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Detection of Low-Frequency Mutations in Exon 8 of the *TP53* Gene by Constant Denaturant Capillary Electrophoresis (CDCE)

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P.O. Ekstrøm A.-L. Børresen-Dale¹, H. Qvist¹, K.E. Giercksky¹ and W.G. Thilly

Massachusetts Institute of Technology, Cambridge, MA, USA and ¹The Norwegian Radium Hospital, Oslo, Norway

ABSTRACT

We extended our development of the means to measure point mutations at the DNA level to the problem of detecting *TP53* gene variants in the area around tumors where mutant fractions are expected to be as low as one mutant per 1000 wild-type (WT) sequences. We met this criterion by using the following methods. The *TP53* exon 8 sequence was amplified from genomic DNA samples under conditions of high polymerase fidelity using a fluorescein-labeled primer. This mixture of WT and mutant sequences was converted to heteroduplexes of mutant and WT sequences by melting and re-annealing. The mixture was resolved by capillary gel electrophoresis under appropriate constant denaturing conditions. Using laser-induced fluorescence, we found that fractions as low as 1/1000 could be detected without any prior enrichment for mutant sequences, and that the melting properties of the amplified DNA will leave "fingerprints" in the electropherogram that can be used to identify the specific mutation. This method is fast and robust and should be applicable to clinical analyses in which rapid scanning for any mutation in an exon-ic sequence is important.

INTRODUCTION

Detection of genetic changes has become an important field in biology and cancer research. A single-base mutation in DNA can lead to altered cellular behavior, which can induce oncogenesis if crucial genes are hit. Detection of such mutations might be beneficial for early diagnosis, prognosis and the evaluation of therapeutic outcome in cancer treatment. The possibility of low-frequency mutation detection is of particular interest both for early detection of malignant diseases, for evaluation of surgical margins and for monitoring relapse. The *TP53* tumor-suppressor gene is commonly mutated in human cancer (11), and 30%–80% of human carcinomas contain sectors with a mutation in this gene, depending on the type and stage of the tumor investigated (19,30). Mutation in *TP53* has been found to be a prognostic factor in several cancers, but whether it is an independent prognostic factor, is not yet known (1,4,22,25). Point mutations in the *TP53* gene can confer characteristics of drug resistance or other phenotypes associated with prognosis in patient response to treatment (1,3,31). It can be important to measure the distribution of such mutant cells in the periphery around excised tumors from the perspective of understanding tumor pathogenesis. It is also important to be able to discover small but potentially lethal sectors of tumors when the tumor area has been removed. Most of the mutations in the *TP53* gene are reported to reside in the core region of the protein coding from exons 5 through 8. This region is the most evolutionary conserved part of the gene (14).

Constant denaturant capillary electrophoresis (CDCE) has previously

been used to analyze low-frequency mutations in the human mitochondrial genome with contiguous high- and low-melting domains (17) and also used for mutation detection in the *N-ras* oncogene with GC-rich primers (18). CDCE itself is an application of principles of cooperative equilibria to the separation of DNA sequences differing by as little as 1 bp in 100. The first demonstration, by Fischer and Lerman (9), used a chemical denaturing gradient on a slab gel that was improved by elimination of the need for a gradient (15). The extension to capillary gel electrophoresis has increased the speed and resolution of the technology, and the use of laser-induced fluorescence has greatly increased general sensitivity (17).

The results from the present study revealed a fast and sensitive method for detection of point mutations in exon 8 of *TP53* gene. This method is general for all DNA isomelting domains of 100–120 bp, and we are now extending the approach to other exons. When applied to the other conserved areas of the gene, we believe that this method can be used in molecular assessment in several clinical settings (6,8,27).

MATERIALS AND METHODS

Instrumentation

The instrumentation was similar to the capillary electrophoresis instrument previously described by Khrapko et al. (17). Modifications were made for detection of tetramethylrhodamine (TMR) and fluorescein simultaneously. Low-power argon laser (Omnichrome, Chino, CA, USA) with 515-nm wavelength was filtered through a 515-nm (10-nm

bandwidth) filter (Corion, Franklin, MA, USA) and focused onto the capillary perpendicular to the microscope objective (Oriol Instruments, Stratford, CT, USA). The microscope objective focused emitted light onto a beam splitter (Type BS-550-S; Corion). Light from the beam splitter was then filtered through a 540-nm (10-nm bandwidth) filter (Corion), then through a filter removing wavelengths above 550 nm (Corion) and onto a Model 70680 Photo Multiplier (Oriol Instruments). The light emitted from the TMR fluorochrome was filtered through a 580-nm (10-nm bandwidth) filter (Corion) before reaching the second photo multiplier. Signals from the photo multipliers were amplified by a Model 70710 Current-Pre Amplifier (Oriol Instruments)

and recorded as previously described (17,18).

Capillary Electrophoretic Conditions

The coating of the fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) is based on the method described by Hjertén (13). Ten meters of capillary (inner diameter 75 μ m and outer diameter 363 μ m) was used for the coating. The capillary was filled with 1M NaOH and sealed off for 2 h, and was then flushed with water before flushing with 1 M HCl for 2 min. The capillary was then rinsed with water and MeOH, injected with Silane (g-methacryloxypropyltrimethoxysilane) and sealed for 18 h. The next day the

capillary was rinsed with MeOH and water. A 6% acrylamide solution (without *N,N'* methylenbisacrylamide) in 1 $\times$ TBE buffer (89 mM Tris-base, 89 mM boric acid, 2 mM ethylenediamine tetraacetic acid, pH ca. 8.3) containing 1 μ L *N,N,N',N'*-tetramethylethylenediamine (TEMED) and 1 mg potassium persulfate per mL solution was injected. After polymerization, the capillary was cut in the desired length (0.3 m), and a window for detection was made with a scalpel. Replaceable linear polyacrylamide (LPA) was made according to Ruiz et al. (26), however, without urea and formamide. LPA was replaced before every run.

Electroinjection of the sample was accomplished by applying 2 μ A for 30 or 60 s. The electrophoretic condition was a constant current of 8 μ A, and the total length of the capillary was 0.3 with 0.1 m effective heated length. The heated zone was made with a metal jacket temperature controlled with circulating water from a water bath. Temperature in the water bath was set to 76°C, but due to heat loss in the tubing, the effective melting temperature in the column was 75.5°C.

Cell Line and Patient Samples

A colon cancer cell line, WiDr (Catalog No. CCL-218; ATCC, Rockville, MD, USA) was cultivated at 37°C in Eagle's minimum essential media (EMEM) medium with 10% fetal calf serum. Cells were frozen and thawed using the described protocol (2). Normal bone marrow cells were obtained from a noncancerous patient and were isolated using Lymphoprep™ (NYCOMED AS Pharma, Oslo, Norway). The WiDr and the normal cells were used in reconstruction experiments. WiDr cells were counted by microscope in a counting chamber and added in ratio of 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ into a total of 10⁷ normal cells.

A second reconstruction experiment was performed by mixing homoduplex mutated and wild-type (WT) polymerase chain reaction (PCR) products in ratio (mutated/WT) 1/10, 1/100, 1/1000 and 1/10 000, followed by formation of heteroduplexes by boiling and re-annealing. The number of PCR copies was estimated from the area under the curve

Figure 1. Detection of heteroduplexes (hd) when tumor cells (WiDr) are mixed with normal cells in ratio 10⁻²–10⁻⁴. The WiDr cells have the mutation C_{GT}→C_{AT} in codon 273 (bp 14 487). The ratio of 1/2 the area under the mutant/WT heteroduplex peaks to the sum of all peaks can be used to estimate the mutant fraction for each mutation. In these examples, these ratios are 0.06/5.9 equals approximately 10⁻², and 0.02/17.2 equals approximately 10⁻³.

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of the electropherogram. To obtain different mutations, DNA from colon carcinomas from a patient cohort previously described (29) were also used in this study. Isolation of DNA was accomplished by standard phenol/chloroform-extraction and ethanol-precipitation using an ABI 340A DNA Extractor (PE Biosystems, Foster City, CA, USA).

PCR

PCR of exon 8 of the *TP53* gene was performed using the high-fidelity enzyme, *Pfu* DNA Polymerase (Stratagene, La Jolla, CA, USA) and the corresponding *Pfu* buffer from Stratagene. We used 20-mer fluorescent-labeled primer (MedProbe, Oslo, Norway) for the amplification (5'-F-ATCCTGAGT-AGTGGTAATCT-3', F = fluorescent, fluorescein or TMR) together with a primer with high-melting domain (GC-rich sequence, 5'-CGCCCGC-CGCGCCCCGCGCCCGTCCCGC-CGCCCCCGCCCGTACCTCGCTT-

AGTGCTCCCT-3') previously described (5). Amplification was performed in a GeneAmp® 9600 Thermal Cycler (PE Biosystems), using the following program: 120 s at 94°C, 35 cycles of 75 s at 94°C, 75 s at 55°C and 60 s at 72°C. Heteroduplex formation between WT and mutation was produced by heating the PCR product to 94°C for 3 min and allowing re-annealing at 65°C for 1 h (28). All samples were processed in duplicates and analyzed twice.

RESULTS

The equilibrium melting temperature of a DNA sequence can be calculated using statistical mechanical principles coupled with knowledge of the free energy for melting any particular base pair (24). The theoretical melting temperature was calculated as 77.5°C for *TP53* exon 8 when attached to a high-melting primer (5). Under separat-

ing condition (i.e., the right temperature in capillary), low-melting domain of the PCR product will unwind, while the high-melting domain will keep the strands together. This will allow for separation of more and less stable mutants and heteroduplexes compared to the WT. The temperature used in CDCE analysis was obtained by increasing the temperature in 0.5°C increments from 72°C until 80°C (data not shown). Variations in temperature between 75° and 77°C affected the separation of the peaks. The final optimal temperature was 76°C and was used in the further CDCE analysis.

Figure 1 displays the detection of mutant PCR fragments when WiDr tumor cells are mixed with normal cells in various ratios. When 10⁴ mutated WiDr cells are mixed with 10⁷ normal cells, giving a ratio 10⁻³ before extraction of DNA, the mutation could be detected repeatedly. Titration down to 10⁻⁴ demonstrated a loss of signal.

Alteration of a single base in the low-melting domain of the fragment to be analyzed will alter the melting behavior of most mutant homoduplexes and all mutant/WT heteroduplexes, respectively. Each mutation will consequently reveal a specific peak (homoduplex) or peaks (heteroduplexes) in the electropherogram, depending on whether or not the mutants have been transformed into heteroduplexes with excess WT DNA. Figure 2 shows some examples of different patterns as a consequence of base pair changes in codon 273 (bp 14 486 and 14 487). DNA from ten other tumor samples harboring different mutations from codon 266 to codon 286 were also analyzed (data not shown). All the mutations we have analyzed so far have displayed a distinct peak pattern with regard to homo- and heteroduplexes.

Provisional identification of mutant homoduplexes can be performed by comparison of the elution time of a sample peak to that of a co-injected, but differentially labeled, authentic standard mutant DNA sequence. In our apparatus, we labeled samples with fluorescein and standards with TMR. By recording at two separate appropriate wave bands, we can differentiate between sample and standard signals. Figure 3 shows an example. In Figure 3, the middle electropherogram displays a

Figure 2 Differences in the peak patterns for different point mutations in codon 273 (bp 14 486 and 14 487) in exon 8 of the *TP53* gene. The DNA was amplified from tumor samples with different known mutations.

WT peak in the TMR channel. This peak co-migrates with the first peak in the samples, indicating that it is the WT homoduplex. Note the cross-talk from the fluorescein in the TMR channel. This is due to the detection of some of the emitted light from the excited fluorescein molecule in the TMR detector. To distinguish between cross-talk and true peaks in the TMR channel, the ratio of the two channel signals are plotted in the lower panel. When the ratio displays a negative peak, this signifies a true signal in the TMR channel. A mutation-specific pattern can also be used to identify unknown mutations under the assumption that there is a specific pattern for each mutation and a database for all mutations is available. In Figure 4, the peak pattern from a sample with an unknown mutation is displayed in the upper panel. In the middle and lower panels, the PCR product from the sample is mixed with PCR products from two known mutant samples. The mutant

in the middle electropherogram has a CCT→CAT base change in codon 278 (bp 14 502). The electropherogram displays one WT peak, two peaks representing mutation and four heteroduplexes. In the lower electropherogram, the test sample was mixed with a mutant with a CGG→TGG base change in codon 282 (bp 14 513). This electropherogram revealed only four peaks, indicating that the same mutation was present in both of the mixed samples. By sequencing the unknown sample, the mutation was identified as CGG→TGG base change in codon 282.

DISCUSSION

In this study, we have applied high-fidelity PCR with fluorescent primer, CDCE and laser-induced fluorescence for detection of mutations in exon 8 in the *TP53* gene. Separation of mutants from WT were performed in a

Figure 3. Provisional peak identification by running WT standard in the TMR channel (middle panel). Mutation is CGG→TGG in codon 282 (bp 14 513) (upper panel). Note the cross-talk from the fluorescein channel onto the TMR channel.

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temperature-controlled denaturing zone. The melting of the DNA (PCR product) was based on heat only, as no chemical denaturant was added. This gave better control over the denaturation conditions in the capillary. The temperature (75.5°C) used for denaturation was slightly lower than the theoretical melting temperature (77.5°C), calculated by a melting program (5). The sensitivity of the CDCE/laser-induced fluorescence (LIF) system for detection of low-frequency mutation in presence of WT was evaluated by mixing tumor cells with normal cells in a ratio from 10^{-2} and as low as 10^{-4} (tumor cells/normal cell). With use of computer software, it is possible to measure the area under the peaks. Comparing the WT area with the area under the mutant plus the area under one heteroduplex peak gives the mutant fraction. The mutation fraction calculated from the peak areas can be used as a quantitative measurement of the mutant fraction in a sample. In Figure 1, we found that the ratio between the area [mutant (mut) /WT] is approximately 10^{-3} . Detection

of low-frequency mutations ($<10^{-3}$) in this setting is limited by that amount of DNA that is possible to put into a PCR. The normal amount of DNA for a 100- μ L PCR is 0.1–1.0 μ g (7). We experienced the upper limit for the amount of DNA possible to have in a 25- μ L PCR to be approximately 300 ng DNA. This amount of DNA gives an estimate of 1×10^5 copies of the *TP53* gene (7,20). Based on this number of copies, the theoretical detection limit is 10^{-4} . This is due to the Poisson distribution of the mutants. Given the ratio 10:100 000 (mut/WT), the probabilities (P) of getting >4 mutants into the PCR is 0.971. With lower frequencies of mutants, the probabilities of getting the mutant into the PCR will decrease (1 mut/100 000 WT, P [mut ≥ 1] = 0.632).

The error rate of the enzyme used in the PCR will give a similar limit of detection for low-frequency mutations. *Pfu* DNA polymerase has an error frequency of 1.6×10^{-6} errors per base pair doubling (21,23). Because our target sequence is approximately 100-bp long, and we carried out some 35 dou-

blings, we expect a PCR-generated mutant fraction of $1/2(1.6 \times 10^{-6})(100)(35) = 2.8 \times 10^{-3}$. This, divided over several polymerase hot spots, gives a PCR-induced mutation rate of 10^{-4} .

The linear detection ranges for laser-induced fluorescence is 3×10^4 to 10^{11} copies (17). Mixing PCR products from WT and mutants in different ratios, brought detection of heteroduplexes down to 10^{-3} (data not shown). This is in accordance with previous findings for human mitochondrial genome and *N-ras* (17,18) with a similar instrument. The sensitivity can be increased twofold by collecting the heteroduplexes before PCR (16), under the condition of a natural existence of high- and low-melting domains in the gene fragment of interest. This is done by restriction cutting the genomic DNA, boiling and re-annealing to create heteroduplexes and loading the DNA onto the capillary and running the electrophoresis. Heteroduplexes are separated and collected at the other end of the capillary. Then, the fluorescently labeled PCRs are performed followed by CDCE analysis.

The effect of single base pair substitution on DNA was first described by Fisher and Lerman (9). Base pair substitution will give changes in the DNA stability due to changes in entropy. The changes are due to loss or gain of a hydrogen bond and differences in base pair stacking (10,24). Melting properties of DNA will leave "fingerprints" in the electropherogram that can be used to identify the mutation (12). Figure 2 displays patterns for base pair substitution in the same codon of exon 8 of the *TP53* gene. Additionally, we have analyzed ten different mutations from codon 266 to codon 286 (data not shown). All the different mutations we have analyzed so far have displayed a distinct peak pattern with regard to homo- and heteroduplexes. Building up a library of all known mutations in the gene of interest and using software to compare the unknown sample, gives a fast and accurate method of determining the exact sequence alteration in the sample without having to perform laborious sequencing techniques. This can be achieved with a complete database over all mutations and assuming that each mutant has a distinct peak pattern. However, it is possible that two differ-

Figure 4. Mutation determination with use of mutation standards. (Upper panel) sample with CCG→TGG codon 282 (bp 14 513), (middle panel) CCT→CAT codon 278 (bp 14 502) and (lower panel) CCG→TGG codon 282.

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ent mutations have a similar peak pattern. This can be circumvented by mixing the two different mutants showing the same peak pattern before analysis to form additional heteroduplexes (12). If the mutants are different, two additional heteroduplexes will appear on the electropherogram (Figure 4).

The determination of the homoduplexes is accomplished by running a sample together with a WT or mutant homoduplex labeled with TMR. The standard will point out which peak of the homoduplexes have the same melting behavior in the capillary. In Figure 3, the WT standard indicates that the first peak in the upper electropherogram is the WT and, therefore, suggests that the mutations have lower melting temperature and, consequently, increased entropy. The mutation is identified by sequencing to be a CGG→TGG in codon 282. As an aide to identification of true peaks in the TMR channel, it is possible to calculate the ratio between

the channels. A decrease in the ratio confirms a signal in the TMR channel.

CONCLUSIONS

This technique can be applied to virtually any known DNA sequence with an iso-melting domain of approximately 100 bp and where a high-temperature melting region can be attached. The size of the PCR product should be approximately 200 bp including a high-temperature melting domain. The sequence used in this study was chosen because of the biological importance in cancer. Under optimal conditions, mutation detection by CDCE can be performed within 20 min after PCR. Combined with a PCR performed in a capillary air thermal cycler, it should be possible to have a sample analyzed for mutation in *TP53* exon 8 within 60 min. This method has a detection range of 10^{-3} , ratio mut/WT, and using com-

puter software, it is possible to calculate the ratio between the WT and the mutation. Recognition of the sequence alteration of the mutants by use of mutation standards can be applied. This method has an advantage over the radioactive manual sequencing in both time and sensitivity and also the advantage of not using radioactive material. With speed and sensitivity, the CDCE can be adapted to investigations of the molecular status of *TP53* in surgical samples surrounding tumors, in premalignant tissue and in tumor cells in the circulation, urine or sputum.

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Address correspondence to:

Dr. Per O. Ekström
Center for Environmental Health Sciences
Massachusetts Institute of Technology
Cambridge, MA 02139, USA
Internet: perolaf@mit.edu