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BRIEF METHOD

Denaturing High Pressure Liquid Chromatography (DHPLC) for the Analysis of Somatic *p53* Mutations

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Denaturing high pressure liquid chromatography (DHPLC) is a relatively new technique, which uses heteroduplex formation between wild-type and mutated DNA strands to identify mutations. Heteroduplex molecules are separated from homoduplex molecules by ion-pair, reverse-phase liquid chromatography on a special column matrix with partial heat denaturation of the DNA strands (O'Donovan et al, 1998; Oefner et al, 1998). DHPLC is potentially a very useful method for the screening of a large number of samples for mutations. So far, it has mainly been used for the analysis of germline mutations in various inherited diseases for which a high degree of sensitivity has been reported (Gross et al, 1999; Holinski-Feder et al, 2001; O'Donovan et al, 1998). With respect to the analysis of somatic mutations in tumors, however, one potential drawback is that DHPLC requires that both wild-type and mutant DNA are present so that heteroduplexes can form. Ideally, these DNA molecules should be present in equal amounts. In actual tumor specimens this is often not the case, because non tumorous cells may be present in various amounts or the normal wild-type allele may be lost (LOH) in tumors with somatic mutations of tumor suppressor genes.

Here we report the use of the DHPLC technique for the analysis of somatic *p53* mutations. We established the separation conditions for exons 5 thru 8 with known *p53* mutations, determined the sensitivity for the detection of heteroduplex by dilution series of wild-type and mutant PCR products, and then analyzed 18 microdissected colorectal carcinomas for *p53* mutations in exons 5 thru 8. DNA from specimens with known mutations in exons 5 thru 8 of *p53* included seven breast carcinomas, two urothelial carcinomas, a DNA sample from a Li-Fraumeni patient and two cell lines (HT29 and MDA-MB-435S), and DNA from blood of an individual with a polymorphism in exon 6. DNA from these tumor samples were either from frozen or paraffin-embedded tumor tissue and consisted almost entirely of tumor cells obtained by touch preparation (Saitoh et al, 1994) or laser microdissection. The DNA was isolated using standard techniques. To establish the separation conditions, DHPLC analysis was performed after mixing the tumor samples and cell lines with wild-type DNA at a ratio of 2:1. The PCR reactions were performed in 25 μl of a reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.0, 1.5, or 2.0 mM $MgCl_2$, 0.01% gelatin, and 200 mM dNTP 0.4 mM of each primer. After an initial denaturation step at 94° C for 4 minutes, 40 cycles were performed consisting of 30 seconds at 55° to 60° C and 30 seconds at 72° C, followed by a final extension of 7 minutes at 72° C. The primers were: exon 5 forward: atgtgttcacttgtgccctg; exon 5 revers: aaccagccctgtcgtctctc; exon 6 forward: agggtccccaggcctctgat; exon 6 revers: cacccttaaccctcctccc; exon 7 forward: ccaaggcgcactggcctcatc, exon 7 revers: cagaggctggggcacagcagg; exon 8 forward: ttccttactgcctcttgctt; exon 8 revers: tgtcctgcttgcttacctcg. Mutation analysis was essentially performed according to the method of Oefner and Underhill (1998) on an automated DHPLC analysis system (Transgenomic, Omaha, Nebraska). The PCR products were denatured for 4 minutes at 94° C and cooled to room temperature at a rate of 1° C/minute, and 3 to 15 μl of PCR products were applied to a preheated reverse phase column (DNA-Sep; Transgenomic). Elution of the DNA was performed in a linear acetonitrile gradient of buffers A and B. Buffer A consisted of 0.1 M triethylammonium acetate (TEAA) and buffer B of 0.1M TEAA and 25% acetonitrile. The temperature for optimal resolution of heteroduplex and homoduplex DNA detection was determined by analyzing the melting behavior of a PCR fragment of each exon while the temperature was increased by 1° C increments from 50° C to 55° C until the fragment was completely melted. The analysis temperature for each fragment was the point at which 75% of the DNA was present as an alpha helix or 1° to 2° C higher. The experimental melting behavior of the DNA fragments was compared with the melting behavior calculated by the Wavemaker software (Transgenomic) included with the DHPLC analysis system. This computer anal-

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Table 1. DHPLC Conditions for Exons 5–8 of the *p53* Gene

Exon	Temperature (°C)	Acetonitril gradient (%B)
5	62	50–63
	65	48–60
	67	48–59
6	61	48–61
7	64	47–59
8	59	49–61
	62	47–59
	64	45–57

DHPLC, denaturing high pressure liquid chromatography.

ysis was included in the interpretation of separate melting domains in the analyzed fragments. Fragments with more than one melting domain were analyzed at additional temperatures. The separation conditions used for each exon are listed in Table 1.

The DHPLC conditions established for *p53* exons 5 thru 8 detected all 10 of the previously characterized sequence variations in exons 5 thru 8 in a blinded analysis (Table 2). In addition, the DNA of the cell lines and of the blood showed aberrant DHPLC patterns in the corresponding exons.

The sensitivity for heteroduplex detection in the presence of various ratios of mutant and wild-type fragments was determined by DNA dilution series of up to 1% using a common polymorphism in exon 6 of the *p53* gene and with DNA of the HT29 cell line, which has an exon 8 mutation (G>A, codon 273). A slightly or clearly aberrant pattern was detected when the amount of one allele was 10% or 20%, respectively, in the case of HT29. In the case of the polymorphism in exon 6, a clear aberrant pattern was detected when the amount was ≥10%. This showed that heteroduplexes can be detected in the presences of 5% to 10% wild-type cells (two alleles/cell) when analyzing a tumor cell population for mutation of a tumor suppressor gene with simultaneous loss of the wild-type allele. Ten percent of normal cells are nearly always present, even when tumor samples are obtained by laser microdissection, making mixing with wild-type DNA unnecessary under most conditions. On the other hand this means that a heterozygous mutation (one normal and one mutated allele/cell) would be detectable in a background of 60% to 80% of normal cells (two normal alleles/cell). Others have reported a high degree of sensitivity for the detection of variant alleles comprising up to 5% of the analyzed DNA (Wolford et al, 2000).

We then analyzed 18 colorectal carcinomas, in which the *p53* mutation status was not known, for mutations in exons 5 thru 8. DNA from these samples was isolated from formalin-fixed, paraffin-embedded specimens after manual microdissection to give a tumor cell content in the range of 60% to 80%. Samples with an aberrant DHPLC pattern were directly sequenced. We identified 10 mutations in nine carcinomas. Exons 5 thru 8, from the samples of the remaining nine carcinomas with normal DHPLC patterns, were also directly sequenced and one mutation

Table 2. Mutations and Polymorphisms Identified by the Established DHPLC Conditions

Exon	Codon	Base change	Type of mutation	Amino acid change	Origin
−1, 5‘ exon 5		G>T	splice site		cc
5	130	CTC>CGC	missense	Leu>Arg	cc
5	135	TGC>TAC	missense	Cys>Tyr	cc
5	163	TAC>AAC	missense	Tyr>Asn	bc[a]
5	175	CGC>CAC	missense	Arg>His	bc[a]
6	209	del 2bp GA	frame shift		cc
6	213	CGA>CGG	silent	Arg>Arg	bl
6	213	CGA>TGA	nonsense	Arg>stop	cc, bc[a]
6	218	del 3bp TGC	in frame deletion		bc[a]
−2, 5‘ exon 7		A>G	splice site		cc
7	233	del 8bp	frame shift		bc[a]
7	235	del 14bp	frame shift		bc[a]
7	240	AGT>AGG	missense	Ser>Arg	uc
7	248	CGG>CAG	missense	Arg>Gln	cc, LF
8	266	GGA>GAA	missense	Gly>Glu	cl
8	266	GGA>GTA	missense	Gly>Val	cc
8	270	TTT>TCT	missense	Phe>Ser	cc
8	273	CGT>CAT	missense	Arg>His	cc, cl
8	275	TGT>TGG	missense	Cys>Trp	bc[a]
8	286	GAA>CAA	missense	Glu>Gln	uc

DHPLC, denaturing high pressure liquid chromatography; cc, colorectal carcinoma; bc, breast carcinoma; uc, urothel carcinoma; cl, cell line; LF, Li Fraumeni patient; bl, blood.

[a] Reported by Saitoh et al, 1994.

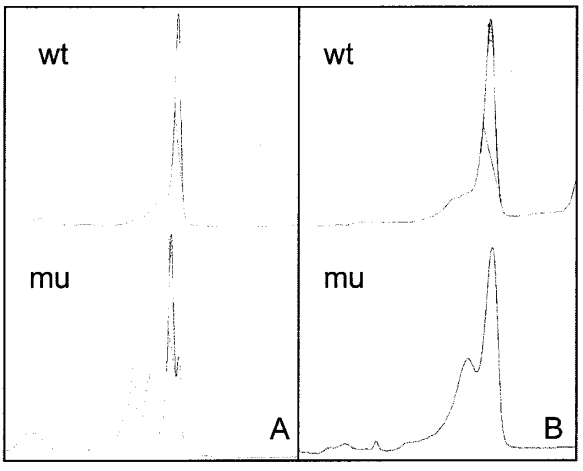

Figure 1.

Examples of an aberrant denaturing high performance liquid chromatography (DHPLC) chromatogram in comparison with the wild type. A, G>T, −1, 5′, exon 5; B, G>A codon 248, exon 7.mu, mutated; wt, wild type.

was found. We analyzed this PCR fragment under additional separation conditions using various temperatures, but no aberrant DHPLC pattern was detected. Taken the analysis of the samples with the known *p53* mutations and the analysis of the colorectal carcinomas together, the DHPLC conditions used detected a total of 19 different mutations and one known exon 6 polymorphism (Table 2). Examples are shown in Figure 1. Out of the 124 PCR fragments that were analyzed, seven DHPLC patterns were judged as suspicious aberrant and no mutation was identified by sequencing. Thus, the overall sensitivity and specificity of our method, used for the detection of *p53* mutations in exons 5 thru 8, was in the range of 95%. Although DHPLC was recently reported for the detection of *p53* mutations in ovarian tumors, DNA sequencing of the tumors that did not show an aberrant DHPLC pattern was not described (Gross et al, 2001). Comparison of sequencing and DHPLC results for other genes has revealed sensitivities for mutation detection ranging from 95% to 100% (Holinski-Feder et al, 2001; Jones et al, 1999; O'Donovan et al, 1998). Other prescreening methods, such as single strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE), have also been widely used for mutation analysis, including for *p53*. However, these techniques are characterized by a lower sensitivity (Hayashi and Yandell, 1993) and are also more labor intensive. Furthermore, the ability of DHPLC to detect mutant alleles, over a broad range of differing wild-type allele concentrations, also makes this method well suited for the analysis of somatic mutations in tissue tumor samples in which the proportion of mutant and wild-type alleles is variable.

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