Genetic Analysis of Multiple Loci in Microsamples of Fixed Paraffin-Embedded Tissue

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Molecular analysis of alterations in genomic DNA is essential for understanding mechanisms by which chemical agents induce or modify tumor development. The assessment of microsatellite polymorphisms, loss of heterozygosity, mutations, and gene rearrangement allows specific comparisons of tumors to premalignant lesions or normal tissue or between similar tumors seen in laboratory species and humans. Utilization of these techniques is frequently limited by minute quantities of available tissue, often restricted to small formalin-fixed tumors or biopsies in paraffin blocks. To address these limitations, we have combined recently developed methodologies for selective recovery, amplification, and analysis of DNA. These techniques provide sufficient materials of high quality for analysis of DNA alterations in microscale amounts of starting material. By combining whole genome amplification through primer extension preamplification with locus-specific heminested PCR, we are able to analyze multiple genetic loci from as little as 1 mm² of a 3-μm-thick formalin-fixed paraffin section. From 10 to greater than 100 loci can be analyzed per tissue section, and locus-specific PCR products may be further evaluated by a variety of techniques (e.g., SSCP, sequencing). Integrating these methodologies into situations where evaluation of very small tissue samples is necessary provides a powerful approach for elucidating molecular events that may be causally related to chemically induced cellular transformation and tumorigenesis. © 1998 Society of Toxicology.

For assessing human risk based on results of experimental studies of carcinogenesis.

Ideally, molecular–genetic analysis of tumors should encompass a wide range of genomic alterations, such as loss of heterozygosity (LOH) at multiple loci, microsatellite instability, mutations in protooncogenes or tumor-suppressor genes, gene rearrangements, and gene amplification, all of which are widely recognized to play context-specific roles in tumorigenesis. Unfortunately, molecular characterization of tumors is frequently hampered by limitations in the availability or quality of the tissue samples to be evaluated. Long-term prospective studies of tissue- or agent-specific carcinogenesis represent an expensive and logistically difficult means to generate fresh tissues for analysis. Thus, the majority of studies of genetic alterations in malignancies rely on post hoc analysis of tumors identified histologically in sections of fixed, paraffin-embedded tissue. Often, the quantity of material available from paraffin sections is quite limited, particularly if a lesion is small. Further complicating the analysis of tumors is the heterogeneous nature of the lesions. Neoplastic and nonneoplastic cells are admixed to some degree in most malignant lesions, necessitating the use of a variety of microdissection techniques to separate the tumor from normal tissue (Shibata et al., 1992; Böhm et al., 1997; Emmert-Buck et al., 1996; Going et al., 1996; Moskaluk and Kern, 1997). Such strategies generally enhance the quality of analysis possible by improving specificity, while reducing the amount of target tissue available for analysis.

In order to overcome the limitations described above, strategies for whole genome amplification have been devised to increase the quantity of target DNA available from small samples (Peng et al., 1994; Zhang et al., 1992; Telenius et al., 1992). Primer extension preamplification (PEP; Zhang et al., 1992) in particular has proven to be a robust method for facilitating analysis of multiple loci, though applications of the technique have been restricted primarily to analysis of freshly isolated cells for preimplantation diagnosis of genetic diseases (Kristjansson et al., 1994; Paunio et al., 1996). In the present study, we describe the use of PEP coupled with heminested, locus-specific polymerase chain reaction (PCR), single-strand conformational polymorphism (SSCP) analysis, and direct sequencing of PCR product, for analysis of alterations in DNA at
multiple loci in microsamples of formalin-fixed, paraffin-embedded tissue.

MATERIALS AND METHODS

Tissue Preparation

Fresh tissue. Fresh liver was obtained from a carbon dioxide-anesthetized male B6C3F1 mouse. Approximately 1 g of liver was minced, twice rinsed by gentle vortexing in phosphate-buffered saline, and twice rinsed by gentle vortexing in Tris-EDTA buffer (10 mM Tris-Cl, pH 8.0). The tissue was then transferred to a glass Potter-Elvehjem-type homogenizer and homogenized by hand in 3 ml of Tris-EDTA buffer. Liver homogenate was transferred to a sterile 15-ml polypropylene screw-capped tube for subsequent DNA extraction.

Paraffin-embedded, formalin-fixed tissue. Formalin-fixed, paraffin-embedded liver tissue from untreated B6C3F1 mice served as control tissue for mutation analysis. Fixed, paraffin-embedded murine hepatocellular carcinomas with known mutations in c-ras genes (Maronpot et al., 1995) was kindly provided by D. Malarkey and R. Maronpot (NIHES, Research Triangle Park, NC). Sections cut from formalin-fixed, paraffin-embedded tissue blocks (typically 3 µm thick) were mounted on polylysine-coated acetate slides. Tissue sections were floated onto the slides and air dried overnight at room temperature prior to heating on a 100°C hotplate for 5 min. Deparaffinization and staining of the sections with hematoxylin and eosin were as described by Shibata et al. (1992). The slides were not coverslipped.

DNA Extraction

Genomic DNA was prepared from fresh liver homogenate by conventional methods (Moore and Strauss, 1995). For analysis of genomic DNA from tissue sections, regions of interest within a section (e.g., tumor tissue) were first identified on the stained acetate slides by light microscopy and then excised from the slide using individual sterile scalpel blades. The excised tissue samples and underlying acetate slide material, which ranged in size from approximately 1 to >4 mm², were placed into 1.5 ml sterile microcentrifuge tubes and digested in 50–100 µl of extraction buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1% Tween 20, and 400 µg/ml protease K) for 12–18 h at 54°C. Larger samples (>4 mm²) were cut into smaller pieces with sterile scalpel blades prior to digestion in up to 100 µl extraction buffer. Following digestion, the protease K was inactivated by incubation at 95°C for 10 min, and the lysates were purified with InstaGene Matrix (Bio-Rad). In order to minimize sample manipulation, a volume of resuspended InstaGene matrix equal to the extraction buffer volume was added directly to the sample extraction tube, the contents were vortexed briefly, and the samples were incubated at 56°C for 30 min. Samples were then vortexed vigorously for 10–15 s and incubated at 100°C for 8 min. The samples were vortexed vigorously again for 10–15 s and then centrifuged at 12,000 rpm for 2–3 min to pellet the acetate slide material and InstaGene matrix. Aliquots of the purified supernatant were taken directly out of the sample extraction tube and used for PEP reactions; the remaining sample was stored at −20°C. We found that InstaGene purification of the acetate slide tissue extracts significantly improved the quality of PEP and subsequent PCR reactions.

Primer Extension Preamplification

PEP was performed as described by Zhang et al. (1992), with minor modifications. The 50-µl PEP reaction mixture consisted of 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 0.2 µM of each of the four dNTPs (Perkin-Elmer/Applied Biosystems), 50 µM random sequence 15-base primers (Operon Technologies), 1.5 mM MgCl₂, 5 units of AmpliTaq DNA polymerase (Perkin-Elmer/Applied Biosystems), and 10 µl of DNA sample. Fifty primer extension cycles were carried out in a Perkin-Elmer 9600 thermocycler after an initial 94°C, 4-min denaturation step. Each cycle consisted of a 1-min denaturation period at 92°C, a 2-min annealing period at 37°C, a programmed ramp of 10 s per degree from the annealing temperature to 54°C, followed by a 4-min primer extension step at 54°C. The PEP reaction products were purified using Microcon 10 microcentrifugators to remove residual 15-mers (30-bp ssDNA/20-bp dsDNA cutoff, Amicon) and reconstituted to their original volume with sterile ddH₂O.

Locus-Specific PCR Amplifications

Amplification of regions of exon 1 and exon 2 of the Ha-ras and K-ras genes (Goodrow et al., 1992a; Yamamoto et al., 1995; Brown et al., 1988), and exons 5–8 of the p53 gene (Goodrow et al., 1992b), were carried out using a two-stage, heminested PCR strategy to maximize the yield and specificity of the locus-specific amplification reactions (Kristjansson et al., 1994). Aliquots (typically 5 µl) of PEP reaction product were used directly or diluted 10- to 1000-fold (as detailed under Results section) for the initial locus-specific PCR reactions. The products from the initial PCR reactions were in turn used directly or diluted 10- to 10,000-fold for use in the subsequent heminested PCR amplifications. Table 1 lists primer sequences, primer concentrations, and cycling conditions used for the locus-specific reactions. Amplification of p53 exons 5–8 utilized intronic primer pairs flank each individual exon to avoid amplifying the pseudogene (Goodrow et al., 1992b).

Analysis of PCR Products

SSCP analysis of Ki-ras exon 1. Locus-specific product from heminested PCR reactions was gel purified to remove unincorporated primers. DNA was recovered from the agarose gel using Ultrafree-MC Centrifugal Filter Units (Millipore) and the manufacturer's "maximum recovery" protocol. Residual ethidium bromide was removed from the purified DNA samples with the QIAquick gel extraction kit (Qiagen). Cold SSCP analysis of PCR product amplified from Ki-ras exon 1 was performed by resolving denatured PCR product at 95°C for 10 min in buffer composed of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, followed by a 5-min incubation in an NaCl/ice bath) on precast 8 × 8 × 0.1 cm, 20% acrylamide/TBE gels (Novex) (Hongyo et al., 1993). Gels were precooled and prerun at 25 mA constant current for 15–30 min prior to electrophoresis of denatured samples. Ki-ras exon 1 samples were separated in a temperature-controlled electrophoresis apparatus (Novex) at 8°C using approximately 25 mA constant current for 4–6 h. DNA was visualized directly in the gel by UV illumination subsequent to staining of the gel with Sybr Green (FMC, Rockland, ME).

Sequencing. Automated sequencing of gel-purified PCR product was performed by the Molecular Biology Core Facility, Adirondak Biomedical Research Center, Lake Placid, New York. DNA sequencing reactions were performed using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer/Applied Biosystems) and AmpliTaq DNA polymerase (type FS: Perkin-Elmer/Applied Biosystems). Sequencing reaction products were analyzed using a Perkin-Elmer/ABI Model 373 automated DNA sequencer.

RESULTS

Sample Extraction

In order to minimize sample handling and avoid the use of organic solvents, we tested a number of protocols that did not rely on organic extractions for preparing genomic DNA from the tissue/acetate matrix. Because the tissue sections were deparaffinized prior to staining, the aqueous DNA extraction
Genetic Analysis in Tissue Microsamples

Table 1: Locus-Specific and Heminested PCR Primer Sequences

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequences (5'→3')</th>
<th>Annealing temp, (specific/hemi)</th>
<th>Primer concentration (specific/hemi)</th>
<th>Amplicon size (specific/hemi)</th>
</tr>
</thead>
</table>
| Ha-ras exon 1 | GAT TGG CAG CCG CTG TAG AA (S)*  
GCT ATG ACA GAA TAC AAG CTT GTG G (SH)  
GGC AGA CTC TAT TAT AG (A) | 50°C/52°C | 0.35 μM/0.35 μM | 143 bp/126 bp |
| Ha-ras exon 2 | CTA AGC CAG TGT GAA TGT CAG (S)  
GAT GTA TGT CCT CTA AGG ACT TG (A)  
ATG ACT GAG TAT AAG CTT GTG (SH)  
AGC GGC GTT ACC TCT ATC GT (A) | Touchdown* | 1.0 μM/0.5 μM | 193 bp/133 bp |
| Ki-ras exon 1 | TTG TAA GCC GTG CTG AAA ATG AC (S)  
ATG ACT GAG TAT AAG CTT GTG (SH) | 46°C/46°C | 1.0 μM/1.0 μM | 139 bp/121 bp |
| Ki-ras exon 2 | TCA GGA CTC CTA CAG GAA AC (S)  
TGG AGA AAC CTG TCT CTG G (SH)  
GAT TTA GTA TTA TTT ATG GC (A)  
AGC GGC GTT ACC TCT ATC GT (A) | 46°C/48°C | 1.0 μM/1.0 μM | 159 bp/126 bp |
| p53 exon 5   | TTC CAG TAC TGT CTC CTC CC (S)  
AGG CTA CCA GTC CTA ACC CCA CAG (A)  
TTG AGA GCC TAT CAT CAC C (AH) | 60°C/60°C | 0.5 μM/0.5 μM | 231 bp/201 bp |
| p53 exon 6   | GCT TCT GAC TTA TTC TTT TCT CTC (S)  
TCT CTA AGA CGC ACA AAC C (A)  
AAT TAC AGA CCT CGG GTG G (A) | 56°C/56°C | 0.5 μM/0.5 μM | 173 bp/147 bp |
| p53 exon 7   | GAG GTA GGG AGC GGT TGC TCT CAC C (S)  
CTC TGA GTA TAC CAC CAT (SH)  
GCT GGG GAA GAA ACA GCC TAA C (A)  
ACT GCC TGG TGC TGG TGT TCT TCT (T)  
ATA GTG GGA ACC TTC TGG (SH)  
ACA GCC TCC TCC GCC TCC TCT (A) | 60°C/60°C | 0.5 μM/0.5 μM | 208 bp/176 bp |

* S, sense primer; A, antisense primer; H, heminested primer.

Buffers were added directly to the samples contained in sterile microcentrifuge tubes. Our initial protocol, boiling the samples in ddH2O, was attempted on the basis of simplicity; however, the procedure repeatedly failed to yield material that could be amplified by PEP or heminested PCR. Variations on extraction schemes that relied on protease K digestions at 37°C for various lengths of time, without or including 0.1% Triton X-100 (Shibata et al., 1992; Peng et al., 1994), produced sample extracts that inconsistently amplified. Sample extraction at 54°C in buffer that included 1% Tween 20 and protease K (Emmert-Buck et al., 1996) proved to be the most reliable method for yielding material that consistently amplified. The addition of an InstaGene purification step (Merkelbach et al., 1997) further improved the PEP/heminested PCR performance of the extracted samples, presumably by removing contaminants that inhibited the PEP or PCR amplifications.

Whole Genome Amplification

In initial experiments, random hexamers were used as primers in attempts at whole genome amplification (Peng et al., 1994). We found that under our test conditions hexamer-based reactions rarely generated significant yields of product, which was assayed by electrophoretic separation of amplification reaction products incorporating [α-32P]dATP (not shown). In contrast, PEP reactions using varying amounts of input high molecular weight genomic DNA generated product that ranged in size from <200 bp to significantly greater than 2000 bp, in yields that were easily detectable by fluorescence-based staining of the DNA (Fig. 1). In the absence of PEP, the lower limit of detection of the input DNA using fluorescent staining was approximately 5 ng (Fig. 1, lane 6). By utilizing PEP, product generated from as little as 50 pg of input DNA, roughly 20 haploid mammalian genomes, was easily detected by fluorescence (Fig. 1, lane 8). Detectable yields of PEP products ranging in size to greater than 2000 bp also were generated from 4 mm of a 3-μm control liver tissue section (Fig. 1, lane 2). Though the 4-mm, 3-μm control liver tissue section theoretically represents about 5000 fixed, paraffin-embedded, 14-μm-diameter cells, the actual yield of DNA extracted from the sample and used as input for the PEP reaction was below the limit of detection of our fluorescence-based gel analysis system (Fig. 1, lane 1). Thus, the PEP reaction substantially increased the pool of DNA available from the extracted tissue section for subsequent analysis by locus-specific PCR.

Locus-Specific Amplification

The extent to which the PEP and primary PCR reactions could be diluted and still yield locus-specific product from the heminested PCR reactions was investigated, using exons 1 and
The eight amplicons of Ki-ras exons 1 and 2, Ha-ras exons 1 and 2, and p53 exons 5-8 from 4 mm$^2$, 3-$\mu$m-thick liver tissue can yield locus-specific product (Fig. 2B). In the case of Ki-ras exon 2, a 1000-fold dilution of the PEP reaction product was still sufficient to generate locus-specific product (Fig. 2B). Similar results were obtained with tissue samples as small as 1 mm$^2$, 3-$\mu$m-thick liver tissue. For example, the 16 reactions pictured in Fig. 2A were derived from a single PEP reaction that utilized only 10% of the DNA extract from a 4-mm$^2$, 3-$\mu$m-thick section. Employing optimized heminested PCR reactions we theoretically could have analyzed $\geq$160 loci from that single 4-mm$^2$, 3-$\mu$m-thick section. This increase in the potential for genetic analysis of small samples of paraffin-embedded tissue is a significant improvement on existing techniques. Previously reported procedures permit at best a few analyses from single tissue sections or pools of serial sections of small lesions to increase sample availability (Moskaluk and Kern, 1992).

Identifying the genetic basis of phenotypes that define the malignant character of tumor cells is critical for understanding the mechanisms by which such cells arise. In our studies of the carcinogenic potential of novel compounds, we are usually restricted to post hoc analysis of fixed, paraffin-embedded tissues because diagnosis of malignancies in long-term carcinogenicity studies is derived from histological examination of tissues. As such, the ability to characterize genetic alterations has been constrained by limited availability of tumor tissue that is processed in a way that significantly hampers extraction and analysis of DNA (Rogers et al., 1990). In order to enhance our ability to characterize genetic alterations in tumors, we sought to develop methods that would expand the number of analyses that could be performed—and thus increase the number of loci analyzed—from individual microsamples of the fixed, paraffin-embedded tissue typically available.

Combining PEP for whole genome amplification with heminested PCR, we were able to perform a minimum of 10 locus-specific amplifications from as little as 1 mm$^2$ of fixed, paraffin-embedded tissue. For example, the 16 reactions pictured in Fig. 2A were derived from a single PEP reaction that utilized only 10% of the DNA extract from a 4-mm$^2$, 3-$\mu$m-thick section. Employing optimized heminested PCR reactions we theoretically could have analyzed $\geq$160 loci from that single 4-mm$^2$, 3-$\mu$m-thick section. This increase in the potential for genetic analysis of small samples of paraffin-embedded tissue is a significant improvement on existing techniques. Previously reported procedures permit at best a few analyses from single tissue sections or pools of serial sections of small lesions to increase sample availability (Moskaluk and Kern, 1992).
Diluted PEP reaction products yield robust locus-specific amplification. One-tenth of the DNA extracted from a single 4-mm², 3-µm-thick tissue section was amplified by PEP. Aliquots of undiluted or serially diluted PEP reaction product were used as template for heminested PCR amplification of exons 1 (A) and 2 (B) of the Ki-ras gene as described under Materials and Methods. Note that all of the amplicons pictured are derived from a single PEP reaction. PEP Dil.: extent of dilution of the PEP reaction product used as template for the initial (1°) locus-specific PCR. For example, a PEP dilution of “10⁻²” indicates that the PEP reaction product was diluted 1:100 prior to serving as template for the 1° locus-specific PCR. A dilution of “1” indicates that undiluted sample was used. 1° PCR Dil.: extent of dilution of the primary locus-specific PCR product used as template for generating the final, heminested, amplicons pictured on the gels. PCR Ctl.: positive (+) and negative (−) controls for the PCR reactions. Heminested PCR amplification of Ki-ras exon 1 from mouse liver genomic DNA was used as the positive (+) control for the PCR reactions, and water blanks served as the negative control. Arrows, expected PCR products. M, DNA molecular weight markers.

Amplification of multiple loci from a single microsample of fixed, paraffin-embedded tissue. An aliquot of DNA extract from 4 mm² of a 3-µm-thick tissue section was amplified by PEP, and locus-specific PCR products were generated from the PEP reaction product using heminested PCR as described under Materials and Methods. Lane 1, p53 exon 5; lane 2, p53 exon 6; lane 3, p53 exon 7; lane 4, Ha-ras exon 1; lane 5, p53 exon 8; lane 6, Ha-ras exon 2; lane 7, Ki-ras exon 1; lane 8, Ki-ras exon 2; M, molecular weight markers. Note that all of the amplicons pictured were collectively derived from approximately 50% of a single PEP reaction representing 5% of the DNA extract.

The reliance of the PEP/heminested PCR technique on Taq thermostable DNA polymerase raises the concern of introduction of artifactual mutations by the polymerase (Cline et al., 1996). We purposely kept our locus-specific amplicons to a small size (<200 bp) to minimize the statistical chance of Taq-based errors being introduced (Brail et al., 1993) and to provide greater flexibility for SSCP methods development (Nollau and Wagener, 1997). Based on our SSCP analyses and direct sequencing of PCR products, we have not found artifactual sequence alterations under the conditions described above. Thus, the procedure appears to be of high fidelity. Nevertheless, use of a proofreading thermostable polymerase instead of, or in addition to, Taq DNA polymerase could further reduce the chance introduction of errors (Cline et al., 1996).

The PEP technique was developed to facilitate preimplantation diagnosis of genetic diseases in freshly isolated single cells (Zhang et al., 1992) and is becoming widely used in that capacity (Kristjansson et al., 1994; Paunio et al., 1996; Schaaff et al., 1996). More recently, PEP has been applied to analysis of mitotic stability of repetitive elements (Zühlke et al., 1997).
and locus-specific DNA methylation analysis (Wong et al., 1997). One of the points of concern with applying PEP to a single diploid genome is the potential for strand or allelic bias in PCR amplifications (Weissensteiner et al., 1996). Serial dilution of PEP reaction product generated from single cells will eventually lead to allelic loss in subsequent PCR analysis. For the fixed tissues we employ, not all of the DNA from a given cell will necessarily be present in the tissue sample (due to sectioning at a thickness less than a nuclear diameter) or available for extraction and amplification (due to crosslinking by the fixation process). Thus, as the sample size gets smaller (approaching one or a few cells), the chance of not amplifying a given locus or differential amplification of alleles from the fixed tissue increases and must be taken into consideration when interpreting results. Both empirical (Zhang et al., 1992) and mathematical (Sun et al., 1995) analysis of the PEP reaction indicate that for amplification of a single haploid genome using conditions described by Zhang et al. (and also in the present procedure), 80% of the genome is amplified at least 30 times. If the PEP reaction is performed starting with a diploid genome, coverage increases to greater than 99.8% (Sun et al., 1995). As such, the potential for differential amplification or allelic loss in analysis of archival tissue using PEP can be minimized by increasing the number of target cell genomes input into the PEP reaction. Advances in microdissection techniques (Shibata et al., 1992; Böhm et al., 1997; Emmert-Buck et al., 1996; Going et al., 1996; Moskaluk and Kern, 1997) should greatly simplify the collection of essentially pure populations of cells of interest from archival tissue samples, allowing the use of PEP/heminested PCR for genetic analysis under conditions that minimize chances for differential allelic amplification or loss.

In summary, we have combined whole genome amplification by PEP with heminested, locus-specific PCR to significantly increase the potential for molecular analysis of multiple loci in individual microsamples of fixed, paraffin-embedded tissue. This approach is a powerful tool for enhancing the quantity and quality of information obtainable from analysis of archival tissues.

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REFERENCES


