for 3 min, and finally with distilled water for 10 s. Biotinylated ddNTP incorporated into the probes was detected by incubation with a 50 mg/l solution of a streptavidin-R-phycoerythrin conjugate (Invitrogen, Cergy Pontoise, France) in detection buffer for 10 min under

dark conditions. Finally, the chips were washed for 10 min with the washing buffer.

Acquisition and analysis of the fluorescent signals
Washing buffer was replaced by detection buffer and the chip was directly analyzed on the Apimager® (Apibio, Grenoble, France).²⁵ Fluorescence levels were allocated to each probe with the analySIS® software (Soft Imaging System GmbH, Munster, Germany).

Results

Accessibility of 3'OH-Ends of Grafted Probes

Before designing the minisequencing assay, the accessibility of 3'OH-ends to the enzymatic reactions was checked using terminal deoxynucleotidyl transferase (TdT). TdT is a template-independent DNA polymerase that catalyzes the irreversible terminal repetitive addition of deoxyribonucleotides to the 3'OH-end of DNA. During this step, probes were extended with one or more biotinylated dATP, which were further detected using the streptavidin-R-phycoerythrin conjugate. Whatever the probes grafted onto the solid support (length, sequence, GC percent), a high specific signal was obtained. The mean fluorescence value for the extended probes was 13- to 27-fold higher than the nonspecific

background value (nonaddressed gold electrodes or electrodes grafted with a homopolymer of pyrrole).

Assay Design

A flow chart of the method is presented in Figure 1.

Reaction controls

In addition to the TP53 codon 72 genotyping probe (P72S probe), several control probes (Table 1) were designed and grafted onto the chip to verify the completion of the various minisequencing steps. First, a PC probe that evaluates the whole genotyping process (target hybridization, extension, and detection) was designed. For each biotinylated ddNTP incorporated, a PCT, whose central sequence was complementary to the PC probe, was designed. The four 50-base-long PCT differed from each other by only one base. Then, two other probes, were added. To test the extension and revelation steps, were added. Four self-elongating probes (SEP)²⁶ were used to validate the extension step. The 3'OH-ends of these probes are self-complementary and able to form dimers directly on the solid support. Each SEP, differing from one another by only one nucleotide, allowed the incorporation of a specific ddNTP in a template-independent extension reaction. A biotinylated probe (biotinylated detection control (DCbio)), corresponding to a sequence from exon 1 of the *K-ras* gene (outside potentially mutated

Figure 1 Method overview.

codons 12 and 13), was then grafted onto the chip to verify the streptavidin-R-phycoerythrin detection step.

Assay optimizations

The key steps of the minisequencing reaction for SNP genotyping in the MICAM[®] format were optimized in preliminary experiments, resulting in the protocol provided in the Materials and methods section. These optimizations covered probe and target design, reaction conditions, and reagent concentrations. First, we analyzed the impact of the pyrrole-labeled probe concentration during the grafting process on the hybridization signal. An increase from the standard 1 μ M concentration, corresponding to an estimated amount of 200 fmol/mm² of linked oligonucleotide,¹⁸ to 3 μ M combined with a 1:20 000 pyrrole-labeled probe/pyrrole monomer ratio resulted in a significant improvement of the hybridization signal (data not shown). Then, to increase the annealing efficiency, several probes were tested by hybridization with biotinylated targets. For minisequencing purposes, the last four bases at 3'OH-end of the oligonucleotide probes must not be changed. Therefore, we focused on the length of both the poly(T) spacer arm and the probe in the direction of the 5'-end. Poly(T) tails from 0 to 15 base long were tested, and the best results were observed with a (T)₁₀ tail (data not shown). The optimal probe length was sequence dependent, and typically, selected probes demonstrating maximal hybridization signals were 20–30 bases long.

Regarding target design, we initially studied the incidence of the nonhybridized 3'OH-end of the target on the hybridization signals. The role of this 3'-dangling end was assessed with a set of oligonucleotides and PCR products designed with or without a 3'-tail. Our results (data not shown) demonstrated that not only did the 3'-dangling end not constrain target annealing but it further stabilized the generated duplex. When the impact of target length was studied, we found an optimal hybridization for short products, less than 150-bp-long. We decided to use 80-bp-long products for subsequent experiments. The use of single-stranded targets obtained by lambda exonuclease digestion of PCR products displaying a 5'-phosphate primer increased the specific signals by 50%, whereas it decreased nonspecific signals by half. Finally, we extensively studied reaction conditions and reagent concentrations (buffer composition, step duration, and reaction temperatures).

Probe specificity

Probe design for a target-dependent solid-phase extension must fulfill several criteria. The principal requirement for the PC and the P72S probes is the absence of terminator incorporation when no target is used in the minisequencing reaction. Potential misincorporations during the extension step, caused by probe hairpins or dimer formations, were studied

for each of the four ddNTPs. Under our optimized minisequencing conditions, no template-independent misincorporation occurred. In the absence of target, only the SEP were extended in the presence of the appropriate ddNTP, whereas the mean fluorescence values for the P72S and PC probes represented less than 1.5% of the specific SEP signals.

Array Minisequencing Characterization

Technical quality

Evaluation of the technical quality of our genotyping process was established by comparing the mean fluorescence signals of the PC probes with the background, that is, the nonaddressed gold electrodes. The absence of nonspecific fixation of biotinylated ddNTP or streptavidin-R-phycoerythrin was observed with low signals on nonaddressed electrodes. For 33 polypyrrole DNA chips reacted, each containing 25 nonaddressed electrodes, the mean background signal ($\pm$ s.d.) was 9 ± 12 fluorescence units (FU), whatever the biotinylated ddNTP used. Regarding the variability of minisequencing PCs, a total of 20 chips, each containing four PC probe electrodes, were reacted with biotinylated ddCTP or ddGTP terminators. The intrachip coefficients of variation were less than 8%. Concerning the interchip analysis, the PC mean fluorescence signal ($\pm$ s.d.) was 1762 ± 249 FU. The low signals on the nonaddressed electrodes and the high signal on the PC electrode resulted in a high signal/noise ratio.

Genotype assignment

Currently, base calling of a biallelic SNP requires two minisequencing reactions on two different polypyrrole DNA chips. To allow comparison between experiments and to prevent chip-to-chip variations, interpretation of results was based on relative data rather than absolute values. For this purpose, the PC probe was used for normalization. To validate the specific incorporation of one biotinylated ddNTP into the *TP53* codon 72 genotyping probe, we calculated the identification (ID) percentage: (P72S signal/PC signal) $\times$ 100, where the P72S signal represents the mean fluorescence value for the P72S probes.

Specificity and sensitivity

To evaluate the ability of the methodology to analyze genomic DNA, minisequencing reactions were performed on two cell lines with known *TP53* codon 72 genotype: SK-BR-3 (homozygous, CGC (Arg)) and MIA PaCa-2 (homozygous, CCC (Pro)). After the generation of single-stranded antisense DNA from each cell line, minisequencing was directly carried out on the MICAM[®] chip. Both genotypes were unequivocally defined. Whereas high ID percentages were obtained when the correct ddNTP was incorporated into the P72S probes, the absence of

specific incorporation was correlated with low ID percentages. For example, using a chip with a defined probe pattern (Figure 2a), when biotinylated ddCTP was used, the ID percentage was 96% for the homozygous CCC codon 72 cell line MIA PaCa-2 (Figure 2b) and 3% for the homozygous CGC codon 72 SK-BR-3 cell line (Figure 2c). Then, to use this ID percentage as a genotyping tool, the threshold value, beyond which a ddNTP incorporation is considered as specific, was determined. For this purpose, we analyzed the minisequencing data (Table 2) obtained with homozygous *TP53* codon 72 targets from both cell lines and human genomic DNA. Our experiments showed that whatever the biotinylated ddNTP used, the ID percentages for the nonspecific signals were always lower than 17%. Finally, to assess the sensitivity of the approach developed, the ability to detect a minor fraction of a mutated allele within a large proportion of wild-type alleles was evaluated by mixing amplicons from the SK-BR-3 and MIA PaCa-2 cell lines. The results (Table 3) demonstrated that a sequence variant, corresponding to less than 25% of the total targets, could be unambiguously detected. Moreover, when only 10% of this allele is present in the reaction, the ID value (23%) is superior to the maximal ID value reported for the nonspecific incorporation (17%). Based on these two ID values, the threshold value was set at 20%.

Application to medical diagnosis

Patient DNA samples extracted from surgically resected normal colorectal mucosa were analyzed by minisequencing on the MICAM[®] DNA chip, and the results were compared with data obtained by

PCR-RFLP and direct sequencing. Figure 3 illustrates the results obtained from three patients with three different genotypes. A total of 23 samples was

Table 2 Specificity of ddNTP incorporation into the *TP53* codon 72 genotyping probe (P72S probe)

Biotinylated ddNTP		Number of experiments	ID ^a (%)	
			Mean	Range
ddGTP	Specific signal ^b	28	80 ± 14	64–108
	Nonspecific signal ^c	16	6 ± 5	1–13
ddCTP	Specific signal ^c	16	112 ± 20	81–147
	Nonspecific signal ^b	32	8 ± 5	1–17

^aIdentification percentage: (P72S signal/PC signal) × 100.

^bMinisequencing reactions using homozygous CGC *TP53* codon 72 targets.

^cMinisequencing reactions using homozygous CCC *TP53* codon 72 targets.

Table 3 P72S probe extension with biotinylated ddCTP as a function of the proportion of amplicons from MIA PaCa-2 and SK-BR-3

Amplicon ratio (MIA PaCa-2: SK-BR-3)	Fluorescent-specific signal ^a (fluorescence units)	ID ^{a,b} (%)
100:0	2358 ± 222	96 ± 9
50:50	2064 ± 313	57 ± 9
25:75	514 ± 90	47 ± 8
10:90	331 ± 34	23 ± 2
0:10	49 ± 29	3 ± 2

^aThe results represent the mean values of eight experiments.

^bIdentification percentage: (P72S signal/PC signal) × 100.

- P72S: *TP53* codon 72 genotyping probe
- PC: positive control probe
- SEPG: extension control probe
- DCbio: detection control probe

Figure 2 Cell line genotyping on the silicon chip. (a) Probe pattern. Minisequencing reactions were performed as described in Materials and methods on SK-BR-3 (b) and MIA PaCa-2 (c) cell lines by using biotinylated ddCTP as chain terminator.

Figure 3 *TP53* codon 72 genotyping by the MICAM[®] assay, PCR-RFLP, and direct sequencing. **(a)** *TP53* codon 72 genotyping by minisequencing on MICAM[®]. **(b)** *TP53* codon 72 genotyping by PCR-RFLP. A 102 bp fragment including *TP53* codon 72 was amplified and then digested by the restriction enzyme *Bst*UI, which recognizes and cuts the restriction site CG▼CG. The site was present in the homozygous G/G codon 72 samples (1). For homozygous C/C genotypes, the CCGG sequence was not cleaved (2). For heterozygous genotypes, three bands were visible (3). **(c)** Direct sequencing of *TP53* exon 4 including codon 72. PCR product sequencing was performed on the automatic DNA sequencer ALFexpress (Amersham Pharmacia Biotech) by using the thermosequenase fluorescent-labeled primer cycle sequencing method.

analyzed by the three approaches, and the genotypes scored by the polypyrrole DNA chip assay were 100% in agreement with conventional techniques. Moreover, because the codon 72 SNP may be used as an intragenic marker to detect loss of heterozygosity (LOH), we analyzed genotypes from paired tumor/normal colorectal mucosa for various heterozygous patients. Typical results are reported in Table 4 for both an LOH and a non-LOH patient. DNA chip assays demonstrated that all ID scores for patient 95B390 were higher than 20%. These values corresponded to a specific extension of the P72 probes by both the ddCTP and the ddGTP chain terminators in normal and tumor tissues. This patient had a heterozygous genotype with no LOH. In contrast, patient 92B439 showed a heterozygous

genotype with an LOH in the corresponding tumor mucosa. This LOH reflects a *TP53* allelic loss, previously determined on this patient by micro-satellite analysis.²³

Discussion

Single nucleotide polymorphisms represent a natural genetic variability with high frequency throughout the human genome. They can convey enhanced or reduced susceptibility towards specific inherited disorders by impairing or improving physiological function. They have been implicated in both monogenic and multigenic diseases.²⁷ A high level of complexity is attained with diseases such as

Table 4 LOH analysis on genomic DNA from colorectal cancer patients

Patient number	Tissue	Biotinylated ddNTP	ID ^a (%)	Genotype	LOH analysis
95B390	Normal	ddGTP	36	G/C	No LOH
		ddCTP	77		
	Tumor	ddGTP	26	G/C	
		ddCTP	66		
92B439	Normal	ddGTP	44	G/C	LOH
		ddCTP	112		
	Tumor	ddGTP	80	G	
		ddCTP	17		

LOH = loss of heterozygosity.

^aIdentification percentage: (P72S signal/PC signal) × 100.

cancer,²⁸ where the disorder results from an accumulation of multiple alterations in a variety of genes regulating cellular growth, apoptosis, angiogenesis, and several other mechanisms. Another important group of genetic variations is those that alter the primary structure of a protein involved in drug metabolism and constitutes a target for pharmacogenomics.²⁹ This approach represents a major advance in the medical field that would certainly help to refine the focus of treatments. This is especially important for oncology, where severe systemic toxicity and unpredictable efficacy are hallmarks of cancer therapies.³⁰ In general, genes do not act in isolation, and response to treatment often relies on complex mechanisms. Therefore, polygenic analytical strategies for SNPs/mutations should be preferred over single gene studies. One can envision a future whereby comprehensive assessment of a set of genetic markers is conducted at the diagnostic stage. To be effective, this (r)evolution needs the development of diagnostic tools adapted to routine use.

To perform multiple clinically valuable tests for SNP/mutation detection, we propose a low-complexity (128 positions) silicon chip that fulfills the criteria of the clinical diagnostic market. This study reports the design of a new DNA chip based on electrocopolymerization of 5'-pyrrole-labeled oligonucleotides and minisequencing allelic discrimination for genotyping of the *TP53* codon 72. Our assay allowed us to distinguish homozygous and heterozygous sequences unambiguously in both cell lines and human colorectal DNA samples. Moreover, application of the developed assay to paired non-tumor/tumor samples allowed the analysis of *TP53* allelic loss.

Traditionally, DNA chip manufacturing has been achieved either by 'on-chip' or 'off-chip' technologies. In the first case, microarrays of oligonucleotides are produced at very high density by combinatorial light-directed *in situ* synthesis on solid surfaces.³¹ Originally intended for ASO hybridization, these chips cannot be directly applied in DNA polymerase-assisted reactions since the oligonucleotide synthesis proceeds in the 3'→5'

direction. To overcome this problem, 3'→5' complementary 'tag' oligonucleotides have been synthesized *in situ* and used as capturing probes for minisequencing products.¹⁴ To obtain DNA chips presenting at their surface free 3'-OH groups, various array *in situ* synthesis strategies have been devised based on the mask or maskless approach.^{32–34} As *in situ* synthesis remains an attractive method for manufacturing high-complexity chips with hundreds of thousands of probes, an 'off-chip' technology is preferable for low-complexity array. In this case, individual oligonucleotides are synthesized prior to immobilization on the chip and may be used as substrates for enzymatic reactions. Enzyme-based methods for SNP/mutation detection are inherently more specific than those based on hybridization.^{11,35} Several approaches such as cleavage (eg the Invader™ assay),³⁶ ligation (eg oligonucleotide ligation assay³⁷ and ligation detection reaction³⁸) or polymerase extension (eg pyrosequencing³⁹ or minisequencing^{26,40}) have been developed. Among these alternatives, minisequencing is becoming the most widely used method for SNP/mutation scoring because of its robustness, accuracy, simplicity, and flexibility. Moreover, the basic procedure has been adopted on several platforms.⁶ For solid-phase-based minisequencing reactions, several immobilization chemistries of oligonucleotides have been tested.⁴¹ In particular, for glass supports, it has been demonstrated that the efficiency of genotyping by minisequencing is largely dependent on the type of oligonucleotide attachment.⁴¹ In our solid-phase minisequencing assay, oligonucleotides are fixed on gold electrodes using pyrrole linkers in an electrochemical addressing process.^{17,18} Combined with a real-time quality control performed at each stage of the production, the developed array, where each electrode corresponds to a different unit of hybridization, is characterized by a homogeneous probe density at the electrode surface with free 3'-OH groups available for enzymatic reactions. Moreover, its microelectronic support structure yields highly predefined hybridization units compatible with easy readout. With regard to chip packaging, the array is easy to handle, and the microreaction

chamber created is suitable for all steps of the genotyping process.

In this study, we have demonstrated that the 3'-OH ends of the grafted probes were accessible to various enzymes such as TdT and DNA polymerase. Minisequencing, like almost all allelic discrimination techniques, has its difficulties when attempting to type all polymorphisms. Nevertheless, the main steps of the minisequencing reaction have been extensively studied and certain general rules can be inferred. Our findings showed that longer probes improved the final signal. Optimal results were obtained with probes of interest ranging from 24 to 30 hybridizable bases. This length is similar to those previously reported in this type of array assay.⁴² For liquid-phase minisequencing, 24- to 30-base-long SNP primers should be used for experiments based on fluorescence polarization, whereas for mass spectroscopy assays, probes as short as 16 bases are preferred. In the absence of a cycling reaction, allowing the generation of large amounts of extension products, the quality of the target is primordial. We ensured optimal signals with short (less than 150 bases) single-stranded targets, presenting 3'- and 5'-dangling ends.

Using the *TP53* codon 72 polymorphism as a study model, minisequencing directly on the polypyrrole DNA chip was characterized by a high signal/noise ratio, and a high degree of reproducibility was obtained when various specific P72S probes were analyzed on the same chip. Nevertheless, despite these technical qualities, signal variations on extended probes were observed from chip to chip. Interchip fluctuations of minisequencing results are related to the individual chip electrocopolymerization process, and work on a wafer format is in progress to address several chips in parallel. In the future, batch production derived from a microelectronic standard will allow on-demand manufacturing of large volumes of biochips at competitive costs.

To allow comparison between experiments, the PC probes were used for normalization, and the ID percentage (probe of interest signal/PC signal) was calculated. Determination of methodology sensitivity showed that an amount of an allelic variant corresponding to less than 10% of the total DNA could be detected. This compares favorably with the sensitivity observed by using minisequencing and fluorescence detection on a glass support.⁴² For mutation genotyping, this detection threshold could be lowered. One option is to reduce the noise level by modifying the optics of the Apimager®. Structured lighting by using liquid crystal matrix may restrict excitation light to the electrodes, thus decreasing the background signal. For base calling, analyses were carried out to determine a threshold, beyond which a ddNTP incorporation is considered as specific. Based on the data obtained from both nonspecific incorporation signals (Table 2) and sensitivity (Table 3), this cutoff value was set at

20%. One should note that all the ddNTPs did not have the same level of incorporation. This observation is not assay format dependent, since it has already been observed.⁴³ Especially, in the assay developed, ddCTP was more efficiently incorporated than ddGTP, and these data were taken into account for result interpretation. This is illustrated for tumor tissues from patients 95B390 and 92B439 (Table 4), displaying two borderline ID values, 26 and 17%, respectively. In the first case, there is no LOH, because the ID percentage (26%) is close to the value of 36% obtained for the normal tissue and two-fold greater than the highest nonspecific signal observed for this ddNTP (13%). In the second case, the ID percentage (17%) is less than the cutoff value of 20% and much lower than the value obtained for normal tissue (112%). This led to the conclusion that there is no specific ddCTP incorporation in this case, meaning that there is an LOH. For LOH analysis, one could not exclude the presence of a small proportion of normal tissue, making result interpretation difficult in some cases.

Application of SNP scoring by the polypyrrole DNA chip assay to 23 human genomic DNA samples demonstrated a 100% correlation with conventional techniques such as direct sequencing and/or PCR-RFLP. Finally, the number of minisequencing reactions required per sample on the developed diagnostic platform could be reduced from four to one by using ddNTPs labeled with four distinct dyes and a multicolor detection system.

In conclusion, the present results demonstrate that this flexible off-chip technology is well adapted for clinical diagnosis. It should be possible to increase significantly the number of polymorphic sites that can be typed per chip, without requiring additional work, by amplifying several separate targets during a multiplex PCR. Moreover, a quantitative application of this silicon device is conceivable because the number of grafted probes is fully controlled. Therefore, one could envisage both genotyping and gene expression analysis on the same support, with the same grafting technology, using the same analysis platform.

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