K-ras Mutation Detection by Hybridization to a Polypyrrole DNA Chip

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Background: Detection of mutations in cancer-related genes is of major importance for both basic knowledge and clinical practice. Several strategies have been developed to diagnose these alterations. We describe a method based on polypyrrole DNA chip technology to detect K-ras gene mutations in tumors.

Methods: An oligodeoxynucleotide array was constructed on a silicon device by copolymerization of 5'-pyrrole-labeled oligodeoxynucleotides and pyrrole. The samples to be analyzed were then amplified by PCR, and the single-stranded biotin-labeled amplified DNA was specifically hybridized to the addressed probes. Perfectly matched duplexes were detected by fluorescence microscopy using R-phycoerythrin as the detection label. The developed methodology was applied to genotype assignment of K-ras in human samples. The genotypes of 75 DNA genomic samples from colorectal cancer patients were analyzed side by side using direct DNA sequencing and a polypyrrole DNA chip.

Results: The chip method unequivocally defined all of the genotypes. Mutations present at <10% of the wild-type DNA concentration could be distinguished.

Conclusions: This probe array assay is a rapid and reliable procedure that may be used to detect mutations.

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The rapid rate of characterization of disease-causing genes has prompted the development of new strategies for nucleic acid sequence analysis. In this field, the combination of solid supports bearing a set of immobilized oligodeoxynucleotide (ODN) probes with the allele-specific oligonucleotide hybridization reaction is a useful tool. Initially, this type of technique was conducted on nylon or nitrocellulose membranes (1). To increase the number of fixed probes and reduce sample volumes, oligonucleotide arrays were developed. Different supports have been used in this way, such as glass (2, 3), polypropylene sheets (4), polyacrylamide gel pads (5), and silicon (6). The precise location of ODNs can be achieved through photochemistry (7) or through micro-mechanical devices (8). We previously described another arraying mode based on the electrosynthesis of a conducting polymer film, including ODNs (9–11).

The K-ras protooncogene is altered by point mutations on codon 12, 13, or 61 in a wide variety of tumors (12). The detection of K-ras mutations enables understanding of cancer biology and pathogenesis. Pancreatic and lung cancers harbor high incidences of K-ras mutant alleles, and these mutations are early events in colorectal tumor development (13). Moreover, alterations involving this oncogene may be of clinical importance because they can provide information for early diagnosis and prognosis (14–16). Activation of the K-ras gene has been detected not only on the tumor, but also in the stools (17) and serum (18) of patients with colorectal cancer. PCR protocols have been described for the diagnosis of pancreatic adenocarcinoma from the presence of mutant K-ras DNA in pancreatic juice (19); therefore, according to the clinical implication of this gene in human tumorigenesis and its potential role as a target for novel therapeutic approaches, reliable methods are needed for the analysis of the K-ras sequence in clinical samples.

Recently, we developed a rapid, large-scale method to detect K-ras gene mutations in tumor samples (20). This approach is based on the use of biotinylated oligonucleotide probes fixed to avidin-coated tubes and a radioactive detection method. In this technology, each tube corresponds to a specific K-ras mutant allele, and to determine the K-ras codon 12 sequence, seven tubes are necessary (one tube for the wild-type sequence, three tubes corresponding to replacement of the first base of the codon, and three other tubes for the mutation on the second base of the codon).

To eliminate the use of radioactive labels and to allow the simultaneous analysis of the seven K-ras codon 12 alleles in a single hybridization, an oligonucleotide array...
on a silicon device allowing fluorescence detection has been developed. The construction of this DNA chip is based on the electropolymerization of 5'-pyrrole-labeled oligonucleotides and pyrrole. Here, we report the technical development and use of this assay for the detection of K-ras mutations in DNA from human colorectal carcinomas.

**Materials and Methods**

**CELL LINES**
Lymph node metastasis of colon adenocarcinoma cell line SW620 [homozygous, GGT(Gly)→GTT(Val)], pancreatic carcinoma cell line Mia PaCa-2 [homozygous, GGT(Gly)→TGT(Cys)], and colon adenocarcinoma cell lines SW1116 [heterozygous, GGT(Gly)→GCT(Ala)] and LS 174T [heterozygous, GGT(Gly)→GAT(Asp)], which display a mutation on the K-ras codon 12 sequence, and the lymph node metastasis of prostate carcinoma cell line LNCaP (wild-type K-ras codon 12 sequence) were purchased from the American Type Culture Collection.

**COLORECTAL SAMPLES**
Samples were obtained during surgery from 75 patients with colorectal cancer (CRLC Val d’Aurelle, Montpellier, France). Colorectal carcinoma and nontumoral mucosa were resected from each patient. According to the Dukes classification, the tumors were staged from A to D (3 stage A, 31 stage B, 18 stage C, and 23 stage D). Fresh samples were immediately frozen in liquid N2 before subsequent nucleic acid extraction.

**DNA EXTRACTION**
High-molecular weight DNA from the cell lines and frozen biopsies was prepared with proteinase K digestion and phenol-chloroform extraction as described previously (21).

**OLIGONUCLEOTIDES**
The oligonucleotide primers and probes were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by reversed-phase HPLC (CIS bio international). For the amplification step, one primer was 5'-labeled with a biotin residue (22), and the other was 5'-labeled with a phosphate residue. Eight oligonucleotides perfectly matched to the oligonucleotides grafted onto the microelectrodes were labeled at their 5' end with a biotin residue. Probes to be addressed were 5'-labeled with a pyrrole residue according to a previously reported procedure using pyrrole phosphoramidite building blocks in the course of oligonucleotide synthesis and a d(T)10 oligonucleotide linker (11).

**DNA SEQUENCING**
Sequence determination was done on amplified (MD1/MD2) DNA using [α-33P]dATP in the dideoxy-chain-termination method as described previously (20).

**SILICON CHIP**
The chips were made using microelectronic technologies on a silicon support (CEA/LETI) (23).

**OLIGONUCLEOTIDE ADDRESSING**
The electropolymerization was carried out as described previously (6). Briefly, each of the 128 microelectrodes (50 μm) was successively switched on, and the electropolymerization was performed by cyclic voltamperometry (−0.35 to +0.85V/saturated calomel electrode) in an aqueous 0.1 mol/L LiClO4 solution containing 20 mmol/L pyrrole and 1 μmol/L of the pyrrole oligonucleotide to be addressed. At the end of the process, the
matrix was rinsed with water and stored at 4 °C until needed.

DETECTION OF K-ras MUTATIONS ON THE CHIP

Generation of the target. The DNA was amplified by PCR. Three primers were used for the different PCR reactions: P3ras1 (5'-GGC CTG CTG AAA ATG ACT GAA TAT-3'), P3ras2, (5'phosphate-P3ras1), and Bio-P3ras2 (5'biotin-TGT TGG ATC ATA TTC GTC CAC AAA ATG-3'). The PCR was carried out in a total volume of 50 μL containing the following: 250 ng of extracted DNA, 200 μM dNTP, 2 U of Taq DNA polymerase (Perkin-Elmer), 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, and 0.1 g/L of Taq DNA polymerase (Perkin-Elmer), 10 mM Tris-

Hybridization reactions were carried out in the microreaction chamber (4 × 3 mm) of the chip created by the packaging. All reactions were performed in a temperature-controlled incubator. A prehybridization step was first conducted for 15 min at 50 °C in a buffer containing the following: 1.8 mol/L tetramethylammonium chloride, 50 mmol/L Tris-HCl (pH 7.4), 2 mmol/L EDTA, 1 g/L sodium dodecyl sulfate, and 5× Denhardt’s solution. Targets (5 fmol of 5'-biotinylated complementary oligonucleotide or 4 μL of PCR products) were then hybridized in a final volume of 10 μL in the above-described buffer for 30 min at 50 °C. Before

the symmetric (P-P3ras1/Bio-P3ras2) amplicons were used, the product was digested with lambda exonuclease (Amersham, Pharmacia-Biotech), whereas the asymmetric (P3ras1/Bio-P3ras2) amplicons were directly hybridized. Two posthybridization washes were carried out, the first wash in 2× saline-sodium phosphate-EDTA containing 1.0 g/L sodium dodecyl sulfate at room temperature, followed by a second wash in 5× saline-sodium phosphate-EDTA containing 1.0 g/L sodium dodecyl sulfate at 50 °C.

Acquisition and analysis of the fluorescent signals. The chip was rinsed in a detection buffer containing phosphate-buffered saline, 0.5 mol/L NaCl, and 5 mL/L Tween 20. The total signal was identified by incubation in a 50 mg/L solution of streptavidin-R-phycocerythrin (Molecular Probes) in detection buffer. This reaction was done at room temperature and in the dark. The DNA chip was then rinsed with the detection buffer and placed between a microscope slide and a microcover glass without the necessity of an additional step. A fluorescence microscope (BH-2; Olympus) was used to observe the fluorescence emission. The phycocerythrin was excited by a mercury lamp, and fluorescent signals were obtained by accumulating the photons for 1 s on a chilled CCD camera (Hamamatsu). Image analysis software (Morphostar; Imaging) was used. For each microelectrode, the mean and the mode of the fluorescent signals were recorded. After detection, the chip was regenerated by a 1-min denaturation step in 0.1 mol/L NaOH.

Results

THE SILICON CHIP

The chip (Fig. 1A) was a 10-mm² active multiplexed device bearing 128 octagonal electrodes with nine gold inputs/outputs. The 50-μm wide microelectrodes were arranged in a rectangular matrix of 8 rows and 16 columns. Each electrode had its own unique address and could be selected individually. The silicon chip was integrated in a package (Fig. 1B) compatible with both the electrocopolymerization step and the biological reactions.

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Amino acid</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>W³</td>
<td>Gly</td>
<td>5'-Py-(T)₁₀-GGA GCT GGC GT-3'</td>
</tr>
<tr>
<td>M₁</td>
<td>Ser</td>
<td>5'-Py-(T)₁₀-GGA GCT GGT GGC GT-3'</td>
</tr>
<tr>
<td>M₂</td>
<td>Arg</td>
<td>5'-Py-(T)₁₀-GGA GCT GGC GT-3'</td>
</tr>
<tr>
<td>M₃</td>
<td>Cys</td>
<td>5'-Py-(T)₁₀-GGA GCT GGC GT-3'</td>
</tr>
<tr>
<td>M₄</td>
<td>Asp</td>
<td>5'-Py-(T)₁₀-GGA GCT GTC GTG GT-3'</td>
</tr>
<tr>
<td>M₅</td>
<td>Ala</td>
<td>5'-Py-(T)₁₀-GTA GCG GCG GGC GT-3'</td>
</tr>
<tr>
<td>M₆</td>
<td>Val</td>
<td>5'-Py-(T)₁₀-GAT GGC GT-3'</td>
</tr>
<tr>
<td>PC</td>
<td>Outside codon 12</td>
<td>5'-Py-(T)₁₀-GCC TTA ACG ATA CAG CTA-3'</td>
</tr>
</tbody>
</table>

* Nucleotides in bold code for the amino acid indicated.

M₅ Wild type; Py, pyrrole; PC, positive control.
The copolymerization process allowed the formation of a homogeneous film (20 nm thick). The amount of hybridizable oligonucleotides grafted onto each electrode was estimated to be 200 fmol/mm² (6). To determine the K-ras codon 12 sequence, a K-ras silicon chip was designed (Fig. 2) with a set of seven mutation-specific oligonucleotides (Table 1) addressed to seven different gold microelectrodes.

**Preliminary Studies on Oligonucleotide Hybridization**

The main steps of the process are described in Fig. 3. The assay was based on the allele-specific hybridization principle, and the aim was to find the hybridization/washing procedure that allowed the detection of all seven possible sequences on codon 12 under the same operating conditions. Biotinylated oligonucleotides that were fully...
matched to those attached to the solid support were used to optimize the reaction conditions. Twenty-base-long oligonucleotides, the sequence that had been optimized previously for the detection of K-ras mutations on a tube format assay based on radioactive detection (mutation tube assay test) (20), were copolymerized to the gold electrodes (data not shown). A reduction of the length of the screening oligonucleotides (probes W and M1–M6) to 14 bases was then made to increase the specificity. Hybridization of a particular oligonucleotide under mild conditions [low temperature (37 °C) and high salt concentration (0.5 mol/L Na\(^+\))] produced substantial nonspecific binding in addition to the signal produced by annealing to the fully match sequence (Fig. 4A). A correlation was found between the amount of fluorescence and the number of mismatches in the duplexes formed. The positive control, the sequence of which is outside the codon 12 region, produced no hybridization signal regardless of the biotinylated probe (M1–M6) used. A 16-base-long sequence for the positive control was kept to obtain a high fluorescent signal that validated the entire process, including PCR, hybridization, and detection. No nonspecific binding of the biotinylated oligonucleotide was observed on the nonaddressed gold microelectrode (Bg electrode) or on the negative control electrode, which was composed of a homopolymer of pyrrole (PP electrode). Better discrimination between codon 12 sequences was obtained when the hybridization temperature was increased from 37 °C to 50 °C, but an increase in temperature did not permit the detection of single-nucleotide mismatches. Specific hybridization was obtained with the introduction in the hybridization buffer of tetramethylammonium chloride at a final concentration of 1.8 mol/L (Fig. 4B). Under these hybridization conditions, (i.e., combining high temperature with an additive), a clear discrimination was made between a fully matched hybrid and a 1-bp mismatched hybrid.

The detection limit was determined under mild hybridization conditions. We determined this value as the amount of biotinylated N probe allowing a mode fluorescence signal on the N electrode equal to twice the mode value on the PP electrode. Within the above-mentioned conditions, 0.1 fmol of N probe could be detected, corresponding to a detection limit of ~10 pmol/L.

**Characterization of the assay with cell lines**

To evaluate the ability of the methodology to analyze PCR products, amplifications were performed on genomic DNA from cell lines. A 117-bp DNA fragment was produced, and its purity was checked by agarose gel electrophoresis. Two ways of obtaining single-stranded DNA were evaluated: (a) asymmetric PCR using a 1:50 ratio of amplification primers, and (b) symmetric PCR using a 5'-phosphorylated primer, followed by digestion with lambda exonuclease. The hybridization results obtained for symmetric digested product were better in terms of fluorescence intensity than those obtained for the asymmetric PCR (data not shown). The operating conditions optimized on the basis of oligonucleotide hybridization permitted detection of homozygous (MIA-PaCa-2 and SW620 cell lines), as well as heterozygous (LS174T and SW1116 cell lines) DNA. The sensitivity of the method was investigated by reconstructing samples with various dilutions of mutant DNA (MIA-PaCa-2 cell line) in wild-type DNA (LNCaP cell line). Samples were amplified through independent PCR reactions, and the amplicons were mixed in different ratios. The generated symmetric amplicons were then digested with lambda exonuclease and hybridized to the chip. A K-ras mutated sequence was unambiguously detected even when the MIA-PaCa-2 cell line represented only 10% of the initial material: a mode value of 42 fluorescence units (FU) on the M3 electrode (Table 2). The background value was 23 FU.

**Table 2. Determination of sensitivity on the silicon DNA chip.**

<table>
<thead>
<tr>
<th>DNA sample ratio (MIA-PaCa-2:LNCaP)</th>
<th>Modal value of the fluorescent signal, FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3 electrode</td>
<td>N electrode</td>
</tr>
<tr>
<td>100:0</td>
<td>136</td>
</tr>
<tr>
<td>50:50</td>
<td>85</td>
</tr>
<tr>
<td>25:75</td>
<td>70</td>
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<tr>
<td>15:85</td>
<td>58</td>
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<tr>
<td>10:90</td>
<td>42</td>
</tr>
<tr>
<td>5:95</td>
<td>27</td>
</tr>
</tbody>
</table>

*Values correspond to the mean of the signals from M1, M2, M4, M5, and M6 electrodes. Signal on Bg electrode, 23 FU.
Genotyping Human Colorectal DNA Samples

To demonstrate the reliability of the procedure, a blind study was conducted on DNA from 75 patients with colorectal cancer. Table 3 shows the characteristics of human tumoral samples in terms of exon 1 (codons 12 and 13) K-ras sequences obtained by direct PCR-product sequencing. Thirty-two percent of patients (24 of 75) had an exon 1 K-ras mutation, and 88% (21 of 24) of the mutations were codon 12 alterations. The only K-ras codon 13 mutated allele found corresponded to GGC(Gly)→GAC(Asp). The genotypes scored by the polypyrrole DNA chip assay were 100% in agreement with conventional DNA sequencing results. All of the K-ras codon 12 mutations detected by direct sequencing were positive by polypyrrole DNA chip analysis, and no false-positive result was obtained with the developed methodology. Moreover, three patients displayed a K-ras codon 13 mutation detected by direct sequencing, and no nonspecific hybridization signal was observed on the electrodes corresponding to the K-ras codon 12 mutated sequences. Fig. 5 shows an example of the data obtained from direct sequencing and DNA chip analysis for a wild-type (GGT) and a mutated (GG/AT) DNA. In both samples, analysis of all controls gave the expected results. Particularly, the positive control probe gave a strong positive signal, whereas the PP control was negative. Moreover, no background was observed on the gold nonpolymerized microelectrode Bg. The genotype assignment of K-ras codon 12 by DNA chip analysis distinguished homozygous and heterozygous sequences unambiguously.

Discussion

The allele-specific oligonucleotide principle has been widely used in developing diagnostic mutation methods (25–27). Currently, this approach relies on a membrane (28, 29) as a solid support and radioactivity as a detection tool. We describe here an allele-specific oligonucleotide method based on the development of a new silicon oligomer array as well as its use for fluorescence measurement and its application to the analysis of K-ras gene mutations.

The reliability of hybridization on ODN chips depends largely on both the quality of the surface-bound oligomer and the type of linkage between the ODN and the solid support. With the methodology described here, the ODNs were characterized and HPLC-purified before grafting. The functionalization of the gold electrodes by electroco-polymerization allows the formation of a controlled thin film of ODN-pyrrole that is very stable. This feature makes it possible to carry out hybridization and washing steps under stringent conditions such as the use of tetramethylammonium chloride and high temperatures. These conditions were neutral toward the polymer film and had no side effects on the packaging compounds. The stability of the ODN-polypyrrole surface is also compatible with NaOH, and a regeneration of the ODN chip after the analysis (hybridization, washing, and detection) is completed is possible without substantial loss of signal. The 5’-end d(T)10 linker makes it possible to increase the hybridization signal by improving the ODN accessibility. Moreover, the 3’ end of the grafted ODN is free, and extension by polymerases of the fixed ODN could be envisaged.

With regard to the geometry of the packaging, the “T”-shaped chip was easy to handle, and the microreaction chamber created was suitable for the hybridization and detection steps. Moreover, all of the steps in the preparation of the functionalized biochip are automated.

The binding and accessibility characteristics of the silicon-grafted ODNs were similar to those observed for nylon membranes because the optimal conditions that allow perfect discrimination of mismatched duplexes were similar. The use of tetramethylammonium chloride, which acts on the melting temperature (30), is especially efficient in equalizing duplex stabilities on both supports.

The methodology described here does not require multiple steps subsequent to PCR, i.e., purification or post-labeling, to obtain reliable biological material. The amplicons to be analyzed are relatively short and are able to reassociate rapidly after heat denaturation; therefore, generation of single-stranded PCR products is important to increase hybridization efficiency with the solid-phase fixed probes. Several approaches for generating single-stranded DNA have been described. Use of asymmetric PCR (31) is not the best way because only relatively small amounts of single-stranded DNA are produced under extensive optimization conditions. The use of T7 gene 6 exonuclease, which digests 5’-phosphorylated and 5’-hydroxylated DNA and is blocked by phosphorothioate (S) linkages, has been reported previously (32). Nevertheless, in our developed technology this method requires modifications in the strand of interest by incorporation of sulfur bridges in one of the amplification primers.

Table 3. K-ras codon 12 and codon 13 sequences of DNA from 75 colorectal cancer patients analyzed by direct sequencing.

<table>
<thead>
<tr>
<th>Codon 12</th>
<th>Codon 13</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>W</strong></td>
<td><strong>M1</strong></td>
</tr>
<tr>
<td>Sequence</td>
<td>GGT</td>
</tr>
<tr>
<td># of patients</td>
<td>54</td>
</tr>
</tbody>
</table>

* a Nucleotides in bold are the mutated nucleotides.
  b W, wild type.
Fig. 5. Comparison of direct dideoxynucleotide sequencing and DNA chip assay in sequence analysis of K-ras codon 12.

Sample 1
K-ras codon 12 sequence: GGT

Sample 2
K-ras codon 12 sequence: GG/AT

The probe pattern of the K-ras DNA chip is as shown in Fig. 2. Sample 1 (top) displays the wild-type K-ras codon 12 sequence (GGT) in sequencing gel and in the DNA chip assay. Sample 2 (bottom) contains a heterozygous missense mutation (GGT→GAT) detected by both techniques.
Lambda exonuclease (33) is able to convert double-stranded amplified DNA to single strands and to enhance specific hybridization. In our assay, this alternative is attractive because (a) the modification (5’ phosphate residue) is not in the hybridizable strand, and (b) the production of single strand is very efficient; moreover, this approach can be applied to multiplex PCR.

The allele-specific mutation detection method on the chip using the same process steps over the entire array was rapid. Starting from genomic DNA, the detection of point mutations can be accomplished in less than 4 h. We are researching ways to develop an automatic station for the hybridization and analysis steps.

The developed DNA chip based on electropolymerization on gold electrodes gives a support with low autofluorescence, allowing the detection of <10% of mutated sequences within a mixed mutant/wild-type population. The sensitivity of direct sequencing performed with radioactive incorporation was ~15%, whereas it was ~5% for our previously reported mutation tube assay test (20) using tubes as solid support and serial hybridizations.

To enable detection of all K-ras mutations, we are developing methods to screen codons 13 and 61 by starting with a multiplex PCR. Moreover, because 128 electrodes are available, we can expect to analyze other genes of interest whose mutations are implicated in the development of cancer. The unambiguous discrimination among the seven potential alleles in the same region suggests that this technology may be useful in multiallelic gene analysis. Finally, a quantitative application of the silicon device we have developed is conceivable because the amount of the grafted ODN is fully controlled.

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