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Rapid method for relative gene expression determination in human tissues using automated capillary gel electrophoresis and multicolor detection

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Abstract

The aim of this study was to evaluate a direct and automated post-polymerase chain reaction (PCR) detection system to simultaneously determine the relative gene expression levels of nine cancer-related human genes. Total RNA was prepared from flash-frozen biopsies derived from human colorectal tumors or normal mucosa and reverse-transcribed to cDNA which was PCR-amplified using primer pairs corresponding to the studied genes. In each reaction, the forward primer was labeled with a fluorescent dye. The PCR products were pooled and an internal size standard with a uniquely colored fluorescent dye was added. The samples were then subjected to automated capillary gel electrophoresis. Fragment analysis software was used to calculate the relative gene expression using β-actin as the reference gene. We found that automated capillary gel electrophoresis with multicolor detection is a rapid, accurate and highly reproducible method for separation and quantification of PCR-amplified cDNA. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Genes; Multicolor detection; Polymerase chain reaction; RNA; DNA

1. Introduction

In order to individualize cancer chemotherapy, information about the gene expression pattern in tumor tissues is crucial. Quantitative analysis of gene expression has proven to be useful in this regard. It has e.g., recently been described that the gene expression level of thymidylate synthase (TS) correlates with the clinical outcome of 5-fluorouracil-based chemotherapy in the treatment of patients with gastric cancer [1]. With the development of polymerase chain reaction (PCR)-based methods for quantitation of cDNA it has now become possible to analyze minute amounts of RNA, e.g., extracted from small needle biopsies [2]. Capillary gel electrophoresis is a relatively novel method which provides fast, high-resolution of PCR products [3]. It has been successfully used for identification of cancer related gene expression patterns in eukaryotic cells and tissues [4], for detection of telomerase activity in somatic cells [5], and for quantitative analysis of HIV-1 cDNA fragments in sera [6].

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The purpose of the present study was to explore the feasibility of using a direct and automated post-PCR detection system for quantitative analysis of reverse-transcribed PCR products. The method is based on an automated capillary gel electrophoresis instrument (Perkin-Elmer ABI Prism 310 Genetic Analyzer) which separates the DNA molecules according to size [7]. Fragments as long as 500 nucleotides can easily be resolved in 28 min with a resolution limit of two nucleotides. If the samples are differentiated by dye color or by PCR product length, multiple samples can be run in a single capillary. The sensitivity of the method makes it possible to detect low abundance transcripts.

Applying the described method we have determined the relative gene expression levels of the cancer-related genes TS, folylpolyglutamate synthase (FPGS), γ-glutamyl hydrolase (GGH), dihydrofolate reductase (DHFR), tumor protein p53 (TP53), and Yamaguchi sarcoma viral oncogene (YES1) in normal and malignant tissues derived from eight patients with colorectal cancer. Furthermore, the low abundance transcripts of the genes deleted in colorectal cancer (DCC) and plasminogen activator inhibitor 2 (PAI2) were detected in the same material by fragment analysis. The methodology that we have used is suitable in different clinical protocols where direct determination of the relative gene expression levels of multiple genes is needed.

2. Experimental

2.1. Preparation of cDNA

Samples of colorectal cancer ($n=8$) together with adjacent normal colorectal mucosa ($n=8$) were excised fresh from operative specimens. The biopsies were snap frozen in liquid nitrogen and stored at −70°C until used. The cDNA was prepared from $4\times4\times2$ mm^3 of tissue by ProGene Lab (Uppsala, Sweden) using the First-Strand cDNA Synthesis Kit and pd(N)$_6$ Primer (Amersham) according to the suppliers instructions.

2.2. PCR primers and ABI Prism dyes

The forward primer of each primer pair was labeled with a fluorescent tag attached to the 5′-end (Table 1). The fluorescent dyes used were 6-carboxyfluorescein (6-FAM), 4,7,2′,7′-tetrachloro-6-carboxyfluorescein (TET), 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein (HEX), or *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (TAMRA). The Gene Scan-500 standard (Perkin-Elmer), sizing fragments between 35 and 500 nucleotides, was used as a size marker. The standard consisted of double-stranded DNA fragments which were single-strand labeled with the fluorescent dye TAMRA.

2.3. PCR conditions

Serial dilutions of the cDNA were PCR amplified in a thermal cycler (Perkin-Elmer Gene Amp PCR system 9600). The compositions of the master amplification mixtures used are described in Table 2. The amplification was initiated by denaturation of the cDNA at 95°C for 3 min followed by a series of five cycles at 96°C for 1 min (denaturation), 65°C for 1 min (annealing), and 72°C for 1 min (extension). Next, a series of 30 cycles at 96°C for 30 s, 65°C for 30 s, and 72°C for 30 s was performed. A 7-min extension step at 72°C was added to the last cycle. There was no need for purification or concentration of the PCR product before detection on the ABI Prism 310 Genetic Analyzer.

2.4. Capillary gel electrophoresis on ABI Prism 310

The different PCR products from each respective dilution series were pooled and mixed in one vial and 1 μl of the mix was added to a tube containing 0.5 μl size standard and 12 μl of formamide. The samples were denatured for 3 min at 95°C, snap-cooled, and mixed briefly before they were analyzed using an automated capillary electrophoresis device (Perkin-Elmer ABI Prism 310 Genetic Analyzer). The 47 cm (36 cm to detector)×50 μm I.D. capillary (ABI Prism 310 Genetic Analyzer Capillary, P/N 402839) was automatically filled with polymer (Perkin-Elmer POP-4, P/N 402838) and a few molecules of the denatured PCR sample were electrokinetically injected from the vial into the capillary for 5 s at 15 kV. The autosampler sequentially loaded 48 samples into the capillary for electrophoresis. As a running buffer, Gene Scan Analyzer Buffer (Perkin-Elmer P/N 402824) containing EDTA was used. Electro-

Table 1
Primer DNA sequences, PCR product length, and forward primer labeling dye used in each of the specific PCR amplifications

Gene	Forward primer	Reverse primer	PCR product (bp)	Forward primer dye	Genbank accession No.
β-Actin	A1: GCGGGAAATCGTGCGTTTGACATT Base position 656–677	A2: GATGGAGTTGAAGGTAGTTTCGTG Base position 864–887	231	6-FAM	M10278
DCC	2834: CCCAGACTAACTGCATCATCATGAG Base position 2207–2231	3151L: CACCTACTGGTGGGAGCAT Base position 2530–2548	281, 341	TET	X76132
DHFR	DHFR: ACATGGGCATCGGCAAGAACG Base position 83–103	DHFR72: GTCGATTCTTCTCAGGAATGG Base position 221–242	159	TET	J00140
FPGS1	24: AGCCCGGACCTCTGGAGTG Base position 1391–1409	242: CTGCCAGTGACTAGCACATGG Base position 1633–1653	262	TET	M98045
FPGS2	224U19: GACCGGCTGAACATCATCC Base position 224–242	535L19: AATGCCCACCTCCACCACT Base position 535–553	332	HEX	M98045
GGH	783U20: CAGTGGCATCCAGAGAAAGC Base position 783–802	1123L21: AGAATCAGAGCCAGGCACATT Base position 1123–1143	360	6-FAM	U55206
PAI2	251U21: TTACCCCCATGACTCCAGAGA Base position 251–271	633L21: ACAGCATTCACCAGGACCATC Base position 633–653	402	HEX	J03603
TP53	P53U: CTTGGGCCTGTGTTATCTCCTAG Base position 13 977–13 999	P53L: CAGGGTGGCAAGTGGCTCCTGAC Base position 14 110–14 132	155	6-FAM	X54156
TS	TS-1: GGAGTTGACCAACTGCAAAGAGTG Base position 11 857–11 880	60: GATGTGCGCAATCATGTACGTGAG Base position 13 589–13 612	254	HEX	D00596
YES1	648U18: AGCGCCTGCAGATTCCAT Base position 648–665	1066L18: CCACACTTCGCCGAAACA Base position 1066–1083	435	6-FAM	M15990

Table 2
Master-mix composition

Gene group	Taq buffer (μl)	25 m*M* $MgCl_2$ (μl)	10 m*M* dNTPs[d] (μl)	12.5 μ*M* primers, forward/reverse (μl)	Water (μl)	Taq polymerase (0.3182 U/μl)	Diluted cDNA (μl)
1[a]	2.5	1.2	0.5	1/1	6.8	2	10
2[b]	2.5	1.9	0.5	2/2	4.1	2	10
3[c]	2.5	1.9	0.5	1/1	6.1	2	10

[a] FPGS1.
[b] DCC and PAI2.
[c] TS, FPGS2, GGH, DHFR, TP53 and YES1.
[d] Deoxynucleotide triphosphates.

phoresis was performed at 15 kV for 28 min at 60°C. All fluorescent dyes were detected by filter set C. A fluorescent dye matrix (Perkin-Elmer Fluorescent Amidite Matrix Standard, P/N 401546) was created and used to compensate the overlap in the fluorescence spectra.

2.5. Calculation of the relative gene expression levels

To size and quantify the DNA fragments the data collected was analyzed by the GeneScan Analysis software. The peak areas of each gene fragment were plotted against the dilution factor and a saturation curve was achieved. The linear range of PCR amplification was determined for each gene and the slope was calculated. The slope of the linear PCR range for each respective target gene was divided with the linear range slope of the reference gene, β-actin [8].

2.6. Statistical analysis

Differences in the gene expression levels between normal and cancerous tissue were statistically tested (two-sided) using the paired Student's *t*-test (Table 3). Statistical values of $P \leq 0.05$ were considered significant.

3. Results and discussion

In the present study we have used the ABI Prism 310 Genetic Analyzer instrument for quantitative analysis of reverse-transcribed PCR products. The method, which is based on separation of DNA molecules multiplexed by size and dye color, makes it possible to distinguish between multiple DNA fragments electrophoresed in a single capillary. Since the standard and the unknown fragments are subjected to exactly the same electrophoretic forces, problems like lane-to-lane or run-to-run variation can be

Table 3
Relative gene expression in colorectal tissue samples (gene/β-actin$\cdot 10^{-4}$)

Gene	Colorectal cancer			Normal mucosa			*P* value
	n[a]	Median	Range	*n*[a]	Median	Range	
FPGS1	8	29.4	23.9–50.4	5	28.5	5.1–34.8	0.36
GGH	8	45.2	17.2–64.5	4	24.2	6.0–45.2	0.0069
TS	8	47.6	22.3–60.5	6	21.1	10.1–42.1	0.0013
DHFR	4	33.0	24.5–39.4	4	45.8	18.3–68.8	0.37
YES1	7	21.7	17.5–67.7	5	33.6	24.5–49.1	0.060
TP53	4	19.5	6.1–143.4	4	16.8	12.8–28.2	0.46

[a] *n*, Number of cDNAs analyzed.

eliminated. The capillary length, the sample injection time and the electric field strength have an effect on the resolution of the DNA fragments [7]. Thus, each of these parameters must be optimized before the fragment analysis is performed.

Using the described method, we have determined the relative gene expression levels of the cancer-related genes TS, FPGS, GGH, DHFR, TP53, and YES1 in normal and malignant tissues derived from patients with colorectal cancer (Fig. 1). Furthermore, since capillary gel electrophoresis is an extremely sensitive method, having a detection limit at the attomole level [9], it was possible to detect the low abundance cellular transcripts of the genes DCC and

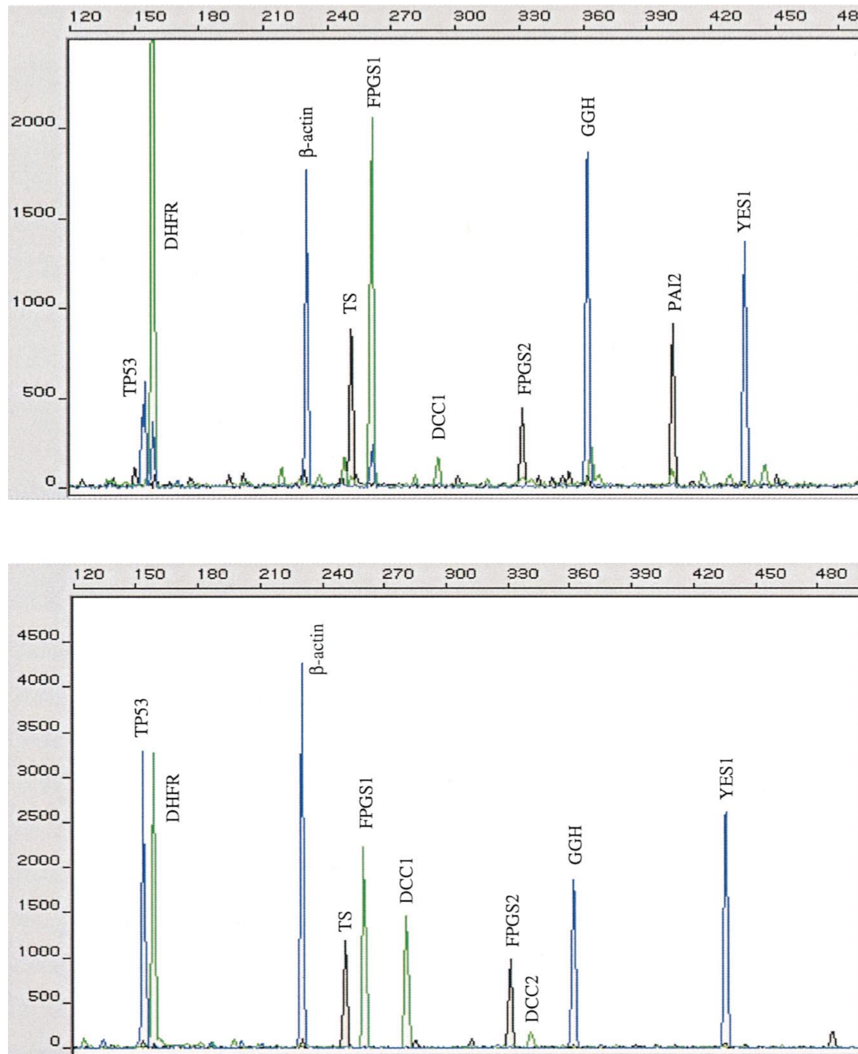

Fig. 1. Electropherogram data showing the expression pattern of the genes TP53, DHFR, β-actin, TS, FPGS, DCC, GGH, PAI2 and YES1 in colorectal tumor tissue (top) and colorectal mucosa (bottom) from one of the patients. FPGS1 and FPGS2 refer to PCR products amplified by different primer pairs. DCC1 and DCC2 refer to alternative splicing products amplified by the same primer pair. The molecular mass in base pairs is displayed across the horizontal axis at the top of the electropherogram and the relative peak fluorescent intensity is along the vertical axis.

PAI2 (Fig. 1). The results showed that DCC gene expression could be detected in normal as well as in tumor tissues whereas PAI2 gene expression could only be detected in tumor tissues. Using the running conditions described above, the expression levels of these two genes were too low to be quantified. Since we were merely interested in finding out whether DCC and PAI2 were active or not in the analyzed tissues we did not optimize the running conditions further. However, enhancement of the fluorescent signal by modification of the injection time and/or the voltage during the run would make it possible to quantify even low abundance transcripts.

The intra-variation of the peak areas for the standard and the samples, calculated as (SD/mean× 100), was found to be 2% (three replicates, n=8). When the same samples were re-analyzed after 24 h, a slight increase in the intra-variation to 6.2% was observed. This increase was presumably caused by a partial DNA renaturation of the samples. There was no difference in the intra-variation whether calculated as the peak area or as the peak height.

The relative gene expression levels were calculated by dividing the slope of the linear PCR range for each respective target gene with the linear range slope of the reference gene, β-actin [10] (Table 3). The intra-assay gene expression variation was found to be 3.2% whereas the inter-assay variation of two discrete measurements of each gene was 17% (n= 42). These results are compatible with those obtained previously by high-performance liquid chromatography (HPLC) (intra-assay variation 2.7% and inter-assay variation 18.5%, n=96) [11,12].

The relative expression levels of cancer-related genes like TS may vary greatly among different individuals [13]. Still, significant differences can often be found between normal and cancerous tissues when large studies are performed. Though the number of patients in the present study was small the result regarding the relative TS gene expression was consistent with those obtained previously by us [11,13] and by others [14] using similar methods. The statistical analysis showed that the relative TS (and GGH) gene expression levels were significantly different between cancerous and normal tissues (Table 3). As seen in the table, the relative TS gene expression was 2.3-times higher in the colorectal cancer tissues than in the normal mucosa. The same result was found when the relative TS gene expression in the same samples (cDNAs) was determined using real-time PCR (unpublished data) [15].

In conclusion, we found that compared with traditional methods, automated capillary gel electrophoresis with multicolor detection is a rapid, accurate and highly reproducible method for separation and quantitation of PCR-amplified cDNA. The major advantages of using this technique for gene expression studies are the following: minute amounts of DNA are required, several samples can be mixed, there is no need for isotope handling, the through-put and sensitivity of the analysis is high, the gel electrophoresis step is completely automated, and the resolution of DNA separation is high [9]. The ABI Prism 310 Genetic Analyzer has proven to be a useful instrument which, in addition to quantitative studies, can be utilized for sequencing, genotyping and sizing of nucleic acids.

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