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# Relative mRNA expression of the lactate dehydrogenase A and B subunits as determined by simultaneous amplification and single strand conformation polymorphism
## Relation with subunit enzyme activity

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**Abstract**

To explore if it is correlated in human tumor cells that the expression of LDH homologous gene and LDH isoenzymes, we used RT-PCR-SSCP technique to measure the relative expression of genes with homologous sequences. The combination of PCR using common primers designed in the highly conserved regions and single-strand conformation polymorphism analysis of the products is used for quantitative determination of the proportions of LDH-A mRNA in human cancer cell lines. The proportion is compared with that of the activities of isoenzymes. The results indicated that the enzyme activity of LDH-A was consistent with mRNA levels in the human tumor cell. The present procedure using a single pair of primers for two fragments can overcome disadvantages in quantitative analysis using multiplex PCR. Template concentrations and PCR cycles did not affect the proportions of LDH-A and LDH-B in the product.


*Keywords:* Expression; Single strand conformation polymorphism; Enzymes; Lactate dehydrogenase; Homologous gene

## 1. Introduction

In mammals, five tetrameric isoenzymes of lactate dehydrogenase (E.C.1.1.1.27; LDH) are found in various proportions in different somatic tissues and are formed by combination of the LDH-A (M) and LDH-B (H) subunits [1]. The LDH-B gene is located on chromosome 12, whereas the LDH-A gene is located on chromosome 11. Determination of the exon–intron organization of human LDH-A and LDH-B genes has shown that the protein-coding sequences of these LDH genes are interrupted by six introns at homologous positions [2,3].

The determination of LDH isoenzymes was the first routine isoenzyme technique to be widely adopted by clinical laboratories. Many reports emphasize the increase in A subunits in tumors of all origins [4,5]. Therefore, LDH isoenzymes may serve

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as a useful tumor marker. However, it is mostly determined at the level of enzyme activity. Sass et al. described that the proportion of LDH-A is similar between mRNA and proteins in rabbit muscle [6]. On the other hand, Marieze et al. [7] reported mRNA abundance was not correlated with the enzyme activity in rabbit muscle during development. In this study, we measured the expression of LDH gene and LDH isoenzymes to explore if it is correlated in human tumor cells. We analyzed the proportion of two mRNA species from various cancer cell lines by RT-PCR-SSCP, and compared with the proportion of the activities of isoenzymes.

## 2. Experimental

### 2.1. Cell lines

The human cancer cell lines used in this study were A549, SPC-A1, PC-12, HL-60, LS-174T, obtained from the Tumor Division of the Xinqiao Hospital (Chongqing, PRC). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum.

### 2.2. RNA isolation and cDNA synthesis

Total RNA was prepared from various cancer cell lines using Tirpure-Isolation-Reagent (Roche, USA). Reverse transcriptase reactions were carried out using AMV reverse transcriptase (Sigma) with random hexamers as primers.

### 2.3. Design and synthesis of primer for LDH

In order to coamplify both LDH-A and LDH-B cDNA, a set of two PCR primers to amplify the conserved regions of LDH cDNA sequences were designed (Fig. 1). Both primers contained several mismatches to the target sequences of either LDH-A or LDH-B cDNA. The nucleotide sequences of the primers thus designed and synthesized were 5′-TGGCAGATGAACTTGCTCTTGT-3′ (forward) and 5′-CTCTCTCCCTCTTGCTGACG-3′ (reverse). The primer sequence corresponded to nucleotide positions 228–249 and 392–411 for LDH-A (188–209 and 352–371 for LDH-B). It yields a product of 184 bp from both LDH-A and LDH-B cDNAs. We also designed specific primers for LDH-A and LDH-B to make cDNA fragments containing sequences of either LDH-A or LDH-B. The nucleotide sequences of the primers were 5′-GCCCGACGTGCATTCCC-GATTCCTT-3′ (forward) and 5′-GACGGCTTTCT-CCCTCTTGCTGACG-3′ (reverse) for LDH-A. This primer sequence corresponded to nucleotide positions 56–80 and 392–416 (361 bp). The nucleotide sequences of LDH-B specific primers were 5′-GGCAACAGTTCCAAACAATA-3′ (forward) and 5′-TAAGAATGTCCACTGGGTTG-3′ (reverse). The primer sequence corresponded to nucleotide positions 102–121 and 368–487 (386 bp).

### 2.4. PCR procedures

The LDH cDNAs were amplified by PCR using a homologous primer. The reaction mixture (25 μl) contained 10 m*M* Tris–HCl, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* each of 4 dNTPs, 10 pmol/ul each of primers, 2.5 Units of ampliTaq DNA polymerase and 2 μl of the cDNA prepared above. PCR conditions are as follows: pre-PCR incubation at 95 °C for 3 min; denature at 95 °C for 30 s; annealing at 60 °C for 30 s; extension at 72 °C for 45 s; 35 cycles; final extension at 72 °C for 10 min. After PCR, 5 μl product was mixed with 1 μl of 10×loading dye and then run on 1% agarose gel. Electrophoresis was carried out at 80 V at ambient temperature. The bands on the gels were visualized by ethidium bromide staining.

### 2.5. SSCP analysis

A 5-μl volume of the amplified products was mixed with 5 μl of denaturing solution (containing 98% deionized formamide, 10 m*M* EDTA, 0.025 bromophenol blue, 0.025% xylene cyanol FF) and heated at 95 °C for 5 min followed by rapid cooling on ice. Then 10 μl of the samples were applied to an 8% polyacrylamide gel. Electrophoresis was performed at 40 W for 8 h at 4 °C using 1×TE buffer as a running solution. After electrophoresis, silver-staining procedure was used to visualize the bands. Electrophoresis profiles were analyzed by the software, Gel-Pro3.1 (Media Cybernetics, USA). The

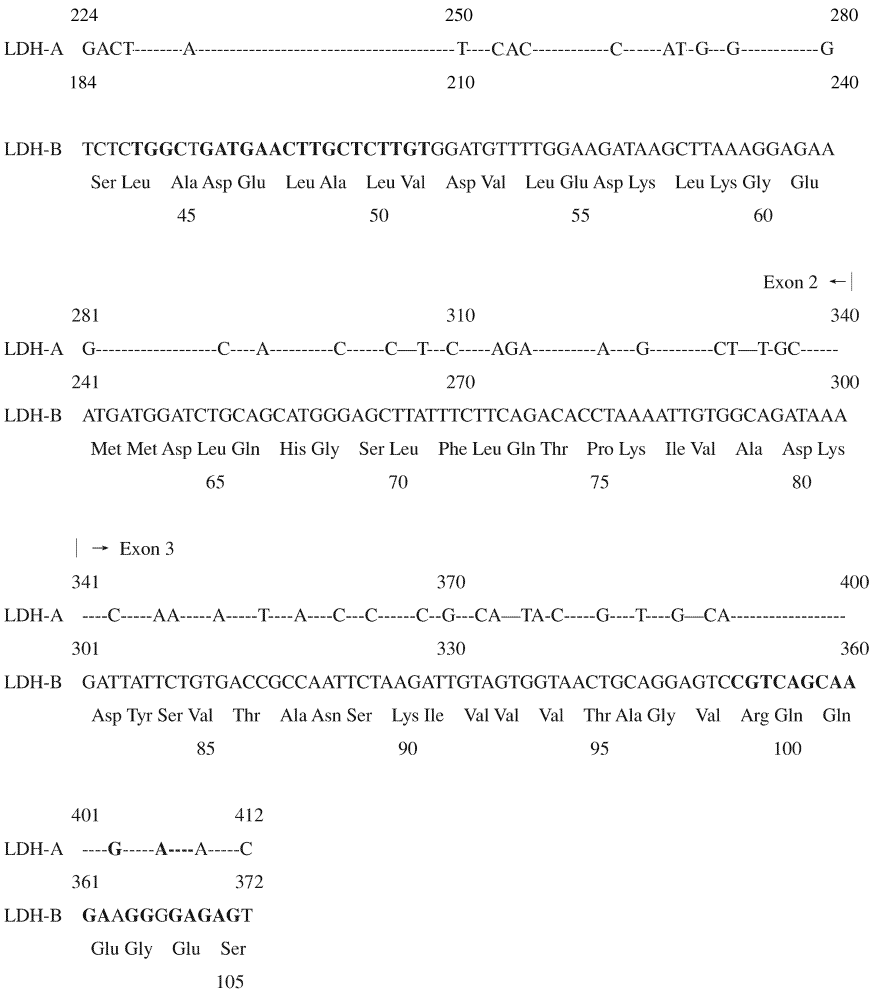


Fig. 1. Homology alignment of the nucleotide and amino acid sequences highly conserved in human LDH-A and PCR primer sequences for LDH cDNA. Part of the nucleotide sequences of LDH-B and those of LDH-A that differ from those of LDH-B are indicated. Identical nucleotides are indicated by hyphens and primer sequences are denoted by bold characters. The numbering of the nucleotide is in the top. The GenBank accession numbers for the LDH-A and LDH-B nucleotide sequences in this paper are X02152 and Y00711 respectively.

proportion of LDH-A cDNA fragments to total LDH cDNA fragments was calculated as LDH-A versus LDH-B plus LDH-A, from the integrated optical density of LDH-A and LDH-B.

### *2.6. Genomic sequencing*

Genomic sequencing was used to confirm the results of SSCP. The two electrophoretic bands were cut from the gels separately, reamplified and sequenced using ABI PRISM (PE Applied Biosystems).

### *2.7. LDH isoenzyme analysis*

Cells in suspension culture were centrifuged and washed with phosphate buffered saline (PBS) three times. The cell pellets were then resuspended in PBS, and lysed by ultrasound on ice. The homogenates were centrifuged at 15 000 rpm for 20 min. Supernatants were collected, and supplied for LDH isoenzyme analysis. LDH isoenzymes were electrophoretically fractionated at 150 V for 30 min with Tris–HCl–Barbital–EDTA buffer (pH 8.6) on terylene cellulose acetate membrane supporting

media [8]. Activity bands were made visible by use of D,L-lactate and NAD as the substrate, phenazine methosulfate (PMS) as the intermediate, and methyl thiazolyl tetrazolium (MTT) as the final hydrogen acceptor. The final concentration of the single-reagent mixture for LDH isoenzyme staining was as follows: 120 m*M* D,L-lactate; 5 m*M* NAD; 2 m*M* MTT; 0.08 m*M* PMS; and 50 m*M* Tris–HCl (pH 7.4). The LDH isoenzyme staining reaction was performed at 37 °C for 25 min (Sandwich method).

### *2.8. The proportion of the activities of two isoenzymes*

Because LDH exists in a tetrameric form by combination of A and B subunits in various proportions in different tissues, forming five isoenzymes, namely, LDH-1 (B4), LDH-2 (B3A1), LDH-3 (B2A2), LDH-4 (B1A3) and LDH-5 (A4), by random tetramer formation. Therefore, the *A* (%) activity level from the percentage of the fractionated five isoenzymes can be calculated using the following formula:

$$A\,(\%) = \mathrm{LDH5}\,(\%) + 0.75 \times \mathrm{LDH4}\,(\%) + 0.5 \times \mathrm{LDH3}\,(\%) + 0.25 \times \mathrm{LDH2}\,(\%)$$

## 3. Results

### *3.1. PCR products by common primers and specific primers*

Fig. 2 is the result of agarose gel electrophoresis of the PCR product.

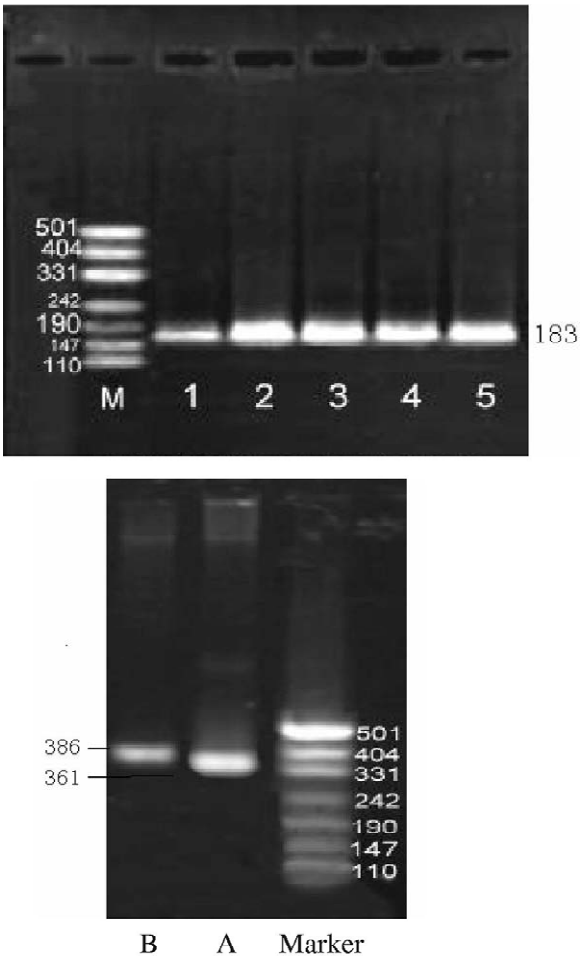


Fig. 2. The results of agarose gel electrophoresis of PCR product. Above: PCR with common primers; below: PCR with specific primers. M, marker; 1, PC-12; 2, LS-174T; 3, HL-60; 4, A549; 5, SPC-A1; A, LDH-A band; B, LDH-B band.

### *3.2. The result of SSCP analysis*

The forward primer anneals LDH-A and LDH-B sequences with zero and one mismatch and reverse primer anneals LDH-A and LDH-B sequences with one and two mismatches, respectively (Fig. 1). The LDH-A or LDH-B could be amplified by the primers described above from solutions containing LDH-A or LDH-B cDNA, respectively. After PCR-SSCP, the bands on the gels were visualized by silver staining procedure. Fig. 3 is the SSCP electrophoresis results.

### *3.3. LDH-A proportion in various cell lines*

Table 1 and Fig. 4 are the LDH-A proportions to total LDH in various cell lines. LDH isoenzyme activity, and LDH-A and LDH-B expressions at mRNA level for five cancer cell lines were analyzed. The *A* (%) calculated from percentage of each isoenzyme ranged from 33.8 to 71.9%. At the mRNA level their LDH-A (%) were calculated to be from 37.9 to 83.7% by the present RT-PCR-SSCP procedure. The proportion of LDH-A mRNA corre-

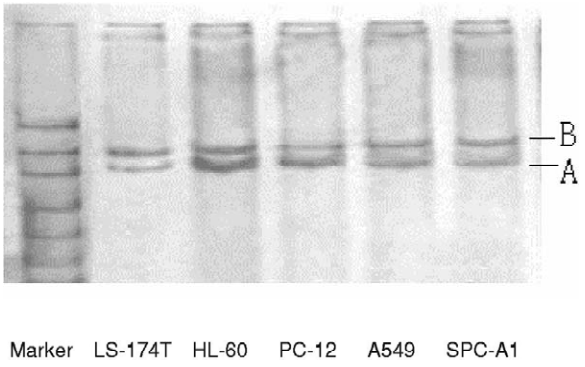


Fig. 3. The SSCP electrophoresis results A: LDH-A; B: LDH-B.

lated well with that determined by zymograms at activity level ($r = 0.9978$).

## 4. Discussion

Many kinds of techniques are being applied to elucidation of genomic or somatic mutations in molecular diagnostic laboratories. Single strand conformation polymorphism (SSCP) analysis has been used to detect genetic polymorphisms, mutations of short nucleotide sequences including single-base substitutions and those of large DNA regions [9–12].

Table 1
Proportions of LDH-A to total LDH in various cell lines

| Cell line | mRNA A (%) | LDH isoenzyme activity (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | A (%) | LDH-1 | LDH-2 | LDH-3 | LDH-4 | LDH-5 |
| A549 | 85.6 | 71.7 | 1.0 | 12.6 | 16.3 | 38.9 | 31.2 |
| SPC-A1 | 83.7 | 71.9 | 1.1 | 11.7 | 16.6 | 39.8 | 31.1 |
| LS-174T | 37.9 | 33.8 | 15.2 | 39.9 | 39.1 | 5.9 | 0.1 |
| HL-60 | 54.2 | 47.9 | 3.4 | 31.6 | 37.6 | 24.7 | 2.7 |
| PC-12 | 57.4 | 48.0 | 8.8 | 27.9 | 31.3 | 26.7 | 5.2 |

A549: lung adenocarcinoma cell; SPC-A1: lung adenocarcinoma cell; LS-174T: colon cancer cell; HL-60: leukemic cell; PC-12: pheochromocytoma cell.

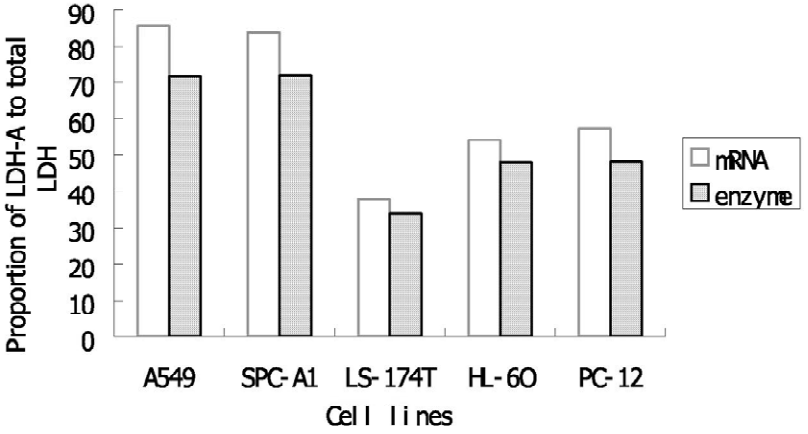


Fig. 4. LDH-A proportions to total LDH in various cell lines. The correlation coefficient of mRNA proportion to enzyme proportion was 0.9978.

Table 2
Dependency of cycle number of PCR and dilution of the cDNA samples on LDH-A proportions

| Cell line | Dilution factor | Cycle number of PCR | | | | | |
|---|---|---|---|---|---|---|---|
| | | 35 | 40 | 45 | Mean | SD | C.V. (%) |
| A549 | 1 | 83.9 | 83.5 | 81.8 | 83.1 | 1.12 | 1.34 |
| | 100 | 84.1 | 86.2 | 85.1 | 85.1 | 1.05 | 1.23 |
| LS-174T | 1 | 39.6 | 36.1 | 37.8 | 37.8 | 1.75 | 4.62 |
| | 100 | 38.4 | 37.2 | 39.5 | 38.4 | 1.15 | 2.99 |
| PC-12 | 1 | 54.5 | 58.2 | 53.6 | 56.5 | 2.44 | 4.32 |
| | 100 | 58.6 | 54.1 | 59.9 | 57.5 | 3.04 | 5.29 |

The procedure, which can separate two or more alleles carrying nucleotide sequence changes, relies on the fact that single-stranded DNA under certain conditions has its own unique conformation and electrophoretic mobility in a non-denaturing polyacrylamide gel. The separated bands can be detected by labeling with radioisotopes or fluorescence, and also by silver staining.

There are some methods of quantitative analyses of mRNA using RT-PCR. Three procedures [13–15] are often used: one is end-point assay in comparison with internal standard; the second is competitive PCR; and the third is real-time monitoring PCR. End-point assay in comparison with internal standard is very simple and there is no effect of efficiency of mRNA extraction and cDNA synthesis. But it has some disadvantages due to the plateau effect of PCR, namely, whether target DNA and internal standard show the same amplification efficiency, and whether they are in their phase of logarithmic increase. Competitive PCR is independent of the plateau effect, but as the name implies, it utilizes synthetic competitor RNA within the same reaction as the sample RNA. It is rather complicated and cumbersome to synthesize the competitor. Real-time RT-PCR technique is very accurate and sensitive to allow a high throughput and can be performed on very small samples [16], but it usually needs a special analyzer.

This study combined RT-PCR with SSCP technique for quantitative detection of expression levels of LDH homologous sequences. The present procedure using a single pair of primers for two fragments can overcome disadvantages in quantitative analysis using multiplex PCR. To understand the influence of template concentrations and PCR cycles to the proportions of LDH-A and LDH-B in the product, we did experiments in different reaction conditions. The amount of cDNA used as a template for PCR may not be constant due to variable efficiency of reverse transcription and stability of cDNA. We checked the effect of diluting the template on the results. We diluted cDNA obtained from three cell lines with distilled water. The proportions of LDH-A in the mixture obtained by the present PCR-SSCP analysis showed satisfactory results. Next, we checked the dependency of results on the number of PCR cycles; 35, 40 and 45 cycles of PCR were carried out. The proportions of LDH-A in the mixture at different cycles of PCR showed similar values. Table 2 shows the influence of PCR cycle numbers and dilution of the cDNA samples on LDH-A proportions. In addition, LDH-A and LDH-B cDNAs amplified by using the specific primers for them were mixed in different ratios. The mixtures were subjected to PCR with common primers followed by SSCP analysis. Determination of the proportion of LDH-A in the mixture confirmed good linearity (Fig. 5).

The character of this method that is different from other quantitative PCR is no internal standards. Many other quantitative PCR techniques have relied on the use of internal standards to monitor the efficiency of the RT-PCR in different reaction tubes. But it is difficult to quantitate mRNA by PCR because small variations in amplification efficiencies among sample tubes can lead to substantial differences in product yield, thereby rendering direct comparisons between samples difficult [17]. The present procedure measures relative ratio of two homologous sequences. The calculated formula is $A/(A+B)$ ($A$,$B$ instead of LDH-A or LDH-B cDNA products). Because all the reaction is in the same tube, the ratio is the same although the quantity of

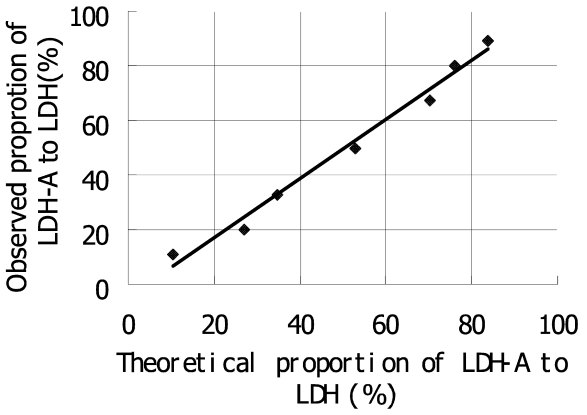


Fig. 5. Quantitative analysis of the proportions of LDH-A cDNA to total LDH cDNA. LDH-A and LDH-B cDNA samples of equivalent molar concentrations were mixed at different ratios (1:8, 1:4, 1:2, 1:1, 2:1, 4:1, 8:1). The DNA fragments were amplified by PCR and analyzed by SSCP to detect proportions of LDH-A mRNA. The correlation coefficient of theoretical proportion to observed proportion was 0.9927.

product may be different. For example, when $A = 1$, $B = 2$, the ratio $= 1/(1 + 2) = 1/3$; when PCR products increase two times, then $A = 2$, $B = 4$, the ratio $= 2/(2 + 4) = 1/3$. Therefore, the result of the present procedure is hardly influenced by the reaction condition. The method also lends itself to the simultaneous examination of multiple samples. The present procedure is not only restricted to applications to investigate genes with homologous sequences, but also may be applied to quantitative determination of relative ratios of mRNA molecules encoded by homologous sequences such as genes for isoenzymes and superfamilies. The primers designed for the present procedure for LDH have several mismatches to the LDH-A and LDH-B sequences. PCR primers with a mismatch at their 3′termini will not be elongated as efficiently as the principle of amplification refractory mutation system [18], while PCR primers with a few mismatches at the position other than the 3′terminus will be elongated successfully as the principle of mismatched PCR [19]. Therefore, a few mismatches in the primer sequence except at the 3′terminus affected little on PCR efficiency.

Previous reports emphasize the increase of LDH-A subunits in many human malignant neoplasms. But it is mostly demonstrated in enzyme activity level. In the investigation of rabbit muscle, the proportion of LDH-A was similar between mRNA and proteins [6]. In the present analysis, it was demonstrated that basic investigations of the procedure were excellent and that the enzyme activity of LDH-A was consistent with mRNA levels in the human tumor cells.

Measurement of protein level or the enzyme activity is the routine test in clinical laboratories. Many factors may influence the test results. By comparing the results of both mRNA and protein test we can understand more about the reason of abnormal results. For example, a cancer patient may have higher ratios of LDH-A mRNA and LDH-A enzyme activity compared with normal control. A cardiac disease patient may have a lower ratio of LDH-A enzyme activity and a normal ratio of LDH-A mRNA. If the ratio would not correlate between mRNA and protein in a patient, then we can deduce that this patient may have two or more kinds of diseases.

In conclusion, this study combined RT-PCR with SSCP technique for quantitative detection of expression levels of LDH homologous sequences and compared with the proportion of the activities of two isoenzymes from various cancer cell lines. The enzyme activity of LDH-A was consistent with mRNA levels in the human tumor cells.

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