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ORIGINAL PAPER

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Observation of circulating tumour cells in patients with non-small cell lung cancer by real-time fluorescent quantitative reverse transcriptase-polymerase chain reaction in peroperative period

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Abstract *Purpose*: To assess whether surgical manoeuvre or resection of lung cancer could lead to haematogenous dissemination of malignant cells. In the mean time, the relationship between the sequence of vessel ligation and the haematogenous dissemination of cancer cells during operation was determined. *Methods*: Exploiting cytokeratin 19 (CK19)/carcinoembryonic antigen (CEA) mRNA as markers, 69 peripheral blood samples were collected from 23 consecutive patients with non-small cell lung cancer (NSCLC) who underwent surgical resection with curative intention in preoperative, intraoperative and postoperative period, respectively. Before the operation, all patients were randomly assigned to one of the two surgical procedure groups according to the order of vessel ligation, PV-first group and PA-first group. Additionally, the ten patients with benign lung disease served as control subjects undergoing surgical resection. The quantity and timing of the shedding of lung cancer cells into the circulation of patients were also monitored by fluorescent quantitative-reverse transcriptase-polymerase chain reaction before, during and after surgery. *Results*: (1) The CK19 diagnostic test: the value of CK19 mRNA in operation was significantly higher than that of preoperation (5.246 ± 0.196 vs. 4.472 ± 0.164, $P = 0.000$) and postoperation (5.246 ± 0.196 vs. 4.694 ± 0.177, $P = 0.013$). The values between adenocarcinoma and squamous carcinoma were strikingly different (4.9110 ± 1.0315 vs. 4.1891 ± 0.4126, $t = 2.364$, $P = 0.028$). The values between PV-first group and PA-first group during perioperative period appear to be different (4.503 vs. 5.085, $P = 0.086$). Before operation, of the 23 cases studied, 14 cases were positive (60.9%). Surprisingly, circulating epithelial cells were detected in two patients resected for benign lung disease. (2) The CEA diagnostic test: the level of CEA mRNA ascended continuously within this period. The postoperative values were significantly higher than those of preoperation (4.874 vs. 4.483, $P = 0.000$) and those of operative day (4.874 vs. 4.537, $P = 0.000$). The values between PV-first group and PA-first group appear to reach statistical significance (4.397 vs. 4.817, $P = 0.075$). At the same time, there was a correlation between preoperative T-stage and perioperative CEA mRNA (4.267 vs. 4.760, $P = 0.025$). Among the 23 cases, 10 cases were positive (43.5%). Both patients with benign lung disease served as control subjects undergoing surgical resection and the volunteers were negative. *Conclusions*: A considerable proportion of patients who appear to have resectable NSCLC might be regarded as having systemic disease, which is often undetectable by current tumour staging method. In terms of a marker used for the NSCLC patients who undergo operation, CEA is more suitable than CK19. The CK19-expressing epithelial cells are released intraoperatively into the circulation, meanwhile CEA-expressing tumour cells are disseminated mostly postoperatively. Surgical manipulation could promote the release of tumour cells into the bloodstream, but the ligation of pulmonary vein before the ligation of the pulmonary artery may partly prevent such release during surgery.

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Keywords Lung neoplasm · Blood · Polymerase chain reaction · Cytokeratin · Carcinoembryonic antigen · Messenger RNA · Peroperative period

Abbreviations FQ-RT-PCR: Fluorescent quantitative-reverse transcriptase-polymerase chain reaction · CK19: Cytokeratin 19 · CEA: Carcinoembryonic antigen · NSCLC: Non-small cell lung cancer · PV: Pulmonary vein · PA: Pulmonary artery · PB: Peripheral blood · ADC: Adenocarcinoma · SCC: Squamous cell carcinoma · PBMNs: Peripheral blood mononuclear cells · ROC: Curve, receiver operator characteristic curve · CTCs: Circulating tumour cells · CI: Confidence interval · Ct: Threshold cycle · CV: Constant of variance · MRD: Minimal residual disease · TNM: Tumour-node-metastases staging

Introduction

Although conventional tumour-staging parameters can provide reliable information about the proportion of a population of patients who will experience a recurrence of the disease, these measures cannot predict which individuals will have a recurrence of disease after primary therapy, particularly if the patient has an early stage disease. Thus, new parameters need to be defined that better identify those patients at the greatest (and at the least) risk of relapse, because this would provide information critical to the subsequent management of the patients. The detection of the earliest manifestations of tumour dissemination is an extremely promising approach that should improve risk assessment and the identification of specific patients who would benefit from adjuvant treatment. During the last 10 years, new immunologic and molecular analytic procedures have been developed to diagnose and characterize minimal residual cancer. Studies are currently in progress to evaluate and standardize these procedures for clinical use (Pantel et al. 1999).

It has been concerned that one may infer the presence of circulating lung cancer cells and hence the potential for metastases if tissue-specific cytokeratin 19 (CK19) mRNA are detected in peripheral blood (PB) of patients with non-small lung cancer. There has been emerging evidence suggesting that CK19 mRNA detection is strongly associated with the presence of metastases or lung recurrence, particularly after surgery (Peck et al. 1998).

The detachment of cancer cells from a primary tumour is one of the early sequential events in the metastatic cascade. Therefore, surgeons always worry that the manual manipulation of a tumour during an operation might enhance the shedding of cancer cells into the bloodstream, resulting in an increase in the incidence of distant metastases. One surgical technique that might prevent such shedding is to ligate the efferent vessels first. In a lung carcinoma operation, ligating the pulmonary vein (PV) before ligating the pulmonary artery (PA) has been postulated as a way to prevent tumour cell dissemination into the bloodstream. Although there has been considerable debate over this recommendation, it has not been rigorously tested.

In the present study, we aim to assess whether surgical manoeuvre or resection of lung cancer could lead to haematogenous dissemination of malignant cells. The quantity and timing of lung cancer cells shedding into the circulation of patients with lung cancer was also monitored by fluorescent quantitative-reverse transcriptase-polymerase chain reaction (FQ-RT-PCR) before, during, and after surgery. To determine the clinicopathological correlation, we analysed the relationship between results and classical parameters.

Materials and methods

Clinical data

With informed consent, 69 PB samples were collected during the preoperative, intraoperative and postoperative period from 23 consecutive patients with lung cancer who underwent surgical resection with curative intention. All blood samples were collected into vacutainer tubes containing sodium heparin. Preoperative blood samples were collected from patients studied at least 1 h before surgery. Intraoperative blood samples were collected immediately after tumour resection. Postoperative blood samples were collected on the seventh day after surgery.

The diagnosis of lung cancer was confirmed histologically on each resected specimen where the tumour size was measured. The postoperative pathologic tumour-node-metastases staging (TNM) stage was determined according to the classification of the International Union Against Cancer.

Before the operation, all patients were randomly assigned to one of the two surgical procedure groups according to the order of vessel ligation, PV-first group (PV ligation preceded the PA ligation) and PA-first group (PA ligation preceded the PA ligation). All the surgical procedures were successfully performed by the same surgical group. Pulmonary vessel mobilization and ligation were the first manoeuvres after entry into the pleural space. Mediastinal lymph node dissection was performed after the completion of lobectomy. All the patients except one had uneventful postoperative recoveries.

To explore whether nontumour cells shed into the circulation during surgery by mechanical spillage, the ten patients with benign lung disease served as control subjects undergoing surgical resection. As negative controls for FQ-RT-PCR, 20 PB samples were collected from healthy subjects.

No patient received any other treatment strategy (e.g. chemotherapy or radiotherapy etc.) before surgery. The clinical and histopathological features of each case were shown (Table 1).

Table 1 Clinicopathological feature of patients with non-small cell lung cancer

Patient no.	Gender/age	Type	Grade	Operation performed	V–A/A–V	pTNM		
						T	*N*	Stage
1	F/52	ADC	Poor	RLL	V–A[a]	2	0	I
2	M/59	ADC	Poor	LUL	V–A	2	2	III
3	M/66	SCC	Poor	RUL	V–A	3	2	III
4	F/70	SCC	Well/mod	RML	V–A	1	0	I
5	M/72	SCC	Well/mod	LLL	V–A	2	0	I
6	M/67	ADC	Well/mod	LLL	V–A	1	0	I
7	M/72	SCC	Well/mod	RUL	V–A	2	1	II
8	M/51	ADC	Well/mod	RLL + RML	V–A	3	2	III
9	M/53	ADC	Well/mod	RUL	V–A	2	0	I
10	M/62	ADC	Well/mod	LUL	V–A	3	0	II
11	F/65	ADC	Well/mod	RUL	V–A	2	0	I
12	M/49	SCC	Poor	LUL	V–A	2	1	II
13	M/50	SCC	Well/mod	RUL	A–V[b]	2	2	III
14	F/54	SCC	Well/mod	RLL	A–V	3	2	III
15	M/71	SCC	Poor	LUL	A–V	1	0	I
16	F/54	ADC	Poor	LUL	A–V	2	1	II
17	F/61	ADC	Poor	LLL	A–V	2	2	III
18	F/66	SCC	Well/mod	RUL	A–V	3	0	II
19	M/73	SCC	Well/mod	RLL	A–V	2	1	II
20	M/54	SCC	Well/mod	LUL	A–V	2	0	I
21	M/54	SCC	Well/mod	RUL	A–V	1	0	I
22	F/57	SCC	Well/mod	RML	A–V	1	1	II
23	M/58	SCC	Well/mod	LUL	A–V	3	2	III

Abbreviation: *F* female; *m* male; *ADC* adenocarcinoma; *SCC* squamous cell carcinoma; *Poor* poorly differentiated; *Well/mod* well/moderately differentiated; *LUL* left upper lobectomy; *RLL* right lower lobectomy; *RUL* right upper lobectomy; *LLL* left lower lobectomy; *RML* right middle lobectomy

[a]Patients whose PV was ligated first followed by PA ligation

[b]Patients whose PA was ligated first followed by PV ligation

Procedure

RNA isolation

Peripheral blood mononuclear cells (PBMNs) were separated with Ficoll-Paque solution. As instructed by the manufacturer, the RNA was extracted from PBMNs by using Trizol reagent (Invitrogen). The concentration of total RNA was quantitated by using UV spectrophotometer (Parmacia-Bitech). The integrity of extracted RNA was verified by migration using gel electrophoresis.

cDNA synthesis

Total RNA was denatured at 70°C for 5 min. Reverse transcriptase reaction was carried out in 18 μl 1× reverse transcriptase buffer [50 mM Tris–HCL (pH 8.3), 75 mM KCL and 3 mM $MgCL_2$] with 0.5 mM deoxynucleotide triphosphates, 1 μl of RNasin and 200 U of Moloney murine leukaemia virus reverse transcriptase (Promega). cDNA were synthesised at 37°C for 30 min.

Primer/probe design

For the development of suitable combinations of Taqman primers and probe, the Primer Express software (PE Applied Biosystems) was used. The resulting primer pair produces a 202 bp fragment. Sequences (from 5′ to 3′) of CK19 were as followed: upper primer GCA GAA CCG GAA GGA TGC T; lower primer TCC GTT TCT GCC AGT GTG TC. The Taqman probe was labelled at the 5′ end with the reporter dye molecular FAM (emission wavelength 518 nm) and was additionally phosphorylated at the 3′ end of the probe to prevent extension during PCR. The sequence of the probe is TGG TTC ACC AGC CGG ACT GAA. The primer-probe set was selected so that the primers were positioned over an intron–exon junction, and were designed to differentiate between the highly homologous pseudogenes. Sequences (from 5′ to 3′) of carcinoembryonic antigen (CEA) were as follows: upper primer GCC TTG ACA AAA CGT TCC TGG; lower primer GAA CGG CGT GGA TTC AAT AGT G. The sequence of the probe is AGT CTC CCT CGG CCG CTC CCC A. The resulting primer pair produces a 199 bp fragment.

Taqman PCR reaction

PCR was conducted in 43 μl 1× PCR buffer [10 mM Tris–HCL (pH 8.4), 50 mM KCL and 1.5 mM $MgCL_2$] with 0.2 mM deoxynucleotide triphosphates, 5 μl of cDNAs, 2 U of Taq DNA polymerase (Promega), 0.4 μM of sense and antisense primers for CK19, respectively. The optimised thermal profile was started with 5 min denaturation at 95°C, followed by 35 cycles

of 95°C for 30 s, 62°C for 20 s and 72°C for 20 s, and a final extension at 72°C for 10 min. LC-5 cell (a lung squamous cell line) RNA standards and multiple water blanks were analysed in parallel with blood samples in each set of PCRs. All reactions were performed in the ABI Prism 7000 Sequence Detection System.

The initial concentration of every sample could be achieved according to the standard curves gained previously. In this study, the values of results were expressed as $\log_{10}$ CK_{19} or $\log_{10}$ CEA mRNA copies per millilitre serum. All reactions were performed in the ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems), which detects the signal from the fluorogenic probe during PCR. The software accompanying the 7000 system calculates threshold cycle (Ct) and determines the starting copy number in the samples.

Spiking experiment using LC-5 cells

LC-5 cells (a squamous cell line of lung cancer) from a monolayer culture were harvested with trypsin-EDTA (Sigma), washed in cold PBS and resuspended. Cell densities were evaluated using a counting chamber and viability by trypan blue staining (Sigma). Densities were adapted to a total of 10^6 cells in 2 ml DEPC-treated water. To simulate the presence of lung cancer cells in the circulation of lung cancer patients, total RNA was first extracted from 10^7 PBMNs from healthy subjects and 10^6 LC-5 cells, respectively. Aliquots of total RNA from 10^6 PBMNs were mixed with LC-5 total RNA, corresponding to 1, 10, 10^2, 10^3, 10^4 and 10^5 LC-5 cells. As for the negative control, only RNA extracted from 10^6 PBMNs was used. The RNA mixtures were then subject to FQ-RT-PCR for construction of the calibration curves and receiver operator characteristic (ROC) curves. Each sample was measured in triplicate.

Statistical analysis

All statistical analyses were carried out after log-transformation of the data. SPSS 10.0 for windows software was used in the statistical analysis. Varied statistical methods were used including repeated measured, independent samples *t* test, one-sample *t* test and Fisher's exact test. A critical alpha level of 0.05 was used for statistical significance.

Results

Evaluation of the CK19 diagnostic test

To define the value of the real-time PCR analysis for the detection of circulating tumour cells (CTCs), we performed an ROC curve analysis using SPSS 10.0 for windows software (Fig. 1).

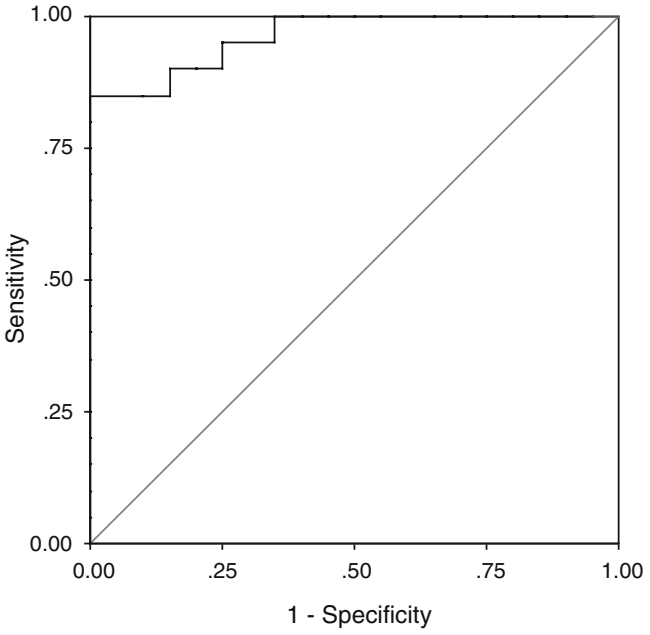

Fig. 1 The ROC curve of CK19 diagnostic test. The curve analysis is based on a plot of sensitivity as a function of 1-specificity. The area under the curve was 0.965, indicating high accuracy. In this study, the upper value of the CI of the median of the volunteer group was considered as the cut-off value, which is 4.120. Under this standard, the sensitivity of the diagnostic test was 90%, and the specificity was 84%

The reproducibility of the technique was established with the Ct value obtained for each dilution (10^5–10^8) of the standard curve in different assays and within an assay: the intraassay and interassay CVs of the Ct were 2.2 and 6.5%, respectively, on an average. Standard curve and amplification plots are showed in Figs. 2 and 3, respectively.

The level of CK19 mRNA in blood during peroperative period

The level during surgery (D_0) was significantly higher than that before surgery (D_{-1}) (5.246 ± 0.196 vs. 4.472 ± 0.164, $P = 0.000$, repeated measures) and after surgery (D_7) (5.246 ± 0.196 vs. 4.694 ± 0.177, $P = 0.013$, repeated measures). While there was no significant difference between the preoperative value and the postoperative one (4.472 ± 0.164 vs. 4.694 ± 0.177, $P = 0.254$, repeated measures) (Fig. 4).

The values between adenocarcinoma (ADC) and squamous carcinoma (SCC) were significantly different (4.9110 ± 1.0315 vs. 4.1891 ± 0.4126, $t = 2.364$, $P = 0.028$, independent samples *t* test) (Fig. 5). As far as gender, grade, age, operation performed and pTNM-stage were concerned, there were no significant difference amongst the values ($P > 0.05$, independent samples *t* test).

The values of CK19 mRNA in blood between PV-first group and PA-first group during perioperative

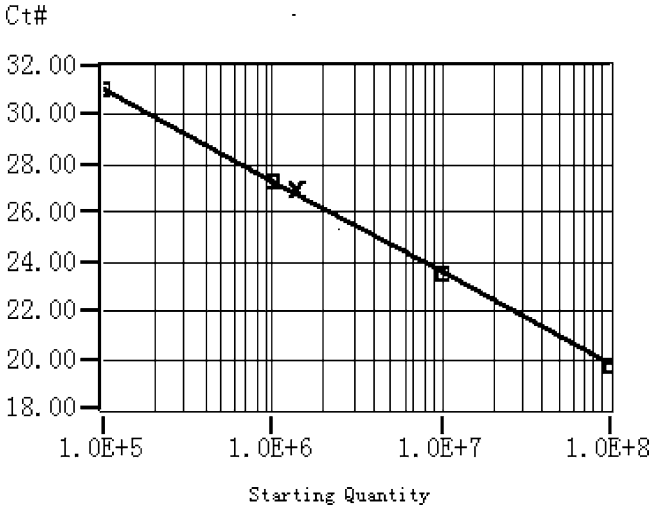

Fig. 2 Standard curve for LC-5 cell line dilutions. The graph shows the Ct value versus the log of the number of CK19 mRNA, measured in triplicate. The standard curve shows four orders of linear dynamic range

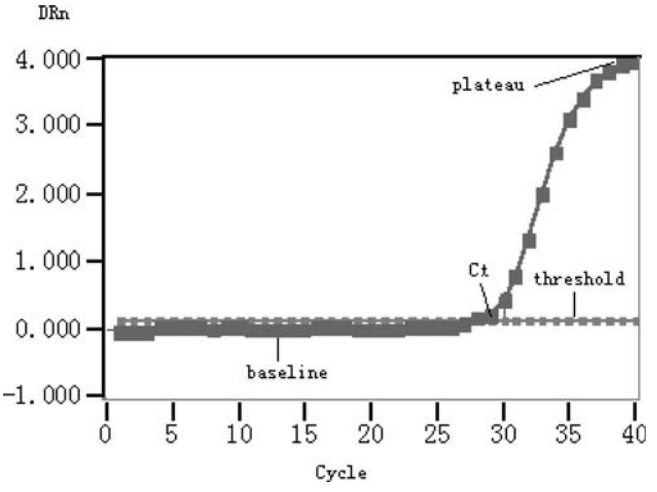

Fig. 3 Typical amplification plot. The graph of the increment of fluorescence reporter signal (ΔRn) versus cycle number during PCR shows three stages: baseline, exponential phase and plateau. The Ct value is calculated by determining the point at which the fluorescence exceeds an arbitrary threshold limit. For each reaction tube, the fluorescence signal of the reporter dye (FAM) is divided by the fluorescence signal of the passive reference dye (ROX), to obtain a ratio defined as the normalized reporter signal (Rn). ΔRn represents the normalized reporter signal (Rn) minus the baseline signal

period seemed to be significantly different (4.503 vs. 5.085, $P = 0.086$, repeated measures) (Fig. 6).

Surprisingly, circulating epithelial cells were detected in two patients resected for benign lung disease ($n = 10$), suggesting that cells from non-malignant bronchial epithelium may also gain entry into the bloodstream during operation. All 20 normal donors were negative for epithelial cells. To address the problem, the CEA was used as a marker to detect the CTCs of same group during the peroperative period.

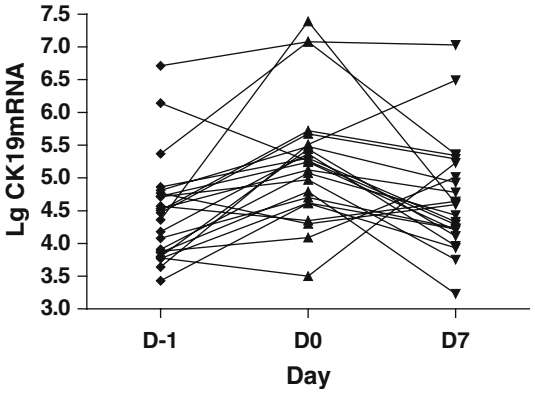

Fig. 4 The comparison of CK19mRNA level in blood at different time points during the perioperative period. The level of CK19 mRNA in PB at different time points. The points show the value of the day before operation, the operating day and the seventh day after operation, respectively. The values of results were expressed as $\log_{10}$ CK19mRNA copies per millilitre serum. The sign D_{-1}, D_0 and D_7 represent the day before surgery, the operating day and the seventh day after surgery, respectively

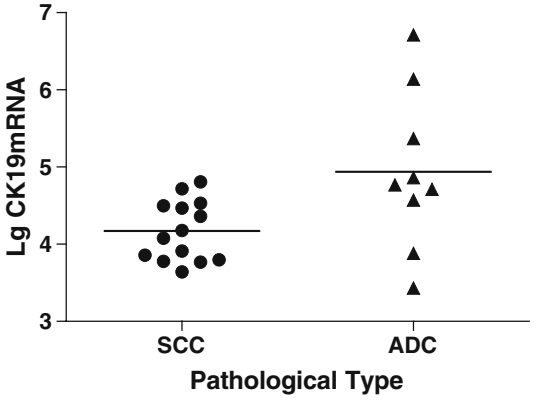

Fig. 5 The comparison of preoperative CK19 mRNA values between lung adenocarcinoma and squamous cell carcinoma

The level of CEA mRNA in blood during perioperative period

The area under the ROC curve in this study was 0.850 (95% CI 0.709–0.991) (Fig. 7). The intraassay and interassay CVs of the Ct value were 2.7 and 5.4%, respectively, on an average.

The level of CEA mRNA ascended continuously within this period. The value of postoperation was significantly higher than that of preoperation (4.874 vs. 4.483, $P = 0.000$, repeated measures) and that of operative day (4.874 vs. 4.537, $P = 0.000$, repeated measures). However, there was no dramatical difference between operative day and preoperation (4.537 vs. 4.483, $P = 0.196$, repeated measures).

The level of CK19 mRNA increased chiefly within the operation and then reduced gradually to the normal domain. Meanwhile the value of CEA mRNA reached the highest point postoperatively. There was no signifi-

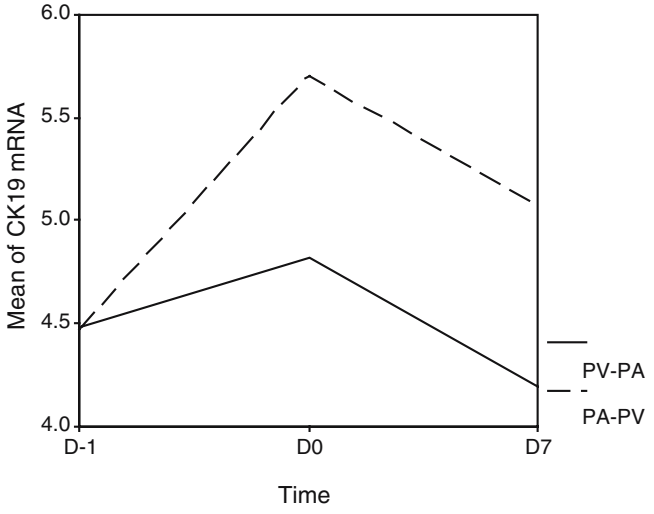

Fig. 6 The effect of sequence of vessel ligation on perioperative CK19 mRNA level. The sign D_{-1}, D_0 and D_7 represent the day before surgery, the operating day and the seventh day after surgery, respectively. PV–PA represent patients whose PV was ligated first followed by PA ligation, meanwhile PA–PV represent patients whose PA was ligated first followed by PV ligation

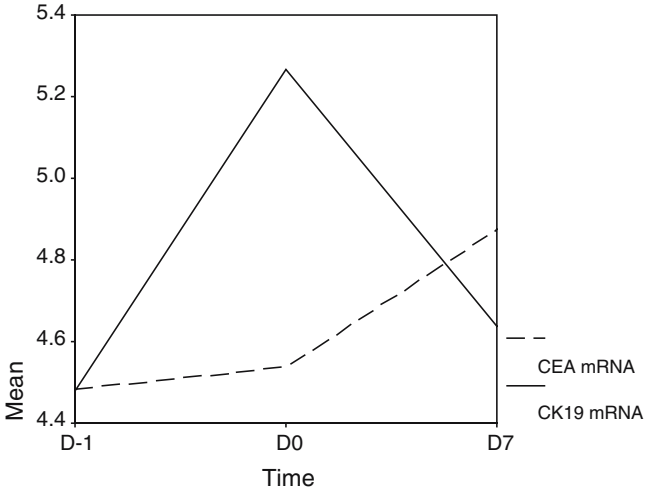

Fig. 8 Comparison of the trend of CK19 mRNA and CEA mRNA in PB within the perioperative period. The sign D_{-1}, D_0 and D_7 represent the day before surgery, the operating day and the seventh day after surgery, respectively

cant difference between preoperative CEA level and operative one (Fig. 8).

There was a striking difference in preoperative CEA mRNA level between ADC and (SCC) (4.6963 ± 0.1628 vs. 4.3455 ± 0.5426, $t = 1.873$, $P = 0.075$, independent samples t test) (Fig. 9). There was no notable diversity with respect of gender, grade, age, operation performed as well as p-TNM ($P > 0.05$, independent samples t test).

The perioperative values of CEA mRNA in blood between PV-first group and PA-first group were strikingly different (4.397 vs. 4.817, $P = 0.075$, repeated measures) (Fig. 10). At the same time, there was a correlation between preoperative T-stage and perioperative CEA mRNA (4.267 vs. 4.760, $P = 0.025$, repeated measures).

The upper value of the confidence interval (CI) of the median of the health volunteer group was considered as the cut-off value. If $\log_{10}$ CK19 > 4.120 (cut-off value), the cases would be classified as positive ones. Of the 23 cases studied, 14 cases were positive before surgery (60.9%). According to the criterion mentioned above, the cut-off value for $\log_{10}$ CEA was 4.6151. Among the 23 cases, 10 cases were positive preoperatively (43.5%).

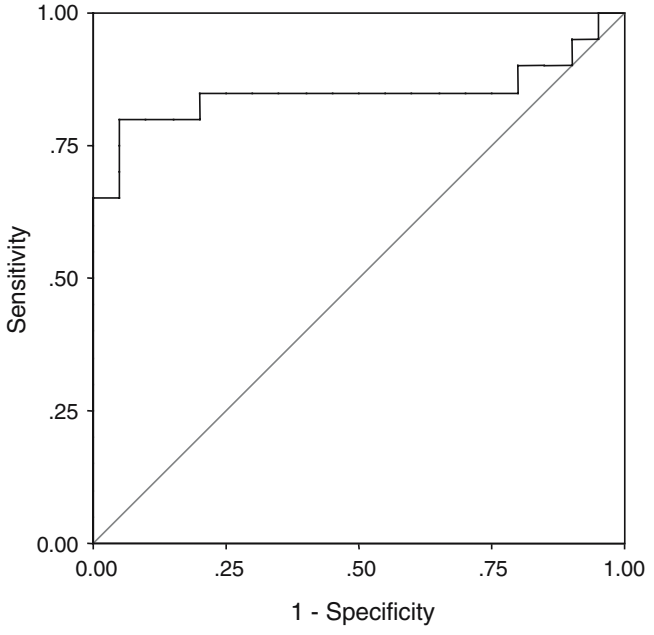

Fig. 7 The ROC curve of the CEA diagnostic test. The curve analysis is based on a plot of sensitivity as a function of 1-specificity. In this study, the upper value of the CI of the median of the volunteer group was considered as the cut-off value, which is 4.6151. Under this standard, the sensitivity of the diagnostic test was 91%, and meanwhile the specificity is 87%

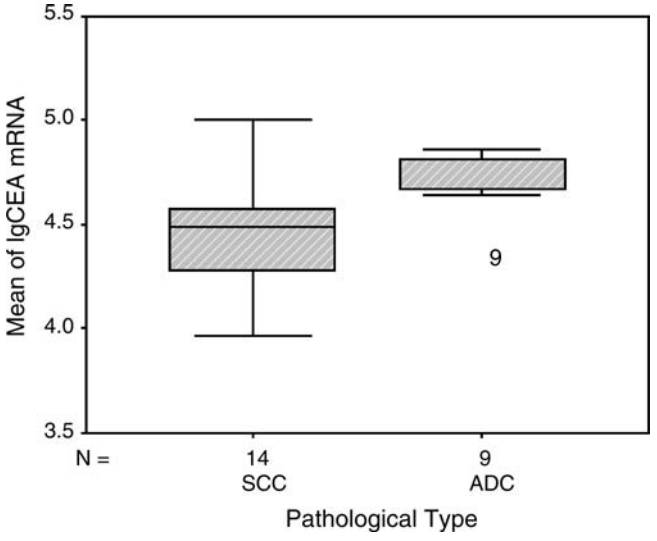

Fig. 9 The comparison of preoperative CEA mRNA values between ADC and SCC

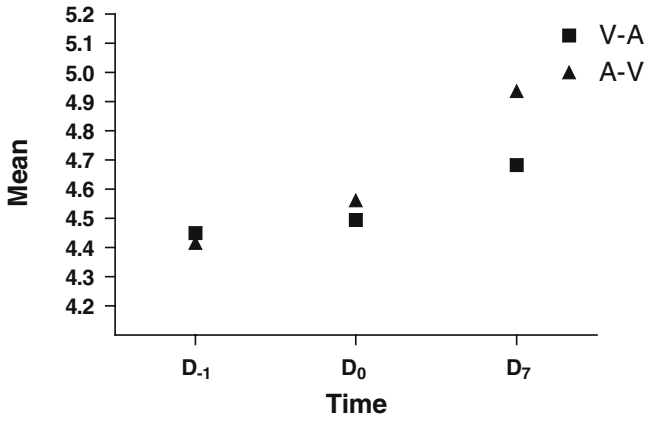

Fig. 10 The effect of sequence of vessel ligation on perioperative CEA mRNA in blood. The sign D_{-1}, D_0 and D_7 represent the day before surgery, the operating day and the seventh day after surgery, respectively. V–A represent patients whose pulmonary vein was ligated first followed by pulmonary artery ligation, meanwhile A–V represent patients whose pulmonary artery was ligated first followed by pulmonary vein ligation

Discussions

Since traditional PCR technology is at best semiquantitative, it has been difficult to differentiate between baseline level of gene expression in normal tissues and increased level of gene expression associated with cancer, raising the concern for false-positive results. In this study, the real-time PCR was exploited to investigate the possibility of using easily accessible body fluids as a source for CTCs detection enabling longitudinal observation of the disease, therapy monitoring and initial diagnosis. Detection and quantitation of CTCs from solid epithelial tumours could become a valuable tool for therapy monitoring.

We assumed that the level of tissue-specific or tumour-specific marker in circulation could approximately reflect the amount of CTCs of solid tumour. This study demonstrated that there was a correlation between the level of circulative CK19/CEA mRNA and the type of non-small cell lung cancer (NSCLC), indicating the biological character of a tumour may be coordinated to the histological type. According to the study, there was more chance of blood dissemination for ADC. We can conclude that many patients with resectable NSCLC are likely to have systemic disease before operation. The clinical practitioner should become aware of the principle—individual-oriented—to that those with more trendy of circulating dissemination, system adjuvant therapy be emphasized after surgery. According to the study, there was no relation between CK19/CEA mRNA and postoperative TNM staging. To a certain extent, the result can explain the contradictory phenomenon—some NSCLC patients, although in early stage according to the conventional TNM criterion, suffered from recurrence or distant metastasis early after ''curable'' operation. Due to the intrinsic fault in the current TNM staging system, it is an urgent agenda to absorb some cellular or molecular factors into the system (Ge et al. 2005).

In accordance with the study involved with CEA, the preoperative T-stage was related to the level of CEA mRNA. It was speculated that the bigger the mass, the more the opportunities of mechanical squeeze for the bigger tumour. With reverse transcriptase PCR technique, Yamashita et al. (2000) found that video-assisted lobectomy was associated with a higher risk of seeding tumour cells into the circulation during operation than open lobectomy. The operator should abide by some basic guidelines during operation. Normally surgeons can resect the visible tumour. Meanwhile, they can squeeze invisible cancer cells into the circulation during the procedure. How to use this ''double-edged'' sword? It was highly recommended that the surgeons treat the tumour gently and avoid turning over and squeezing the mass as much as possible.

This study demonstrated that many patients with ''resectable'' NSCLC are likely to have systemic disease before surgery. Metastatic relapse after the complete resection of an apparently localized primary tumour indicates that at the time of surgery disseminated cancer cells are often undetectable by current tumour staging procedures and the distant metastases could develop from the occult CTCs present at the time of surgery, or before. Although the technique does not allow for specific identification of tumour cells and a positive finding of CK19/CEA mRNA merely indicates the presence of epithelial cells, our results suggest that a considerable proportion of patients who appear to have respectable NSCLC might be better regarded as having systemic disease. The presence of CTCs should be incorporated in the UICC staging nomenclature by including isolated tumour cells (Mi) in the M-category of the Tumour-Node-Metastases classification. Patients with occult dissemination of viable tumour cells are not cured by surgery alone and may benefit from additional adjuvant therapy (Hosch et al. 2000).

In this study, both CK19 and CEA were used as markers to monitor the fluctuation of CTCs of the same group. The result showed that the cells expressing CK19 mRNA were mainly released into the blood stream during operation, while the cells expressing CEA mRNA chiefly shedded into circulation after operation. We maintained that the diversity of specificity should be responsible for the different results. The expression of tissue-specific mRNA transcripts is retained during carcinogenesis. Gene transcripts specific for the tissue of cancer origin are indicative for disseminated cells when the gene is normally not expressed at the site of investigation. Cytokeratin are constituents of the intermediate filaments of epithelial cells. Among this gene family, cytokeratin 19 is one of the widest distributed keratin (Moll 1994). Because most markers of CTCs and micrometastases in solid tumours are tissue specific (i.e. expressed in tumour and their normal tissue of origin), the mechanical introduction of normal or benign cells in the circulation after invasive procedures may

lead to false-positive PCR results (Hardingham et al. 2000). The presence of tissue-specific mRNA in PB does not necessarily reflect the existence of malignant cells or the clinical status of patients, suggesting that cancer-specific mRNA should be applied as a marker for detecting micrometastases. In haematological malignancies such as chronic myelogenous leukaemia, tumour-specific molecular abnormalities are well characterised and the development of detection techniques for minimal residual disease (MRD) proved to be of significant importance in clinical practice (Hughes et al. 1991). However, such disease-specific chromosomal alterations in solid tumours are rare, and therefore other strategies are required for most tumour types. Generally, CEA is highly expressed in cells of the foetal colon and in a variety of neoplasms where its presence is a negative prognosticator of clinical outcome (Bockmann et al. 2001). CEA was analysed as a marker gene for the identification of CTCs in the blood of lung cancer (Castaldo et al. 1997; Kurusu et al. 1999). Some researchers also found that there were low-level CEA expression in normal tissues and insisted on controversy of its specificity (Bockmann et al. 2001). Using real-time quantitative RT-PCR, CEA is absolutely cancer cell specific in Giesing et al. (2000) observation. It was hypothesized that CEA mRNA was expressed in normal tissues but at significantly lower levels than in the tissues containing metastatic lung cancer in the present study. If assumed level of CEA mRNA is detected in blood samples, this implies the presence of ectopic epithelial cells. Of the patients with resectable benign lung disorder, two cases where CK19 mRNA increased intraoperatively. Nevertheless all the healthy volunteers were negative. We concluded that CK19 mRNA is not the best marker of CTCs during invasive procedures (e.g. operation); however, it is still suitable for non-invasive procedures (e.g. monitoring the effect of chemotherapy or radiotherapy). Our observation shows that the CK19-expressing bronchus epithelial cells are shedded by mechanical instruction into the blood during operation, and CEA-expressing lung cancer cells are released mostly postoperatively. This observation was in concordance with the reported study, which presented evidence that alb-expressing hepatocytes are released intraoperatively into the circulation, and AFP-expressing tumour cells are disseminated mostly postoperatively and that may potentially be the source of recurrence or metastasis (Wong et al. 1999).

In studies of patients who had a solid tumour, perioperatively obtained PB samples were examined by molecular methods. Results showed that a temporary intraoperative dissemination of tumour cells into the circulation could occur (Hansen et al. 1995; Weitz et al. (1998). The study demonstrated that surgical manipulation can promote the release of tumour cells into the blood stream, but the ligation of the PV before the ligation of the PA may partly prevent such release during surgery.

The relationship between CTCs and the development of metastatic disease is not fully understood, and the presence of tumour cells in the blood does not necessarily indicate the subsequent risk of clinical systemic disease. It is generally believed that very few tumour cells that shed into the bloodstream succeed in establishing secondary tumours (Fidler 1991). Thus patients who have persistently positive blood samples after lobectomy may be those at risk for systemic relapse. According to the reported studies of Pachmann et al. (2005) and Rolle et al. (2005), the patients with lung ADC and a continuous increase of circulating epithelial cells after complete resection of lung cancer are at an increased risk of early relapse. Our findings should alert surgeons to the possible danger of tumour cell dissemination during an operation and suggest that the PV should be ligated first, before the ligation of the PA, in patients undergoing a lobectomy for NSCLC. Distant metastases come into being through not only haematogenous dissemination, but also lymphatic spread. Therefore, ligating the PV, first, possibly lessens intraoperative haematogenous dissemination. However, it cannot prevent the tumour cells from spreading to an ectopic site through a lymphatic channel. We support the basic principle of turning over and squeezing the mass as rarely as possible during surgery. Future follow-up studies in a larger cohort of patients with NSCLC should be done to examine whether the patients with positive CK19/CEA mRNA might have more metastases, shorter survivals, or shorter disease-free intervals than those who did not have positive results.

To distinguish between the presence of CTCs and normal PBMNs in blood, we have exploited the sufficiently sensitive and well-optimised molecular protocol in this study. In order to define criteria to identify true positivity, the upper value of the 95% CI of the median for the blood sample of the volunteer group was considered as the upper limit for ''normal expression''. It can overcome the default of conventional RT-PCR that can only produce a positive or negative result. To some extent, the specificity of the marker might be improved. Using this technique, both the CTCs and the micrometastases in lymph nodes or bone marrow can be assessed quantitatively. It is extremely useful for real-time monitoring of systemic disease. We performed a single-point blood sampling for patients before operation in this study. However, cancer cells may be intermittently shedding into the bloodstream. Thus sampling errors may have occurred in that patients who had negative blood samples when the initial blood was drawn, that is, they might have had CTCs intermittently at another time or under other circumstances.

After escape from the primary tumour, further genetic alteration may occur in disseminated cells as a result of positive selection (e.g. escape from immune response, escape from therapy). This is in agreement with the observed genomic heterogeneity between primary tumours and occult metastases as well as between the individual metastases of one patient (Offner et al. 1999; Zhang et al. 1997). Therefore, quantification, characterisation and assessment of the potential of CTCs are essential in further studies, which will possibly provide more infor-

mation about metastatic potential, drug sensitivity and the development of a therapy resistance than analysis of the primary tumour and will eventually help to identify subgroups of patients receiving benefit from a particular therapy (Curry et al. 1999). The detection of disseminated tumour cells has introduced a new opportunity to evaluate which of the diverse biological characteristics of the primary tumour might favour the early dissemination of its cells.

In conclusion, the results of current studies suggest that micrometastases may happen at an early stage in lung cancer. These results support the concept that NSCLC are systemic rather than local-regional diseases. Improved staging can be expected with the information of micrometastases, and a subgroup of patients who will benefit from adjuvant therapy might be identified. Although reliable and standard methods need to be developed before detection of micrometastasis is incorporated into the routine clinical practise, we suggest that it be considered as an important correlate in clinical trials in NSCLC.

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