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Automated Constant Denaturant Capillary Electrophoresis Applied for Detection of *KRAS* Exon 1 Mutations

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ABSTRACT

In this study, we have applied automated constant denaturant capillary electrophoresis (ACDCE) for the detection of KRAS exon 1 mutations. Samples from 191 sporadic colon carcinomas previously analyzed for KRAS mutations with allele-specific PCR (ASPCR), temporal temperature gradient electrophoresis (TTGE), and constant denaturant capillary electrophoresis (CDCE) were analyzed. In ACDCE, an unmodified ABI PRISM™ 310 genetic analyzer with constant denaturant conditions separated fluorescein-labeled PCR products. Temperature in combination with a chemical denaturant was used for separation. The optimal separation conditions for PCR-amplified KRAS exon 1 fragments were determined by adjusting the temperature before electrophoresis. In the ACDCE analysis, the sequence of a mutant was determined by comparing the electropherogram of the fragment to that of known mutations followed by mixing the sample with control mutations before reanalysis. In a titration experiment mixing mutant and wild-type alleles, the sensitivity for mutation detection was shown to be 0.6% in this automated CDCE technique. The automation of CDCE allowed rapid analysis of a large number of test samples over as short period of time and with a commercially available apparatus.

INTRODUCTION

Mutations in the *KRAS* gene have been found in a variety of different tumors. The frequency of *KRAS* mutations varies depending on the tumor type. Mutations in *KRAS* exon 1 are frequently detected in cancer of the pancreas and the colon but are rare in cancer of the breast and liver (2). The presence of *KRAS* mutations is associated with a lack of response to adjuvant chemotherapy in patients with colorectal cancer

(1). Thus, simple and automated techniques for mutation detection in this gene may find use in pretreatment mutation screening in large groups of patients. To automate the analysis of point mutations in *KRAS*, an unmodified ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was used for constant denaturant capillary electrophoresis (CDCE). Automated CDCE (ACDCE) is based on the same separation principle as in denaturant gradient gel electrophoresis (DGGE) described by Fischer and Lerman (6), constant denaturant gradient electrophoresis (CDGE) (8), and temporal temperature gradient electrophoresis (TTGE) (3,4). The separation principle of these methods is based on the melting behavior of the double helix of a given fragment. In DGGE, PCR products of test samples are run parallel to a denaturant gradient in the gel. CDGE analysis is performed in a constant denaturant gel, while, in TTGE, a constant denaturant gel combined with a linear increase in the temperature during electrophoresis is used for the separation of PCR products (3,4).

In the original paper describing the CDCE technique (9) and in reports on mutation detection in *NRAS*, *KRAS* exon 1, and *TP53* exon 8 (3,5,11) with CDCE, heat alone or in combination with a chemical denaturant was used as denaturant for separation of fragments. In ACDCE, heat in combination with a chemical denaturant was used to screen for *KRAS* mutations. A series of 191 colorectal carcinomas, previously analyzed for *KRAS* mutations with allele-specific PCR (ASPCR), TTGE, and CDCE, was used to compare the hands-on time needed, analysis time, and sensitivity of TTGE, CDCE, and ACDCE (3). The method described here utilizes a conventional commercially available capillary instrument with no modifications as a tool for mutation status analysis by CDCE. This result is to our knowledge novel ease the acquisition of CDCE by a wider circle of researchers.

MATERIAL AND METHODS

Material and DNA Extraction

Tumor tissues from 191 patients with colorectal carcinomas were snap

frozen in dry ice and stored at -70°C until analyzed. The patients were from a consecutive series from Uppsala and Falun County, Sweden, and have previously been described (10). Control samples with known sequences in *KRAS* exon 1 were analyzed in parallel with the test samples. These control samples were cell lines from the ATCC cell repository: HT 29 (ATCC HB 8245) wild-type, HCT 116 (ATCC CCL 247) heterozygous for a GGC to GAC mutation in codon 13, and SW480 (ATCC CCL 228) with homozygous GGT to GTT mutation in codon 12. Six previously sequenced DNA tumor samples with *KRAS* mutations were also used as controls.

DNA was extracted from cell lines and fresh frozen tissue samples by cutting the tissue into small pieces or after homogenization with a Polytron (Brinkmann Instruments, Westbury, NY, USA). Lysis buffer (75 mM NaCl, 24 mM EDTA) containing 1–2 mg proteinase K and 1%–2% SDS was added, and the mixture was incubated at room temperature overnight or at 55°C for 4 h. After phenol and chloroform extraction, the DNA was precipitated with ammonium acetate (pH 7.5) and ethanol and thereafter washed in ethanol and dissolved in TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA).

PCR Amplification

All PCRs were performed on a PTC-200™ terminal cycler (MJ Research, Waltham, MA, USA).

PCR was performed by mixing 100 ng genome DNA with 25 µM each dNTP (Applied Biosystems), 10× *Pfu* buffer, 2 U cloned *Pfu* (Stratagene, La Jolla, CA, USA), and 5 pmol each primer (MedProbe, Oslo, Norway) in a final volume of 50 µL. PCR primers for *KRAS* exon 1 were as follows: 5'-fluorescein-ATGACTGAATATAAACTTGTG-3' and 5'-CGCCCGCCGCGCCCGCGCCCGTCCCGCGCCCCGC-CCGCCTCTATTGTTGGATCATATT-3'. Cycling parameters were 35 cycles of denaturation for 1 min at 94°C, annealing at 53°C for 1 min, and elongation at 72°C for 1 min. All PCR products were thereafter denatured for 5 min at 94°C and incubated 1 h at 65°C for heteroduplex formation.

ACDCE Analysis

ACDCE analyses were performed on an unmodified ABI PRISM 310 Genetic analyzer. Uncoated capillaries, 47 cm long with an internal diameter of 50 μ m (Applied Biosystems) and denaturant gel POP4 (Applied Biosystems) were selected for separation of the fragments. Amplified PCR products were diluted 1:100 in distilled water before loading. The ABI 310 has automated sieving matrix replacement and load of 48 samples as standard. Loading of the amplified fragments was achieved with 15.0 kV for 5 s. The fluorescein-labeled PCR products were electrophoresed at 15.0 kV (319 V/cm), and the laser power was 9.9 mW. The optimal electrophoresis conditions for the separation of mutant fragments were determined by running a PCR product from the cell line HCT 116 at different temperatures with 1°C intervals and by analyzing the theoretical melting profile of *KRAS* using MacMelt computer program (MedProbe). During the analysis of PCR fragments, the temperature of the denaturing zone was held constant to achieve optimal separation. Electropherograms were analyzed with the standard GeneScan® software supplied for the ABI 310 Genetic Analyzer. A sample with abnormally migrating bands were reanalyzed together with known mutations with a similar migration pattern to determine the mutation status of that sample (7). The analyses of *KRAS* of the 191 colorectal carcinomas were done blindly to be able to compare the results with that previously reported (3).

RESULTS AND DISCUSSION

For optimization of peak separation, the cell line HCT 116 with a heterozygous mutation in codon 13 of *KRAS* was run at temperatures from 30°C to 60°C. The best separation of homoduplex and heteroduplex peaks was achieved at about 20°C lower than the theoretical melting profile (3). With only temperature as denaturant (CDCE), separation of a mutant in *KRAS* exon 1 was achieved between 69°C and 71°C, with maximum separa-

tion at 70°C (3). The unmodified ABI 310 apparatus cannot be adjusted to temperatures above 70°C, and, therefore, a chemical denaturant in addition to temperature is required. The chemical denaturant in POP4 lowers the temperature needed for separation with about 20°C. Separation was achieved between 47°C and 51°C, giving an optimal separation at 49°C. Samples showing more than one peak after the primer peaks on the electropherogram when run at the optimal separation temperature were accordingly scored as mutant samples. Using these conditions, 66/191 (34.6%) samples were found to have mutations in *KRAS* exon 1. Electropherograms of two samples are shown in Figure 1. All homoduplexes except samples with a G-to-C transversion in codon 12 were separated. The two heteroduplexes were well separated in all mutated samples. It proved unreliable to assign to a mutant a tentative sequence based on the migration patterns on the electropherogram alone. Instead, the mutant samples were mixed with DNA from control samples with a similar pattern on the electropherogram, boiled, re-annealed, and reanalyzed to determine the correct mutation. Of the mutations found with ACDCE, 55 (83.3%) were at codon 12 and 11 (16.7%) were at codon 13. The mutations detected were the same as those found with CDCE (3).

Sensitivity, analysis time, and hands-on time needed to analyze 100 samples with TTGE, CDCE, and ACDCE were calculated. The analysis time for 100 test samples with TTGE and ACDCE was two days; with CDCE, the test samples were analyzed in five days. With ACDCE, 1 h of hands-on time was needed, while two days were needed for TTGE analysis and five days for CDCE analysis.

For sensitivity analysis, cells from the cell line HCT 116 were titrated with *KRAS* wild-type cell line HT 29, giving allele ratios from 1:1 down to 1:1000 (mutant allele:wild-type allele). The PCR samples were diluted before ACDCE analysis, the ratio under the peaks were measured with the GeneScan software provided, and the wild-type to mutant peak ratio was calculated. In the automated CDCE analyses, heteroduplex peaks could be detected

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in samples containing 0.6% mutant alleles, and a mutant homoduplex peak could be seen in samples containing down to 4.2% mutant alleles (Figure 2). For manual CDCE, a mutant homoduplex could be detected in down to 1%, and mutant heteroduplexes could be detected in down to 0.1% (9). In TTGE analysis, a mutation can reliably be detected in down to 10% at the homoduplex level and down to 1% at the heteroduplex level (9).

In this ACDCE approach, fluorescein-labeled PCR products were used, but other fluorescence labels can be used for visualization. We analyzed a few samples labeled with rhodamine or 6FAM, and the results were comparable with those found with fluorescein-labeled PCR products. The manufacturer of the instrument recommends the fluorescent dyes 6-fam, het, hex, and tamra when the apparatus is used for sequencing.

Analysis time for one fragment was 20 min, and we analyzed 48 samples in a row at one fixed temperature. We consistently observed that when a mutant sample was electrophoresed in different runs, reproducible electropherograms were not obtained. This was due to small temperature differences between the runs in the denaturing zone of the ABI 310 apparatus. When a sample scored as a mutant was analyzed together with known mutations, the results were reproducible as previously reported for gel analysis (7).

Compared to CDCE, in which each sample has to be loaded manually, ACDCE analysis can be performed on 48 or 96 samples in a row without any intervention during analysis. We analyzed more than 300 samples successively, without changing buffers, electrophoresis gel, or capillary, and were able to reproduce results with control samples at different intervals in this series.

With the temperature controller on the ABI 310 apparatus, we were able to separate all heteroduplexes, but it proved difficult to separate some mutant homoduplex bands with a G-to-C mutation. This is probably due to the somewhat imprecise temperature control, with increments of 1°C. To be able to resolve all possible mutants at the homoduplex level, a better temperature unit is needed for the instrument. The

Figure 1. Electropherograms of *KRAS* exon 1. Upper part (A) with wild-type sequence and (B) with a codon 12 mutation with GGT to GAT. Optimal separation temperature was shown to be 49°C.

Figure 2. Six electropherograms with samples selected from the reconstruction sensitivity experiment. The mutant fraction (MF) in each electropherogram is denoted in the figure. The heteroduplexes in the lower three electropherograms are enlarged to visualize the peaks. The mutant homoduplexes in the electropherograms are not enlarged. A mutant homoduplex could be detected in a sample with 4.2% mutated alleles, and the heteroduplex peaks could be detected in a sample with down to 0.6% mutated alleles. The sensitivity calculations are based on wild-type to mutant peak ratios.

software available for ABI 310 Genetic Analyzer is not designed for this type of mutation analysis. With the existing programs, we found it difficult to obtain the raw data recorded by the computer. The electropherograms shown in this report are non-manipulated printouts of the results presented by the GeneScan software. It was not possible to align different electropherograms to compare electrophoretic patterns of different samples.

In conclusion, the ABI 310 Genetic Analyzer proved to be sensitive in *KRAS* exon 1 mutation detection, rapid, time saving, and less labor demanding than conventional CDCE. A better temperature unit is required to be able to adjust the temperature more precisely. Adapted software programs for the analysis of the electropherograms on the ABI 310 Genetic Analyzer will turn this instrument into a valuable tool for routine mutation analysis based on the melting characteristic of PCR-amplified DNA fragments.

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This work was supported by grants from the Norwegian Cancer Society. Address correspondence to Dr. Jens Bjørheim, Department of Immunology, Section for Immunotherapy, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway. e-mail: j@bjorheim.net

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J. Bjørheim, P.O. Ekstrøm, E. Fossberg, A.-L. Børresen-Dale, and G. Gaudernack
The Norwegian Radium Hospital
Oslo, Norway