Comparisons for detecting NY-ESO-1 mRNA expression levels in hepatocellular carcinoma tissues

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Abstract. NY-ESO-1 is a cancer/testis (CT) antigen expressed in normal adult tissues solely in the testicular germ cells of normal adults and in various cancers. It induces specific humoral and cellular immunity in patients with NY-ESO-1-expressing cancer. We compared the expression of NY-ESO-1 mRNA in hepatocellular carcinoma (HCC) patients by using various primers and DNA polymerases to optimize RT-PCR conditions and to evaluate the correlations among the expression levels of NY-ESO-1, LAGE-1 and SSX-1 and clinical parameters. We determined differences in the abilities of the various primers and DNA polymerases to amplify the NY-ESO-1 gene at different exons. Primers designated as P3 detected targeted sequences better than primers P1 and P2; AmpliTaq Gold® DNA polymerase was more effective than Platinum® pfx DNA polymerase and Taq DNA polymerase. NY-ESO-1, LAGE-1 and SSX-1 mRNAs were detected in 29.7, 45.3 and 37.5%, respectively, of the 64 HCC specimens. No CT antigen mRNAs were detected in the 64-paired adjacent non-cancerous tissues. The frequency for the co-expression of one, two or three antigens of NY-ESO-1, LAGE-1 and SSX-1 was 57.8, 35.9 and 18.8%, respectively. We also analyzed the relationships among the CT antigen expression levels and several clinical parameters. There were no significant differences between CT antigen expression levels and clinical parameters, except the correlations between the expression of SSX-1 and the age of the patients.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide; because of its poor prognosis, the number of HCC-related deaths is almost identical. Thus, HCC represents the third most common cause of cancer-related deaths world-wide. Eighty-two percent of all cases (and hence deaths) are in developing countries (55% in P.R. China alone) (1). The mortality of HCC is the second highest among that caused by malignant tumors in P.R. China. The annual death toll for HCC in P.R. China is ranked at number one in the world. Hepatic surgery is a principal treatment for HCC and has achieved significant advances in recent years; however, the recurrence and metastasis of HCC are still the major problems associated with its high mortality (2,3).

Ideally, cancer therapy should possess sufficient potency to eradicate systemic tumors in multiple body sites and the specificity to discriminate between neoplastic and non-neoplastic cells. In both of these respects, immunotherapy is an attractive approach. The first step of active immunotherapy is to identify cogent antigen targets for HCC. Among tumor antigens identified to date, cancer/testis (CT) antigens have been recognized as a group of highly attractive targets for cancer vaccine development. CT antigen-encoding genes comprise 44 families, including the NY-ESO-1 (4), MAGE (5) and SSX (6) gene groups. In view of the expanding list of CT genes and their significance to antigen-specific cancer vaccine development, a CT gene database to accumulate relevant data at a single web interface has been created (7). These CT genes are widely expressed in a variety of human cancers, such as melanoma, breast cancer and esophageal cancer (8).

NY-ESO-1 is a CT antigen identified originally from the serological analysis of the recombinant cDNA expression cloning (SEREX) method, in the context of esophageal cancer. This antigen is relatively ubiquitous in a number of cancers, including melanoma, breast cancer (9), lung cancer (10), synovial sarcoma (11) and HCC (4). Spontaneous humoral and cellular immune responses to NY-ESO-1 are detected frequently in cancer patients whose tumors express this molecule (12-14). Indeed, NY-ESO-1 is the most immunogenic CT antigen discovered thus far, making it a highly promising therapeutic vaccine component. However, the frequency of NY-ESO-1 mRNA expression in HCC patients varies widely from 0 to 42.5% (4,15-20). The variability may be due to different regions and ethnicity distributions. Additionally, non-standard detection methods could contribute to differences in its detection efficiencies. The present study was designed to compare detection levels of NY-ESO-1 mRNAs expressed in HCC using the various commercial DNA polymerases and

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Table I. Sequences and evaluations of specific primers.

Primer	Sequence	Tm	% GC	Hairpin	Dimer	Full length
NY-ESO-1 P1						
Upstream	5'-atggatgctgcagatgcgg-3'	59.7	57.9%	-	+	329
Downstream	5'-ggcttagcgcctctgccctg-3'	66.0	70.0%	+	+	
NY-ESO-1 P2						
Upstream	5'-cagggctgaatggatgctgcaga-3'	63.7	56.5%	+	+	332
Downstream	5'-gcgcctctgccctgagggagg-3'	69.7	76.2%	+	+	
NY-ESO-1 P3						
Upstream	5'-agttctacctcgccatgcct-3'	59.9	55.0%	-	+	386
Downstream	5'-tcctcctccagcgacaaacaa-3'	60.0	52.4%	-	-	
LAGE-1						
Upstream	5'-ctgcgcaggatggaaggtgcccc-3'	69.1	69.6%	-	+	332
Downstream	5'-gcgcctctgccctgagggagc-3'	69.7	76.2%	+	+	
SSX-1						
Upstream	5'-ctaaagcatcagagaagagaagc-3'	58.4	43.5%	-	-	422
Downstream	5'-agatctcttattaatcttctcagaaa-3'	54.1	26.9%	+	+	

published primer sequences cited frequently in the pertinent references.

Materials and methods

Patients and samples. HCC and adjacent non-cancerous tissue specimens were obtained from 64 patients undergoing surgery at the Guangxi Province in South P.R. China. The HCC patient group consisted of 59 males and 5 females of the average age, at diagnosis, of 48.9±12.8 years (mean ± standard deviation; range, 22-76 years). HCC diagnosis was confirmed by pathological examination. The tumor-node-metastasis (TNM) stage was determined according to the criteria issued by the UICC in 1997 (21). Tissue specimens were collected from both tumor regions and adjacent non-cancerous areas (>5 cm from the tumor) from resected HCC patients during surgery. All samples were dissected into sections of ~0.5x 0.5x0.5 cm and immediately stored in liquid nitrogen. Simultaneously, we collected routine venous blood without anticoagulation and separated the serum for the hepatitis-related serological tests and α-fetoprotein (AFP) detection. Informed consent was obtained from all patients for the use of specimens and sera.

Isolation of total RNA. Total RNA was isolated from 30 mg frozen tissue samples of HCC using an RNeasy mini kit (Qiagen), following the manufacturer's instructions. The RNA was eluted in 50 μl RNase-free water. Potentially contaminating DNA was removed by treating the sample with an RNase-free DNase set (Qiagen, cat. No.79254). The resulting RNA concentration was measured spectrophotometrically (Hitachi) and RNA quality was assessed by electrophoresis on a 1% agarose gel.

Synthesis of cDNA. The RNA samples (2 μg) were mixed with 500 ng random primers (Promega) and 500 ng oligo$(dT)_{15}$ (Promega). Diethyl pyrocarbonate (DEPC)-treated water was added to achieve a total volume of 12 μl. The samples were degenerated at 70˚C for 10 min, followed by incubation on ice for 2 min. An 8 μl reaction mix was added, including 1 μl 0.1 M dithiothreitol (DTT) (Invitrogen), 1 μl 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, and dTTP; Promega), 40 U RNaseOUT™ Reconbinant RNase inhibitor (Invitrogen), 200 U SuperScript™ III reverse transcriptase (Invitrogen) and 4 μl 5X first-strand buffer (Invitrogen). Samples were placed in a water bath at 42˚C after mixing for 1 h and inactivated at 70˚C for 15 min.

Specific primers, sequences and evaluations. The sequences of primers and the results evaluated with the software Primer Premier (version 5.0, Premier, Canada) are listed in Table I. All primers were synthesized by Shanghai Sangon Biotechnology Co. Ltd.

Amplification of ß-actin. The ß-actin cDNA PCR was performed for each sample as quality control checks to ensure proper amplification of cDNA. The primer sequences used were: 5'-ggcatcgtgatggactccg-3' and 5'-gctggaaggtggacagcga-3'. The PCR products of 613 bp were identified by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

Amplification of NY-ESO-1 with three primers and three DNA polymerases. The NY-ESO-1 cDNA PCRs were performed with three primer pairs and three DNA polymerases. Testis tissues and blank reagents were used as positive and negative controls, respectively. The total volume was 25 μl and included 200 μg cDNA and components recommended in the different DNA polymerase instructions for 35 cycles with a pre-degeneration cycle at 94˚C for 5 min (95˚C for 10 min using AmpliTaq Gold® DNA polymerase) and a final extension at 72˚C for 7 min in a thermal cycler (PTC-100, MJ Research). The amplification products were identified by electrophoresis on a 1.5% agarose gel and visualized by

Table II. Conditions and components for PCR amplification of NY-ESO-1.

	Taq DNA polymerase	Platinum® *pfx* DNA polymerase	AmpliTaq Gold® DNA polymerase
Reaction component (25 μl)	1X PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$ 0.5 μM each primer 1.0 units polymerase	2X PCR buffer 0.3 mM dNTP 1.0 mM $MgSO_4$ 0.5 μM each primer 1.0 units polymerase	1X PCR buffer 0.2 mM dTNP 1.5 mM $MgCl_2$ 0.5 μM each primer 0.625 units polymerase
PCR condition	94˚C, 60 sec 60˚C, 60 sec 72˚C, 60 sec	94˚C, 15 sec 60˚C, 30 sec 68˚C, 60 sec	94˚C, 60 sec 60˚C, 60 sec 72˚C, 60 sec

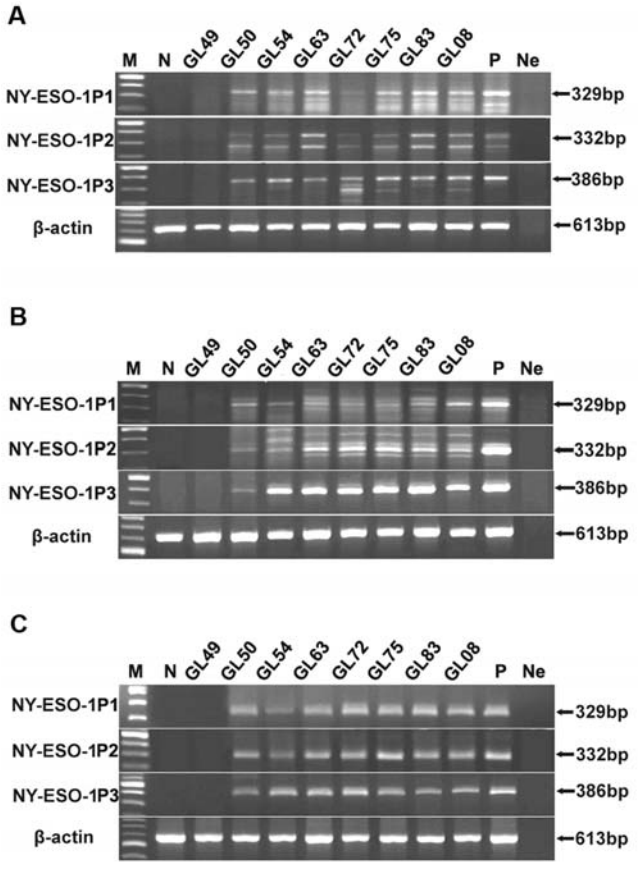

Figure 1. Comparison of NY-ESO-1 expression amplified with three primers and three DNA polymerases. (A) Amplification of NY-ESO-1 with three primers and Taq DNA polymerase; (B) amplification of NY-ESO-1 with three primers and Platinum *pfx* DNA polymerase; (C) amplification of NY-ESO-1 with three primers and Amplitaq Gold DNA polymerase. M, Molecular marker, 100 bp DNA ladder (Gibco); N, adjacent non-cancerous; P, positive control, testis tissues; Ne, Negative control, PCR amplification in the absence of template; ß-actin: 613 bp cDNA quality control.

ethidium bromide staining. Reaction conditions and components are listed in Table II.

Sensitivity of DNA polymerase. To compare the sensitivity of the three DNA polymerases, 1 μl testis cDNA was diluted serially to amplify ß-actin and NY-ESO-1 with *Taq* DNA polymerase (Invitrogen), Platinum® *pfx* DNA polymerase (Invitrogen) and AmpliTaq Gold DNA polymerase (Applied Biosystems), respectively. Conditions and components for PCR amplification are provided in Table II. Duplicates of amplification reactions were performed and three times were repeated.

Amplification of NY-ESO-1, LAGE-1 and SSX-1. The NY-ESO-1 and LAGE-1 cDNA PCRs were performed with AmpliTaq Gold DNA polymerase and annealing temperatures of 60˚C and 65˚C, respectively, for 60 sec. The SSX-1 PCR was performed with *Taq* DNA polymerase and annealing conditions of 58˚C for 60 sec. The primer sequences are listed in Table I and the reaction components are listed in Table II.

Sequencing and analysis of PCR products. All positive PCR products were recovered from the agarose gels and sequenced in two directions using a direct sequencing technique and the original PCR primers. The sequence data were analyzed by comparing with similar sequences in GenBank. GenBank access numbers: NY-ESO-1 (NM_001327), LAGE-1 (NM_172377), SSX-1 (NM_005635).

Statistical analysis. The χ^2 test and independent sample t-test were employed. All tests were performed using SPSS software version 11.0 (SPSS Inc., Chicago, IL). P-values <0.05 were regarded as being statistically significant.

Results

Amplification of NY-ESO-1 with three primers and three DNA polymerases. Non-specific products were amplified with the primers NY-ESO-1 P1 and NY-ESO-1 P2 and *Taq* DNA polymerase. These amplicons could not be sequenced. The positive PCR products that amplified with the primer NY-ESO-1 P3 (for simplicity, shortening these primer names to P1, P2 and P3) and *Taq* DNA polymerase were specific and confirmed by sequencing.

Although the non-specific products were yielded, the positive PCR products, amplified with primers P1 and P2 and Platinum *pfx* DNA polymerase, were confirmed by sequencing. The positive PCR products, amplified with primer P3 were specific and confirmed by sequencing.

The positive PCR products, amplified with primers P1, P2 and P3 and AmpliTaq Gold DNA polymerase were specific and confirmed by sequencing (Fig. 1).

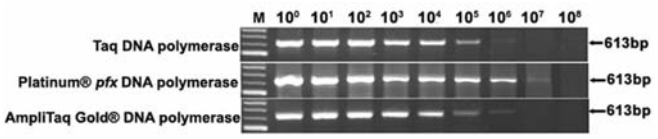

Figure 2. Comparison of sensitivity of three DNA polymerase to amplify ß-actin. M, Molecular marker, 100 bp DNA ladder (Gibco); 10^0-10^8: testis cDNA serial dilutions from 10^0 to 10^8.

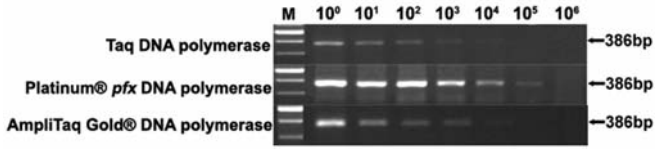

Figure 3. Comparison of sensitivity of three DNA polymerase to amplify NY-ESO-1. M, Molecular marker, 100 bp DNA ladder (Gibco); 10^0-10^6: testis cDNA serial dilutions from 10^0 to 10^6.

Table III. The relationship of the expression of NY-ESO-1 and LAGE-1.

	LAGE-1		
	Positive	Negative	Total
NY-ESO-1			
Positive	14	5	19
Negative	15	30	45
Total	29	35	64

Sensitivity of DNA polymerase. The lowest dilution at which ß-actin could be detected was 10^6 times using *Taq* DNA polymerase, 10^7 times using Platinum *pfx* DNA polymerase and 10^6 times using AmpliTaq Gold DNA polymerase (Fig. 2). The lowest dilution at which NY-ESO-1 could be detected was 10^4 times using *Taq* DNA polymerase, 10^5 times using Platinum *pfx* DNA polymerase and 10^4 times using AmpliTaq Gold DNA polymerase (Fig. 3).

Expression of NY-ESO-1, LAGE-1, SSX-1 in HCC. The expression frequencies of NY-ESO-1, LAGE-1, SSX-1 were 29.7% (19/64), 45.3% (29/64) and 37.5% (24/64). The three CT antigens were not expressed in adjacent non-cancerous tissues (Fig. 4).

Co-expression of NY-ESO-1, LAGE-1 and SSX-1 in HCC patients. The frequencies for the co-expression of one, two or three antigens were 57.8% (37/64), 35.9% (23/64), and 18.8% (12/64), respectively (Fig. 5). The expression frequencies of NY-ESO-1 and LAGE-1 were 29.7% (19/64) and 45.3% (29/64), respectively. Among them, 21.9% (14/64) of the HCC samples expressed both genes and 53.1% (34/64) expressed at least one of the genes (Table III).

Correlation between CT antigen expression and clinical parameters. The relationships between CT antigen expression

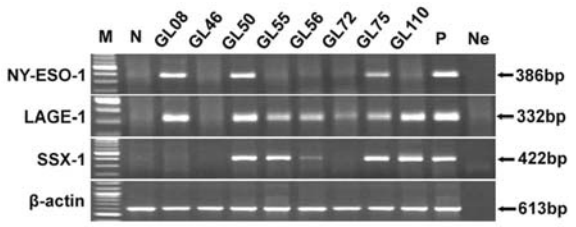

Figure 4. Expression of NY-ESO-1, LAGE-1, SSX-1 in HCC. M, Molecular marker, 100 bp DNA ladder (Gibco); N, adjacent non-cancerous; P, positive control, testis tissues; Ne, negative control, PCR amplification in the absence of template; ß-actin: 613 bp cDNA quality control.

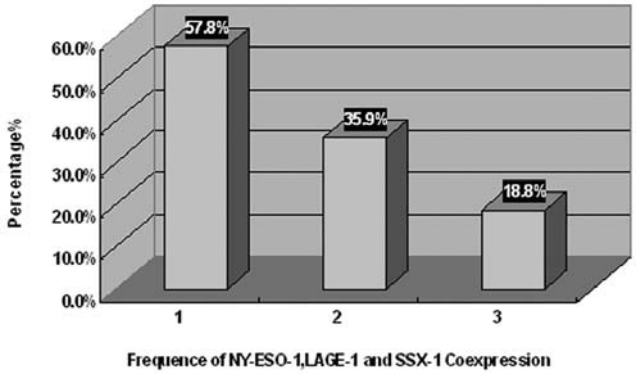

Figure 5. Co-expression of NY-ESO-1, LAGE-1 and SSX-1 in HCC. 1-3, number of co-expressed CT genes.

and the associated clinical parameters, including age, gender, HBV infection, serum AFP level, tumor stage and tumor size, were analyzed and reported herein. In our study, the results from three CT antigen expression analyses consistently showed that the CT antigen-positive patients were generally older than the antigen-negative patients (Table IV). Statistical analyses revealed that mRNA the expression of SSX-1 correlated with older age ($P<0.05$). There were no significant differences between the CT antigen expression levels and the other clinical parameters.

Discussion

Chen *et al* (4) identified NY-ESO-1 with the SEREX method on an esophageal carcinoma cDNA library using autologous patient sera. NY-ESO-1 is a 22 kDa hydrophobic protein encoded by a gene in the Xq28 region (22,23). RT-PCR analysis showed that NY-ESO-1 mRNA expression occurred at variable levels in a wide array of human cancers, including melanoma, breast cancer, bladder cancer, prostate cancer and HCC (8). This is particularly true in HCC patients, in which Chen *et al* (4) reported NY-ESO-1 expression in 2/7 (28.6%) of tested American HCC patients, Luo *et al* (15) reported undetectable levels of NY-ESO-1 in 21 (100%) tested Chinese HCC specimens and Peng *et al* (20) reported NY-ESO-1 expression in 31/73 (42.5%) of Chinese HCC patients. Additionally in the Asian region, Chen *et al* (19) demonstrated the expression of NY-ESO-1 in 11/30 (36.7%) Taiwanese HCC patients. Because these mRNA expression frequencies were obtained under non-standardized RT-PCR conditions, we conducted similar studies to detect the NY-ESO-1 expression

Table IV. Correlation between CT antigen expression and clinical parameters.

	NY-ESO-1		LAGE-1		SSX-1	
	+	-	+	-	+	-
Cases	19	45	29	35	24	40
Gender						
Male	18	41	28	31	22	37
Female	1	4	1	4	2	3
HBsAg						
Positive	18	37	27	28	21	34
Negative	1	8	2	7	3	6
Serum AFP level (ng/ml)						
≤20	6	7	6	7	7	6
>20	13	38	23	28	17	34
Tumor diameter (cm)						
≤5	6	17	10	13	9	13
>5	13	28	19	22	15	27
Tumor stage						
I or II	8	13	10	11	10	11
III or IV	11	32	19	24	14	29
Average age (year)	49.3±11.3	48.8±13.5	49.7±10.9	48.2±14.3	54.3±12.6[a]	45.7±11.9[a]

[a]T, 2.682 and P=0.01.

frequencies in HCC patients using the different DNA polymerases and primers used frequently in the references.

Many factors are important for amplifying the correct gene using PCR. Selecting the proper primers and polymerases are the most important steps in designing a PCR experiment. Some guidelines of primer design should be considered, including (but not restricted to) assurance of a high (45-55%) guanine and cytosine (GC) content and the absence of potential primer-dimers or primer-hairpin configurations. In our study, we selected three primer pairs used most frequently in the CT literature and we identified them as NY-ESO-1 P1, NY-ESO-1 P2 and NY-ESO-1 P3 (for simplicity, shortening these primer names to P1, P2 and P3). The three primer pairs were all designed to recognize and bind different exons of the NY-ESO-1 gene and were chosen to exclude and distinguish false-positive amplicons from any contaminating DNA in the RNA preparation. Evaluating the primer sequences with the Primer Premier 5.0 software, we found that P3 was better than P1 or P2 as follows. The GC content of P3 was 55 and 52.4% in the upstream and down primers, respectively. Thus the two PCR primers possessed similar annealing temperatures. Neither primer contained any potential to form primer-hairpins, thus avoiding any internal secondary structures. Although the upstream primer of P3 has the capacity to form a primer-dimer, the complementary bases are at the 5' ends.

In our study, we used *Taq*, Platinum *pfx* and AmpliTaq Gold DNA polymerases. Each DNA polymerase has unique characteristics that affect the efficacy of the PCR. *Taq* DNA polymerase is purified from *E. coli* expressing a cloned *thermos aquaticus* DNA polymerase gene. This enzyme has both 5' to 3' DNA polymerase and 5' to 3' extronuclease activities, but lacks a 3' to 5' extronuclease activity. Non-specific products were yielded when the PCRs were performed using *Taq* DNA polymerase with the P1 and P2 primers. The positive products could not be sequenced because the PCR had a relatively high error rate because the polymerase lacked the 3' to 5' exonuclease proofreading function, the primers had an insufficient melting temperature and retained the capacity to form internal structures. However, the positive products yielded by the PCRs using *Taq* DNA polymerase and P3 primers were specific, as confirmed by sequencing. The results indicated that the choice of primer is very important when using *Taq* DNA polymerase in detecting NY-ESO-1.

The Platinum *pfx* DNA polymerase is complexed with specific monoclonal antibodies to inhibit DNA polymerase and 3' exonuclease activities during PCR assembly and initial denaturation. This configuration significantly reduces or eliminates pre-PCR misprimings, primer-dimers, artifacts and any other non-specific amplification products. Additionally, this enzyme provides high amplification specificity, sensitivity and amplicon yield. Platinum *pfx* offers the benefit of proof-reading activity. Its 3' to 5' exonuclease activity eradicates mismatched base pairings, so it offers high sensitivity and yield. The positive products obtained with Platinum *pfx* DNA polymerase and the P1 and P2 primers could be sequenced, even in the presence of non-specific products. The positive

products yielded with NY-ESO-1P3 were specific and confirmed by sequencing. The results indicated that Platinum *pfx* DNA polymerase should be employed for producing high yields of NY-ESO-1.

AmpliTaq Gold DNA polymerase is a chemically modified form of AmpliTaq DNA polymerase, which renders the enzyme inactive at room temperature. Upon thermal activation (typically 5-10 min at 95˚C), the modifier is permanently released, resulting in an active enzyme. This pre-heat step allows activation of the polymerase, which helps eliminate the non-specific primer-template complex formations at lower temperatures. Hence, consistent amplification results were seen routinely with AmpliTaq Gold DNA polymerase and the three primer pairs.

Detecting the lowest working dilutions of ß-actin and NY-ESO-1 enabled us to compare their amplification sensitivities of the three DNA polymerases. The sensitivity of Platinum *pfx* DNA polymerase was higher than that of *Taq* DNA polymerase and AmpliTaq Gold DNA polymerase. False-positive and false-negative PCR results are unexpected in detecting the expression of NY-ESO-1. Thus, specificity and sensitivity of the technology should be considered. The results that the seven positive specimens were coincident detecting with three DNA polymerases indicated the sensitivity of the technology was adequate. Our results suggest that AmpliTaq Gold DNA polymerase should be coupled with P3 primers to provide the most consistent results in detecting NY-ESO-1 with high specificity.

In this study, the expression levels of NY-ESO-1, LAGE-1 and SSX-1 were detected by RT-PCR in 64 HCC patients. The primers were confirmed from different exons to avoid false-positive results caused by potential contamination with genomic DNA. We identified all PCR products by sequencing, using the same primers used for the respective amplifications. The NY-ESO-1 and LAGE-1 amplifications were performed with AmpliTaq Gold DNA polymerase and the SSX-1 amplification was performed with *Taq* DNA polymerase. LAGE-1 and NY-ESO-1 are highly homologous and have similar nucleotide compositions in which the G+C content is 63.5 and 64.6%, respectively. Therefore, the LAGE-1 amplification was also performed with AmpliTaq Gold DNA polymerase. SSX-1 was amplified with *Taq* DNA polymerase and the result was highly successful. It is not necessary that all CT genes be amplified with AmpliTaq Gold DNA polymerase. The expression frequencies of NY-ESO-1, LAGE-1 and SSX-1 were 29.7% (19/64), 45.3% (29/64) and 37.5% (24/64), respectively. The three CT antigens were not expressed in adjacent non-cancerous tissues. The co-expression of at least one, two and three of the NY-ESO-1, LAGE-1 and SSX-1 genes was 57.8% (37/64), 35.9% (23/64) and 18.8% (12/64) in HCC tissues, respectively. The high frequencies of co-expression of the numerous CT antigens in HCC suggests the possibility of polyvalent vaccinations for HCC. Polyvalent vaccinations with multiple antigens, which prevent the heterogenous expression of tumor cells, may provide improved clinical results over a single antigen-based vaccination in cancer immunotherapy. The opportunity for cancer-specific immunotherapy can thus be expanded (9).

We were particularly interested in the expression of NY-ESO-1 and LAGE-1. By representive difference analysis, Lethe *et al* (24) identified and cloned CTAG2, which they called LAGE-1. The deduced 180-amino acid protein shares 84% identity with NY-ESO-1 and contains two glycine-rich regions and a hydrophobic stretch near the C-terminus. The correlation between the expression of LAGE-1 and NY-ESO-1 in the tumor samples has been noted and the expression of these two genes has been reported in a wide variety of cancer types other than HCC (24). Because of homologies at both the mRNA and protein levels, there is the potential for cross-detection with molecular methods and antibodies against NY-ESO-1 in LAGE-1 detection assays. In our study, the expression frequencies of NY-ESO-1 and LAGE-1 were 29.7% (19/64) and 45.3% (29/64), respectively. Among them, 21.9% (14/64) of the HCC samples expressed both genes and 53.1% (34/64) expressed at least one of the genes. This finding was comparable to that reported by Xing *et al* (25), in which they determined that nine (26.5%) out of the 34 HCC samples were NY-ESO-1 mRNA-positive and 12 (35.3%) were LAGE-1 mRNA-positive. Among them, 7 (20.6%) HCC samples expressed both genes and 14 (41.6%) expressed at least one of the genes. NY-ESO-1 or LAGE-1 mRNA is expressed at a high frequency in HCC tissue samples. NY-ESO-1 and LAGE-1 proteins share the same epitopes recognized by cytotoxic T lymphocyte (CTL). It is thus possible that these genes can serve as targets for antigen-specific immunotherapy in HCC patients.

The correlation between the CT antigen and tumor clinical parameters is a long-standing topic of interest. Understanding the relationships among CT antigen expression and clinical parameters, such as gender, age, HBV infection, serum AFP levels, tumor stage and tumor size, is the focus of our current study. Our results from three CT antigen expression analyses consistently showed that the CT antigen-positive expressing patients were generally older than the antigen-negative group. Statistical analyses revealed that the expression of SSX-1 was correlated with older patient age ($P<0.05$). There were no significant differences between CT antigen expression and any of the other clinical parameters. Our study that the expression of CT gene mRNA transcripts increased with the age may be due to the hypomethylation of the cellular genome, which is a possible mechanism of CT antigen mRNA expression (26). In this study, the serum AFP was normal (<20 ng/ml) or slightly elevated (<40 ng/ml) in 29.7% (19/64) of the HCC patients. In these 19 patients, 13 maintained NY-ESO-1, LAGE-1 or SSX-1 mRNA in their HCC tissues, implicating these molecules as tumor-specific markers of HCC cell detection in circulation for diagnosis. This assay combined with the examination of serum AFP levels may improve the diagnosis of HCC patients.

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