METHODS

Harmonized Microarray/Mutation Scanning Analysis of TP53 Mutations in Undissected Colorectal Tumors

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Both the mutational status and the specific mutation of TP53 (p53) have been shown to impact both tumor prognosis and response to therapies. Molecular profiling of solid tumors is confounded by infiltrating wild-type cells, since normal DNA can interfere with detection of mutant sequences. Our objective was to identify TP53 mutations in 138 stage I–IV colorectal adenocarcinomas and liver metastases without first enriching for tumor cells by microdissection. To achieve this, we developed a harmonized protocol involving multiplex polymerase chain reaction/ligase detection reaction (PCR/LDR) with Universal DNA microarray analysis and endonuclease V/ligase mutation scanning. Sequences were verified using dideoxy sequencing. The harmonized protocol detected all 66 mutations. Dideoxy sequencing detected 41 out of 66 mutations (62%) using automated reading, and 59 out of 66 mutations (89%) with manual reading. Data analysis comparing colon cancer entries in the TP53 database (http://p53.curie.fr) with the results reported in this study showed that distribution of mutations and the mutational events were comparable. Hum Mutat 24:63–75, 2004.

KEY WORDS: zip-code addressing; TP53; p53; mutation detection; microarray; endonuclease V; thermostable ligase; mismatch recognition

DATABASES:
TP53 – OMIM: 191170; GenBank: X54156.1
http://p53.curie.fr (TP53 Database)

INTRODUCTION

Loss of TP53 (MIM# 191170) is observed in approximately one-half of all human cancers, making it the most commonly inactivated tumor suppressor gene [Soussi, 2003; Soussi and Béroud, 2001]. By disrupting TP53 function, cellular stress signals such as DNA damage, oxidative stress, hypoxia, and nucleotide depletion [Vogelstein et al., 2000; Vousden and Lu, 2002] go unheeded, creating a permissive environment for sequence errors that lead to oncogenic mutation. Disruption of TP53 activity breaches a second line of defense, as TP53 also responds to unregulated growth signals caused by the overexpression of certain oncogenes [Vousden, 2002]; thus, TP53 disruption can also contribute to uncontrolled proliferation of cells harboring activated oncogenes. Normally, TP53 will respond to such signals either by arresting the cell cycle to permit DNA repair in mildly damaged genomes or by inducing apoptosis to eliminate cells with severely damaged genomes. In addition to preventing propagation of genomic errors, TP53 is also implicated in the regulation of genes that inhibit angiogenesis and metastatic disease progression. The tumorigenic potential of a cell is greatly influenced by the functional status of TP53.

Many prospective cancer therapy studies indicate that the functional status of TP53 will also influence a tumor's response to therapy. For example, the commonly used cancer drug, 5′-fluorouracil, is ineffective in TP53-deficient human cells; however, the DNA damaging agent, adriamycin, induces apoptosis irrespective of the TP53 status [Bunz et al., 1999]. Similarly, cells with transcriptionally inactive forms of TP53 are less sensitive to vinca alkaloids, but become more sensitive to

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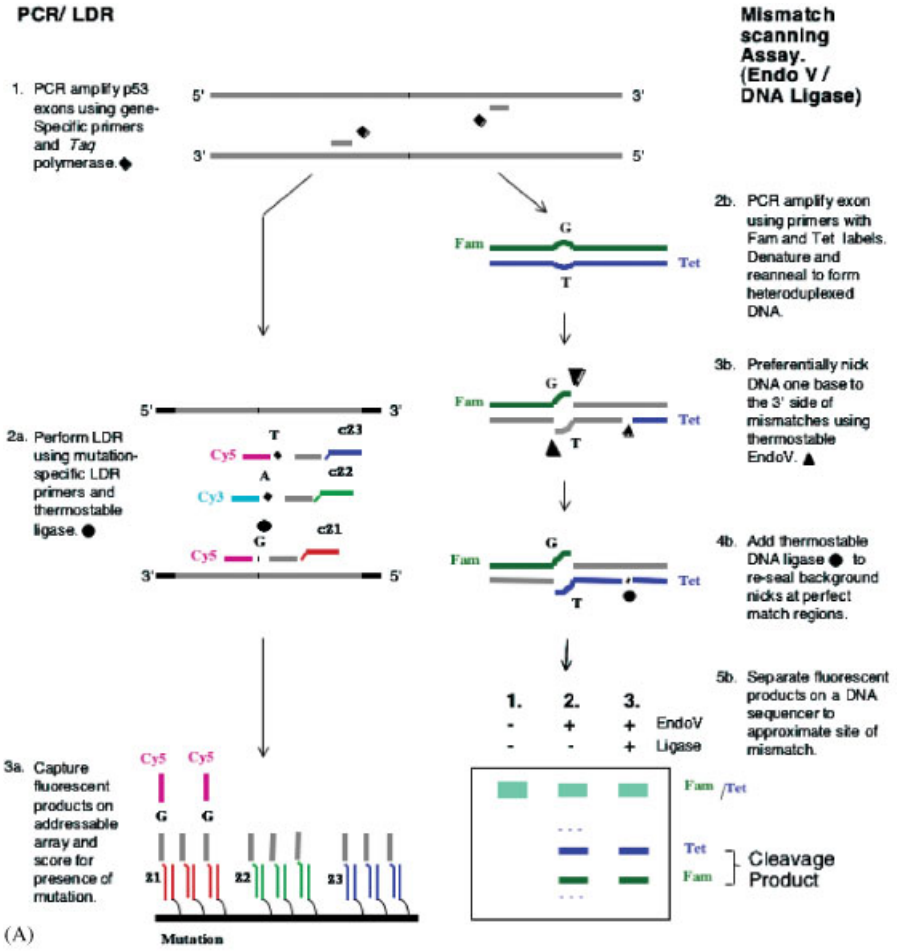
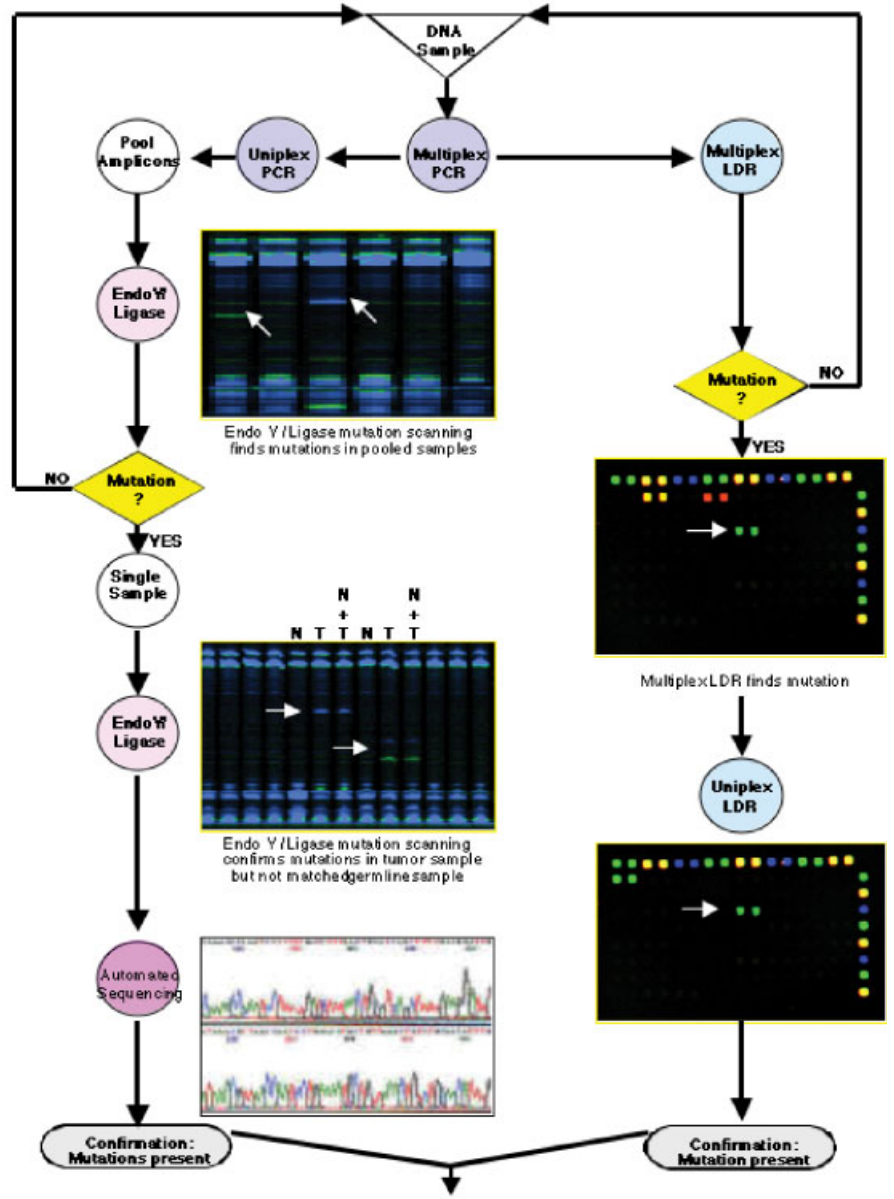

PCR/ LDR
Mismatch scanning Assay. (Endo V / DNA Ligase)
1. PCR amplify p53 exons using gene-Specific primers and Taq polymerase.
2a. Perform LDR using mutation-specific LDR primers and thermostable ligase.
3a. Capture fluorescent products on addressable array and score for presence of mutation.
2b. PCR amplify exon using primers with Fam and Tet labels. Denature and reanneal to form heteroduplexed DNA.
3b. Preferentially nick DNA one base to the 3' side of mismatches using thermostable EndoV.
4b. Add thermostable DNA ligase to re-seal background nicks at perfect match regions.
5b. Separate fluorescent products on a DNA sequencer to approximate site of mismatch.
Fam
Tet
Cy5
Cy3
c23
c22
c21
Mutation
EndoV
Ligase
Cleavage Product
(A)


DNA Sample
Pool Amplicons
Uniplex PCR
Multiplex PCR
Multiplex LDR
Endo V Ligase
Mutation ?
NO
YES
Single Sample
Automated Sequencing
Endo V/Ligase mutation scanning finds mutations in pooled samples
Endo V/Ligase mutation scanning confirms mutations in tumor sample but not matchedgermlinesample
Multiplex LDR finds mutation
Uniplex LDR
Confirmation: Mutations present
Confirmation: Mutation present
Optimize each assay to detect mutations that the other misses to achieve high sensitivity and high specificity
(B)

paclitaxel [Zhang et al., 1998]. The presence of wild-type TP53 activity is essential for therapies dependent on the anti-angiogenic agent, TNP-470 [Zhang et al., 2000] and anti-angiogenic combination therapy [Yu et al., 2002], while the "ONYX-015" adeno-like virus depends on the complete absence of wild-type TP53 activity in order for transduction to occur. Recent studies have indicated that breast cancer patients harboring TP53 mutations have significantly worse prognoses than those with wild-type status [Borresen-Dale, 2003]. Thus the functional status of TP53 may influence treatment outcome.

While the functional status of TP53 in a cell affects response to therapies and tumor prognosis, several studies have suggested that not all TP53 mutations are alike. TP53 mutant proteins with a flexible conformation correlate with poor prognosis, and different missense mutations are known to have differing effects on the conformational stability of TP53 [Chen et al., 2001]. It has also been demonstrated that cell lines ectopically expressing various mutant forms of TP53 are insensitive to different chemotherapeutic agents [Blandino et al., 1999]. In addition, different TP53 mutations have different cellular consequences. Finally, small synthetic molecules have been generated that are capable of restoring wild-type TP53 function both in vitro and in vivo [Bullock and Fersht, 2001; Bykov et al., 2002]. Thus, the specific TP53 missense mutation in a cell impacts response to therapies and tumor prognosis.

These initial studies indicate that knowledge of the specific TP53 mutation is of growing importance. Although current methods for mutation detection possess some very desirable characteristics (e.g., denaturing gradient gel electrophoresis [DDGE], DHPLC, SSCP, dideoxy-fingerprinting [ddF], restriction endonuclease fingerprinting [REF]), these methods are of limited utility in large-scale prospective clinical trials involving solid tumors [Elsaleh et al., 2001; Kimler et al., 2000; Nabholtz et al., 1999, 2002; Soong et al., 2000] due to low throughput and/or sensitivity (see Kirk et al. [2002] for review). Immunohistochemical analysis of 142 colorectal tumors demonstrated that only 51% of tumors that significantly overexpressed the TP53 protein contained DNA mutations [Kaserer et al., 2000]. Likewise, 32% of tumors that contained a mutated TP53 gene did not concordantly overexpress the TP53 protein. Direct sequencing and gene hybridization chips fail to identify mutations in TP53 over 20% of the time, due in part to dilution of mutant alleles by infiltrating stromal cells in solid tumors [Ahrendt et al., 1999]. Thus, the functional status of TP53 does not necessarily correlate with immunostaining, sequencing, or hybridization chip results [Kaserer et al., 2000].

For effective drug therapy of solid tumors, there is an urgent need to accurately assess TP53 functional status and to precisely determine the nature of the TP53 mutation. In order to substantiate that certain factors are of major effect in influencing outcome, it is necessary to establish statistical significance by surveying a large number of tumors. In this study, we sought to improve both the accuracy and the throughput of TP53 mutation detection by developing a harmonized protocol that combines the strengths of two sensitive enzymatic assays. Rapid analysis is promoted by creating two complementary, parallel tracts with facility for efficient throughput. Endonuclease V (EndoV)/ligase mutation scanning can detect unknown mutations and allows sample pooling [Huang et al., 2002]. This method has been shown to detect substitutions, insertion/deletion mutations varying in size from one to three bases, and scanning ability in amplicons up to 1.7 kb [Huang et al., 2002]. The polymerase chain reaction/ligase detection reaction (PCR/LDR) [Khanna et al., 1999] has substantial multiplexing capability for predetermined mutations, which is extended further by coupling analysis to a Universal DNA microarray [Favis et al., 2000; Gerry et al., 1999]. Both enzymatic assays have sufficient sensitivity to allow analysis of undissected solid tumors, which substantially improves throughput. Figure 1 illustrates how this harmonized protocol functions (Fig. 1A) and provides a flow chart of how the two parallel tracts advance (Fig. 1B). The current study provides the first report of the TP53 Universal DNA microarray and details the application of an assay that combines the utility of two sensitive enzyme systems to analyze mutations in undissected solid tumors.

MATERIALS AND METHODS

Tumor Procurement and DNA Extraction

Control DNA samples with known TP53 mutations were obtained from preexisting samples archived in T. Soussi's collection. All patients recruited from Memorial Sloan Kettering Cancer Center underwent surgical resection for primary adenocarcinoma of the colon. Written informed consent was obtained from each subject. The majority (95%) of patients were identified as Caucasian, while 5% were identified as non-Caucasian. A total of 120 primary colon tumors (15 Stage I, 22 Stage II, 41 Stage III, and 42 Stage IV) were collected at the time of surgical resection in accordance with Institutional Review Board approved protocols. Two to four viable portions of the tumor were harvested by sharp

FIGURE 1. **Mutation detection using PCR/LDR and EndoV mutation scanning assays. A: Schematic explaining PCR/LDR and EndoV/Ligase mutation scanning assays. B: Flow chart of combined PCR/LDR and EndoV mutation scanning analysis of TP53 mutation status. DNA extracted from undissected primary tumors is subjected to multiplex PCR amplification of exons 4–8. PCR/LDR analysis (right side) subsequently uses multiplex LDR to simultaneously query for the presence of 110 mutations. The ligation products are analyzed on a universal DNA microarray and samples containing TP53 mutations (indicated by the white arrow) are confirmed using uniplex LDR. The uniplex LDR reaction targets only the specific candidate mutation and the control reaction for the amplicon of interest. EndoV mutation scanning (left side of figure) draws from the same multiplex PCR reaction and specific amplicons are selectively amplified. Amplicons for the same exon that are derived from different tumor samples are pooled in groups of three and subjected to EndoV mutations scanning. If mutations are found (indicated by the white arrows), the amplicon harboring the mutation is reanalyzed in each individual sample. Automated sequencing identifies the specific mutation.**

dissection and snap frozen in liquid nitrogen within 15 min of removal. From each tumor, four core samples containing a mean normal cell count between 30–50% were taken from different regions, combined, and DNA was extracted using the QIAamp Tissue Kit (Qiagen, Chatsworth, CA) according to manufacturer's guidelines.

Multiplex PCR Amplification

DNA extracted from undissected primary tumors was subjected to multiplex PCR amplification of exons 5, 6, 7, and 8 for gel-based assays, while exon 4 was added to the multiplex for microarray assays in order to include the codon 72 polymorphism in the analysis of exons 4–8. PCR was performed in a 50 μl reaction using 100 ng DNA, 100 μM dNTP, 1× PCR Buffer II (Applied Biosystems, Foster City, CA) supplemented with 1.5 mM final concentration of $MgCl_2$, 2.5 units Ampli*Taq* Gold (Applied Biosystems), and 0.4 μM of each primer. Primer sequences, in 5′ to 3′ orientation, were as follows: exon 4 forward = CCGGACGATATTGAACAATGGTTC; exon 4 reverse = GCAAGAAGCCCAGACGGAAAC; exon 5 forward = CTGTTCACTTGTGCCCTGACTTTC; exon 5 reverse = CCAGCTGCTCACCATCGCTATC; exon 6 forward = CCTCTGATTCCTCACTGATTGCTCTTA; exon 6 reverse = GGCCACTGACAACCACCCTTAAC; exon 7 forward = GCCTCATCTTGGGCCTGTGTTATC; exon 7 reverse = GTGGATGGGTAGTAGTATGGAAGAAATC; exon 8 forward = GGACAGGTAGGACCTGATTTCCTTAC; exon 8 reverse = CGCTTCTTGTCCTGCTTGCTTAC. PCR was performed by heating the reaction for 10 min at 95°C, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min. Amplification was verified by examining the products on a 3% agarose gel. *Taq* Polymerase was eliminated by incubating the reaction for 10 min at 70°C with 50 μg/ml Qiagen proteinase K. This treatment was followed by incubation at 90°C for 15 min to denature proteinase K. The TP53 sequence used was GenBank Accession X54156.1. Version X54156.1 GI:35213.

Mutation Detection and Analysis

Using the UMD (Universal Mutation Database) software described by Béroud and Soussi [2003], we analyzed the frequency of TP53 mutational events in colon cancer. Among the 1,427 TP53 mutations that are described for colon cancer, there are 375 different variant classes, ranging from a high frequency of occurrence (such as g.13203G>A [p.R175H] found 177 times) to 230 variants that are found only one time. From this information, it was possible to devise 58 ligase detection reactions (LDR) that allow the detection of 70% of TP53 mutation in colon cancer. Oligonucleotide design and synthesis, ligase detection reaction (LDR), and *Tth* DNA ligase production were performed as previously described [Barany and Gelfand, 1991; Khanna et al., 1999]. LDR primers were divided into two tubes, based on whether the ligation was directed against the upper or lower strand of DNA. LDR primers designed for gel assays are published elsewhere [Dong et al., 2001], while those for array experiments are available in Supplementary Table S1 (available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat).

Following a multiplex PCR amplification of the regions of interest, each mutation is simultaneously detected using a thermostable ligase that joins pairs of adjacent oligonucleotides complementary to the sequences of interest. Ligation occurs only when there is perfect complementarity at the junction between the paired oligonucleotides. Ligation products are distinguished based on differential labeling and migration on a polyacrylamide gel, or hybridization to specific addresses on the universal DNA microarray. The reaction was performed as previously described [Favis et al., 2000], except 3 μl of PCR reaction was used. Quality control for LDR was performed using a synthetic template for each mutation to test the ability of the full mix of upper or lower ligation primers to produce the expected, specific signal on the DNA microarray. In addition, 100 DNA samples derived from various types of cancers and known to contain TP53 mutations targeted in the PCR/LDR assay were analyzed in a blinded fashion.

EndoV/ligase mismatch scanning was performed as described elsewhere [Huang et al., 2002]. Briefly, heteroduplexed variant and wild-type PCR amplicons from tumor DNA are generated using 6FAM and TET labeled primers. *Thermotoga maritima* (*Tma*) EndoV recognizes and primarily cleaves heteroduplex DNA one base 3′ to a mismatch. Since matched DNA is also nicked at low levels, a highly specific thermostable DNA ligase is used to reseal just those nicks. This lowers background signal and improves the signal-to-noise ratio. Fragment mobility of cleavage products on a DNA sequencing gel reveals the approximate position of the mutation. Amplicons from different tumors corresponding to specific exons were pooled in groups of three. Mutation scanning was performed, and samples identified as containing TP53 mutations were analyzed individually to confirm the mutation and then sequenced. Amplicons for exons 5, 6, 7, and 8 were generated using the PCR primers described for PCR/LDR above and were sequenced using the dRhodamine Terminator Cycle DNA Sequencing kit (Applied Biosystems, Foster City, CA) according to manufacturer's guidelines.

Mutations in the TP53 gene were analyzed with the UMD software [Béroud and Soussi, 2003]. The TP53 database version used for this analysis (December 2002) contains 14,968 mutations, including 1,516 for colon cancer. Genetic alterations reported herein use nucleotide numbering identical to Accession X54156.1.

Microarray Fabrication and Hybridization

Fabrication and hybridization were performed as previously described [Favis et al., 2000; Gerry et al., 1999], except hybridization was carried out in the presence of 100 μg/ml sheared salmon sperm DNA. Arrays were reused three times and were stripped between uses by submerging for 1 min in a solution of boiling 100 mM Bicine/0.1% SDS. Stripped arrays were rinsed in nanopure water, excess water was removed using forced air, and the arrays were stored in slide boxes at room temperature.

Quality control for array fabrication was performed on representative arrays by staining with SYBR Green II (Molecular Probes, Eugene, OR) to determine whether all 64 zip-code addresses had spotted. To verify that no cross-contamination of addresses occurred during spotting, selected arrays were subjected to four hybridizations (stripping between hybridizations) using 6FAM-labeled complementary zip-codes. Arrays were hybridized such that odd rows, even rows, odd columns, or even columns were selectively targeted to produce specific signals without extraneous signals.

RESULTS

Validation of the PCR/LDR Tract: 58 Different TP53 Mutations Can Be Detected Using a Gel-Based Assay

The TP53 gene is mutated in hundreds of positions, and thus represents an enormous challenge to mutation detection strategies [Soussi and Béroud, 2001]. In addition, different cancers exhibit different preferential sites of mutation in this gene [Hussain et al., 2000]. To circumvent problems associated with molecular heterogeneity in TP53, we initially developed a gel-based assay to detect high frequency mutations in colon cancer and established validation criteria for the assay. (Validation of the EndoV tract can be found elsewhere [Huang et al., 2002].) By focusing on a single cancer and using a bioinformatics approach to dictate the assay design, it

was possible to engineer the assay sensitivity such that a significant percentage (approximately 70%) of database-predicted mutations for colon cancer could be detected [Iacopetta, 2003].

LDR is a versatile method for discriminating single-base mutations or polymorphisms and is ideal for multiplexing, since several primer sets can ligate along a gene without the interference encountered in polymerase-based systems (see Fig. 1 for description). Additionally, LDR readily discriminates between wild-type and frameshift or point mutation sequences [Favis et al., 2000; Gerry et al., 1999; Khanna et al., 1999; Zirvi et al., 1999].

A total of 111 LDR primers were designed for this initial assay and the reaction was optimized to achieve multiplex PCR amplification of exons encompassing the DNA binding domain (exons 5, 6, 7, and 8), followed by the multiplex LDR detection of 58 mutations and four amplicon controls (see Dong et al. [2001] for the list of targeted mutations and primer sequences). To validate the assay's ability to detect mutations in clinical samples, 100 blinded tumor DNA samples containing TP53 mutations known to be targeted by the assay were analyzed (see Fig. 2B). No information regarding the origin or the nature of the samples was provided until after results were submitted and samples were unblinded, hence all samples were treated identically and subjected to multiplexed PCR followed by multiplex LDR. The tumor DNA was derived from a variety of cancers, from both fresh frozen and paraffin-embedded tissue and included both normal and mutant samples. In addition, artificial mixes of tumor genomic DNA diluted 1:20 (p.R273H (g.14487G>A), double mutant p.R196X+p.R248Q ([g.13346C>T]+[g.14070G>A]), and p.R175H (g.13203G>A)) and 1:100 (p.R175H (g.13203G>A) and p.R273C (g.14486C>T)) in normal genomic DNA were included. The results demonstrated that the PCR/LDR TP53 assay could detect all mutations that were represented by LDR primer sets, even when diluted at 1:100 (Fig. 2C). (In natural tumor samples where it was found that 5% of cells were tumorigenic, we have demonstrated successful mutation detection using PCR/LDR [Fouquet et al., 2003].) The two mutant samples that were refractory contained mutations that were not included in the LDR primer sets.

Although PCR error was not expected to affect assay sensitivity, this was verified by comparing the EndoV mutation scanning results using the proofreading polymerases. Different polymerases for PCR achieve different rates of fidelity, with *Taq* polymerase error rates of 1.3×10^{-5} to 3×10^{-6} per base and proofreading polymerases on the order of 5×10^{-7}. These error rates are very small compared to the target sensitivity of 1 in 100, and were not expected to create a signal on the sequencing gel comparable to a true mismatch, even at a dilution of 1:100. As confirmation, we experimentally compared *Taq* polymerase with commercially available high-fidelity mixes containing *Pfu* or *Tgo* polymerases, with no difference in background observed (data not shown).

Validation of the PCR/LDR Tract: PCR/LDR Coupled to Analysis on a Universal DNA Microarray to Accommodate Analysis of 110 Different TP53 Mutations

To be of clinical utility, it is necessary to be able to survey a large number of mutations. In deference to the requirement that LDR products have precisely-defined mobility, analysis using a gel-based system places a practical limit of approximately 60–80 alleles per lane of a sequencing gel. To overcome these limitations, analysis of the LDR products was transferred to a universal microarray system. We expanded the number of LDR primers for universal microarray analysis so that the reaction could in toto accommodate 70% of all mutations found in the TP53 database that were associated with colon cancer (exon 4 was added to PCR multiplex to accommodate codon 72 polymorphisms), 65% associated with osteosarcomas (14 mutations and two polymorphisms added with 30 new primers), and 80% associated with lung cancer (38 mutations added using 71 primers). The mutations detected and the universal microarray design are shown in Supplementary Figure S1 (available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat) while the LDR primer sequences are shown in Supplementary Table S1. The universal array (Fig. 3A) was validated as described for the gel-based assay and then tested for the ability to detect mutations in undissected tumor samples (Fig. 3B). Each mutation is uniquely identified based on the color of the fluorescent signal, the address emitting the signal, and whether the signal was observed for the "upper" or "lower" strand reaction.

Dideoxy Sequencing Is Insufficient for Mutation Analysis in Undissected Colon Tumors

DNA was extracted from 120 stage I–IV colorectal tumors and was examined for TP53 mutations and the status of amino acid 72. The TP53 gene was analyzed using the harmonized protocol and DNA sequencing. As described above, throughput for the EndoV tract was facilitated by pooling amplicons from specific exons in groups of three, these were scanned, and those bearing mutations were rescanned individually and sequenced (Fig. 1).

Table 1 shows a comparison between the harmonized protocol and dideoxy-sequencing. The harmonized protocol identified all 66 TP53 mutations found in the tumor set. We can achieve this high level of sensitivity and specificity because the two tracts of the harmonized protocol complement each other. Of the 66 mutations, 14 were mutations not represented in the LDR primer set, and two mutations fell below the limits of detection for this approach (p.H193R (g.13338A>G) and p.G245S (g.14060G>A) were detectable using synthetic template controls), however EndoV mutation scanning succeeded in detecting these mutations. Similarly, EndoV mutation scanning was unable to detect 20 of the mutations in four specific GC-rich sites known to be refractory to cleavage by this enzyme [Huang et al.,

EXON	MUTATION	LANE	SIZE	EXON	MUTATION	LANE	SIZE
7	M237I G-A/T	U	56				
5, 8	A161T, C275Y, C275F	U	55				
8	V272M, V272L	U	54				
7,8	G245V, F270I	U	53	6, 7	Y205S, Y205C	L	53
8	G244C, G244D, G244V	U	52	6	P190L, T211A, T211I	L	52
8	C238Y	U	51	8	R306X	L	51
				6	R213X	L	50
6	WT	U	49	7, 5	S241F, S241C, K132R	L	49
8	R273H	U	47	7	WT	L	47
5	C135Y, C135F	U	46				
5, 8	C141Y, C141F, R280K, R280I	U	45	8	R282W	L	45
5	C176Y, C176F	U	44	8	R273C	L	44
5	R175H	U	43	6	R196X	L	43
5	V173M, V173L, V143M, V143A	U	42	6	Y220C	L	42
5	G245S	U	41	7	I254V, G154S	L	41
5	G245D	U	40	5	P152L	L	40
5	R248Q	U	39	5	R175C, R175L, H179Y	L	39
				7	R248W	L	38
8	WT	U	37	7	R249W, R249G	L	37
				7	R249S	L	36
5	R158H	U	35	5	H179R, H179L	L	35
				5	WT	L	33
				5	V157F, V157L	L	32

(A)

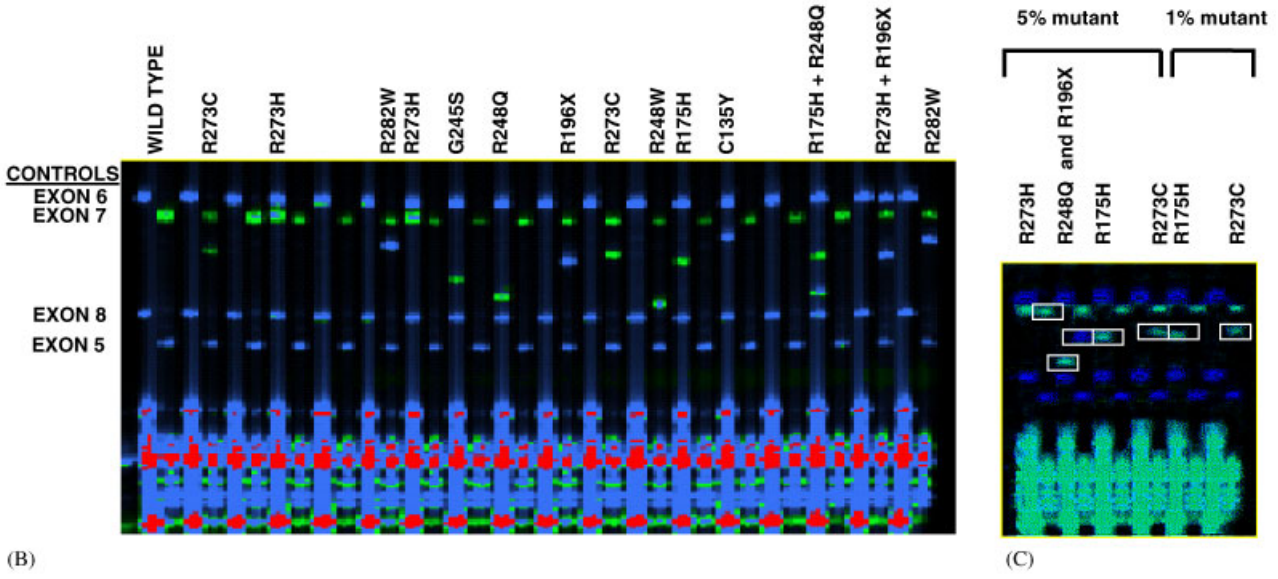


FIGURE 2. **Gel-based assay can simultaneously query for 58 different TP53 mutations. A:** Diagram of gel results for PCR/LDR assay. The gel-based assay can simultaneously query for 58 different TP53 mutations. LDR reactions rely on ligation of primers identical to either the upper strand (U) or lower strand (L) of the DNA sequence. U and L reactions are performed in separate tubes and loaded into separate lanes. The columns to the left of the bold red line represent the two lanes of a 10% sequencing gel. The boxed products are the amplicon controls for TP53 exons 5, 6, 7, and 8. The TP53 exon containing the mutation and the size of each product (bases) is shown in the first and last columns of each lane, respectively. **B:** Gel results of a mutation detection experiment using 100 blinded samples of DNA isolated from tumors. Amplicon controls for each exon are designated on the left of the image (identical to boxed regions in A). Mutation status of the sample is indicated over the second of the two lanes dedicated to each sample. For both panels, blue products are FAM-labeled reactions, while green products are reactions labeled with TET. Mutations detected are represented by amino acid and position. **C:** Detection of mutant DNA diluted 1:20 and 1:100 in normal DNA. DNA samples with known TP53 mutations were diluted into wild-type DNA and provided in a blinded manner for TP53 mutation detection. The samples were subsequently PCR amplified and subjected to PCR/LDR. The mutations found (listed above the corresponding two gel lanes for each sample) were confirmed to match the known mutation in the diluted DNA. LDR products for mutations are indicated with boxes. Dilution appears at the top of the figure over corresponding gel lanes.

2002], but PCR/LDR readily detected these mutations. Because PCR/LDR/Universal array can find mutations that are refractory to cleavage by EndoV mutation scanning, while the latter finds deletions or uncommon mutations not covered by our LDR primers, this harmonized protocol can find all mutations present in

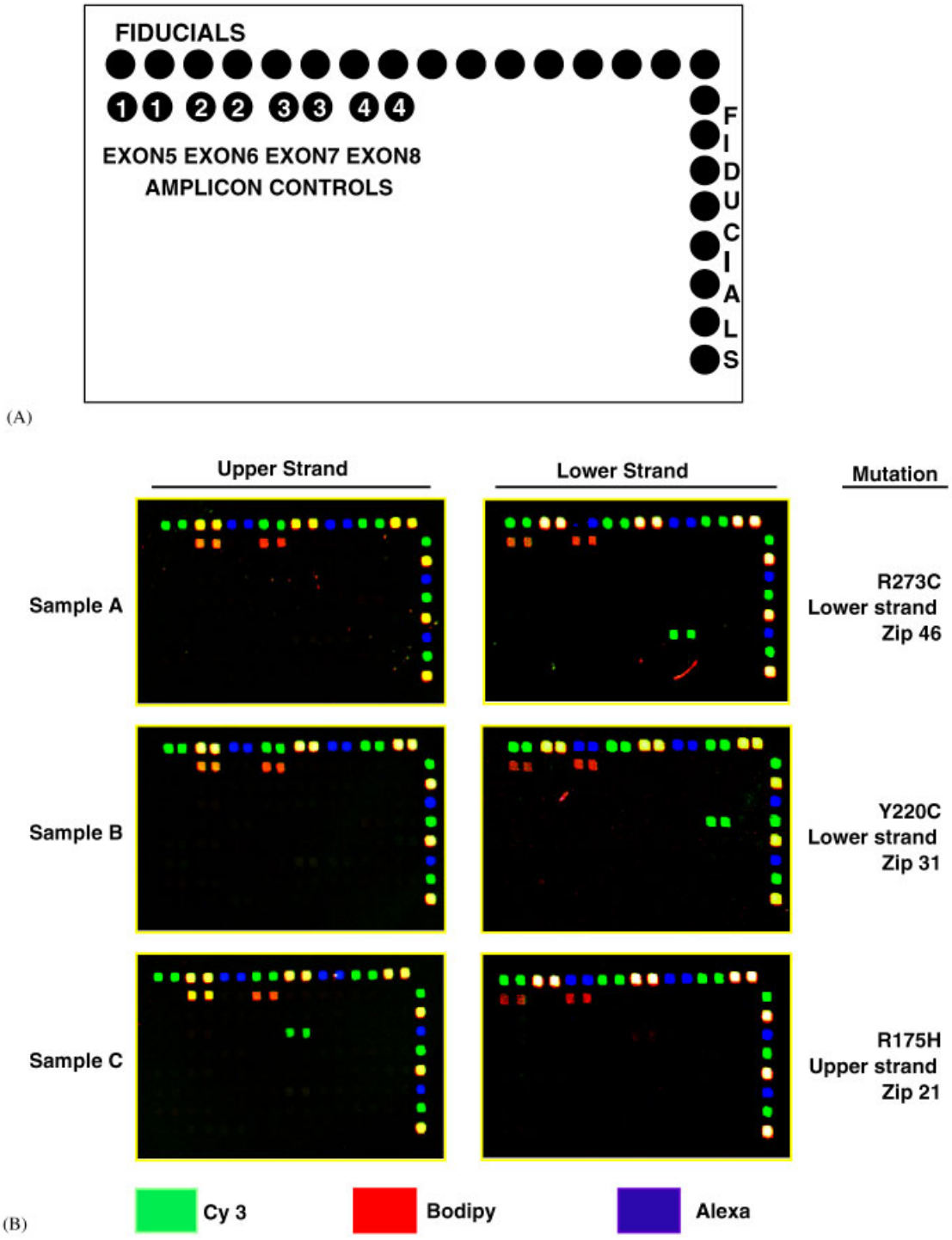


FIGURE 3. **Universal DNA microarray analysis can detect 110 different TP53 mutations. A:** Diagram of chip layout for the TP53 assay. The assay uses two 64-address arrays, but for simplicity, a composite diagram is presented. Addresses are double-spotted on to a three-dimensional surface comprised of a loosely cross-linked polymer of acrylamide and acrylic acid. The three-dimensional surface combined with the zip-code system allows hybridized arrays to be stripped of target and reused. Fiducials labeled with fluorochromes Cy3, Bodipy, and Alexa are spotted along the top and the right side of the array to provide orientation. Amplicon controls for exons 5, 6, 7, and 8 are directed to zip-codes in the first row of the array and labeled with both Cy3 and Bodipy. The zip-code addresses occupied by these controls are indicated by the numbers in the circles: upper strand control reactions hybridize to zip-code 2 (exon 6) and zip-code 4 (exon 8) (later versions of the array include zip-code 5 (exon 4)); lower strand control reactions hybridize to zip-code 1 (exon 5) and zip-code 3 (exon 7). A maximum of three different products can be directed to each address. Cy3 signal is represented in green, Bodipy-labeled products are red, and Alexa signals are blue. **B:** Results of PCR/LDR/Universal DNA microarray analysis of undissected colon tumor DNA. Three different samples are depicted. LDR reactions directed against the upper (left side) or lower strand (right side) of DNA are performed in separate tubes and hybridized to different arrays. Mutations found in each sample are named on the far right of the panel and are identified by the location of the zip-code exhibiting the signal, the color of the fluorochrome associated with the signal, and whether the signal appears on an array for upper or lower strand reactions.

TABLE 1. Comparison Between the Harmonized Protocol and Dideoxy-sequencing

Sample #: (138 total)	Combined Assay	DNA sequencing, Automated read	Resequencing of both strands, Manual read	TP53 Mutation
1	(7) Y234C (W)*	ND	ND	g.14028A>G
2	(5) R175H (REV)*	(5) R175H	NN	g.13203G>A
4	(8) R273C (REV)*	(8) R273C	NN	g.14486C>T
6	(7) R248Q*†	ND	(7) R248Q	g.14070G>A
7	(5) R175H (REV)*	(5) R175H	NN	g.13203G>A
9	(7) R248Q*†	ND	(7) R248Q	g.14070G>A
13	(5) R175H (REV)*	(5) R175H	NN	g.13203G>A
15	(6) R213X (W)*†	(6) R213X	NN	g.13397C>T
17	(7) Y234C (W)*	ND	ND	g.14028A>G
18	(8) R282W*†	(8) R282W	NN	g.14513C>T
20	(8) R273C (REV)*	(8) R273C	NN	g.14486C>T
24	(7) R248W*†	ND	(7) R248W	g.14069C>T
26	(8) R273H*†	(8) R273H	NN	g.14487G>A
27	(7) R248Q*†	ND	(7) R248Q	g.14070G>A
29	(8) R273C (REV)*	(8) R273C	NN	g.14486C>T
31	(8) R273H*†	(8) R273H	NN	g.14487G>A
32	(5) E171X*†	(5) E171X	NN	g.13190G>T
33	(5) G154G (LF, S)†	(5) G154G (S)	NN	g.13141C>A
35	(5) G154D (LF)†	(5) G154D	NN	g.13140G>A
36	(5) Y163C (LF)†	(5) Y163C	NN	g.13167A>G
38	(5) R175H (REV)*	(5) R175H	NN	g.13203G>A
40	(8) R306X (REV)*	ND	(8) R306X	g.14585C>T
41	(6) E224E (LF, S)†	ND	(6) E224E (S)	g.13432G>A
42	(5) R175H (REV)*	(5) R175H	NN	g.13203G>A
43	(6) H193R†	(6) H193R	NN	g.13338A>G
44	(5) H179Y*†	(5) H179Y	NN	g.13214C>T
45	(7) R248Q*†	ND	(7) R248Q	g.14070G>A
46	(7) R248Q*†	ND	(7) R248Q	g.14070G>A
49	(5) R175H (W, REV)*	(5) R175H	NN	g.13203G>A
53	(5) R175H (REV)*	(5) R175H	NN	g.13203G>A
55	(8) E285K*†	(8) E285K	NN	g.14522G>A
59	(8) R273C (REV)*	(8) R273C	NN	g.14486C>T
60	(8) R282W,*	ND	(5) K164X	g.14513C>T
	(8) R306X (REV)*		(8) R306X	g.14585C>T
	(5) K164X†			g.13169A>T
65	(5) R175H (REV)*	(5) R175H	NN	g.13203G>A
66	(7) R248Q*†	ND	(7) R248Q	g.14070G>A
67	(8) R282W*†	ND	(8) R282W	g.14513C>T
68	(6) Y205F (LF)†	ND	(6) Y205F++	g.13374A>T
71	(8) R273C (REV)*	(8) R273C	NN	g.14486C>T
73	(5) Q167 insA†		(5) Q167 insA†	g.13178_13179insA
	(8) R282W*†	(8) R282W	(8) R282W	g.14513C>T
77	(7) S261R (LF)†	(7) S261R	NN	g.14108A>C
78	(7) R248W*†	ND	(7) R248W	g.14069C>T
79	(7) S240 delA (LF)†	ND	(7) S240 delA++	g.14045delA
80	(5) R175H (REV)*	(5) R175H	NN	g.13203G>A
81	(5) R175H (REV)*	(5) R175H	NN	g.13203G>A
84	(7) S261R (LF)†	(7) S261R	NN	g.14108A>C
89	(6) Q192X (LF)†	(6) Q192X	NN	g.13334C>T
90	(6) H214 insA (LF)†	ND	(6) H214 insA++	g.13401_13402insA
91	(7) G245S†	(7) G245S	NN	g.14060G>A
93	(5) R175H (REV)*	(5) R175H	NN	g.13203G>A
94	(6) H214 insA (LF)†	ND	(6)H214 insA++	g.13401_13402insA
96	(8) R273H*†	(8) R273H	NN	g.14487G>A
97	(8) R267G (LF)†	(8) R267G	NN	g.14468C>G
98	(8) R282W*†	(8) R282W	NN	g.14513C>T
102	(7) R248Q*†	ND	(7) R248Q	g.14070G>A
103	(7) R248Q*†	ND	(7) R248Q	g.14070G>A
114	(6) R213R (LF, S)†	(6) R213R (S)	NN	g.13399A>G
116	(6) P190L (LF)†	(6) P190L	NN	g.13329C>T
117	(6) R213R (LF, S)†	(6) R213R (S)	NN	g.13399A>G
122	(7) R248W*†	ND	(7) R248W	g.14069C>T
123	(7) E258D (LF)†	(7) E258D	NN	g.14102A>T
124	(8) R273C (REV)*	(8) R273C	NN	g.14486C>T
126	(6) E224E (LF, S)†	ND	(6) E224E (S)	g.13432G>A
137	(8) R273C (REV)*	(8) R273C	NN	g.14486C>T
(75 samples)	ND	Not determined #	Not determined #	
p53 Mutants	66/66	41/66	59/66	
% correct	Set as standard	62%	89%	

(5), (6), (7), (8) = exon 5, 6, 7, or 8; * = detected by PCR/LDR; † = Detected by EndoV; ++ = Required gel purification of PCR product to obtain sequencing result; # = Sequencing of 5 random samples of 75 called negative by harmonized protocol reveal no new mutations; ND = Not detected; LF = Current PCR/LDR universal array primer set does not contain primers to detect this low frequency mutation; NN = Not necessary since mutation was identified using automated DNA sequencing; REV = Refractory to EndoV cutting; (S) = Silent mutation; and (W) = Weak signal. All nucleotide numeric designations use numbering identical to, GenBank Accession X54156.1.

this cohort of undissected tumor samples. By comparison, direct sequencing with automated reading of the sequence found only 41 out of 66 mutations (62% sensitivity) and consistently failed to identify mutations in codon R248, which is the most frequently mutated TP53 codon for all cancers. When direct sequencing with automated reading was supplemented with resequencing of both strands and manual reading, the score improved to 59 out of 66 mutations identified (89% sensitivity). However, for five of the samples, the mutation could only be detected by gel purifying the PCR product prior to sequencing. In addition, prior knowledge of the site of mutation was required when manually reading sequencing results to identify certain mutations that were present at low levels.

Analysis of 138 Colorectal Tumors and Liver Metastases Using the Harmonized Protocol

A summary of the colorectal tumor data can be found in Table 2. We found that 44% of the samples were mutant for TP53. The frequency of mutation for TP53 was in agreement with previous findings for colorectal cancer [Iacopetta, 2003; Soussi et al., 2000]. For TP53, 60 samples had point mutations, four samples exhibited deletions, five samples contained polymorphisms, one sample was double mutant (insertion of A in codon Q167+p.R282W ([g.13178_13179A]+[g.14513C>T])), and one sample was identified as a triple mutant (p.K164X+p.R282W+p.R306X ([g.13169A>T]+[g.14513C>T]+[g.14585C>T])).

As a quality control step before data release, the first pass results from the present study were compared to the 1,516 mutations found in colon cancer that were stored in the database. The distribution of the mutations along the TP53 gene and the pattern of mutation events were quite similar (see Supplementary Figure S2, available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat), reinforcing the accuracy of the harmonized protocol. In addition, by performing detailed comparisons of the current results with the TP53 database, we were able to identify and quickly eliminate a spurious weak LDR signal corresponding to p.R273S (g.14486C>A). This mutation had never been detected in the 1,620 entries for colorectal cancer and only 11 times in the database overall. When preliminary PCR/LDR results suggested the low-level presence of this mutation in seven samples, the finding was considered highly improbable; the results from the EndoV tract were closely inspected and DNA sequencing in both directions was pursued. Failure to confirm the borderline LDR finding led us to carefully scrutinize the p.R273S (g.14486C>A) reaction. It was revealed that human error led to the synthesis of LDR oligonucleotides that did not correspond to the correct signal. Since our synthetic template to test ligation reactions was designed as reverse complements of the joined LDR oligonucleotides, early tests of the chip did not identify this reaction as problematic. As a result of this finding, we will in the future design synthetic templates de novo, without referring to the LDR product for the reaction. Thus, by relying on a bioinformatics approach, spurious signals can be readily distinguished from authentic mutations and processes can be continuously refined and improved. In contrast, mutation detection at the very same R273 codon by gene chip hybridization was unable to distinguish a weak signal resulting from false hybridization of wild-type sequence [Wen et al., 2000] from the presence of low-level mutant allele.

A common polymorphism in TP53 is located at codon 72. Since LDR had been shown to be sufficiently sensitive to detect the presence of a variant sequence when diluted 1:100 in wild-type sequences (see description of gel-based assay above), determination of the status of both codon 72 alleles was possible, even in the event of 17p loss of heterozygosity (LOH) in the tumor cells, due to the 30 to 50% estimated stromal contamination. In agreement with previous studies that showed the allele frequencies in Caucasians to be approximately 70% arginine allele and 30% proline allele [Beckman et al., 1994; Harris et al., 1986], we found allele frequencies of 75 and 25% for the R and P alleles, respectively.

DISCUSSION

We have designed a harmonized protocol that relies on two sensitive enzymatic reactions to detect the presence of mutations in undissected solid tumors, in which contaminating wild-type stroma can account for the majority of DNA template present in a sample. The universal microarray-based tract uses a thermostable ligase enzyme to detect predetermined mutations by discriminating between wild-type and mutant templates, resulting in the separation of the mutation detection and array hybridization. The mutation scanning tract detects unknown mutations and relies on thermostable EndoV and ligase to produce high sensitivity. The harmonized protocol successfully detected all 66 mutations found in the present study, many of which were missed by DNA sequencing.

Although DNA sequencing using manual reading performed significantly better than automated reading (89 and 62% sensitivity, respectively), identification of mutated bases ultimately relied on prior knowledge of mutation position, as indicated by results from the harmonized protocol. Overall, the fusion of PCR/LDR/Universal array and EndoV mutation scanning proved to be a rapid means of identifying mutation. While it may be argued that other combinations of mutation detection methods might result in equal sensitivity (e.g., SSCP + sequencing), these applications still fall short. Whereas electrophoretic mobility assays can detect low level mutations, these approaches may miss a significant portion of mutations: SSCP misses 30% of possible mutations [Bjursell et al., 2000; Hayashi, 1991; Korn et al., 1993; Makino et al., 1992; Suzuki et al., 1990]; methods such as CSGE, DGGE, CDGE, and DHPLC, which look for differential electrophoretic migration between homo- and heteroduplexes, have been shown

TABLE 2. Summary of Colorectal Tumor TP53 Mutation Analysis

Variable	Outcome	Frequency	Percent
Tumor Stage	I	15	11
	II	22	16
	III	41	30
	IV	42	30
	Metastases	18	13
	Total	138	100
TP53 Mutation/Polymorphism Summary	WT	75	54
	Mutations	61	44
	Polymorphisms	5	4
	Total	138	100
TP53 Amino Acid 72 Polymorphism Status	P homozygote	10	7
	Heterozygote	55	40
	R homozygote	73	53
	Total	138	100
TP53 Nonsense & Missense Mutations	Missense	51	84
	Nonsense/indel	10	16
	Total	61	100
TP53 Structural Motifs with Mutation	DNA contact	28	46
	L2 loop	18	30
	Beta sandwich	4	7
	H2 helix	6	10
	C-terminus	2	3
	H1 helix	1	2
	L3 loop	2	3
	Total	61	100
TP53 Mutations and Polymorphisms*	R175H g.13203G>A	11	17
	R248Q g.14070G>A	8	12
	R273C g.14486C>T	7	11
	R282W g.14513C>T	5	8
	R248W g.14069C>T	3	5
	R273H g.14487G>A	3	5
	R306X g.14585C>T	2	3
	S261R g.14108A>C	2	3
	Y234C g.14028A>G	2	3
	H214 g.13401_13402insA	2	3
	E224E g.13432G>A	2	3
	R213R g.13399A>G	2	3
	G154G g.13141C>A	2	3
	E171X g.13190G>T	1	2
	E258D g.14102A>T	1	2
	E285K g.14522G>A	1	2
	G245S g.14060G>A	1	2
	G154D g.13140G>A	1	2
	H179Y g.13214C>T	1	2
	H193R g.13338A>G	1	2
	R267G g.14468C>G	1	2
	P190L g.13329C>T	1	2
	Q192X g.13334C>T	1	2
	R213X g.13397C>T	1	2
	Q167 g.13178_13179insA	1	2
	K164X g.13169A>T	1	2
	S240 g.14045delA	1	2
	Y163C g.13167A>G	1	2
	Y205F g.13374A>T	1	2
	Total	66	100

*All nucleotide numeric designations use numbering identical to GenBank Accession X54156.1.

to miss 11% of polymorphisms [Chen and Thilly, 1994; Fodde and Losekoot, 1994; Ganguly, 2002; Ganguly et al., 1993; Guldberg and Guttler, 1994; Khrapko et al., 1994; Kozlowski and Krzyzosiak, 2001; Larsen et al., 2001; Mitchelson, 2001; Ridanpaa and Husgafvel-Pursiainen, 1993; Rozycka et al., 2000]. DNA sequencing, on the other hand, has low sensitivity and will miss low-level mutations. To optimize paired reactions based on these approaches, microdissection would be required. The major strength of the harmonized assay is that mutation detection may be implemented in the absence of microdissection to enrich for tumor DNA.

One advantage of the harmonized protocol is that the two tracts of this combined process use the same multiplex PCR reaction, thus this approach does not consume excessive amounts of limited tumor DNA sample. Also, the two tracts of the process advance in parallel and there is no need for microdissection on either tract, thus this approach is also less time consuming. A current limitation to the wider application of the harmonized assay may be the lack of comprehensive databases for other important tumor suppressor genes and oncogenes. To program the PCR/LDR/Universal array tract in the present study, we used a bioinformatics selection process to guide us to the most significant TP53 mutations in colon cancer. The legitimacy of the programmed mutations is supported by a recent report [Kato et al., 2003], in which 2,314 TP53 mutations were evaluated for functional impact using a yeast-based assay. With the exception of A161 mutations, TP53 mutations included on the universal array showed a loss of activity, demonstrating the efficacy of a bioinformatics selection process. If similar database resources are lacking for other genes of interest, it will first be necessary to build the databases that can clarify which mutations should be targeted for analysis. The rapid advances in sequencing and mutation scanning technology over the past few years will undoubtedly assist in expanding web-based databases of both germline and inherited mutations in cancer-associated genes. Additional bioinformatics resources will likely be launched if it can be shown that tumor profiling can be successfully applied to the clinical decision process.

In comparison to previous analyses using direct hybridization to gene oligonucleotide arrays to detect TP53 mutations in frozen tissue, tumors deficient in neoplastic cells required selective microdissection. Although the intent of the gene hybridization chip was to detect all TP53 mutations, it failed, detecting only 81% [Ahrendt et al., 1999], 84% [Wikman et al., 2000], and 92% [Wen et al., 2000] of TP53 mutations. In all cases, insertion/deletion mutations proved intractable to this detection scheme, and significantly reduced the sensitivity values. Further, gene hybridization arrays required statistical considerations on background to improve specificity from 34 to 86%, but at the cost of reduced sensitivity from 92 down to 84% [Wikman et al., 2000]. In contrast, the LDR primers were predicted to accommodate roughly 70% of colon cancer mutations (as estimated by prevalence in the TP53 database) and PCR/LDR/Universal array succeeded in identifying 68% of TP53 mutations found in the undissected colorectal adenocarcinomas analyzed (Table 1). By creating a harmonized protocol involving both PCR/LDR/Universal array and EndoV mutation scanning, all TP53 mutations in the targeted exons are detected, including insertion/deletion mutations, thus achieving high sensitivity with high specificity. This result demonstrates that the PCR/LDR-bioinformatics approach to universal chip development combined with EndoV mutation scanning presented here out-performs approaches that attempt to target all possible mutations, such as gene chip hybridization or automated sequencing. The added value of our assay is that time-consuming microdissection is eliminated.

In conclusion, rapid and accurate mutation analysis of tumors is critical to resolve differences in prognosis and response to therapy. Due to the comparatively advanced state of understanding, the TP53 gene is a strategic starting point to demonstrate proof of principle and to advance the translational research to achieve this goal. Approaches that prove successful for TP53 mutation detection (e.g., curation of comprehensive mutation databases, bioinformatics-based approaches to assay design, and development of rapid, sensitive assays) may also be applied to characterizing the mutation status of other tumor suppressor genes and oncogenes. In time, the ability to perform molecular profiling of tumors may facilitate tailoring individualized treatments for individual patients.

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