Mol. Cells, Vol. 13, No. 3, pp. 481-487

Molecules and Cells

Quantification of Tumor Suppressor mRNA Expression by Poly-competitive RT-PCR Using a TS-IS that Contained Multiple Internal Competitors

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(Received February 2, 2002; Accepted March 22, 2002)

Despite the recent introduction of real-time PCR methods and cDNA microarrays, competitive PCR techniques continue to play an important role in nucleic acid quantification because of the significantly lower cost of equipment and consumables. In this study, we developed a construct, termed tumor suppressor-internal standard (TS-IS) that produced poly-competitive RNA templates as an internal standard to quantify cellular RNA concentration of tumor suppressor genes. This construct is composed of not only sets of primers for detecting the expression of several tumor suppressor genes (such as pRB, $p16^{INK4A}$, $p15^{INK4B}$, $p14^{ARF}$, p53, and $p21^{WAF1}$), but also HPRT as an endogenous marker. Using an internal standard RNA that was synthesized from the TS-IS construct, we were able to establish optimized conditions for the quantification of tumor suppressor genes with minimal amounts (50 ng) of cellular RNA. In addition, the usefulness of this method was confirmed by analyzing the expression levels of tumor suppressor genes in fourteen hepatoma cell lines as a model. The TS-IS assay that we used was inexpensive and a widely applicable method that permitted the reliable and accurate quantification of tumor suppressor genes.

Keywords: Hepatocellular Carcinoma Cells; Poly-competitive RT-PCR; Quantification of Gene Expression; Tumor Suppressor Genes; Tumor Suppressor-internal Standard (TS-IS).

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Introduction

Cancer is a disease that has an abnormal and unregulated proliferation of cells. The changes in its genome results in the formation of a malignant tumor (Hanahan and Weinberg, 2000). Although there is a lot of information on cancer biology, present cancer therapies are seriously limited in overcoming the complexity of cancer. In order to have a good cancer treatment, it is necessary to make an early diagnosis and analysis of the cancer-related genetic status of patients. An analysis of the developmental mechanism that governs the transformation of normal human cells to malignant cancers at the molecular and genetic levels will mark a departure point in several directions for the treatment and prevention of cancer.

According to advanced genetic research, multiple genetic alterations that occur in human cancer target both oncogenes and tumor suppressor genes (Biden *et al.*, 1997; Hollstein *et al.*, 1991; Levine *et al.*, 1991; Sherr, 1998). Recessive functional loss of tumor suppressor genes, such as $p14^{ARF}$, $p15^{INK4B}$, $p16^{INK4A}$, $p21^{WAF1}$, p53, and pRb, is central to the tumorigenesis process (Levine *et al.*, 1991; Weinberg, 1995). The expression level and pattern of tumor suppressor genes depend on tumor types and their progressiveness (Kamijo *et al.*, 1997; Markl and Jones, 1998). Therefore, a rapid and accurate analysis of the expression profile of tumor suppressor genes and their genetic status will provide valuable tools for the diagnosis and determine the direction of medical treatment.

Abbreviations: DTT, dithiothreitol; FBS, fetal bovine serum; HPRT, hypoxanthine phosphoribosyl transferase; Rb, Retinoblastoma; RT-PCR, reverse transcription-polymerase chain reaction; TS-IS, tumor suppressor-internal standard.

PCR is one of the many tools that have commonly been used for the accurate quantification of DNA or RNA. Competitive PCR techniques in particular continue to play an important role in nucleic acid quantification because of its significantly lower cost in equipment and consumables. The procedure relies on the co-amplification of the sequences of interest with a serially diluted synthetic DNA fragment of known concentration (competitor) using a single set of primers (Gaiger *et al.*, 1995; Lion *et al.*, 1992). The initial quantity of target molecules in the sample can be calculated from the ratio of the competitor- and target-derived amplicons that are generated during PCR, provided that the target and competitor sequences are amplified with equivalent efficiency (Liu and Burt, 1998; Vu *et al.*, 2000; Wang *et al.*, 1989).

In order to detect the expression level and patterns of tumor suppressor genes with reproducibility, accuracy, rapidity, and high sensitivity, we constructed a TS-IS vector that contained primer sequences against not only 6 essential tumor suppressor genes, but also HPRT as an endogenous marker. The devised TS-IS construct provides a multi-specific cDNA template to detect and quantify the expression of these genes. A competitive RT-PCR assay using the TS-IS construct was efficiently carried out with fourteen human hepatocellular carcinoma cell lines as a model.

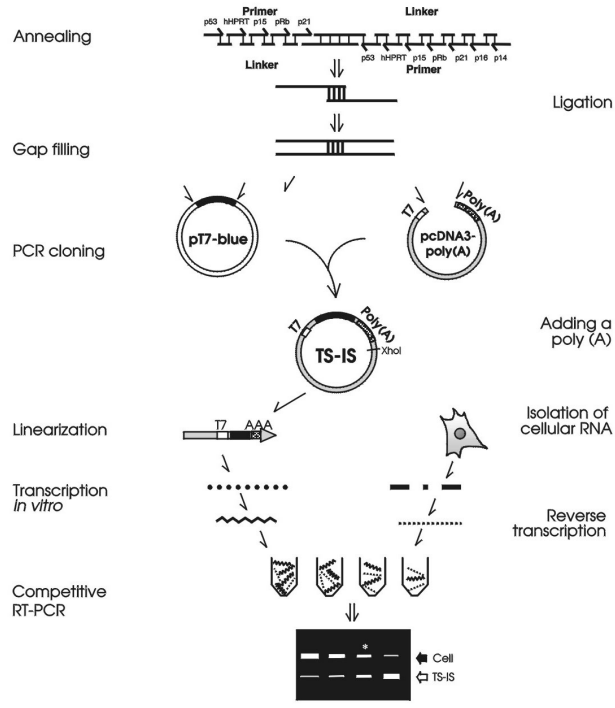


Fig. 1. A flow chart showing the generation and use of the tumor suppressor-internal standard (TS-IS) construct for the quantification of tumor suppressor mRNAs by poly-competitive RT-PCR assay.

Materials and Methods

Designing tumor suppressor gene-specific primers For designing primers specific for each tumor suppressor gene, the following considerations were made: (i) Primers should be 19–20 bases in length. (ii) The primers should have a G/C content between 55 and 70% and terminate in one or more G or C bases. (iii) There should not be any obvious secondary structures and internal complementary sequences. (iv) The primers should be selected from separate exons to distinguish between the products that were derived from cDNA and the contaminated genomic DNA. In addition, we generated primers for the HPRT gene as an internal marker. Forward and reverse primers were named 1R and 2R, respectively, except for p15 4R that is a universal reverse primer for all of p14, p15, and p16.

Construction of the TS-IS plasmid A schematic diagram that demonstrates the construction of the TS-IS vector is shown in Fig. 1. The linker oligomers that were used for joining each of the two neighboring primers are as follows: (i) Linker oligomers have 6 bp complementary sequences with two adjacent primers for tumor suppressor genes to be linked. (ii) Linker oligomers, located in the middle of the TS-IS sequences (i.e., between forward primer of p21 and reverse primer of p53), have complementary sequences to each other with the $(GA)_4$ or $(TC)_4$ as well as 6 bp complementary sequences of p21 and p53 (Fig. 1). All of the forward or reverse primers and two middle linkers were phosphorylated separately by T4 polynucleotide kinase (Stratagene, USA) before annealing with the matching pair of primers. The annealed primers were ligated, gap-filled, then subjected to 28 cycles of PCR amplification. The PCR reactions were composed of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min. The PCR product that contained the TS-IS sequences was inserted into the pT7 Blue vector (Novagen, USA). DNA sequencing in both directions verified accurate sequences of the TS-IS construct. The DNA fragment that contained the TS-IS sequences in the pT7 Blue vector was subcloned into pcDNA3-poly (A) (Invitrogen, USA), so that the TS-IS sequences were located between the T7 polymerase promoter and the poly (A) tail. This construct was named pcDNA3-TS-IS-poly (A).

The cRNA transcription from the TS-IS The pcDNA3-TS-IS-poly (A) plasmid was linearized with *Xho*I and transcribed *in vitro* using T7 RNA polymerase (Boehringer Mannheim, Germany). The *in vitro* transcription mixtures were digested with RNase-free Dnase I to remove the template DNA. The cRNA was purified with RNAzol™ B (TEL-TEST, USA) and separated by size exclusion chromatography. The amount of cRNA was estimated by spectrophotometry. Its size and integrity were examined by agarose gel electrophoresis (Park *et al.*, 1996).

Cell culture and RNA preparation The fourteen human hepa-

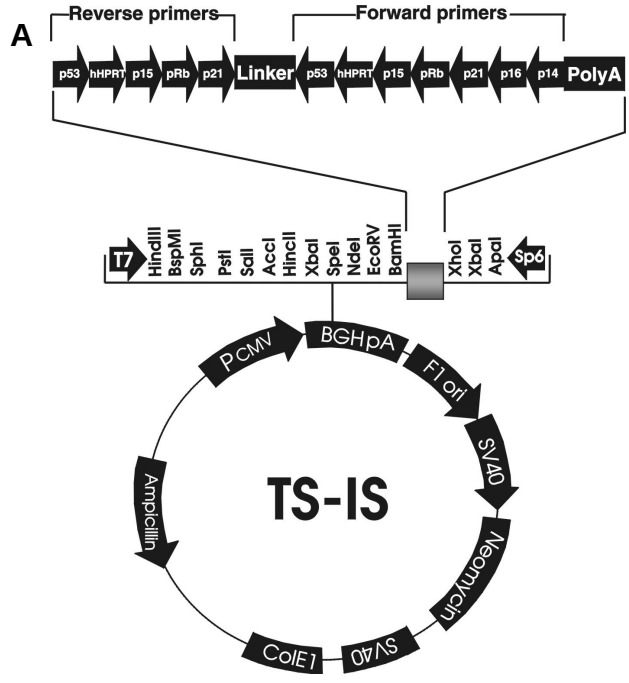
tocellular carcinoma cell lines - SK-Hep1, HepG2, HepG2.2.15, Hep3B, Chang liver, PLC/PRF5, SNU182, SNU354, SNU368, SNU387, SNU398, SNU423, SNU449, and SNU475 - were maintained in a RPMI medium 1640 (Gibco-BRL, USA) that contained 10% fetal bovine serum (Hyclone, USA) (Kim *et al.*, 2001). The human primary mesengial cells (a kind gift from Dr. Ku Kong, Hanyang University School of Medicine) and the peripheral blood mononuclear cell (PBMC) were separated from the blood of five normal humans. The normal liver tissue was obtained from Hepatocellular carcinoma (HCC) tumor tissues by micro-dissection in a cryostat. Total RNA was purified from the cells or tissues with RNAzol™ B (TEL-TEST, USA). The purity and quantity of the total RNA were spectrophotometrically estimated.

Reverse transcription and poly-competitive PCR Five hundred ng of TS-IS cRNA and 1 μg of total cellular RNA from hepatoma cell lines were separately reverse-transcribed. The total cDNA that was derived from the cellular RNA of each cell line was used for a competitive PCR reaction along with the TS-IS cDNA as an internal control. The amount of TS-IS RNA that was used is as follows: 10^{-3}–10^{-6}ng for HPRT and p21; 10^{-4}–10^{-7} ng for $p14^{ARF}$ and $p16^{INK4A}$; 10^{-5}–10^{-8} ng for $p15^{INK4B}$, p53, and pRb. Thirty-five cycles of the PCR reaction were carried out at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min in the presence of 1 μCi of [α]P^{32} dCTP. The PCR products were separated by electrophoresis in 5% polyacrylamide gel and exposed to the X-ray film after gel drying. Relative fluorescent intensity for each PCR product was determined with the BIO-PROFIL® image analysis software, BIO-1D (Vilber Lourmat).

Results and Discussion

Construction of the TS-IS plasmid We made a construct pcDNA3-TS-IS-poly(A) to produce internal competitor standards for the quantitative analysis of the expression levels of key tumor suppressor genes. The schematic drawing of this construct is shown in Fig. 2A. Specific primers for the 6 tumor suppressor genes, $p14^{ARF}$, $p15^{INK4b}$, p16 ^{INK4a}, p21, p53, and pRB, were designed with considerations that are explained in **Materials and Methods**. In addition, in order to monitor the quality and quantity of the total cellular RNA that was prepared from the diverse samples, primer sequences for an internal control marker (HPRT gene) were also incorporated into the construct. The relative positions of the primer pairs were arranged in order to yield 56–335 bp length differences between the PCR products of cellular target RNAs and the TS-IS cRNA (Figs. 2A and 2B). The correct size of the PCR product was observed when TS-IS cRNA was subjected to RT-PCR using primer pairs of the tumor suppressor genes (Fig. 2C).

Reliability test of competitive RT-PCR using TS-IS To



B

Gene	Size of RT-PCR Product (bp)	
	TS-IS	mRNA
p14	208	152
p15	128	259
p16	188	130
p21	128	244
p53	128	416
pRB	128	463
HPRT	128	270

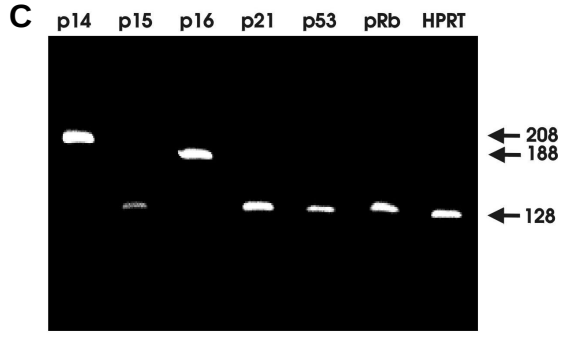
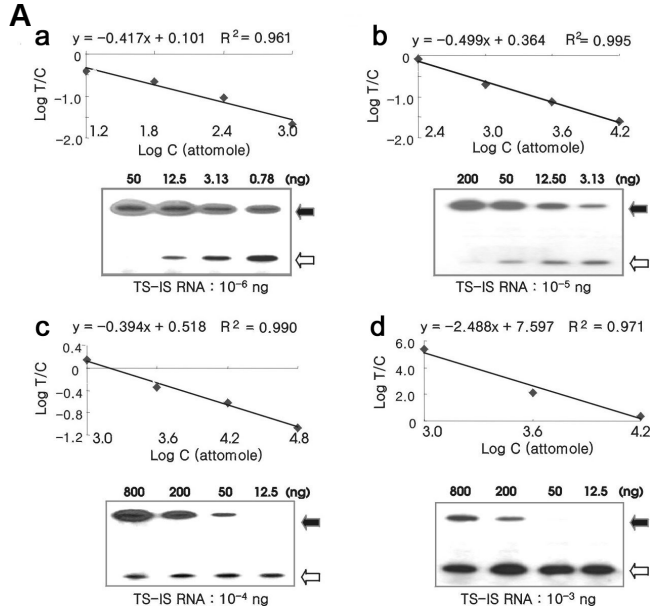


Fig. 2. A pcDNA3-TS-IS-poly(A) vector efficiently synthesizes the internal competitive standard RNA to quantify the expression of tumor suppressor genes. **A.** A schematic drawing of the construct pcDNA3-TS-IS-poly(A). **B.** Expected PCR product sizes from the endogenous cellular and TS-IS RNAs for each tumor suppressor gene in the poly-competitive RT-PCR assay. **C.** A RT-PCR data showing the expected size of each PCR product that was synthesized from the TS-IS internal standard. The cRNAs were synthesized *in vitro* from the pcDNA3-TS-IS-poly(A) using T7 RNA polymerase. Then the first-strand cDNA was obtained by a reverse transcription reaction. PCR was performed using a set of tumor suppressor or HPRT gene specific primer.

Table 1. Estimation of numbers of total mRNA in reactions containing fixed amounts of TS-IS by method A

TS-IS(ng)	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Numbers of HPRT molecule[a]	4.8×10^3	4.8×10^4	4.8×10^5	4.8×10^6
Log C[b]	0.74	2.20	3.20	4.20
C (attomole)	0.55×10	1.59×10^2	1.59×10^3	1.59×10^4
Number of total mRNA[c]	3.3×10^6	9.6×10^7	9.6×10^8	9.6×10^9
HPRT RNA/ mRNA	1.45×10^{-3}	0.5×10^{-3}	0.5×10^{-3}	0.5×10^{-3}

[a] TS-IS of 10^{-6} ng corresponds to 4.8×10^3 molecules of TS-IS RNA that consists of 367 bp.
[b] To compensate the band intensity between the cellular and TS-IS PCR products due to the size difference, we multiplied 2.1 to the TS-IS value (i.e., 270 bp/128 bp = 2.1). In the log T/C versus log C plot as shown in Fig. 3A, the log C values were obtained where log T/C is equal to 0.
[c] Numbers of total mRNAs = (C attomole) × (10^{-18}) × (6×10^{23}).



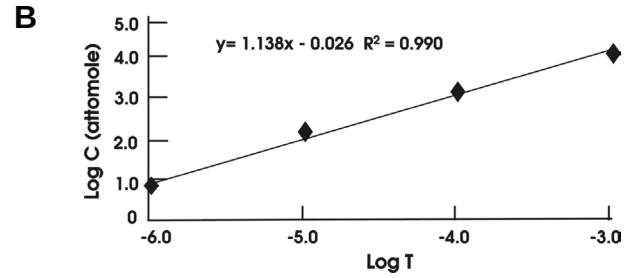


Fig. 3. Competitive RT-PCR analysis for the quantification of the cellular mRNA level in the reactions containing fixed amounts of TS-IS (method A). **A.** Plots of the ratio (log T/C) of the PCR products on a log-log scale as a function of the indicated amounts of log C (upper panels) and the representative competitive RT-PCR data (lower panels). Concentration of cellular HPRT mRNA was estimated from the value of log C in the X-intercept. Filled arrows indicate the PCR products that were produced from the cellular RNA; open arrows represent those of the internal standard TS-IS. **B.** A linear regression plot of the values (log T vs. log C) that were obtained from panel A shows that the estimation of endogenous cellular HPRT mRNA concentration is reliable when any amount of TS-IS RNA is employed.

test whether or not competitive RT-PCR using TS-IS is reliable, two kinds of experiments were performed on the HPRT gene as a control. In the first method (method A), a competitive RT-PCR was carried out with fixed amounts of the TS-IS RNA (10^{-6}, 10^{-5}, 10^{-4}, or 10^{-3} ng) and various amounts of the cellular total RNA from 800 to 0.78 ng. Figure 3A shows the represented competitive RT-PCRs and the plots of the ratio (log T/C) of PCR products on a log-log scale as a function of indicated amounts of log C. Because the X-axis is the log value of the cellular total mRNA (log C) and the Y-axis is the log T/C, the value in the X- intercept is the value of log C in which the number of cellular total mRNA molecules is equal to that of TS-IS RNAs. These values were 0.74, 2.2, 3.2, and 4.2; i.e., 3.3×10^6, 9.6×10^7, 9.6×10^8, and 9.6×10^9 molecules of cellular RNA, respectively (Table 1). When the values of log C are plotted against log T (Fig. 3B), a linear regression line was obtained. Therefore, this method is reliable whenever any amount of TS-IS is employed in the reaction. The average ratio (HPRT RNA/ total mRNA) is 0.73×10^{-3} [{($4.8 \times 10^3/3.3 \times 10^6$) + ($4.8 \times 10^4/9.6 \times 10^7$) + ($4.8 \times 10^5/9.6 \times 10^8$) + ($4.8 \times 10^6/9.6 \times 10^9$)} × 1/4].

In the second method (method B), a competitive RT-PCR was carried out with fixed amounts of the cellular total RNA (50 ng) and various amounts of TS-IS RNA from 10^{-3} to 10^{-6} ng. Figure 4 shows a representative competitive RT-PCR and a plot of values of log C/T versus log T. The log T value on the X-intercept is − 3.14; therefore, the amount of TS-IS RNA is equal to $10^{-3.14}$ ng (i.e., 3.5×10^6 molecules of TS-IS RNA) (Table 2). The ratio (HPRT RNA/total mRNA) that is estimated from method B is 1.2×10^{-3} (= $3.5 \times 10^6/2.9 \times 10^9$), and this value is quite consistent with the average value of method A (0.73×10^{-3}). As the discrepancy of estimation between these two methods is less than two fold (i.e., 1.634), both methods will be useful for the quantification of the tumor suppressor mRNA. It is noteworthy, however, that method A requires more cellular RNA than method B. Therefore, method B is a better choice.

Expression profile of tumor suppressor genes in hepatoma cell lines As a sample of competitive RT-PCR, we selected fourteen human hepatocellular carcinoma cell

Table 2. Estimation of numbers of HPRT RNA in a reaction containing 50 ng cellular RNA by method B

Numbers of mRNA molecule[a]	2.9×10^9
Log T[b]	−3.14
T	$10^{-3.14}$
Numbers of HPRT RNAs[c]	3.5×10^6
HPRT RNA/mRNA	1.2×10^{-3}

[a] We assumed that mRNA corresponds about 5% of total cellular RNA and the average size of mRNA is 1.5 kb.
[b] To compensate the band intensity between the cellular and TS-IS PCR products due to the size difference, we multiplied 2.1 to the TS-IS value (i.e., 270 bp/128 bp = 2.1). In the log C/T versus log T plot as shown in Fig. 4, the log T value was obtained where log C/T is equal to 0.
[c] TS-IS of 10^{-3} ng corresponds to 4.8×10^6 molecules of TS-IS RNA that consists of 367 bp.

Table 3. Expression levels of tumor suppressor genes in normal and hepatoma cells. The numbers of RNA molecules were estimated by poly-competitive RT-PCR assay. Each numbers represent number of corresponding RNA molecules in 50 ng of total RNA. ND, not detected in this assay.

Status	Cell line/tissue	Gene					
		p14	p16	p21	p53	pRB	HPRT
Normal	Normal liver	ND	ND	4.8×10^7	4.8×10^5	4.8×10^5	4.8×10^6
	PBMC	ND	ND	4.8×10^7	4.8×10^5	4.8×10^5	4.8×10^6
	Mesangial cell	ND	ND	4.8×10^5	4.8×10^3	4.8×10^3	4.8×10^6
Tumor	Chang liver	4.8×10^3	4.8×10^3	4.8×10^6	4.8×10^3	4.8×10^4	4.8×10^6
	HepG2	$<4.8\times10^2$	4.8×10^3	4.8×10^6	4.8×10^3	4.8×10^3	4.8×10^6
	HepG2.2.15	4.8×10^4	4.8×10^4	4.8×10^7	4.8×10^4	4.8×10^5	4.8×10^6
	SNU182	4.8×10^3	4.8×10^3	4.8×10^5	4.8×10^4	4.8×10^2	4.8×10^6
	SNU368	4.8×10^2	4.8×10^3	4.8×10^6	ND	48	4.8×10^6
	Hep3B	4.8×10^3	4.8×10^3	4.8×10^5	ND	ND	4.8×10^6
	PLC/PRF5	4.8×10^3	4.8×10^2	4.8×10^5	48	48	4.8×10^6
	SNU449	ND	ND	4.8×10^6	4.8×10^3	4.8×10^3	4.8×10^6
	SNU423	4.8×10^3	ND	4.8×10^6	ND	ND	4.8×10^6
	SNU354	4.8×10^2	ND	4.8×10^4	ND	ND	4.8×10^6
	SNU475	4.8×10^3	ND	4.8×10^6	ND	ND	4.8×10^6
	SNU398	ND	ND	4.8×10^5	4.8×10^3	ND	4.8×10^6
	SK-Hep1	ND	ND	4.8×10^6	48	ND	4.8×10^6
	SNU387	ND	ND	4.8×10^6	ND	ND	4.8×10^6

lines in which the expression and genomic status of the tumor suppressor genes are well known (Table 4). In this test, we also prepared total cellular RNAs from normal liver tissue, peripheral blood mononuclear cells (PBMC), and human primary mesengial cells (HPMC). Cellular RNAs and TS-IS cRNA were individually reverse-transcribed, then competitive PCR was performed with

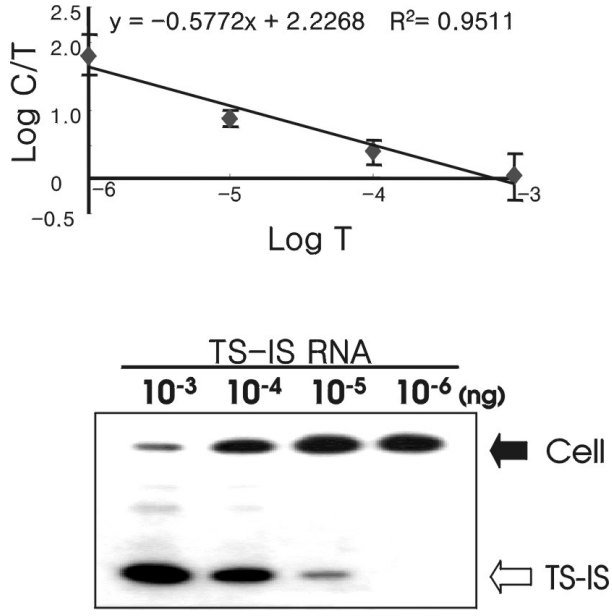


Fig. 4. Competitive RT-PCR analysis for the quantification of the cellular mRNA level in the reactions containing fixed amounts of cellular RNA (Method B). A plot of the ratio (log C/T) of the PCR products on a log-log scale as a function of the indicated amounts of log T (upper panel) and the representative competitive RT-PCR data (lower panel).

method B. The competitive RT-PCR of the HPRT gene was carried out with every sample to monitor whether an assay would properly perform and make a standard curve of a fixed amount of target cDNA against different amounts of TS-IS cDNA. The absolute concentration of the tumor suppressor mRNA was estimated from the concentration of TS-IS that would have given the same intensity to the tumor suppressor mRNA when analyzed (see **Materials and Methods**).

Each cell line exhibited a distinct expression pattern of six tumor suppressor genes. These results were simplified and summarized in Table 3. In the RNAs that were prepared from normal liver tissue, PBMC and HPMC, the expression of $p14^{ARF}$, $p15^{INK4B}$, and $p16^{INK4A}$ was not observed, although the expression of p21, p53, and pRB was detected. On the contrary, in hepatocellular carcinoma cell lines, the expression of $p14^{ARF}$ and $p16^{INK4A}$ was found in 10 of 14 cases (71.4%) and 7 of 14 cases (50.5%), respectively. Their expression levels were variable within a range of 10- to 100-fold difference (Table 3). Among the 7 hepatocellular carcinoma cell lines in which the p16 expression was not detected, 3 cases had genomic deletions in the INK4 locus encoding $p14^{ARF}$, $p15^{INK4B}$, and $p16^{INK4A}$. The remaining 4 cases had methylation at their promoters (Baek *et al.*, 2000; Table 4). The expression of pRb was detected in 7 of 14 cases (50.0%), but the level

Table 4. Expression and genomic status of tumor suppressor genes in normal and hepatoma cell lines

Cell line	p14		p15		p16		p21		p53		pRb	
	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Normal	ND	-	ND	-	ND	-	ND	+	ND	-	ND	-
Chang liver	ND	+	ND	+	ND	+	ND	+	+	+	+	+
HepG2	ND	+	ND	+	+	+	ND	+	+	WT[b]	+	+
HepG2.2.15	+[a]	+	+[a]	+	HM[a]	+	ND	+	+	WT[c]	+	+
Hep3B	+[a]	+	+[a]	+	WT[a]	+	ND	+	+	MUT[b]	+	-
SNU182	+[a]	+	+[a]	+	+[a]	+	ND	+	+	MUT[d]	+	+
PLC/PRF5	ND	+	ND	-	ND	+	ND	+	+	MUT[e]	+	-
SNU368	ND	+	ND	+	ND	+	ND	+	+	MUT[d]	+	-
SNU449	-[a]	-	-[a]	+	-[a]	-	ND	+	+	MUT[d]	+	+
SNU423	+[a]	+	+[a]	+	M[a]	-	ND	+	+	MUT[d]	+	-
SNU354	ND	+	ND	+	ND	-	ND	+	+	MUT[d]	+	-
SNU475	+[a]	+	+[a]	+	M[a]	-	ND	+	+	MUT[d]	+	-
SNU398	+[a]	-	+[a]	+	M[a]	-	ND	+	+	MUT[d]	+	-
SK-Hep1	-[a]	-	-[a]	-	-[a]	-	ND	+	+	WT[c]	+	-
SNU387	-[a]	-	-[a]	+	-[a]	-	ND	+	+	MUT[d]	+	-

+, expression was detected; -, expression was not detected; WT, wild-type; MUT, mutant; ND, not determined; M, methylation of promoter; HM, hemimethylation of promoter.
[a] Baek *et al*., 2000; [b] Okamoto *et al*., 1994; [c] Lee *et al*., 1995; [d] Kang *et al*., 1996; [e] Wang *et al*., 1994; [f] Mitry *et al*., 2000.

of its expression, even in the detected cells, was much lower compared to normal cells. It is noteworthy that, although mutations of p53 were found in 10 of 13 cases (Table 4), p53 mRNA was detected in 8 of 14 cases (57.1%) in this study. We detected the p21 expression in all of the 14-cell lines that were tested, regardless of the presence of wild-type p53.

The $p14^{ARF}$ was repressed by p53 and its expression was not detected in several normal tissues (Stott *et al.*, 1998; Zindy *et al.*, 1997). Therefore, the $p14^{ARF}$ expression that was detected in most of the hepatoma cells in our study may be due to the mutation of p53. It is remarkable, however, that the $p14^{ARF}$ RNA was over-expressed in the HepG2 and HepG2.2.15 cells in which the wild-type p53 was present. It suggests that there are some mechanisms that cause the over-expression of $p14^{ARF}$ and $p16^{INK4A}$ in the presence of p53 and/or pRb function.

These findings suggest that the expression levels of tumor suppressor genes in hepatocellular carcinoma cells are very distinct from that of normal liver cells. Moreover, hepatocellular carcinoma cells showed very heterogeneous expression patterns on tumor suppressor genes, which is consistent with intra-tumor heterogeneity. Tumor induction is a multi-step process that involves the activation of oncogenes and the inactivation of tumor suppressor genes. Like other cancer, the loss of p53 and/or pRB functions, the deletion of genomic INK4 locus that encodes $p14^{ARF}$, $p15^{INK4b}$, and $p16^{INK4A}$ genes, were thought to be major causes of hepatoceullar carcinomas (Baek *et al.*, 2000; Biden *et al.*, 1997; Chin *et al.*, 1998; Hollstein *et al.*, 1991; Kamb, 1995; Kang *et al.*, 1996; Ko and Prives, 1996; Newcomb *et al.*, 2000; Okamoto *et al.*, 1994). It is intriguing that more than one tumor suppressor gene shows abnormal expression profiles due to changes in their own genomic status or other transcriptional regulators (Hanahan and Weinberg, 2000; Sherr, 1996; 1998; Stott *et al.*, 1998; Weinberg, 1995; Zhang *et al.*, 1998). The question of whether or not the abnormal expression pattern of tumor suppressor genes in hepatocellular carcinoma cells can explain the prognosis of hepatoceullar carcinoma development or their heterogeneous origins is still unanswered. To confirm the significance of these possibilities, more investigations are needed, including our TS-IS-based poly-competitive RT-PCR method.

Conclusion The poly-competitive PCR method that is presented here is efficient for the quantification of even the low frequency of mRNAs in a sample (Table 3). The synthetic internal standard, TS-IS, which contains multiple primer sets, allows the quantification of a number of different mRNAs in parallel. It could, therefore, partially reduce experimental errors. Since the TS-IS vector contains the HPRT gene-specific primer set, it provides additional reliability and accuracy to the experimental results. As demonstrated by the quantification of the tumor suppressor mRNAs in the fourteen human hepatocellular carcinoma cell lines (Table 3), this technique could be useful as a tool for cancer typing and/or diagnosis in clinical research and treatment.

Acknowledgments We thank Dr. Eun-Ho Park for providing us with equipment and facilities, as well as for his thoughtful suggestions. We thank Brian Watson, Bo Mi Jung, and Jong Joo Lee for their critical reading of our manuscript and valuable criticisms. This work was supported by a grant (No. HMP-98-B-3-0021) from the 1998 Good Health R&D Project, Ministry of Health and Welfare of Korea. J. H. J., C. J. H., and L. J. S. were supported in part by the Brain Korea 21 Project from the Ministry of Education and Human Resources of Korea through the Research Group of Stem Cell and Early Development.

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