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Newly Identified Biomarkers for Detecting Circulating Tumor Cells in Lung Adenocarcinoma

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Circulating tumor cells (CTCs) have been implicated in cancer prognosis and follow up. Detection of CTCs was considered significant in cancer evaluation. However, due to the heterogeneity and rareness of CTCs, detecting them with a single maker is usually challenged with low specificity and sensitivity. Previous studies concerning CTCs detection in lung cancer mainly focused on non-small cell lung carcinoma. Currently, there is no report yet describing the CTC detection with multiple markers in lung adenocarcinoma. In this study, by employing quantitative real-time PCR, we identified four candidate genes (mRNA) that were significantly elevated in peripheral blood mononuclear cells and biopsy tissue samples from patients with lung adenocarcinoma: cytokeratin 7 (CK7), Ca^{2+}-activated chloride channel-2 (CLCA2), hyaluronan-mediated motility receptor (HMMR), and human telomerase catalytic subunit (hTERT). Then, the four markers were used for CTC detection; namely, positive detection was defined if at least one of the four markers was elevated. The positive CTC detection rate was 74.0% in patients with lung adenocarcinoma while 2.2% for healthy controls, 6.3% for benign lung disease, and 48.0% for non-adenocarcinoma non-small cell lung carcinoma. Furthermore, in a three-year follow-up study, patients with an increase in the detection markers of CTCs (CK7, CLCA2, HMMR or hTERT) on day 90 after first detection had shorter survival time compared to those with a decrease. These results demonstrate that the combination of the four markers with specificity and sensitivity is of great value in lung adenocarcinoma prognosis and follow up.

Keywords: Ca^{2+}-activated chloride channel-2; circulating tumor cells; cytokeratin 7; hyaluronan-mediated motility receptor; telomerase catalytic subunit
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Introduction

Adenocarcinoma is the most common form of lung cancer (Miao et al. 2012). In the absence of metastasis, lung adenocarcinoma is largely treatable; therefore, early diagnosis is of great value in reducing the mortality and morbidity associated with this disease (Wang et al. 2012). Current diagnostic methods often result in diagnosis after metastasis, including physical examination, X-ray and computer tomography, histological examination of the tumor or biopsy and evaluation of mediastinal and sentinel lymph nodes (Sher et al. 2005). Thus, early diagnostic methods are needed.

The release of tumor cells into the blood stream is critical for metastasis, which gives rise to circulating tumor cells (CTCs) (Allan and Keeney 2010). The presence of CTCs in peripheral blood has been associated with poor prognosis in multiple types of cancer (Jacob et al. 2007; Helo et al. 2009; Mostert et al. 2009; Mavroudis 2010). The majority of studies on CTCs in lung cancer focused on non-small cell lung carcinoma (NSCLC) (Hayes et al. 2006; Okumura et al. 2009). Lung adenocarcinoma is a type of NSCLC in non-smokers. However, the CTC detection in lung adenocarcinoma is rarely investigated separately (Yu et al. 2013). Lung adenocarcinoma cells migrate more easily and are different in biological characteristics compared with other non-adenocarcinoma NSCLC.

The challenge of CTCs detection is the requirement of high sensitivity combined with high specificity (Nagrath et al. 2007). CTCs are highly heterogeneous and present at very low frequencies, about 1-10 CTCs per ml of whole blood in patients with metastatic disease. Thus, CTCs are

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usually enriched by employing density gradient centrifugation (Ficoll-Hypaque separation), immunomagnetic and size filtration procedures (Maheswaran and Haber 2010), and analyzed by polymerase chain reaction (PCR) and flow cytometry. CTCs detection by a panel of markers is an important supplement to the current tumor-node-metastasis (TNM) staging system for improved prognosis and rapid assessment of therapeutic response, facilitating the design of better therapeutic strategies for the treatment of NSCLC (Sher et al. 2005).

Fluorescence-based quantitative real-time PCR (qPCR) allows detection and measurement of minute amounts of nucleic acids in a wide range of samples from numerous sources, and it has become a leading technique for nucleic acid quantification (Paterlini-Brechot and Benali 2007). The sensitivity of qPCR makes it possible to identify one target cell in 10^6-10^7 normal cells, which corresponds approximately to one cell in 0.1-1 ml of blood. In addition, multi-marker qPCR has shown an acceptable sensitivity with well specificity for the detection of CTCs in advanced NSCLC patients (Mavroudis 2010). To date, qPCR has been widely used to detect CTCs in various cancers, including breast, lung and prostate cancers (Ghossein et al. 1999). In this study, we identified CTCs in lung adenocarcinoma patients with multi-marker qPCR and assessed its clinical significance.

Materials and Methods

Study design

Phase I: Identification of gene markers for CTCs in lung adenocarcinoma. Samples were collected from primary biopsies and adjacent non-cancerous tissues from 12 patients with stage I-III lung adenocarcinoma (including 2 minimally invasive adenocarcinoma, 9 invasive adenocarcinoma, and 1 variant of invasive adenocarcinoma). Peripheral blood samples were collected from 12 age-matched patients with stage IV lung adenocarcinoma and from 10 healthy subjects as normal controls. Peripheral blood mononuclear cells (PBMCs) were prepared as described below. The mRNA expression patterns were compared between lung adenocarcinoma tissues and adjacent normal tissues and between patients' PBMCs and control PBMCs. The gene markers, whose expression was elevated in both blood samples and adenocarcinoma tissues, were further analyzed in the subsequent phase II.

Phase II: Validation of gene markers. Blood samples were collected from 30 patients with stage IV lung adenocarcinoma prior to therapy and from 20 healthy subjects as normal controls. PBMCs were prepared as described below. The putative gene markers, identified in phase I, were verified in these PBMC samples. Gene markers, which showed significant difference between patient samples and controls, were further verified in phase III.

Phase III: Large-scale validation and clinical outcome analysis. PBMCs were prepared from an independent group of 100 patients with lung adenocarcinoma and from 48 healthy subjects as normal controls. The gene markers identified in phase II were verified in these independent samples. All of 100 patients with lung adenocarcinoma were followed up for 3 years to assess the relationship between CTCs and clinical outcome. In addition, PBMCs from 48 patients with benign pulmonary disease and 100 patients with non-adenocarcinoma NSCLC were also included to examine the specificity of the makers.

Selection of tumor marker genes

Gene markers were compiled from recent literature with reference to CTC detection in lung cancer (Sher et al. 2005; Hayes et al. 2006; Jacob et al. 2007; Xi et al. 2007; Okumura et al. 2009; Mavroudis 2010; Sanchez-Palencia et al. 2011; Pak et al. 2012; Young et al. 2012). Accordingly, the following 48 genes were selected: genes upregulated in lung cancer, including carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5), collagen type XI alpha 1 (COL11A1), ceruloplasmin (CP), chemokine (C-X-C motif) ligand 13 (CXCL13), gremlin 1 (GREM1), marker of proliferation Ki-67 (MKI67), matrix metallopeptidase 1(MMP1), matrix metallopeptidase 12 (MMP12), serine peptidase inhibitor, Kazal type 1 (SPINK1), secreted phosphoprotein 1 (SPP1), topoisomerase (DNA) II alpha 170kDa (TOP2A), TOX high mobility group box family member 3 (TOX3); tumor metastasis genes, including annexin A5 (ANXA5), dual specificity phosphatase 6 (DUSP6), v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3 (ERBB3), hepatocyte growth factor (HGF), hyaluronan-mediated motility receptor (HMMR), interferon regulatory factor 4 (IRF4), lymphocyte-specific protein tyrosine kinase (LCK), met proto-oncogene (MET), monocyte to macrophage differentiation-associated protein (MMD), neurofibromin 1 (NF1), signal transducer and activator of transcription 1 (STAT1), signal transducer and activator of transcription 2 (STAT2); genes differentially expressed in adenocarcinoma vs. squamous cell carcinoma, including Ca^{2+}-activated chloride channel-2 (CLCA2), desmoglein 3 (DSG3), keratin 5 (KRT5), keratin 14 (KRT14), small proline-rich protein 1A (SPRR1A), anterior gradient 2 (AGR2), lens protein with glutamine synthetase domain (LGSN), NK2 homeobox 1 (NKX2-1), surfactant associated 3 (SFTA3), serine peptidase inhibitor Kazal type 1 (SPINK1); other genes relating to CTCs in lung cancer, including cytokeratin 7 (CK7), thyroid Transcription Factor-1 (TTF-1), carcinoembryonic antigen (CEA), human telomerase reverse transcriptase (hTERT), baculoviral IAP repeat containing 5 (BIRC5 or Survivin), surfactant protein B (SFTPB), cytokeratin 20 (CK20), BMI1 polycomb ring finger oncogene (BMI-1), mucin 16, cell surface associated carbohydrate antigen (CA125), fibronectin 1 (FN1), epithelial cell adhesion molecule (EpCAM), snail zinc finger protein (SNAIL), cytokeratin 19 (CK19), and basic helix-loop-helix transcription factor twist (TWIST).

Sample collection and processing

Samples were collected from the Affiliated Tumor Hospital of Harbin Medical University (Harbin, China). Biopsies of lung adenocarcinoma and adjacent normal tissues were obtained from thoracic surgery patients. Peripheral blood samples were obtained from patients with lung adenocarcinoma, non-adenocarcinoma NSCLC, and benign lung disease including tuberculosis and false-positive tumors from pneumonia. Written informed consent was obtained from all patients prior to the study. Control peripheral blood samples were provided by volunteers from the laboratory of Cancer Institute of Heilongjiang Province (Harbin, China). The study was approved by the ethics committee of the Affiliated Tumor Hospital of Harbin Medical University and the Second Hospital of Heilongjiang Province.

Fresh tissue samples (< 300 mg) were grinded into powder in liquid nitrogen immediately. Total RNA was prepared with about 1

ml Trizol (Invitrogen, Carlsbad, California, USA) and stored at −80°C until use.

Peripheral blood samples were collected and processed as described below. To avoid contamination with epithelial skin cells, all blood samples (7.5 ml) were collected with the first 2 ml blood discarded. Peripheral blood mononuclear cells (PBMCs) containing CTCs were prepared with Red Blood Cell Lysis Buffer (Solarbio, Beijing, China) according to previous reports (Hayes et al. 2006; Xi et al. 2007; Okumura et al. 2009). Briefly, blood samples were processed within 30 min. After centrifugation at 2,000 g for 15 min, buffy coat was collected and washed once with phosphate buffered saline (containing 0.14 g/L KH_2PO_4, 9 g/L NaCl, 0.8 g/L Na_2HPO_4, pH 7.4). Then, cells were pelleted by centrifugation and lysed with TRIzol Reagent (Invitrogen) for RNA extraction. Total RNA of PBMCs was collected as described for the tissue samples.

RNA extraction and cDNA synthesis

According to the Trizol-manufacturer's instruction, RNA was extracted from tissue or PBMC samples and dissolved in 22.5 μl RNase-free water. The concentration of RNA was determined using NanoDrop 1000 (Nanodrop, Wilmington, Delaware, USA). Typical RNA concentration range was 525-3,809 ng/μl from PBMCs, and 1,018-4,712 ng/μl from tissue samples. Ultraviolet spectrophotometry and polyacrylamide electrophoresis (PAGE) were used to determine the purity and integrity of the extracted RNA. RNA extraction was standardized, based on the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009). First-strand cDNA synthesis was performed using 1 μg RNA with Oligo(dT) primers in a 20-μl reaction system (Promga, Madison, USA).

Quantitative real-time PCR (qPCR)

The mRNA levels of genes of interest were measured by real-time PCR (7500 by Applied Biosystems, Foster City, CA, USA) using SYBR Green for Phase I and TaqMan probes for Phase II and III. The primers and probes were designed and synthesized by Shanghai Generay Biotech (Shanghai, China). Sequences of primers and TaqMan probes for phase II and phase III are listed Table 1. Optimal annealing temperatures were determined by gradient PCR. Each qPCR reaction was performed in a final volume of 20 μl using SYBR Green PCR Master mix or TaqMan probe PCR Master mix (TaKaRa). All experiments were carried out according to MIQE standards (Bustin et al. 2009).

Total amount of mRNA was normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using the following formula: $\Delta Cq = Cq^{(GAPDH)} - Cq^{(marker\ gene)}$. The differential expression ratios from adenoma samples relative to control samples were calculated using the formula: $2^{\Delta Cq-(mean\ \Delta Cq\ normal)}$.

To prepare an artificial CTC model as positive control, a trace amount of A549 human lung adenocarcinoma cells were mixed with peripheral blood samples (five A549 cells in 7.5 ml peripheral blood). Then, the total RNA was extracted from the mixture and used as the positive control in qPCR. The relative mRNA expressions of the samples were calculated as follows: $Q = 2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq = \Delta Cq^{(sample)} - \Delta Cq^{(positive\ control)}$; $Cq^{(sample)} = Cq^{(marker,\ sample)} - Cq^{(GAPDH,\ sample)}$; $\Delta Cq^{(positive\ control)} = Cq^{(marker,\ positive\ control)} - Cq^{(GAPDH,\ positive\ control)}$.

Statistical analysis

The characteristics of participants, distribution of tumor stage, and individual number of positive CTCs in phase I, II and III were determined by Student's *t*-test, analysis of variance (ANOVA), chi-square test (χ^2 test), or Fisher's exact probability test, where appropri-

Table 1. Sequences of primers and TaqMan probes for phase II and phase III studies.

Primer	5′-3′ sequence	Size of PCR product
Survivin (sense)	AAGAACTGGCCCTTCTTGGA	253 bp
Survivin (antisence)	CAACCGGACGAATGCTTTT	
Survivin taqman probe	CCAGATGACGACCCCATTGGGCCGG	5′ FAM 3′ TAMARA
hTERT (sense)	TACGTCGTGGGAGCCAGAAC	86 bp
hTERT (antisense)	TTCCGCAGAGAAAAGAGGGCCGA	
hTERT taqman probe	TTCCGCAGAGAAAAGAGGGCCGA	5′ FAM 3′ TAMARA
CK7 (sense)	GACATCGAGATCGCCACCTAC	162 bp
CK7 (antisense)	ATTGCTGCCCATGGTTCCC	
CK7 taqman probe	AATGCCACCGCCACTGCTACTGCC	5′ FAM 3′ TAMARA
TTF-1 (sense)	CTTCGCCTTCCCCCTCTCC	156 bp
TTF-1 (antisense)	CCCTCCATGCCCACTTTCTTG	
TTF-1 taqman probe	TCTTCCTTCCTCCTCCAGCCGCCG	5′ FAM 3′ TAMARA
CLCA2 (sense)	ATGCCAATGTGAAACAGG	
CLCA2 (antisense)	CTGCTCCATCATCAAGGA	
CLCA2 taqman probe	AATGCCACTGTCACTGCCAC	5′ FAM 3′ TAMARA
HMMR (sense)	GCAAACACTGGATGAGCTTGATAAA	146 bp
HMMR (antisense)	CCAGTTCAGCCTCCTTCCCTTT	111 bp
HMMR taqman probe	TACAGCAAAAGGAGGAACAAGCTGAAAG	
GAPDH (sense)	GAAGGTGAAGGTCGGAGTC	225 bp
GAPDH (antisense)	GAAGATGGTGATGGGATTTC	
GAPDH taqman probe	CAAGCTTCCCGTTCTCAGCC	5′ FAM 3′ TAMARA

ate. The Wilcoxon Rank sum test was used to determine the relative expression difference of the mRNA markers between patients with lung adenocarcinoma and normal controls. The sensitivity and specificity were calculated according to standard formulas. Receiver operating characteristic (ROC) curves and logistic regression analysis were established for discriminating between patients with or without CTCs in lung adenocarcinoma. The Kaplan-Meier's survival curves and Log-Rank tests were used to analyze patient survival. Statistical analyses were performed using SPSS v.17.0 software and R-2.15.2 software. All *p*-values were two-sided and $p < 0.05$ was considered statistically significant.

Results

Characteristics of participants

A total of 380 participants were recruited, including 154 patients with lung adenocarcinoma, 48 patients with benign lung disease, 100 patients with non-adenocarcinoma NSCLC, and 78 healthy controls. The clinical characteristics of participants are shown in Table 2. There was no significant difference in age and gender between different groups ($p > 0.75$ for both).

Identification of lung adenocarcinoma-associated genes in PBMCs and biopsy tissue samples

As described in study design phase I, the mRNA expression patterns of 48 tumor markers in PBMCs and biopsy tissue samples were compared. All of the 48 tumor markers were increased in biopsy tissue samples. However, the expression levels of only six mRNAs were elevated in PBMCs of lung adenocarcinoma patients when the cutoff value was set at 2-fold. The six markers were cytokeratin 7 (CK7), Ca^{2+}-activated chloride channel-2 (CLCA2), hyaluronan-mediated motility receptor (HMMR), human telomerase catalytic subunit (hTERT), Survivn, and thyroid transcription factor-1 (TTF-1). Consistently, these mRNA levels were increased more than 500-fold in biopsy tissue samples (Table 3). In contrast, several markers that have been reported to be expressed by CTCs in NSCLC (Hayes et al. 2006; Pak et al. 2012) showed no significant difference, e.g., cytokeratin 19 (CK19) and epithelial cell adhesion molecule (EpCAM) (Table 4).

Validation of selected markers in a small set of PBMC preparations

The expression of the six putative markers identified in phase I was validated by qPCR. We found that the expression of the six mRNAs was detectable in the majority of PBMC samples from 30 patients with Stage IV lung adenocarcinoma and 20 healthy controls; especially, four of the six markers (CK7, CLCA2, HMMR and hTERT) were significantly elevated in PBMCs from lung adenocarcinoma patients compared to healthy controls (CK7, $p < 0.0001$; CLCA2, $p = 0.0034$; HMMR, $p = 0.006$; hTERT, $p < 0.0001$) (Fig. 1A-D). Since no significant difference was observed for Survivin (BIRC5) or TTF-1 ($p = 0.0692$ and $p = 0.0580$, respectively), they were excluded from further analyses.

The areas under the ROC curves were 0.8492 (0.7375-0.9609, 95.0% CI) for CK7; 0.7467 (0.6068-0.8866, 95.0% CI) for CLCA2; 0.7883 (0.6573-0.9194, 95.0% CI) for

Table 2. General information of participants and distribution of tumor stages.

Characteristic	Adenocarcinoma ($n = 154$)	Other types of NSCLC ($n = 100$)	Healthy controls ($n = 78$)	Other pulmonary diseases ($n = 48$)
Gender (number)				
Male	78	52	37	25
Female	76	48	41	23
Age (year)				
Mean	64	64	62	61
Median (range)	64 (49-91)	64 (40-76)	62 (45-85)	61 (51-70)
TNM stage in				
Phase I				
I	5	–	–	–
II	6	–	–	–
III	1	–	–	–
IV	12	–	–	–
Phase II				
IV	30	–		–
Phase III				
I	20	9	–	–
II	17	20	–	–
III	30	38	–	–
IV	33	33	–	–

Table 3. Identification of genes up-regulated in lung adenocarcinoma compared with healthy controls (expression difference: ≥ 2-fold in PBMCs; > 500-fold in tissue samples) by real-time PCR.

Tumor tissues vs. adjacent normal biopsies from 12 patients with stage I-III lung adenocarcinoma		PBMCs from 12 patients with stage IV lung adenocarcinoma vs. 10 healthy subjects	
mRNA	Average differential change	mRNA	Average differential change
CK7	**1,041**	**CK7**	**5.20**
CLCA2	**985**	**hTERT**	**5.13**
hTERT	**974**	**CLCA2**	**4.99**
HMMR	**885**	**HMMR**	**4.67**
Survivin	**896**	**Survivin**	**2.95**
DUSP6	784	**TTF-1**	**2.98**
TTF-1	**558**	ERBB3	1.98
TOX3	550	MMP1	1.98
CA125	513	CA125	1.93
ANXA5	427	ANXA5	1.76

Table 4. Gene markers that were found to be unsuitable for this study.

Tumor tissues vs. adjacent normal biopsies from 12 patients with stage I-III lung adenocarcinoma		PBMCs from 12 patients with stage IV lung adenocarcinoma vs. 10 healthy subjects	
mRNA	Average fold change	mRNA	Average fold change
LCK	189	CK19	0.850
TOP2A	189	EpCAM	0.843
STAT2	176	STAT2	0.625
CXCL13	173	CXCL13	0.555
NF1	167	TOP2A	0.475
STAT1	153	STAT1A	0.320
MMD	56	MMD	0.225
HGF	26	LCK	0.240
SPP1	13	SPINK1	0.060
MK167	10	MK167	0.075

HMMR; and 0.8725 (0.7666-0.9784, 95.0% CI) for hTERT, respectively (Fig. 2A). The cutoff value was CK7 (0.2398), CLCA2 (0.3237), HMMR (0.2877), hTERT (0.1912); sensitivities of each marker was: CK7 (76.7%), CLCA2 (76.7%), HMMR (70.0%), hTERT (56.7%); and specificities: CK7 (100.0%), CLCA2 (95.0%), HMMR (95.0%), hTERT (100.0%).

Due to heterogeneity of tumor cells, the expression of at least one of the four markers (a combination of the four markers) was considered as CTC positive. Thus, we determined the effect of the combination of four markers, by constructing a ROC curve and fitting a logistic model with parameters: CK7, HMMR, hTERT and CLCA2. The area under the ROC curve was 0.9217 (0.8341-1.0000, 95.0% CI), the sensitivity and specificity were 86.7% and 100.0%, respectively, when the optimal threshold was 0.2449 (Fig. 2B). These results indicated that CTCs detection using the combination of four markers was superior to individual of the four markers. Therefore, the value of the combination of four markers was used for large-scale validation in phase III.

Independent large-scale validation in PBMC samples and clinical outcome analysis

To further evaluate the clinical significance of the combination of four markers, the expression of the four markers was analyzed in PBMCs from 100 patients with stage I-IV lung adenocarcinoma and 48 healthy controls as described in phase III. The CTC positive rate was 74.0% in patients while it was only 2.0% in healthy controls.

The specificity of the combination of four markers for detection of CTCs was assessed in lung adenocarcinoma by analyzing the expression of four genes in PBMCs from 48 patients with benign lung disease and 100 patients with non-adenocarcinoma NSCLC. The CTC positive rate was 6.3% for patients with benign lung disease and 48.0% for patients with non-adenocarcinoma NSCLC. As shown in Fig. 3A, there was no significant difference in the CTC positive rate between benign lung disease and healthy controls ($p = 0.6170$). Furthermore, the CTC positive rate of non-adenocarcinoma NSCLC patients was significantly lower than that of lung adenocarcinoma patients ($p = 0.0002$; stage I, $p = 0.2422$; stage II, $p = 0.1473$; stage III, $p =$

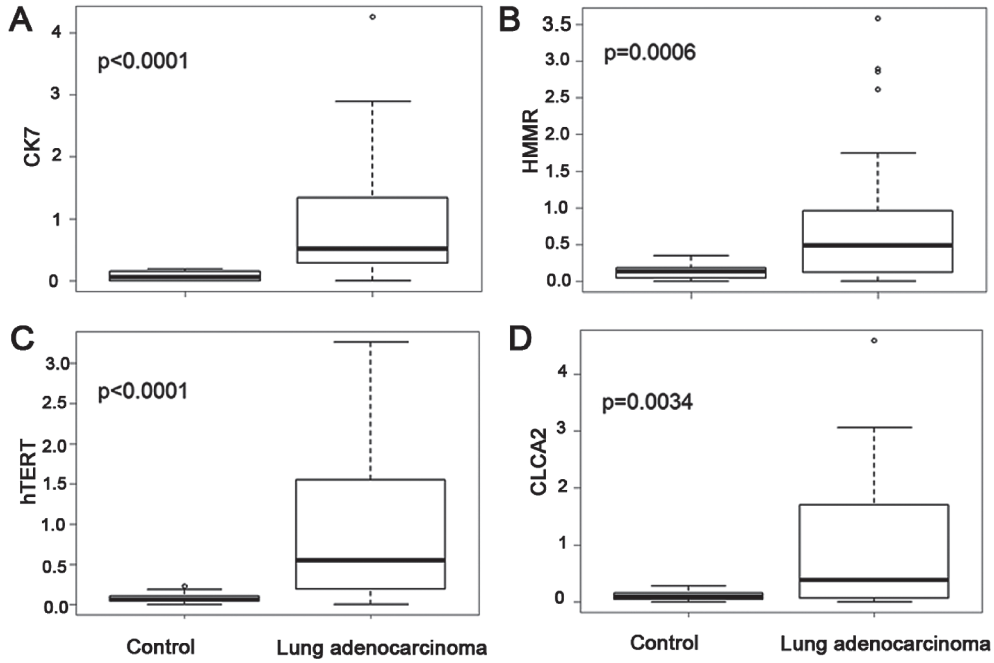

Fig. 1. Validation of the putative markers by qPCR analysis (Phase II).
Box plots show the relative expression levels of mRNAs, normalized to GAPDH, for 30 lung adenocarcinoma patients and 20 healthy controls. (A) CK7; (B) HMMR; (C) hTERT; (D) CLCA2. The lines inside the boxes denote the medians; boxes mark the interval between the 25th and 75th percentiles; whiskers denote the interval between the 10th and 90th percentiles; circles indicate data points outside the 10th and 90th percentiles.

0.0242; and stage IV, $p = 0.0004$, Fig. 3B).

We also assessed the significance of the change of CTC levels in monitoring the efficacy of therapy. Of the 100 patients with lung adenocarcinoma, 43 underwent surgical resection, 53 received non-operative therapies, and 4 abandoned treatment; 2 patients were lost 30 days after first detection. As mentioned above, the expression of at least one of the four markers was regarded as positive detection. The first detection was performed on day 0 (d 0) after the first detection. In 74 patients with positive CTC detection, a decreased CTC level was observed in most patients having surgery and some receiving non-operative therapy. As expected, the CTC level was increased in all patients who abandoned treatment. The follow-up outcome of 10 randomly selected patients was shown in Fig. 4A. Analysis of the CTCs levels and survival time showed that patients who had an increase in the CTC level after 90-day follow-up had shorter survival times compared to those who had a decrease ($p < 0.001$) (Fig. 4B). In contrast, at the end of the three-year follow-up, the survival time of patients with CTCs was significantly shorter than those without ($p = 0.001$) (Fig. 4C); however, no significant difference was observed between those with high and low CTC levels (data not shown). The data of patients in Fig. 4B and C were divided into two groups (operable and non-operable) and reanalyzed as in Fig. 5A and B. We found that the survival time of both patients with operable and non-operable showed similar trend that is all patients with negative CTC detection and patients with a decrease in CTCs live longer. These results demonstrated that the combination of four markers we defined provides satisfactory significance in lung adenocarcinoma prognosis and rapid evaluation of treatment response.

Discussion

In this study, we defined a combination of four markers for diagnosis of metastasis of lung adenocarcinoma and evaluated the significance of the combined marker in cancer prognosis and evaluating therapy efficacy. We initially selected 48 candidate tumor markers after extensive exploration of the literature and found they were all detectable in both tissue and plasma samples of lung adenocarcinoma. In regard to the high heterogeneity and low frequency (Sergeant et al. 2008; Bunn 2012; Cen et al. 2012), we employed qPCR in this study. We determined the expression of the 48 potential marker mRNAs, six of which (CK7, CLCA2, HMMR, hTERT, Survivn, and TTF-1) showed significant differential expression ≥ 2-fold in PBMCs and > 500-fold in tissues compared to healthy controls. However, only four markers (HMMR, CLCA2, CK7, and hTERT) were significantly elevated in PBMCs of lung adenocarcinoma patients compared to controls.

CK7, a member of the CK family, has been reported in breast cancer, renal carcinoma, bladder cancer, and 97.0%-100.0% of lung adenocarcinoma cases, including primary tissues, metastatic organs, and cavity liquid (Camilo et al. 2006; Serpil et al. 2009; Dukic et al. 2011). Immunohistochemical examination of CK7 and CK20 has been used

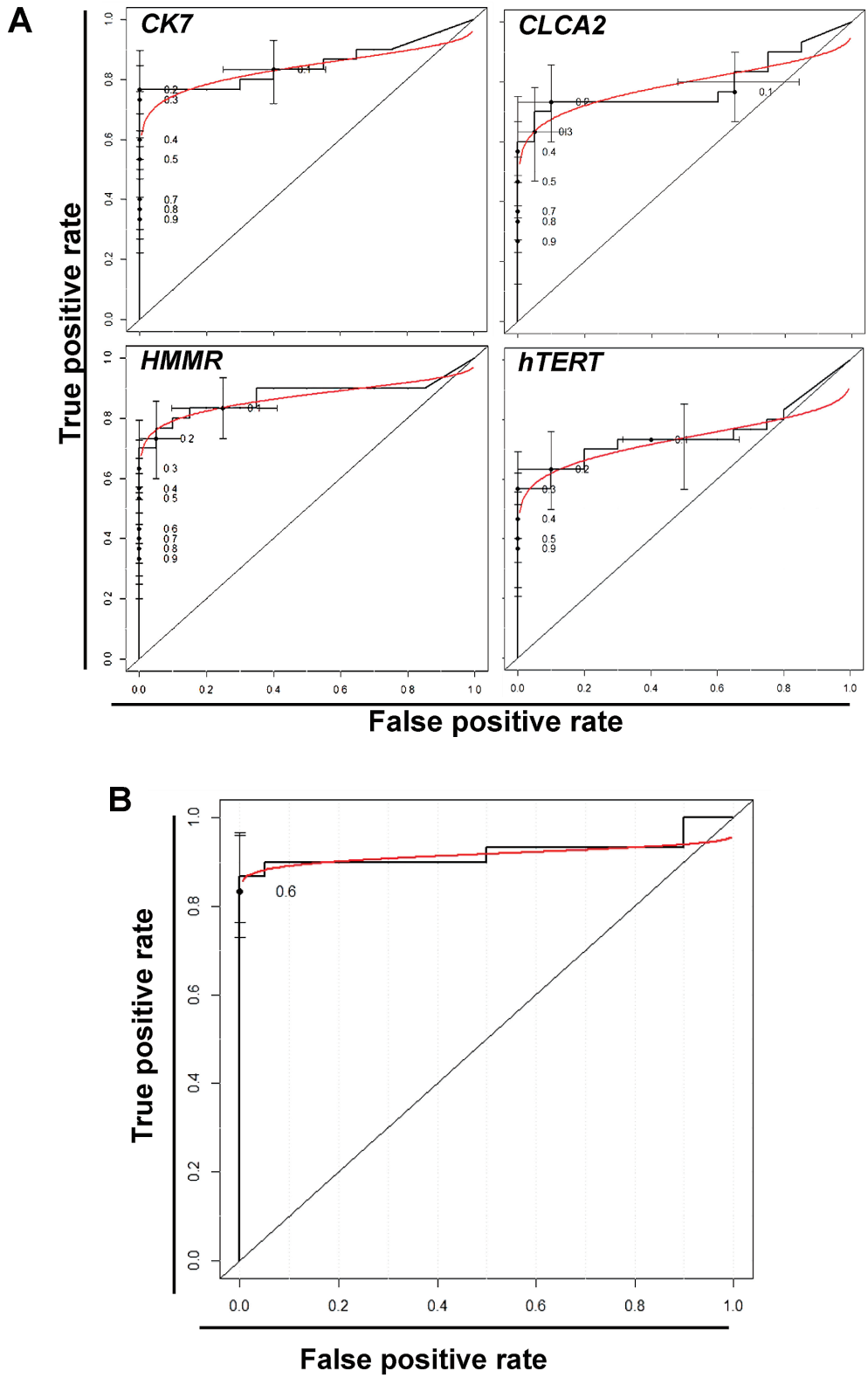

Fig. 2. Empirical and smooth receiver operating characteristics (ROC) curve analysis.
The areas under the ROC curves are as follows: (A) CK7, 0.8492 (0.7375-0.9609, 95.0% CI); CLCA2, 0.7467 (0.6068-0.8866, 95.0% CI); HMMR, 0.7883 (0.6573-0.9194, 95.0% CI); hTERT, 0.8725 (0.7666-0.9784, 95.0% CI); (B) the combined four markers (the expression of at least one of the four markers), 0.9217 (0.8341-1.0000, 95.0% CI).

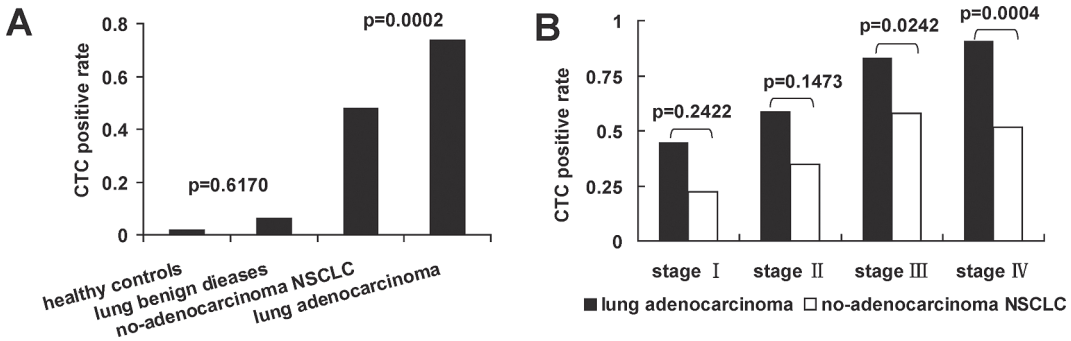

Fig. 3. Validation of the combination of the four markers in independent large-scale PBMC samples.
A. Histograms showed the positive CTCs detection rates by the combined marker in 296 PBMC samples from patients with lung adenocarcinoma (74.0%), healthy controls (2.0%), benign lung disease (6.3%), and non-adenocarcinoma NSCLC (48.0%). B. Histogram of the positive CTCs detection rates by the combined markers for lung adenocarcinoma patients at different stages of disease compared to non-adenocarcinoma NSCLC.

diagnostically for discrimination of primary lung adenocarcinoma and lung metastasis of colorectal cancer (Ikeda et al. 2006). Of the four markers, CK7 was the most suitable marker for CTC detection of lung adenocarcinoma, with an optimal threshold, sensitivity and specificity of 0.3237, 76.7% and 100.0%, respectively.

CLCA2 belongs to the CLCA family of calcium-activated chloride channels and was initially identified in human lung, trachea, and mammary glands as hCLCA2. Loss of CLCA2 expression has been associated with tumorigenicity in breast cancer (Gruber et al. 1999; Toonkel et al. 2010); whereas, overexpression has been reported in squamous cell lung cancer. In addition, overexpression of CLCA2 has been reported to be specifically associated with lung cancer and squamous cell tissues (Xi et al. 2007). Our study determined that the optimal threshold, sensitivity and specificity of CLCA2 in lung adenocarcinoma were 0.3237, 0.8% and 95.0%, respectively. This is the first study about CLCA2 in lung adenocarcinoma and suggests that CLCA2 is a suitable marker for CTC detection in lung adenocarcinoma by qPCR.

HMMR, a cell surface oncogenic protein, is widely up-regulated in human cancers and related with cell motility and invasion. However, the molecular mechanisms of regulating of HMMR in cancers remain unknown (Maxwell et al. 2008). HMMR is a novel breast cancer susceptibility gene and a tumor-associated antigen found in solid and blood tumors. It has been widely reported that the overexpression of *HMMR* is invovled in cell invasion and migration of various advanced cancers, including breast cancer, prostate cancer, bladder cancer, and acute myeloid leukemia (Liu et al. 2012). Tano et al. (2010) found that MALAT-1 enhanced the motility of lung adenocarcinoma cells by influencing the expression of motility-related genes, *CTHRC1*, *CCT4*, *HMMR and ROD1*. Here we found that HMMR was detectable in the majority of lung adenocarcinoma samples and was significantly elevated in patient plasma compared to controls (p = 0.006). Validations in phase II and phase III confirmed *HMMR* as a suitable candidate for CTC detection in lung adenocarcinoma.

hTERT, which replicates the ends of linear DNA, is expressed in almost 90.0% of human cancers (Melin et al. 2012). hTERT has been included in CTC detection in peripheral blood by multi-marker qPCR analysis. Its role in diagnosis of metastasis and assessment of therapy efficacy and prognosis (Wu et al. 2006; Uen et al. 2007) has been illustrated in advanced biliary tract malignancies (Leelawat et al. 2012), breast cancer (Strati et al. 2011) and digestive tract tumors. In this study, the sensitivity and specificity of hTERT were 56.7% and 100.0%, respectively, indicating that hTERT was a suitable marker for combined diagnosis of CTCs in lung adenocarcinoma.

In common with other tumors, lung adenocarcinoma is heterogeneous with respect to histological and biological characteristics (Samuel and Hudson 2013). Due to the heterogeneity of tumor cells, the biological characteristics of tumor cells of different patients vary from each other. Even if the tumor cells from the exact same patient express varied marker genes. Thus, the reliability of single marker based detection of CTCs is limited. Recent studies showed that the CTC detection by a panel of markers using PCR provided satisfactory effects. In this study, by employing multiplex-PCR, we found that combining *CK7*, *CLCA2*, *HMMR*, *hTERT* provided an optimal threshold, sensitivity and specificity of 0.2449, 86.7% and 100.0%, respectively, which were superior to those obtained by single markers. As mentioned previously, the expression of at least one of the four genes was a positive CTC detection. In independent large-scale validation using the combination of four markers, we observed that the positive CTC detection rates in patients with lung adenocarcinoma of 74.0% (45.0%, 58.8%, 83.3% and 90.9 % for stage I, II, III and IV, respectively), indicating the sensitivity of this combined marker.

To further validate the specificity of the combination of four genes for CTC detection in lung adenocarcinoma, plasma samples from patients with benign lung disease and non-adenocarcinoma NSCLC were also examined. The positive CTC detection rates in these patients were 6.3%

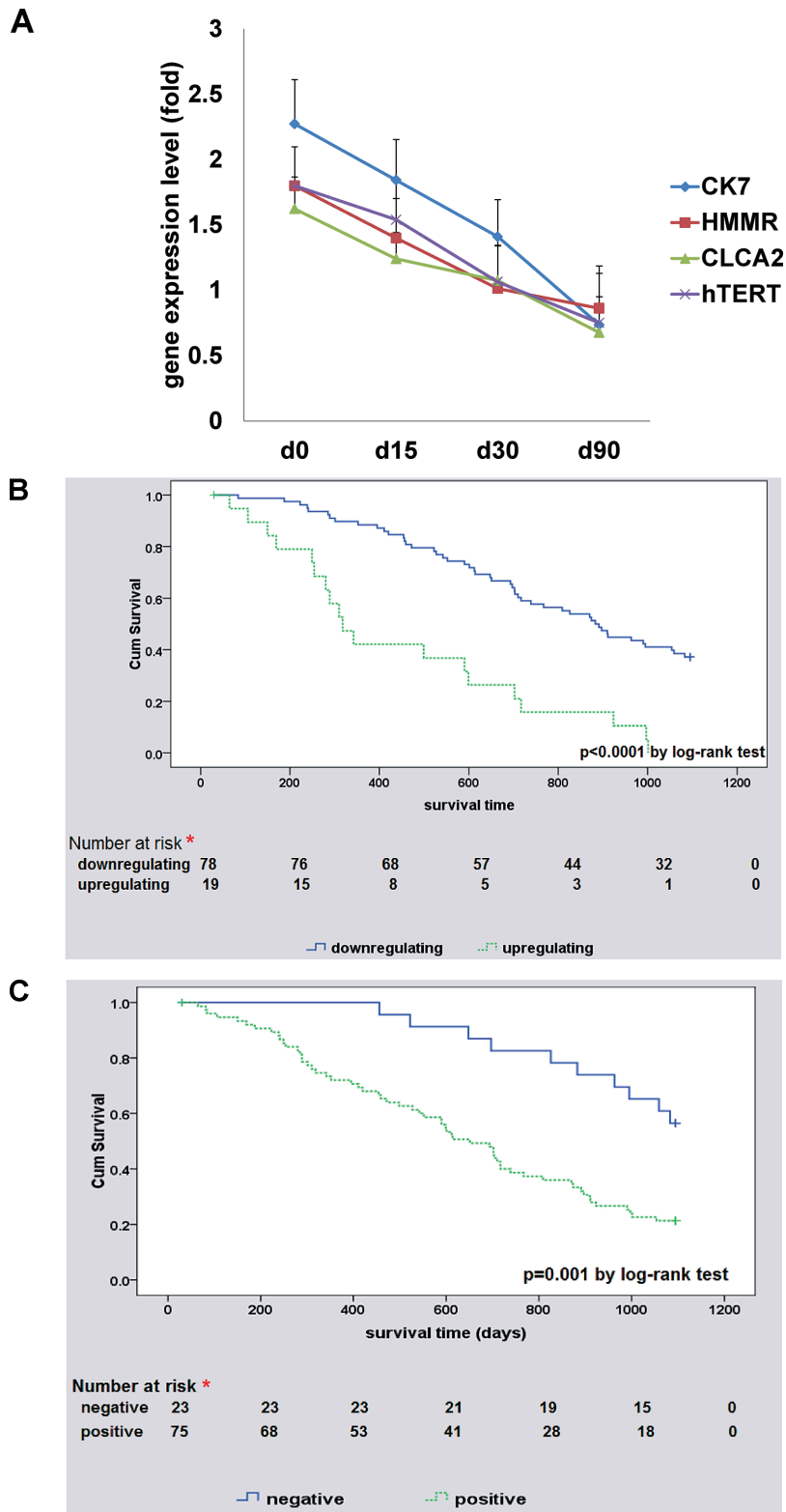

Fig. 4. Assessment of the treatment response and prognosis of lung adenocarcinoma patients with CTCs detection by the combined markers.

(A) The follow-up outcome of 10 randomly selected patients with lung adenocarcinoma: dn is n days after first detection. (B) The median survival time of patients with up-regulated and down-regulated CTCs are 318 days and 883 days, respectively. The Kaplan-Meier's survival curves and Log-Rank test ($\chi^2 = 24.319$, $p < 0.001$) show that the survival time of patients with up-regulated CTCs is significantly lower than those with down-regulated CTCs. (C) The median survival time in patients with CTCs (+) and CTCs (−) are 651 days and 972 days, respectively. The Kaplan-Meier's survival curves and Log-Rank test ($\chi^2 = 11.961$, $p = 0.001$) show that the survival of patients with positive CTCs is significantly shorter than those with negative CTCs. *The data indicate the numbers of survived patients at each survival time.

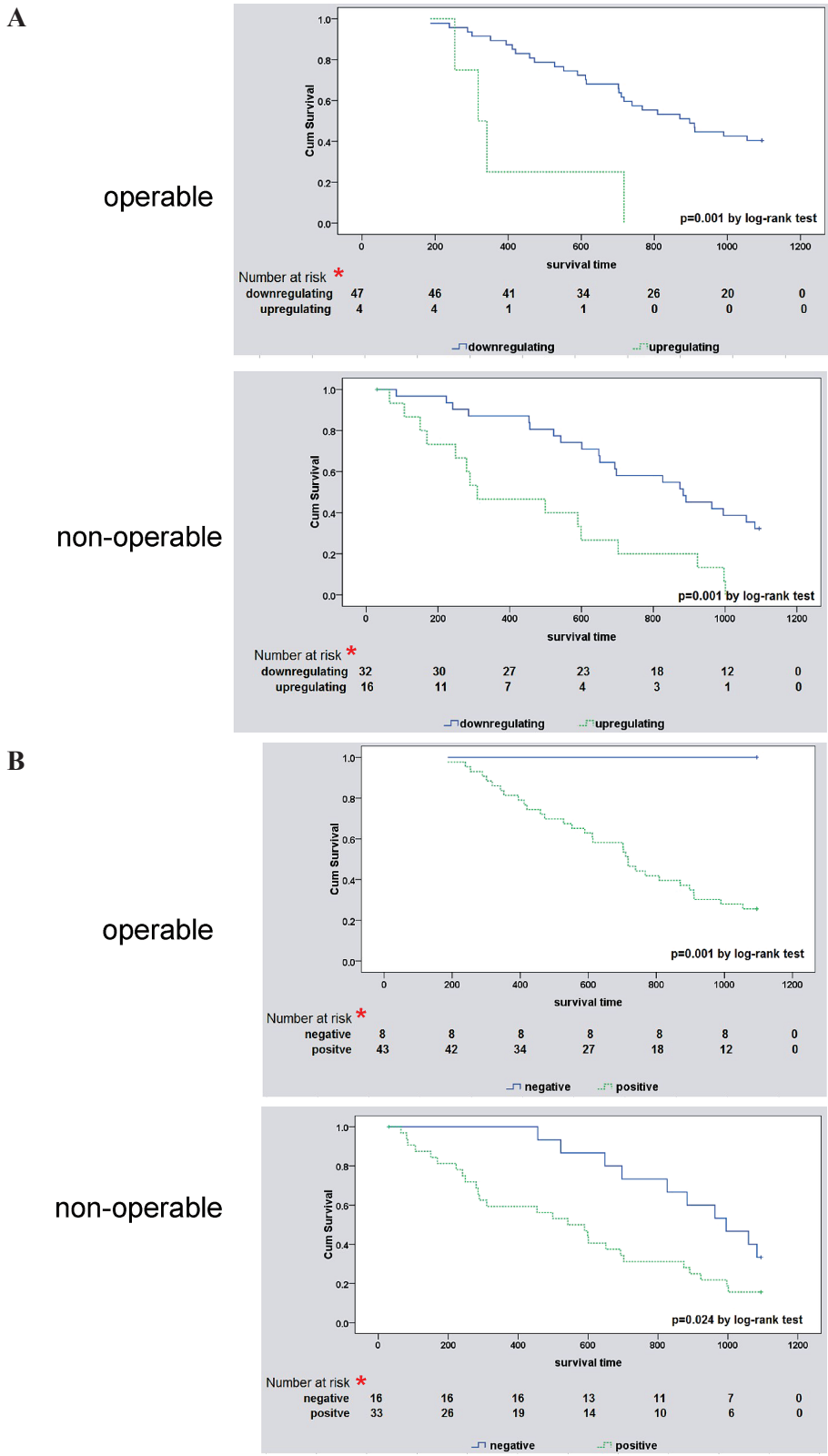

Fig. 5. Assessment of the treatment response and prognosis of lung adenocarcinoma patients with CTCs detection by the combined markers.
The data of patients analyzed in Fig. 4B and C were divided into two groups (operable and non-operable) and reanalyzed. (A) The data of patients analyzed in Fig. 4B were divided into two groups (operable and non-operable) and reanalyzed. (B) The data of patients analyzed in Fig. 4C were divided into two groups (operable and non-operable) and reanalyzed. *The data indicate the numbers of survived patients at each survival time.

and 48.0%, respectively, indicating that the combined marker was specific for CTC detection of lung adenocarcinoma.

Several reports have indicated that CTCs can be used to evaluate treatment response in certain cancer patients. As reported, we found that CTCs decreased in most patients after surgical resection and in some patients received non-operative therapy, while CTCs increased in patients who abandoned treatment. A 90-day follow-up study showed that patients with a rise in CTCs had shorter survival times than those with a decline in CTCs, highlighting this alternative approach for rapidly assessing the treatment response and prognosis of lung adenocarcinoma patients instead of clinical imaging. In addition, in regard to the three-year follow-up study, we found that the survival time of patients with CTCs was significantly shorter than those with negative CTC detection ($p = 0.001$).

Currently, no published reports are available describing the detection of CTCs by multi-gene qPCR in patients with lung adenocarcinoma. Our data have shown that CTC detection using a combined marker is promising for lung adenocarcinoma patients, in terms of both specificity and sensitivity, and could supplement the traditional TNM method for cancer staging to provide better prognosis.

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Conflict of Interest

The authors declare no conflict of interest.

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