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Technical Advances

A Polymerase Chain Reaction-Based Microsatellite Typing Assay Used for Tumor Cell Line Identification

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In this report we describe the application of a polymerase chain reaction (PCR)-based DNA typing assay to the analysis of tumor cell line identity. We have applied the technique to analyze four tumor cell lines purchased from the American Type Culture Collection (SK-OV-3, SK-BR-3, OVCAR, HeLa) and four lines isolated from the ascites fluids of ovarian cancer patients (YAOVBIX1, YAOVBIX3, OC194, and OC346). In this assay, three polymorphic tetranucleotide microsatellite loci (GABARB1, TH01, and HPRTB) were amplified from tumor cell line DNAs in radioactive PCR reactions. The products were resolved in polyacrylamide gels and exposed to film to produce individual-specific patterns for five of the cell lines (HeLa, SK-BR-3, OVCAR, YAOVBIX3, and OC194). However, three of the cell lines, SK-OV-3, YAOVBIX1, and OC436 had identical "fingerprints" at all three loci. The probability that the observed profile match could occur between three randomly selected heterologous cell lines was calculated to be 1.32 × 10^{-13}. On the basis of this analysis, we have identified two independent cross-contamination events involving the SK-OV-3 ovarian adenocarcinoma cell line. The PCR-based analysis of tetranucleotide microsatellite loci is technically straightforward and produces discrete allelic bands associated with known population frequencies, allowing for the unequivocal interpretation of typing patterns. (Am J Pathol 1994, 144:486–491)

Cell line cross-contamination is a potential problem of major significance in biological research. The most widely documented case of mistaken cell line identities involved the HeLa cervical tumor cell line, which overtook cell lines in laboratories around the world.[1–4] Many of these contamination events went undetected for years, resulting in estimated financial losses of millions of dollars.[5] The relatively recent development of DNA fingerprinting and typing techniques[6–9] and their subsequent application to cell line analysis[10–22] now allows for the rapid and accurate validation of cell line identity. Most of the techniques applied to cell line fingerprinting have been Southern blot-based, involving the digestion and hybridization of genomic DNAs with repetitive DNA probes.[10–22] In this report we describe the application of a polymerase chain reaction (PCR)-based DNA typing technique to analyze polymorphic microsatellite loci for the purpose of verifying the origins of eight tumor cell lines growing in our laboratories.

Microsatellites are genetic loci comprised of tandemly aligned repetitions of 1–6 bp nucleotide motifs, the number of which is often subject to allelic variation throughout the population. Allelic profiles of multiple loci can be individual-specific, making it possible to determine genetic identity on the basis of microsatellite length polymorphisms. In our analysis we amplified the TH01, GABARB1, and HPRTB tetranucleotide microsatellite loci from the DNAs of eight of the

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tumor cell lines growing in our laboratories and resolved the products on polyacrylamide gels. On the basis of these "fingerprints," we concluded that the SK-OV-3 ovarian adenoma carcinoma cell line, used widely in ovarian cancer research,[23–32] has cross-contaminated cell cultures in two separate laboratories.

Materials and Methods

Cell Lines and Genomic DNA Preparation

SK-OV-3 and OV-CAR ovarian, HeLa cervical, and SK-BR-3 breast carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and processed for analysis at passages 26, 18, 93, and 313, respectively. YAOVBIX1 and YAOVBIX3 ovarian carcinoma cells were isolated as morphologically distinct colonies originating from the cultured ascites fluid of an ovarian cancer patient at the Yale New Haven Hospital.[30] The OC436 and OC194 cell lines were isolated from the cultured ascites fluids of patients diagnosed with Meigs' syndrome and ovarian carcinoma, respectively, at the UCLA Medical Center.[26,32] All cells were grown to confluence in T-75 flasks for genomic DNA preparation. Monolayers were lysed (1% sarcosyl, 10 mmol/L EDTA in PBS) and treated successively with RNase (100 μg/ml, Boehringer Mannheim, Indianapolis, IN) and proteinase K (100 μg/ml, Boehringer Mannheim). Lysates were phenol/chloroform-extracted and genomic DNAs were ethanol-precipitated in 0.3 mol/L sodium acetate. After pelleting and drying, the precipitates were resuspended in Tris EDTA, pH 8.

Microsatellite PCR

Description of the TH01, GABARB1, and HPRTB microsatellite loci, their primers, and their allele frequencies are presented in Table 1. PCRs were performed according to the specifications of the Perkin-Elmer Cetus GeneAmp PCR reagent kit (Norwalk, CT) with minor modifications. Briefly, 1 μg of genomic DNA from each of the cell lines was used for each 50-μl PCR reaction containing 1X reaction buffer, 1.25 U AmpliTaq DNA polymerase, 10 ng of each primer, and 200 μmol each of dCTP, dGTP, and dTTP. The concentration of cold dATP per reaction was reduced to 50 μM, and 2.5 μCi [^{35}S]dATP (DuPont, NEN Products, Boston, MA) were added. The reaction mixtures were cycled in a Perkin-Elmer Cetus DNA thermal cycler for 30 cycles consisting of a 1-minute denaturing step at 94 C, a 1-minute annealing step at 55 C, and a 1-minute extension step at 72 C. Ten μl of each completed PCR reaction mixture were then mixed with 5 μl of stop solution (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, United States Biochemical, Cleveland, OH), and heat-denatured at 80 C for 3 minutes. Three-μl volumes of the denatured samples were resolved in 6% polyacrylamide sequencing gels (Sequagel, National Diagnostics, Manville, NJ) subjected to 1500 V for 2.5 hours. The gels were fixed in a 10% methanol/10% acetic acid solution for 1

Table 1. *Features of the TH01, GABARB1, and HPRT Microsatellite Loci Used for Cell Line Typing Analysis*

	Microsatellite Locus		
	GABARB1	TH01	HPRTB
Reference	Dean et al[34]	Edwards et al[35]	Edwards et al[35]
Gene	γ-Aminobutyric acid receptor	Tyrosine hydroxylase	Hypoxanthine phosphoribosyl-transferase
Chromosome	4p	11p15.5	Xq26
Primer sequences	5′-TGA-TAG-CTA-GAA-AGC-TAG-CAA-G-3′ 5′-GCT-CAT-TAA-ACA-CTG-TGT-TCC-T-3′	5′-GTG-GGC-TGA-AAA-GCT-CCC-GAT-TAT-3′ 5′-ATT-CAA-AGG-GTA-TCT-GGG-CTC-TGG-3′	5′-ATG-CCA-CAG-ATA-ATA-CAC-ATC-CCC-3′ 5′-CTC-TCC-AGA-ATA-GTT-AGA-TGT-AGG-3′
Amplified product	139–163 bp	183–207 bp	263–299 bp
Repeat motif	(GATA)*n*	(AATG)*n*	(AGAT)*n*
Number of alleles in population	7	8	11
Number of repeats found in alleles of our PCR products	11, 6	10, 9	14, 12
Published population frequencies of above alleles	0.12, 0.02	.35, .14	.16, .34
Calculated genotype frequencies	2 (0.12 × 0.02) = 0.0048	2 (0.35 × 0.14) = 0.0980	2 (0.16 × 0.34) = 0.1088

The number of repeats in all of the alleles was determined by sequencing the PCR products (GABARB1 alleles shown in Figure 2), or by measurement of relative band position on autoradiographs. Published allele population frequencies[34,35] were used to calculate the genotypic frequencies.

hour, heat-dried in a vacuum gel drying apparatus, and then exposed to film for 1 to 7 days.

Microsatellite sequencing

PCR reactions for sequencing were performed in larger 300-μl reaction volumes. The proportion of all reagents was the same as described above, except that [^{35}S]dATP was eliminated and 200 μM dATP was used. The completed reaction mixtures were combined with DNA sample loading buffer (6X TBE, 50% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol) and resolved electrophoretically in 6% NuSieve GTG agarose gels (FMC BioProducts, Rockland, ME) run at 20 W for 5 hours. Bands containing the resolved alleles were excised from the gel and suspended in equal volumes of 2.5X TAE buffer/0.5 mol/L NaCl solution and incubated at 65 C for 10 minutes. The melted mixtures were phenol/chloroform-extracted and the DNAs were ethanol-precipitated in 0.3 mol/L sodium acetate, pelleted, dried, and resuspended in 20 μl TE.

The sequencing reactions were performed by the method of Simon et al.[33] Ten μl of each DNA sample were combined with 1 μl of the appropriate primer (10 ng/μl), denatured at 95 C for 3 minutes, and then annealed at room temperature for 2 minutes. 3.2 μl of labeling reaction mixture (9.6 μl 5X Sequenase buffer, 5.5 μl 0.1 mol/L DTT, 2 μl dH_2O, 0.5 μl [^{32}P]dCTP [Amersham, Arlington Heights, IL, 3.8 μl sequenase, US Biochemical, Cleveland, OH]) were then added to each annealed sample and incubated at room temperature for 2 minutes. 3.4-μl aliquots of the labeling reactions were combined with 2.5-μl aliquots of termination mixture, incubated at 37 C for 5 minutes, and then stopped with 2.5 μl stop solution. Completed reaction mixtures were denatured at 80 C for 3 minutes, and then 2 μl of each sample were resolved in an 8% polyacrylamide sequencing gel run at 1500 V for up to 5 hours. Gels were then exposed to film overnight.

Statistics

Population frequencies[34,35] of the microsatellite alleles identified in our PCR products are shown in Table 1 and were used to calculate genotypic frequencies. The probability that three randomly selected heterologous cell lines would all have identical genotypes at the three microsatellite loci studied was calculated as the cube of the product of the individual locus genotype frequencies.[36]

Results

Autoradiographs of the resolved microsatellite PCR products are shown in Figure 1, demonstrating that the allelic profiles at the TH01, GABARB1, and HPRT loci are identical for SK-OV-3, YAOVBIX1, and OC436 cells. These three cell lines also had identical allelic profiles at seven other microsatellite loci (data not shown). The fingerprints for SK-BR-3, OVCAR, HeLa, YAOVBIX3, and OC194 cells demonstrate the variability of profiles for heterologous cell lines.

Using DNAs from SK-OV-3 cells, the sequence of each microsatellite locus was determined to verify the identity of the loci in our PCR products and to determine the number of repeats for the purpose of

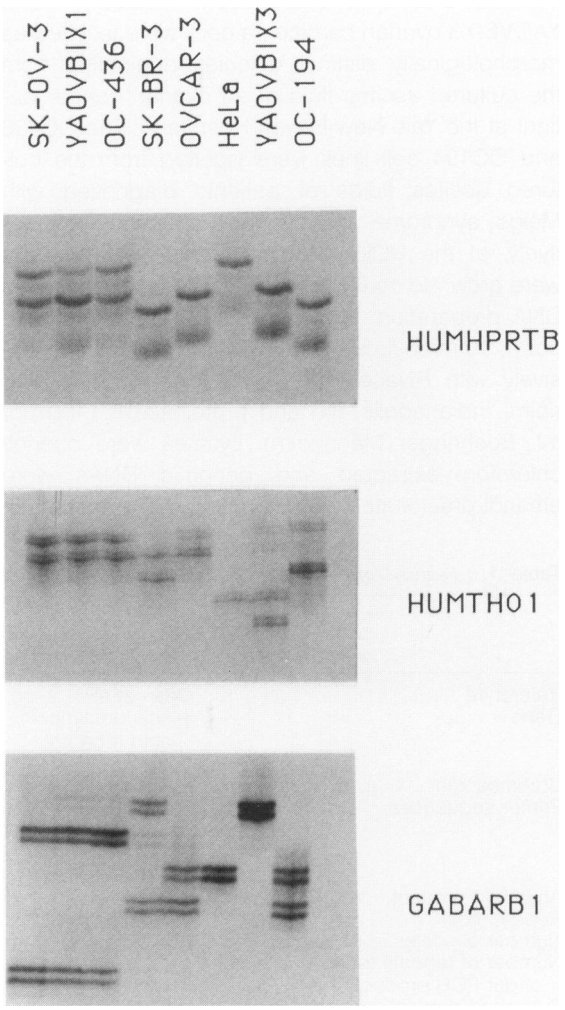

Figure 1. *PCR-based DNA typing analysis showing identical profiles for the SK-OV-3, YAOVBIX1, and OC436 cell lines at three microsatellite loci. Panels at* top, center, *and* bottom *show the allelic profiles at the HPRTB, TH01, and GABARB1 loci, respectively. SK-BR-3, OVCAR, HeLa, YAOVBIX3, and OC194 cells demonstrate the variability of profiles for heterologous cell lines.*

allele identification (Table 1). Figure 2 shows the sequence and number of repeat motifs found at the GABARB1 locus in SK-OV-3 cells. At the GABARB1 locus, at which both alleles were sequenced, the (GATA)*n* sequence was repeated 11 times in one allele and 6 times in the other. At the TH01 and HPRTB loci, the longer alleles were sequenced and the lengths of the shorter alleles were deduced by measuring relative band positions on the autoradiographs. At the TH01 locus the (AATG)*n* sequence was repeated 10 times in the longer allele and 9 times in the shorter allele. At the HPRTB locus the (AGAT)*n* sequence was repeated 14 times in the longer allele and 12 times in the other.

The probability of finding a given allele in a cell line equals the population frequency of that allele.[36] Population frequencies for the alleles identified in our PCR products were obtained from previously published studies,[34,35] and are listed in Table 1. These allele frequencies were used to calculate the genotypic frequencies for each of the three loci studied. Assuming Hardy-Weinberg equilibrium, the genotypic frequency for a heterozygote locus is defined as $2(PaPb)$, where P is the probability (population frequency) associated with alleles a and b. The genotypic frequencies of the GABARB1, TH01, and HPRTB allelotypes found in our PCR products are thus $2(0.12 \times 0.02) = 0.0048$, $2(0.35 \times 0.14) = 0.098$, and $2(0.16 \times 0.34) = 0.1088$, respectively. With alleles at multiple loci, the probability of a profile match is the product of the individual locus genotype frequencies.[36] Therefore, the probability that three randomly selected heterologous cell lines would have identical profiles at all three microsatellite loci studied is $[(0.0048) \times (0.098) \times (0.1088)]^3 = 1.32 \times 10^{-13}$. On the basis of the ob-

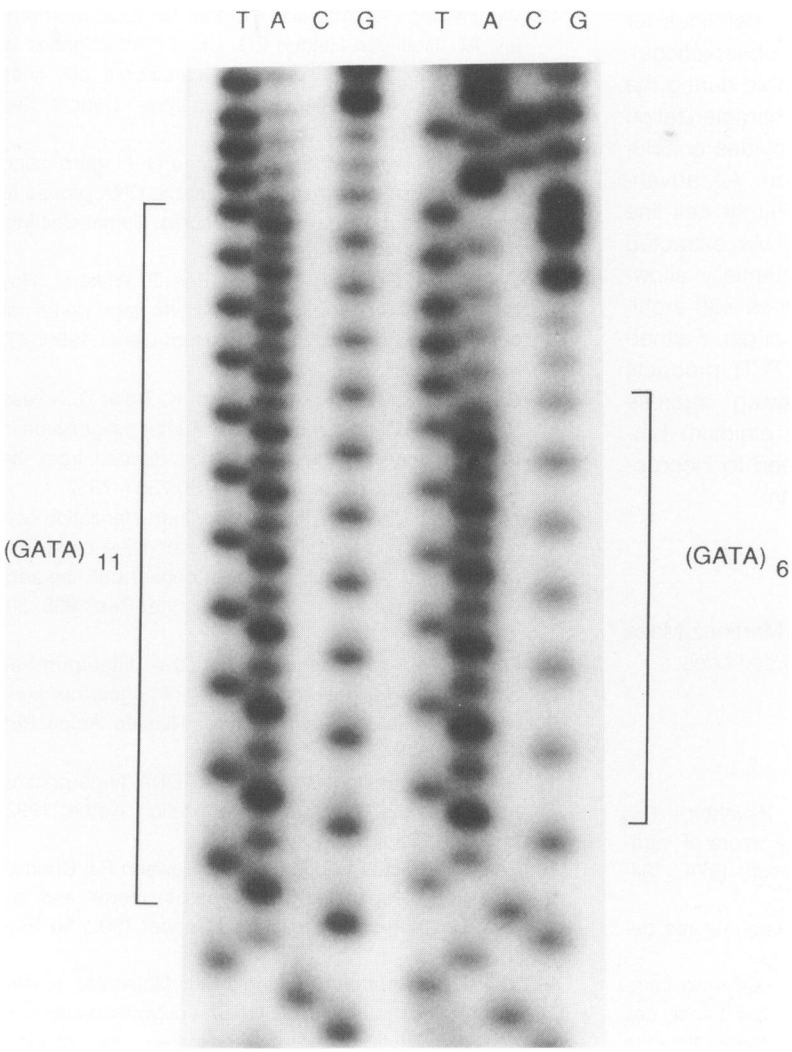

Figure 2. *Sequences showing the number of GATA repeats in both alleles at the GABARB1 locus in SK-OV-3 cells.*

served profile match, the SK-OV-3, YAOVBIX1, and OC436 cells all appear to be from the same individual.

Discussion

We have found the PCR-based analysis of microsatellite loci to be a technically straightforward method for determining cell line identity and have used it to confirm suspected cell contamination events involving the SK-OV-3 ovarian tumor cell line in two isolated laboratories. The resolution of discrete alleles in this system allows for the unequivocal interpretation of autoradiographs and allows for a definitive calculation of match probabilities between allelic profiles. Although microsatellites have recently been shown to be unstable in "replication error positive" (RER^+) tumors and tumor cell lines,[37–42] we have found the tetranucleotide loci analyzed in this study to be stable in cell lines for up to 400 passages (unpublished observations). Thus, microsatellites appear to be stable during the passage of RER^- cell lines, and the characterization of identical alleles at multiple loci provides conclusive evidence of cross contamination. An advantage of the PCR-based method for tumor cell line identity is that it can be used on DNAs extracted from paraffin-embedded tissues, potentially allowing for identity comparisons of cell lines with archival material containing the tissue of origin. Furthermore, tetranucleotide microsatellite PCR products can also be resolved in high-sieving agarose gels,[43] allowing for visualization by ethidium bromide staining and eliminating the need to incorporate radioactivity into the PCR reaction.

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