Technical Advance

Multiple Mutation Analyses in Single Tumor Cells with Improved Whole Genome Amplification

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Combining whole genome amplification (WGA) methods with novel laser-based microdissection techniques has made it possible to exploit recent progress in molecular knowledge of cancer development and progression. However, WGA of one or a few cells has not yet been optimized and systematically evaluated for samples routinely processed in tumor pathology. We therefore studied the value of established WGA protocols in comparison to an improved PEP (I-PEP) PCR method in defined numbers of flow-sorted and microdissected tumor cells obtained both from frozen as well as formalin-fixed and paraffin-embedded tissue sections. In addition, the feasibility of I-PEP-PCR for mutation analysis was tested using clusters of 50–100 unfixed tumor cells obtained by touch preparation of ten breast carcinomas by conventional sequencing of exon 7 and 8 of the p53 gene. Finally, immunocytochemically stained microdissected single disseminated tumor cells from bone marrow aspirates were investigated with respect to mutations in codon 12 of *Ki-ras* by restriction fragment length polymorphism (RFLP)-PCR after I-PEP-PCR. The modified I-PEP-PCR protocol was superior to the original PEP-PCR and DOP-PCR protocols concerning amplification of DNA from one cell (efficiency rate I-PEP-PCR 40% versus PEP-PCR 15% and DOP-PCR 3%) and five cells (efficiency rate I-PEP-PCR 100% versus PEP-PCR 33% and DOP-PCR 20%). Preamplification by I-PEP allowed 100% sequence accuracy in > 4000 sequenced base pairs and *Ki-ras* mutation detection in isolated single disseminated tumor cells. For reliable microsatellite analysis of I-PEP-preamplified DNA, at least 10 unfixed cells from fluorescence-activated cell sorting, 10 cells from frozen tissue, or at least 30 cells from formalin-fixed and paraffin-embedded tissue sections were required. Thus, I-PEP-PCR allowed mul-

tiple reliable microsatellite analyses suited for microsatellite instability and losses of heterozygosity and mutation analysis even at the single cell level, rendering this technique a powerful new tool for molecular analyses in diagnostic and experimental tumor pathology. (Am J Pathol 1999, 154:83–95)

Today it is generally accepted that molecular analyses in tumor pathology should be performed in precisely defined homogeneous tumor cell populations with low or even no contamination by nontumor cells.^{1,2} Accordingly, it has been shown that loss of heterozygosity (LOH) can reliably be detected in tumor samples only if the content of tumor cells exceeds 70–80%.³ Therefore, cell sorting^{4,5} and microdissection techniques^{2,6–10} have been used with increasing frequency in molecular oncology. Most recently, laser microdissection was introduced as a novel method to dissect single tumor cells and cell groups without any nontumorous contamination.^{1,2,11,12} This technique is a useful tool that may help provide new insights into the molecular basis of neoplasia, in particular of carcinogenesis and tumor heterogeneity, as possible predictors of tumor biology and therapy response.

A prerequisite for molecular analyses of single or few cells is a reliable and reproducible method for DNA and RNA preparation for subsequent polymerase chain reaction (PCR) analyses. Whole genome amplification (WGA) methods have been introduced as powerful tools, initially for sperm typing^{13,14} and DNA analysis in single or few blastomeres^{15–17} and nucleated erythrocytes from maternal blood¹⁸ for the diagnosis of fetal inherited diseases. These techniques have recently been extended for the use in tumor pathology where preneoplastic and neo-

Supported by Wilhelm Sander-Stiftung, München, Germany (93.055.2) and the Dr. Mildred Scheel Foundation for Cancer Research (10–1096-Ha I).

Accepted for publication September 12, 1998.

Data have been presented in part at the 89th Annual Meeting of the American Association for Cancer Research, March 28–April 1, 1998, New Orleans, Louisiana.

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plastic fluorescence-activated cell sorter (FACS)-sorted aneuploid esophageal tumor cells were analyzed for microsatellite instability.^{5,19} In contrast to single specific PCR²⁰ and subchromosomal analysis by *in situ* hybridization²¹ this procedure allows multiple molecular analyses in only few or even single tumor cells.

By WGA, the entire genome is randomly amplified and can then be analyzed by multiple specific PCR analyses. WGA is performed using either nondegenerated or degenerated primers.²² The latter turned out to be more effective for DNA amplification and can be done in at least two different ways. One approach is degenerate oligonucleotide primer-PCR (DOP-PCR),^{23,24} often used as the first step in *in situ* hybridization with flow-sorted chromosomes,^{24,25} microdissected chromosomes, and, more recently, for comparative genomic hybridization.^{26,27} DOP primers have defined sequences at the 5' end and the 3' end. Between these regions is a random hexamer sequence. PCR is performed under low-stringency conditions during the first 5 cycles followed by 35 cycles with a more stringent annealing temperature. An additional method of WGA has been introduced by Zhang et al¹³ and is known as primer-extension-preamplification PCR (PEP-PCR). In contrast to DOP-PCR, totally degenerated PCR primers 15 nucleotides long are used in PEP-PCR. In each of 50 cycles the template is first denatured at 92°C. Subsequently, primers are allowed to anneal at a low stringent temperature (37°C) which is then continuously increased to 55°C and held for another 4 minutes for polymerase extension. It has been shown by microsatellite analysis of markers on all chromosome arms, hybridization of the PEP-PCR product to metaphase chromosome spreads, and investigation of the telomere length that PEP-PCR is indeed able to amplify the complete genome.²⁸⁻³⁰

However, the reliability of WGA, particularly in single or a few microdissected formalin-fixed and paraffin-embedded solid tumor cells, has not yet been systematically evaluated. The number of cells is especially critical for both microsatellite instability (MSI) and LOH detection because too few cells or too little DNA may result in unequal allelic amplification.^{13,19,23,31} Barret et al⁵ examined 1000 flow-sorted esophageal tumor cells rather than single cells to prevent an artifactual dysequilibrium between the two different allelic fragments due to low cell number. The limiting cell number of microdissected tissue sections has not been systematically investigated for MSI or LOH analyses.

Another critical point refers to the intrinsic error rate of *Taq* polymerase, which is prone to introduce AT-to-GC transitions³² as well as to generate deletion mutations due to secondary structures of the DNA templates.³³ Thus, there is a potential risk of getting incorrectly amplified sequences during WGA and subsequent specific PCR because 100 or more amplification cycles are being performed. At present, neither the applicability of WGA to various cell preparations and tissue sections nor the requirements and limits of WGA with respect to cell number, cell processing, and amplification conditions are unequivocally established. In our work we have studied different WGA methods and showed that the efficiency of

PEP-PCR is higher than that of DOP-PCR. Furthermore, we significantly enhanced the efficiency of PEP-PCR by several modifications, especially for the investigation of tumors from tissue sections used in routine pathology. Using this improved PEP-PCR variant (I-PEP-PCR), we were able to reliably perform multiple microsatellite and sequencing studies with a single or few cells.

Materials and Methods

Tumor Cell and Tissue Processing

The applicability of DNA preamplification was tested in whole cells and tissue sections. First, two different whole cell preparations were tested: FACS-sorted SW480 cells obtained from the American Type Culture Collection (Manassas, VA) and stained with fluorescein diacetate and immunocytochemically stained cells from cytospots from bone marrow aspirates.

SW480 Cells

SW480 cells were resuspended in phosphate-buffered saline containing fluorescein diacetate (0.1 μ g/ml, Sigma, Munich, Germany) and stained for 10 minutes at room temperature. Sorting was performed with a FAC-Star^{plus} cell sorter (Becton-Dickinson, Heidelberg, Germany). Cells were analyzed at a rate of approximately 200 cells per second and sorted with a drop drive frequency of 25,000 Hz directly into Eppendorf cups containing cell lysis buffer (see below). Cell lysis was performed in parallel in 5 μ l alkaline buffer (200 mmol/L KOH; 50 mmol/L dithiothreitol) followed by a 10-minute incubation at 65°C and subsequent addition of 5 μ l neutralizing buffer (900 mmol/L Tris-HCl, pH 8.3, 300 mmol/L KCl, according to Zhang et al¹³) or in 10 μ l Expand Lysis buffer (1 $\times$ Expand HiFi buffer No. 3 (Boehringer Mannheim, Mannheim, Germany) including 4 mg/ml proteinase K and 0.5% Tween 20 (Merck, Darmstadt, Germany) as detergent) followed by a 14-hour incubation at 48°C and a 10-minute inactivation step at 94°C. Alternatively (in comparing experiments), *Taq* lysis buffer (1 $\times$ *Taq* PCR buffer (Life Technologies GmbH, Eggenstein, Germany) including 4 mg/ml proteinase K and 0.5% Tween 20 (Merck) was tested.

Bone Marrow Aspirate

Bone marrow aspirates (10 ml) were separated by Ficoll density gradient and a total of 2×10^6 bone marrow cells were used specifically to detect epithelial cells by immunostaining with CK18 monoclonal antibody (clone CK2, Boehringer Mannheim) according to the manufacturer's instructions. Positive cells were isolated from cytospots using a laser microdissector as previously described¹¹ and lysed in 10 μ l EL buffer (see the preceding subsection).

Second, the feasibility of DNA preamplification was examined in tissue sections after histochemical or immunohistochemical staining. Four types of sectioned tis-

sue were tested: methylene blue-stained frozen sections, hematoxylin/eosin (H&E)-stained sections from formalin-fixed and paraffin-embedded tissue, sections from formalin-fixed and paraffin-embedded tissue after immunohistochemical staining for p53, and tumor touch preps.

Methylene Blue-Stained Frozen Sections

Frozen sections of the urinary bladder 5 μm thick were prepared and superficial cancer cells as well as normal muscle cells from the bladder wall ($n_{\text{cell}} = 10, 25, 50, 100, \sim 250$, and ~ 500 each) were laser-microdissected.¹¹

H&E-Stained Tissue Sections

Sections of colonic carcinoma tissue 5 μm thick, fixed approximately 24 hours in phosphate-buffered saline buffered formalin (3.7%) and embedded in paraffin, were deparaffinized by incubating the slides in xylene for 2×15 minutes, in 99.9% ethanol for 2×10 minutes, in 96% ethanol for 2×10 minutes, and in 70% ethanol for 2×10 minutes and stained with H&E. Laser-microdissected tumor and normal muscle cell groups ($n_{\text{cell}} = 30, 50, 100, \sim 250, \sim 500$, and ~ 1000 each)¹¹ were lysed in alkaline buffer¹³ or EL buffer as described above.

Tissue Sections after Immunohistochemical Staining for p53

Sections of formalin-fixed and paraffin-embedded colon cancer 5 μm thick were stained with p53 antibody (Clone Bp53-12; Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions. Positive cells ($n_{\text{cell}} \approx 100$) were microdissected and lysed in EL buffer as described.

Tumor Touch Preparations

Touch preparations of breast tumors were done as described by Kovach et al.³⁴ In brief, the partly thawed surface of frozen breast cancer tissue was lightly pressed against the surface of a sterile silanized microscope slide. Clusters of 50–100 pure tumor cells adhering to the surface of the slide were stained by brief dipping in a methylene blue/toluidine blue solution and transferred into 10 μl EL buffer by sterile needles (Microlance3, Becton Dickinson, Franklin Lakes, NJ).

Whole Genome Amplification by PEP-PCR, I-PEP-PCR, and DOP-PCR

PEP-PCR was performed according to Zhang et al.¹³ using a MJR PTC200 thermocycler (Biozym, Oldenburg, Germany), *Taq* polymerase (Life Technologies GmbH), and 15 mer random primers (MWG Biotech GmbH, Ebersberg, Germany).

I-PEP, the modified PEP-PCR variant, differs from original PEP protocol¹³ in the following three modifications: (i) cell lysis in EL buffer, (ii) use of 3.6 U of a mix of *Taq*

polymerase and proofreading *Pwo* polymerase (Expand High Fidelity PCR System, Boehringer Mannheim) in whole genome amplification, and (iii) an additional cyclical elongation step at 68°C for 30 seconds before the denaturation step at 94°C. Fifty amplification cycles were performed, each consisting of a 1-minute step at 94°C, a 2-minute step at 37°C, a ramping step of 0.1°C/sec to 55°C, a 4-minute step at 55°C, and a 30-second step at 68°C. I-PEP-PCR was set up by adding 50 μl I-PEP mix (final concentration 0.05 mg/ml gelatine, 16 $\mu\text{mol/L}$ totally degenerated 15-nucleotide-long primer, 0.1 mmol/L dNTP, 3.6U Expand High Fidelity polymerase, 2.5 mmol/L MgCl_2 , in $1 \times$ PCR buffer No.3) to 10 μl lysed cells.

DOP-PCR was performed using the DOP-PCR Master kit (Boehringer Mannheim) according to the manufacturer's protocol. Efficiency rates (ER) of the different WGA methods were assessed in terms of the percentage of successful amplification of at least two microsatellite markers (see Table 2) and a 540-bp β -globin fragment in subsequent single-round PCRs.

Single-Round Multicycle PCR after I-PEP

Specific single round PCR (0.2 mmol/L dNTP; 0.3 $\mu\text{mol/L}$ primers; 0.5 U *Taq* (Life Technologies) or Expand High Fidelity polymerase) rather than nested PCR was done using 2- μl or 5- μl aliquots of the preamplified DNA in a final volume of 20 μl or 30 μl , respectively, in a PTC100 Thermocycler (MJ Research, Watertown, MA) for 50 cycles: 94°C/1 minute, 50–60°C/1 minute, 72°C/1 minute, followed by a final extension at 72°C for 8 minutes as described previously.^{35,36} Primer sequences are given in Table 1. Amplified microsatellites (3 μl) were detected as described³⁷ and β -globin amplification products (5 μl) were electrophoretically separated on 2.5% agarose gels (FMC BioProducts, Rockland, ME) and stained with ethidium bromide.

Detection of p53 Gene Mutations after Preamplification by I-PEP

Clusters of approximately 50 tumor cells were lysed and preamplified according to I-PEP protocol. One-thirtieth aliquots (2 μl) were used for subsequent amplification of exon 7 and 8 of the p53 gene (final volume 50 μl ; final concentration 200 nmol/L dNTPs, 1.25U Expand High Fidelity polymerase, 0.4 $\mu\text{mol/L}$ E7 or E8 first-round PCR primers) (see Table 1), 1.5 mmol/L (exon 7) or 2 mmol/L (exon 8) MgCl_2 . PCR was performed with an initial incubation of 94°C for 2 minutes, followed by 35 cycles of 94°C for 1 minute, 50°C for 2 minutes and 72°C for 3 minutes, with a final elongation for 10 minutes at 72°C. DNA (50–150ng) purified by polyethylenglycol precipitation (equal volume of PCR product and PEG-Mix containing 26% PEG8000, 0.6 mol/L Na acetate, pH 5.3, 6.6 mmol/L MgCl_2) was taken for cycle sequencing in both directions using the E7 and E8 first-round primers in a PTC200 MJR thermocycler (MJ Research; initial incubation at 96°C for 2 minutes, 25 cycles: 96°C/15 seconds, 50°C/15 seconds, 60°C/4 minutes) using the PRISM

Table 1. Primer Sequences

Ki-ras	
5'BstNI	5' ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT 3'
3'WT	5' TCA AAG AAT GGT CCT GCA CC 3'
3'BstNI	5' TCA AAG AAT GGT CCT GGA CC 3'
β -globin up	5' GGT TGG CCA ATC TAC TCC CAG G 3'
β -globin down	5' GCT CAC TCA GTG TGG CAA AG 3'
Microsatellite primers:	
TP53.PCR up	5' AGG GAT ACT ATT CAG CCC GAG GTG 3'
TP53.PCR down	5' ACT GCC ACT CCT TGC CCC ATT C 3'
TP53.ALS up	5' TCG AGG AGG TTG CAG TAA GCG GA 3'
TP53.ALS down	5' AAC AGC TCC TTT AAT GGC AG 3'
D2S123 up	5' AAA CAG GAT GCC TGC CTT TA 3'
D2S123 down	5' GGA CTT TCC ACC TAT GGG AC 3'
D5S346 up	5' ACT CAC TCT AGT GAT AAA TCG 3'
D5S346 down	5' AGC AGA TAA GAC AGT ATT ACT AGT T 3'
D17S250 up	5' GGA AGA ATC AAA TAG ACA AT 3'
D17S250 down	5' GCT GGC CAT ATA TAT ATT TAA ACC 3'
CyclinD1 up	5' TTA CAA TGT CAT ATA CTG CCA 3'
CyclinD1 down	5' GTA TTG TGA AAC AGC AAC CT 3'
D3S1283 up	5' GGC AGT ACC ACC TGT AGA AAT G 3'
D3S1283 down	5' GAG TAA CAG AGG CAT CGT GTA TTC 3'
1p32 up	5' TGG CGA GAC TCC ATC AAA G 3'
1p32 down	5' CCT TTT AAG CTG CAA CAA TTT C 3'
BAT26 up	5' TGA CTA CTT TTG ACT TCA GCC 3'
BAT26 down	5' AAC CAT TCA ACA TTT TTA ACC C 3'
D9S283 up	5' TGC TGG ATT TCA GGT AGG G 3'
D9S283 down	5' ATG GTT ATG CGG GTG TAT TTC TC 3'
Primers for Amplification and Sequencing of <i>p53</i> Gene	
<i>p53</i>	
E7 first round up	5' AAAGGCCTCCCCTGCT 3'
E7 first round down	5' GAGCAGTAAGGAGATT 3'
E7 second round up	5' CTCCCCTGCTTGCCA 3'
E7 second round down	5' GATGGGTAGTAGTATG 3'
E8 first round up	5' GACAGGTAGACCTGAT 3'
E8 first round down	5' TCTGAGGCATAACTGC 3'
E8 second round up	5' AGACCTGATTCCTTAC 3'
E8 second round down	5' TAACTGCACCCCTGGTC 3'

Ready Dye Terminator Cycle Sequencing Kit (Applied Biosystems GmbH, Weiterstadt, Germany) and an Applied Biosystem 373 sequencer according to the manufacturer's instructions. Nonpreamplified DNA from parallel samples was sequenced in parallel after nested PCR of exon 7 and 8 of *p53* gene (nested PCR primer, see Table 1; template DNA: 2 μ l of first-round PCR products; sequencing primer: E7 and E8 second-round primers; PCR for 35 cycles of 94°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes, with a final elongation for 10 minutes at 72°C).

Ki-ras Mutation Analysis by RFLP-PCR

Detection of *Ki-ras* mutation in tumor tissue and in CK18-positive tumor cells from bone marrow of one pancreatic carcinoma patient and one rectal carcinoma patient was performed by enrichment PCR according to Trümper et al³⁸ with minor modifications. Corresponding normal or CK18-negative cell DNA was used as a negative control; the CAPAN-1 cell line (American Type Culture Collection) served as a positive control. Briefly, a 157-bp fragment of the *Ki-ras* gene was amplified with 0.5 μ mol/L each of the primer 5'BstNI (PCR primers are given in Table 1) and 3'

WT in a volume of 50 μ l for 16 cycles (annealing temperature 57°C) using 1 U Expand HiFi polymerase (Boehringer Mannheim). A 16- μ l aliquot was subsequently digested with 20 U *Mva*I (Boehringer Mannheim) in a volume of 20 μ l for 6 hours at 37°C, resulting in fragmentation of the wild-type and enrichment of the mutated *Ki-ras* gene. Digested DNA (10 μ l) was used for a second round of PCR in the presence of 0.5 μ mol/L each of primers 5'BstNI and 3'BstNI (see Table 1) for 35 cycles (annealing temperature 60°C) in a volume of 50 μ l. Finally, 30 μ l were digested with *Mva*I in a volume of 36 μ l for 4 hours at 37°C, 7 μ l nondenaturing loading buffer were added, and 20 μ l of this mix were analyzed on a 10% nondenaturing polyacrylamide gel.

Results

Efficiency Rates of Different WGA Methods

The efficiency rates (ER) of the different WGA methods were assessed in terms of the percentage of successful amplification of the D2S123 (197–227 bp), D17S250 (140–155 bp), TP53ALS (130–150 bp), and TP53PCR

Table 2. Amplification Efficiency after Preamplification of DNA Using Different Whole Genome Amplification Methods

A. Cultured SW480 cells																		
Number of cells	I-PEP-PCR						PEP-PCR						DOP-PCR					
	β -Globin		D2S123		CyclinD1		β -Globin		D2S123		CyclinD1		β -Globin		D2S123		CyclinD1	
	ER*	%	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%
1	5/10	50	2/10	20	5/10	50	0/10	0	1/10	10	2/10	20	0/10	0	0/10	0	1/10	10
5	10/10	100	10/10	100	10/10	100	1/10	10	4/10	40	5/10	50	3/10 [†]	30	0/10	0	3/10 [†]	30
10	10/10	100	10/10	100	10/10	100	9/10	90	10/10	100	10/10	100	7/10	70	3/10	30	10/10	100
100	9/10	90	9/10	90	10/10	100	9/10	90	9/10	90	10/10	100	10/10	100	7/10	70	10/10	100

B. Frozen microdissected tissue (normal urinary bladder)																		
Number of cells	I-PEP-PCR						PEP-PCR											
	D17S250		D2S123		β -Globin		D17S250		D2S123		β -Globin							
	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%
10	5/5	100	5/5	100	n.d.		4/5 [‡]	80	5/5 [‡]	100	n.d.							
25	5/5	100	5/5	100	n.d.		4/5 [‡]	80	5/5 [‡]	100	n.d.							
50	5/5	100	10/10	100	4/4	100	5/5 [‡]	100	10/10 [‡]	100	3/4 [‡]	75						
100	n.d.		2/2	100	4/4	100	n.d.		2/2	100	4/4	100						
200	n.d.		2/2	100	2/2	100	n.d.		2/2	100	2/2	100						
500	n.d.		2/2	100	2/2	100	n.d.		2/2	100	2/2	100						

C. Formalin-fixed and paraffin-embedded microdissected tissue (normal colonic mucosa)																		
Number of cells	I-PEP-PCR						PEP-PCR											
	TP53ALS		TP53PCR		D2S123		TP53ALS		TP53PCR		D2S123							
	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%	ER	%
30	3/3	100	3/3	100	3/3	100	3/3 [‡]	100	0/3	0	2/3	66						
50	3/3	100	3/3	100	3/3	100	1/3 ^{‡,‡}	33	0/3	0	1/3	33						
100	3/3	100	3/3	100	3/3	100	2/3 ^{‡,‡}	66	1/3 [‡]	33	2/3	66						
250	3/3	100	3/3	100	3/3	100	2/3	66	1/3 [‡]	33	2/3	66						
500	2/2	100	2/2	100	2/2	100	1/2	50	1/2	50	1/2	50						
1000	2/2	100	2/2	100	2/2	100	2/2	100	2/2	100	2/2	100						

*ER (efficiency rate): number of successful amplifications per number of trials

†Nonspecific bands visible

‡Weak amplification signals.

Lengths of PCR products: β -Globin: 540 bp, D2S123: 197–227 bp, CyclinD1: 130 bp, TP53PCR: 103–135 bp, TP53ALS: 130–150 bp, D17S250: 140–155 bp.

(103–135 bp) microsatellite markers, a 130-bp fragment of the CyclinD1 gene, and a 540-bp β -globin fragment by single-round PCR. The exact number of experiments for every marker and every investigated cell number of the different cell and tissue types is given in Table 2.

First, SW480 cells sorted by FACS were examined as single, five, ten, and 100 cells (ten samples in parallel, each). ER of DOP-PCR was inferior to PEP-PCR and tended to generate nonspecific bands (Table 2A; Figure 1A, bottom *versus* middle, amplification of β -globin fragment). Because the efficiency of PEP-PCR and subsequent single-round PCR in single- and five-cell assays was not satisfying, the original PEP protocol was modified. For this purpose, the influence of proofreading polymerase (Expand High Fidelity polymerase), enzymatical cell lysis, and introduction of an additional elongation step during preamplification was systematically tested. It turned out that the ER of WGA could be enhanced by combining these modifications (Table 2A; Figure 1A, top). In single-cell assays, ER for I-PEP ranged from 20% to 50%; in samples of five or more cells ER was constantly 100%. Thus, PEP-PCR could be markedly improved by

these three modifications, making the I-PEP-PCR particularly suitable for multiple single-round PCR analyses of one or a few intact cells.

Second, ER of I-PEP-PCR and PEP-PCR was assessed in cell clusters of 10–1000 cells obtained from frozen as well as formalin-fixed, paraffin-embedded tissue sections using the β -globin fragment PCR and four microsatellite markers (D2S123, TP53PCR, TP53ALS, and D17S250). As shown in Table 2B and Figure 1B, I-PEP enabled reliable amplification of the three investigated fragments in all tested frozen section samples (ER: 100%). In comparison, the original PEP-PCR resulted in an ER of 75–100%. However, in formalin-fixed and paraffin-embedded tissue sections I-PEP-PCR was drastically superior to the original PEP-PCR protocol. As shown in Figure 1C, in microdissected clusters of H&E-stained colonic cancer sections consisting of 30, 50, 100, 250, 500, or 1000 tumor cells, amplification of p53 marker (TP53.PCR) after I-PEP-PCR resulted in strong bands even in 30-cell clusters, whereas the original PEP-PCR protocol failed to reliably amplify DNA in samples with < 1000 cells. These

Figure 1. Efficiency of I-PEP-PCR compared to unmodified PEP-PCR and DOP-PCR. **A:** SW480 cells were sorted as single, five, ten, and 100 cells by FACS in ten parallel samples, respectively. The cells were lysed and DNA was preamplified either by I-PEP, PEP-PCR or DOP-PCR. A 1/30th aliquot was used for specific amplification of a 540-bp β -globin fragment. Positive controls were 10 ng SW480 genomic DNA preamplified (first +) and nonpreamplified (second +). Negative controls (–) were H₂O in preamplification PCR followed by specific PCR. PCR products were analyzed by ethidiumbromide-stained 2.5% agarose gels. **B:** Cell clusters consisting of 50, 100, 200, 500, and 1000 cells microdissected from methylene blue stained frozen bladder tissue sections were lysed and preamplified by I-PEP-PCR and PEP-PCR, respectively. One-tenth DNA aliquots were used for specific amplification of a 540-bp β -globin fragment. PCR products were analyzed by ethidiumbromide-stained 2.5% agarose gels. **C:** Cell clusters consisting of 30, 50, 100, 250, 500, and 1000 cells were microdissected from a H&E stained formalin-fixed and paraffin-embedded colonic tissue section, lysed and preamplified according to I-PEP-PCR and PEP-PCR protocol, respectively. One-tenth aliquots were used for specific amplification of TP53 PCR microsatellite marker (120-bp fragment, uninformative alleles). As positive controls a cell cluster of > 1000 cells was used. PCR products were analyzed by silver-stained denaturing polyacrylamide/urea gels.

results could be confirmed using markers TP53PCR and D2S123 (Table 2C).

Demonstration of Genomic Instability (MSI, LOH) in Single Cells: Requirements and Limitations

The reliability of microsatellite analysis of I-PEP-preamplified DNA was examined using FACS-sorted SW480 cells and microdissected tumor cell clusters of frozen tissues, as well as formalin-fixed and paraffin-embedded tissue sections stained by H&E or p53 immunohistochemistry.

Ten samples of FACS-sorted SW480 cells were analyzed after being sorted into samples of one, five, ten, and 100 cells. Each group was used for I-PEP-PCR and subsequent single-round D2S123 amplification. Although a 100% amplification ER was seen already in the five-cell sample, preferential amplification of one of the two alleles was evident in 40% of the samples in this group (see

lanes 1, 4, 5, and 6 in the five-cell experiment in Figure 2A). Artificial monoallelic amplification was observed in two of these four samples. Reproducible and unbiased amplification of both microsatellite alleles was achieved only with 10 or more cells (Figure 2A and Table 3A). These results were confirmed by five other microsatellite markers (D17S250, D5S346, 1p32, D3S1283, and BAT 26) (for data see Table 3A).

In contrast to FACS-sorted cells or other intact cells, microdissected cells or cell clusters from tissue sections are much more difficult to analyze due to variation in DNA quality after surgery and specimen processing. Furthermore, cells in tissue sections are cut and may have lost parts of their genomic DNA. To determine the limits of unbiased biallelic microsatellite amplification after I-PEP-PCR in frozen tissue (urinary bladder, immediately frozen after biopsy and kept at –80°C until DNA preparation) we have assessed the D17S250, D2S123, and D5S346 markers in samples consisting of 10, 25, 50, and > 1000

Figure 2. Microsatellite analysis by silver-stained denaturing polyacrylamide/urea gels. **A:** SW480 cells were sorted into samples of 1, 5, 10, or 100 cells by FACS in ten parallel samples, respectively. The cells were lysed enzymatically and DNA was preamplified by I-PEP-PCR. A 1/30th aliquot was used for amplification of D2S123 microsatellite marker (200- to 230-bp fragments). **B:** Methylene blue-stained and microdissected cell clusters of 10, 25, and 50 cells from frozen tissue were preamplified either by I-PEP-PCR (top) or PEP-PCR (bottom) and a 1/12th aliquot was used for amplification of D2S123 microsatellite marker. **C:** Microdissected cell clusters of 30, 50, 100, and 250 cells from H&E stained sections from formalin-fixed and paraffin-embedded tissue (right) were preamplified either by I-PEP-PCR (upper part) or PEP-PCR (lower part) and a 1/12th aliquot was used for amplification of D2S123 microsatellite marker. **D:** Microdissected cell clusters of 100 cells from immunohistochemically p53-positive (p53 IHC+) and -negative (p53 IHC-) stained cells from sections of formalin-fixed and paraffin-embedded tissue were preamplified by I-PEP-PCR and a 1/12th aliquot was used for amplification of TP53ALS microsatellite marker.

nonneoplastic cells, respectively. As shown in Figure 2B, strong amplification of both alleles (D2S123 locus) was achieved after preamplification only by I-PEP-PCR, whereas specific PCR after PEP preamplification resulted in much weaker amplification products. The other two microsatellite markers tested showed also biallelic amplification for all tested samples after I-PEP-PCR preamplification (for exact results see Table 3B). Furthermore, microsatellite markers TP53ALS and D9S283 were amplified from microdissected tumor cells from bladder cancer samples (~250 cells per sample) after I-PEP-PCR preamplification (Table 3B). Of 262 samples, 254 (97%) were successfully and accurately amplified.

PCR amplification from formalin-fixed and paraffin-embedded tissue is a widely used method but the DNA quality can vary considerably. The limits of biallelic amplification were investigated with the TP53ALS, TP53PCR, D2S123, 1p32, D3S1283, and BAT26 markers in microdissected samples of nonneoplastic cells of the colon mucosa consisting of 30, 50, 100, 250, 500, and > 1000 cells (Table 3C). As shown in Figure 2C (top), I-PEP-PCR allowed accurate biallelic amplification even in all 30-cell samples. In contrast, original PEP-PCR led to artificially biased amplification of one allele in six of the seven successfully amplified PCR products (Figure 2C, bottom). The ER was considerably lower for formalin-fixed, paraffin-embedded tissue than for fresh frozen tissue. The analyses of microsatellite markers 1p32, D3S1283, and BAT26 using microdissected cell clusters of approximately 250 cells revealed an accurate amplification after

I-PEP-PCR in 190 of 288 samples (66% efficiency rate; Table 3C).

Furthermore, I-PEP-PCR allowed reliable p53 LOH (TP53ALS) detection in clusters of about 100 cells dissected from p53 immunostained routinely processed tissue sections (Figure 2D).

Sequencing Analysis after I-PEP-PCR: Error-Free Amplification and Reliable Mutation Detection

To evaluate the sequence fidelity of the amplified DNA after I-PEP-PCR and subsequent gene-specific amplification, we examined the sequence accuracy in exon 7 and 8 of the p53 gene in touch preparations of frozen breast cancer tissue in eight and ten individual breast cancers, respectively, which had been shown previously to carry four mutations in exon 7 or 8 according to direct sequence analysis without preamplification.^{39,40}

Remarkably, sequencing I-PEP-PCR-preamplified DNA from clusters of 50–60 cells revealed exactly the same mutations as sequencing nonpreamplified DNA. Figure 3 shows a heterozygous 8-bp deletion in exon 7 (Figure 3A), a hemizygous C/G to G/C transversion (Figure 3B), a heterozygous A/T to C/G transversion (Figure 3C), and a heterozygous A/T to T/A transversion in exon 8 (Figure 3D), respectively, after preamplification by I-PEP-PCR. Furthermore, no artificial mutation was found in

Table 3. Accuracy of Microsatellite Amplification after Preamplification with I-PEP-PCR

A Cultured SW480 cells				B Frozen tissue (urinary bladder)				C Formalin-fixed and paraffin-embedded tissue (Colon)			
Locus	Number of cells	Monoallelic amplification	biallelic amplification	Locus	Number of cells	monoallelic amplification	biallelic amplification	Locus	Number of cells	monoallelic amplification	biallelic amplification
D2S123*	1	2/10 [†] (20%)	—	D17S250*	10	—	5/5 (100%)	TP53ALS [‡]	30	3/3 (100%)	—
	5	2/10 [†] (20%)	8/10 (80%)		25	—	5/5 (100%)		50	2/3 (66%)	—
	10	—	10/10 (100%)		50	—	2/2 (100%)		100	3/3 (100%)	—
	100	—	9/10 (90%)		>1000	—	2/2 (100%)		250	2/3 (66%)	—
D17S250 [†]	1	3/10 (30%)	—	D2S123*	10	—	5/5 (100%)		500	2/2 (100%)	—
	5	7/10 (70%)	—		25	—	5/5 (100%)		>1000	2/2 (100%)	—
	10	10/10 (100%)	—		50	—	2/2 (100%)	TP53PCR [‡]	30	3/3 (100%)	—
	100	10/10 (100%)	—		>1000	—	2/2 (100%)		50	3/3 (100%)	—
D5S346*	1	2/10 [†] (20%)	—	D5S346*	10	—	5/5 (100%)		100	3/3 (100%)	—
	5	2/10 [†] (20%)	8/10 (80%)		25	—	5/5 (100%)		250	2/3 (66%)	1/3 [‡]
	10	—	10/10 (100%)		50	—	2/2 (100%)		500	2/2 (100%)	—
	100	—	10/10 (100%)		>1000	—	2/2 (100%)		>1000	2/2 (100%)	—
1p32*	10	—	124/126 (98%)	TP53ALS*	250	—	70/75 (93%)	D2S123*	30	—	3/3 (100%)
D3S1283 [†]	10	123/126 (98%)	1/126 [‡] (<1%)	TP53ALS [†]	250	56/56 (100%)	—		50	—	2/3 (66%)
BAT26 [†]	10	119/126 (94%)	—	D9S283*	250	—	81/82 (99%)		100	—	3/3 (100%)
				D9S283 [†]	250	47/49 (96%)	—		250	—	2/3 (66%)
									500	—	2/2 (100%)
									>1000	—	2/2 (100%)
								1p32 [†]	250	61/96 (64%)	5/96 [‡] (5%)
								D3S1283*	250	7/96 [‡] (7%)	70/96 (73%)
								BAT26 [†]	250	59/96 (61%)	5/96 [‡] (5%)

*informative biallelic locus with respect to the sample analyzed.

[†]noninformative monoallelic locus with respect to the sample analyzed

[‡]biased amplification: preferential amplification of one allele in biallelic loci and artificial amplification products in monoallelic loci
 — no amplification

Lengths of PCR products: β -Globin: 540 bp, D2S123: 197–227 bp, CyclinD1: 145 bp, TP53PCR: 103–135 bp, TP53ALS: 130–150 bp, D17S250: 140–155 bp, BAT26: 80–100 bp, 1p32: 140–209 bp, D5S346: 96–122 bp, D3S1283: 150–160 bp, D9S283: 120–150 bp

the wild-type exons of p53 gene, demonstrating an accurate amplification of DNA by I-PEP-PCR.

Ki-ras Mutation Analysis of I-PEP-Amplified DNA from Single Disseminated Tumor Cells and Paraffin-Embedded Tumor Tissue

We have examined the feasibility of I-PEP-PCR for mutation analysis in single disseminated tumor cells from bone marrow aspirates from a pancreatic and a rectal carcinoma patient. Disseminated tumor cells were detected immunocytochemically by CK18 staining, picked from immunospots as single or few cells ($n_{\text{cell}} = 3\text{--}5$), and used for detection of Ki-ras mutation by RFLP-PCR according to Trümper et al.³⁸ The mutation analysis was also performed with microdissected tumor cells ($n_{\text{cell}} \sim 200$) from the formalin-fixed, paraffin-embedded primary tumors of these patients.

As shown in Figure 4, Ki-ras amplification of 18 of 19 investigated preamplified DNA samples was successful, corresponding to an ER of 95%. Mutated Ki-ras genes were represented by 143-bp fragments after a restriction digest with *Mva*I, whereas wild-type genes were demonstrated by 114-bp fragments. All CK18 immunocytochemical positive cells showed the mutation-specific

143-bp fragment that was also detected in the parent primary tumors indicating their atypia and origin (Figure 4B). All immunocytochemical negative controls (preamplified DNA from normal tissue or hematopoietic cells, Figure 4A, lanes 8–11) showed a 114-bp wild-type-specific fragment that could not be detected in the tumor cells.

Discussion

An improved PEP-PCR protocol enables an efficient and accurate WGA of intact and sectioned microdissected tumor cells with respect to microsatellite analysis as well as mutation analysis by conventional sequencing and RFLP-PCR.

In diagnostic and experimental tumor pathology, microsatellite analysis is a rapid and reliable tool for the characterization of the allele status at almost any chromosomal locus demonstrating LOH or MSI.^{35,41–43} However, MSI analysis and in particular detection of LOH require a homogeneous population of tumor cells, because any contamination by adjacent normal cells (eg, lymphocytes or stromal cells) would lead to erroneous underestimation of the LOH frequency.³ Thus, only pure tumor cell populations should be analyzed, requiring ac-

Figure 3. Sequence analysis of p53 gene after I-PEP-PCR. Tumor cell DNA from tumor touch preparations³⁴ of breast cancer patients were preamplified by I-PEP-PCR. One-thirtieth aliquots were used for nested PCR amplifying exon 7 and exon 8 of p53 gene. PCR products were subsequently used for cycle sequencing. The positions of an 8-bp deletion (A) and of mutated nucleotides (nt) are indicated (A-D). Mutated nucleotides are shown in italics.

curate microdissection techniques best provided by laser microdissection. On the other hand, after microdissection the number of tumor cells is usually very low and the corresponding amount of DNA allows only one or few specific (nested) PCR amplifications. Because MSI and LOH studies must be done with multiple markers, preamplification of the entire DNA by WGA would be very helpful. Although this technique has already been shown useful in intact sperm cells,¹³ blastomeres,¹⁵⁻¹⁷ and fetal nucleated erythrocytes,¹⁸ the method has not yet been optimized for samples routinely used in tumor pathology. Our intention was to establish a protocol that allows multiple DNA analyses of single cells or small cell groups in a single-round locus-specific PCR after preamplification. For this purpose we improved preamplification allowing subsequent single-round PCR and compared the efficiency of our modified I-PEP-PCR with the original standard PEP protocol and DOP-PCR.

As first step, we replaced nested PCR, which includes a total of 50–60 cycles (25–30 cycles each in the first and second rounds), with a single-round multicycle (SRMC, $n_{\text{cycles}} = 50$), gene-specific PCR. Under standard conditions, saturated amplification, ie, amplification that reaches the plateau level, is usually achieved after 30 cycles according to the equation $N_f = N_0 (1 + Y)^{n-1}$, where N_f is the final copy number of double-stranded target sequence, N_0 is the initial copy number, Y is the

efficiency of primer extension per cycle (*Taq* polymerase ≈ 0.88),^{32,44} and n is the number of PCR cycles under conditions of exponential amplification.^{45,46} When the plateau is reached, about 10^{12} – 10^{13} final copies are generated and more PCR cycles can result in amplification of nonspecific bands. This calculation refers to an initial target copy number of $\approx 10^5$ molecules ($0.5 \mu\text{g}$ genomic DNA $\approx 1.5 \times 10^5$ copies of mammalian genome). Assuming that PEP-PCR results in at least 30-fold amplification of a single cell's DNA¹³ and a 1/30th aliquot of PEP-DNA is used for subsequent locus-specific amplification, theoretically only two target copies are present. In this case the saturated amplification plateau (ie, 10^{12} – 10^{13} amplified copies) is not reached before about 50 cycles. Although this computation also depends on other parameters (eg, gene-specific amplification factors), it provides a useful general rule of thumb for designing PCR conditions. In fact, our results are quite concordant with this estimate because amplification of a β -globin DNA fragment from single-cell or 5-cell DNA preamplified by I-PEP-PCR was successful with a 50-cycle specific PCR but not with 30 cycles. A reduction to 40 cycles led to either faint bands or negative results (data not shown).

Cell lysis turned out to be another critical step influencing the preamplification efficiency. Proteinase K digestion in $1\times$ PCR buffer containing Tween 20 as the detergent allowed successful amplification (100%) of

Figure 4. Ki-ras mutation analysis in isolated CK18-stained disseminated tumor cells in bone marrow. Microdissected cells were preamplified by I-PEP-PCR and 1/10th aliquot was used for subsequent RFLP-PCR. Undigested (157-bp fragments) and *Mva*I-digested DNA (143-bp fragments in mutated codon 12; 114-bp fragments in wild-type codon 12) are given in parallel for each sample (A, 1–11; B, 1–8). A: CK18 immunocytochemically positive stained cells were examined as single (Lanes 1, 2, 4, and 5) or few ($5 < n < 10$) cells (Lanes 3, 6, and 7). Lanes 1 and 2: cell samples from a pancreatic carcinoma patient. Lanes 3–7: cell samples from a rectum carcinoma patient. Lanes 8–11: negative controls (unstained hematopoietic cells, $n \sim 1000$ each). B: Tumor tissue. Lanes 1–3: pancreatic carcinoma. Lane 4: normal pancreatic tissue. Lanes 5 and 6: rectum carcinoma. Lane 7: normal rectal tissue. Lane 8: CAPAN-1 cell line as positive control.

β -globin fragment in preamplified DNA of five cells, whereas the amplification rate was only 60% when cells were lysed in alkaline buffer (data not shown). This difference was especially drastic in formalin-fixed paraffin-embedded tissue (Figures 1C and 2C). Most probably, protease is capable of degrading nuclear proteins involved in chromosomal packaging (eg, histones) and thereby allows more efficient delivery of DNA. This might be especially true in fixed tissue, where DNA packaging proteins are covalently cross-linked with each other or with DNA/RNA.

Preamplification could be further improved by use of Expand High Fidelity polymerase, which resulted in an enhanced efficiency of 40% compared to *Taq* polymerase (tested in five-cell assays; data not shown). The higher efficiency of Expand High Fidelity polymerase may be due to its 3'–5' exonuclease proofreading function, which removes mismatched nucleotides at the 3' ends of the random primers and makes them suitable for elongation and amplification. In contrast, *Taq* polymerase cannot extend primers containing mismatches at the 3' end as efficiently as perfectly matched primers.^{47,48} Because one can assume that many random primers are not perfectly matching their target sequences, the number of amplification products will be much higher when Expand High Fidelity polymerase instead of *Taq* polymerase is used during WGA. Further improvement, particularly in formalin-fixed tissue, was achieved by an additional cyclical elongation step at 68°C rather than 55°C during preamplification (data not shown).

Serious problems occur when tissue sections are microdissected and used for DNA analysis by PCR because the cell and nucleus volume is diminished by cutting. As a result, chromosomes or parts of chromo-

somes could get lost, leading to artificial allelic losses (pseudo-LOH). Indirect evidence for this is provided by, eg, control fluorescence *in situ* hybridization experiments with centromeric probes on normal tissue sections showing loss of chromosomes in 15 to 20% of all nuclei. Our results showing preferential allelic amplification in microdissected samples of few cells ($n < 100$) from sectioned tissue samples could reflect the possible loss of genetic material. For this reason and according to our experience, microsatellite allele typing is not possible with single cells of sectioned tissues. To find out the critical cell number, we conducted a systematic investigation using tissue sections from both frozen and formalin-fixed paraffin-embedded tumor samples. Our results clearly show that the reliability of microsatellite analyses depends largely on tissue preparation. The sensitivity of microsatellite amplification after preamplification using frozen tissue sections is about threefold to 50-fold higher than it is using paraffin-embedded tissues. Furthermore, in microdissected frozen tissues reliable amplification of both microsatellite alleles was possible even in clusters of 10 cells. In our experience this marks the lowest limit of cell numbers for homogeneous biallelic amplification and lies slightly below the value reported by Barrett et al (50- to 100-cell equivalents).¹⁹ However, in contrast to our studies, Barrett et al used DNA dilution series. Thus, cell lysis has not been considered in their studies. However, we could not achieve the 90% ER reported by Zhang et al,¹³ who investigated FACS-sorted sperm cells. A possible explanation for this discrepancy in the sensitivity could be that sperm cells have a higher survival rate during FACS sorting than the cultured colonic tumor cells used in our study.

In general, success of WGA and subsequent locus-specific PCRs requires quick handling of fresh or thawed tissues, especially during microdissection, to avoid DNA degradation by nucleases. In our hands, the experiments succeeded when preparation time was < 30 minutes. Compared to frozen tissues, corresponding experiments using paraffin-embedded tissues led to quite variable results. In some cases multiple microsatellite analyses succeeded with cell clusters containing 30 microdissected cells (lowest limit), whereas other ones were completely negative, depending on time of fixation and storage.

As reported by Zhang et al,¹³ biased allele amplification can occur not only due to low cell numbers but also due to insufficient amounts of preamplified DNA used in specific PCR. Our study confirms this finding as we have observed a heterogeneous microsatellite pattern in specific PCR of preamplified DNA from five cells, using a 1/30th aliquot PEP-DNA for D2S123 amplification. Homogeneity (ie, uniform pattern of PCR products produced in multiple parallel experiments with the same samples) could be achieved either by increasing the number of cells used for I-PEP-PCR or by increasing the amount of I-PEP-PCR DNA. The use of 1/10th PEP-DNA aliquot in subsequent single-round D2S123 amplification lead to homogeneity of 50% in single cells. The same result was obtained when we performed a nested PCR of the D2S123 microsatellite locus with 5 μ l preamplified DNA.

This corresponds roughly to reports of others¹⁴⁻¹⁷ who also used a 1/10th rather than a 1/30th aliquot of preamplified DNA for specific nested PCR.

Whereas MSI can be detected relatively easily due to the presence of new alleles of different sizes, LOH analysis is much more difficult. The most critical point here is the contamination by normal cells due to inexact microdissection or intratumoral genetic heterogeneity. However, I-PEP-PCR and subsequent single-round amplification proved to be suited also for LOH analyses in microdissected cells even after immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections.

For mutational analysis of preamplified genomic DNA the intrinsic error rate of *Taq* polymerase (error rate: 10^{-4} /bp) and the high total number (100 and more) of PCR cycles during WGA and subsequent PCR must be taken into consideration. So far, conventional sequencing of DOP- or PEP-preamplified DNA has not been published. The solid-phase minisequencing of preamplified DNA reported by Paunino et al⁴⁹ describes a method for detection of specific point mutations but is not comparable to sequencing methods.

In our study we used a polymerase mix containing *Taq* polymerase for high amplification efficiency and *Pwo* polymerase with proofreading activity to minimize artificial mutagenesis during amplification. Although proofreading polymerases (*Pfu* polymerase) have already been used in PEP-PCR,⁵⁰ the sequence accuracy has not yet been evaluated. Incorrect amplification is, however, especially critical when low numbers of target copies are used, because a polymerase-induced mutation during the first few cycles would result in a major mutant population in the final PCR products. Theoretically, when *Taq* polymerase is used, a 10^6 -fold amplification (eg, 20 cycles) of a 200-bp fragment will lead to an estimated PCR-induced mutant fraction of 33% using the equation $F = 1 - e^{-b \cdot f \cdot d}$; $F = 1 - e^{-(200) \times (10^{-4}) \times (20)} = 0.33$ where F is the mutant fraction, b is the length of target sequence, f is the error rate, and d is the number of doublings.³² Assuming a randomly distributed mutation pattern, the resulting error frequency per base, on average, would be 1.7×10^{-3} (0.33/200 bp). Thus, a higher number of cycles (eg, 100) should result in error frequency of 86%. The corresponding error rate per base of a 200-bp fragment would be 4.3×10^{-3} , ie, one mutation per 233 bp. As we have sequenced a total of > 4000 bp ($8 \times$ p53-exon 7, 210 bp; $10 \times$ p53-exon 8, 240 bp) and found no artificially introduced mutations, one can assume that preamplified genomic DNA under the conditions used here is actually suited for further amplification and sequence analysis, at least for DNA fragments of up to 240 bp. This size range allows exonwise sequencing of frequently mutated tumor-associated genes (eg, p53). Furthermore, sequencing of specific DNA fragments after preamplification is obviously sufficient to discriminate between heterozygous and hemizygous mutation status as seen in mutations in exon 7 and 8 of the p53 gene (Figure 3, C and D). This is an important prerequisite for mutation detection in germline DNA.

The usefulness of our PEP-PCR variant was also confirmed by RFLP-PCR. Although detection of a Ki-ras mutation in codon 12 is generally critical due to the artificial introduction of *Taq* polymerase-introduced mutations,³⁸ this problem seems to be largely solved now, as we could demonstrate accurate mutation detection in codon 12 of the Ki-ras gene both in microdissected paraffin-embedded cells and microdissected disseminated tumor cells from bone marrow after immunostaining on cytospins. In contrast to the detection of tumor cells in blood or bone marrow by RT-PCR,⁵¹⁻⁵⁵ we have established a technique to characterize isolated disseminated tumor cells by multiple DNA analyses. This allows the examination of several gene defects that may predict therapy response.⁵⁶⁻⁵⁸

In summary, I-PEP-PCR is a very reliable method for WGA that supports microsatellite analysis, accurate sequencing, and mutation detection methods like RFLP-PCR. I-PEP-PCR is more effective than the conventional PEP-PCR and DOP-PCR protocols, especially in microdissected fixed tissue samples. In combination with laser or manual microdissection techniques, I-PEP-PCR provides a powerful new tool to study the molecular changes underlying carcinogenesis, clonal expansion, and tumor dissemination in one or a few cells.

Acknowledgments

We thank Petra Wegele for excellent technical assistance and Prof. R. Fishel (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia) for revising the manuscript. We also thank the LASER-Center Grosshadern (Munich) for giving us the opportunity to use the laser microdissection device.

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