Detection of circulating tumor cells (CTCs) in patients with lung carcinoma by real-time fluorescent quantitative-PCR approach before and after chemotherapy

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ABSTRACT

Circulating tumour cells (CTCs) are referred to the tumour cells that disseminated from the primary tumour and survive in circulating during the proceeding of tumour growth. As surgical treatment evolves and local control has improved, the failure of cancer treatment has largely remained the result of systemic metastasis. Selection of patients most likely to benefit from adjuvant strategies remains problematic. In order to develop a new standard of curative effect, this study was designed to track the number of CTCs in patients with lung cancer during chemotherapy. Methods: Samples of peripheral blood was taken from each lung cancer patients (n=32) on the day before chemotherapy as well as the third week after the chemotherapy cycle. The samples were subjected to real-time fluorescent quantitative reverse-transcriptase polymerase chain reaction (fqRT-PCR). Meanwhile the tumour size was determined by chest X-ray or computed tomograghy. Results Compared to that of pre-chemotherapy, the expression level of cytokeratin (CK) 19 in the patients significantly declined after chemotherapy (t=4.659,P=0.000). The level of CK19 mRNA in patients with small cell lung cancer (SCLC) was higher than that of patients with non-small cell lung cancer (NSCLC) (t=1.944, P=0.061). The decrease of CK19 mRNA level correlated well with the type during the treatment. Relatively the decrease of SCLC is more obvious (t=6.073,P=0.000). The variation of CK19 mRNA level before and after chemotherapy was positively related to the disparity of tumour burden (r=0.593). There was also a significant association between the type (NSCLC vs. SCLC) and the change of tumour size (t=3.686,

P=0.001).The positive rate before chemotherapy was 71.9% (23/32), while that after chemotherapy was 37.5% (12/32), indicating that 11 patients converted into negative after chemotherapy. Of the 16 patients which were in Ⅳ-stage, 11 cases were positive (11/16,68.8%). Surprisingly, of the remaining 16 patients which were Ⅱ/Ⅲ stage, 12 cases were regarded as positive according to the criteria (12/6,75%). Conclusions: The real-time fluorescent quantitative-PCR approach is useful for measuring the relative number of CTCs in a patients' peripheral blood to monitor the effectiveness of treatment, and for designing more comprehensive and reasonable therapeutic regimes at earlier dates for patients. The treatment response can be immediately assessed by serial quantitation of CTCs after chemotherapy, and therefore this method highlights an alternative approach to rapidly access the patient's response to treatment.

Keywords: Lung Neoplasm; Blood; Polymerase Chain Reaction; Cytokeratin; Messenger RNA; Chemotherapy

1. INTRODUCTION

Traditionally clinical response criteria for solid tumors were defined according to the change of the mass during therapy; briefly, complete response (CR) is defined as complete disappearance of all measurable and available clinical evidence of cancer; partial response (PR) is defined as at least a 50% reduction in the size of all measurable tumour areas, progressive disease (PD) is defined as an increase of = or $>$ 25% (compared to baseline or best response) in the size of all measurable tumour areas; And stable disease (SD) is defined as neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD. If the mass was resected, how do we as-

Table 1. Clinicopathological featurse and CK19 mRNA expression in peripheral blood before and after chemotherapy for patients with lung cancer.

Patient	Gender /Age	Type	Stage	Tumour size (cm^2)		CK19 mRNA*	
				Before	After	Before	After
1 #	F/54	ADC	II	8.4	1.6	6.03	4.26
2 #	M/59	SCLC	IV	19.8	9.6	5.19	4.05
3 #	F/48	SCLC	IV	5.2	3.4	5.61	4.09
4 #	M/62	SCC	III	10.1	9.8	4.04	4.40
5 #	M/63	ADC	IV	35.3	26.7	4.23	3.81
6 #	M/67	SCLC	III	34.4	15.8	4.86	2.28
7 #	M/68	SCC	II	5.2	3.4	4.22	3.40
8 #	M/60	SCC	III	8.3	6.4	3.51	4.30
9 #	F/53	ADC	IV	5.1	5.0	3.62	3.78
10 #	M/58	ADC	IV	9.3	8.7	3.55	3.50
11 #	M/45	ADC	II	19.6	12.6	4.60	3.89
12 #	M/63	SCC	III	22.5	10.4	6.15	4.75
13 #	M/62	SCC	IV	14.4	8.7	3.31	3.29
14 #	M/49	SCC	III	36.1	33.2	3.93	3.73
15 #	F/54	ADC	IV	42.6	33.6	5.90	4.74
16 #	F/47	SCLC	IV	10.5	0.0	5.72	3.29
17 #	M/52	SCC	III	19.3	18.0	6.78	5.71
18 #	M/62	SCC	III	3.6	3.4	4.12	4.71
19 #	M/39	SCLC	III	60.8	11.9	5.99	3.98
20 #	M/33	SCC	IV	16.5	17.2	5.06	5.08
21 #	F/50	SCC	IV	10.9	11.8	4.64	4.26
22 #	M/60	SCLC	III	7.3	0.0	4.94	2.86
23 #	M/56	SCC	III	10.9	12.5	5.06	5.02
24 #	F/66	SCC	IV	7.8	3.1	5.14	3.99
25 #	F/45	SCC	IV	15.1	13.9	4.25	4.16
26 #	M/62	SCC	IV	2.97	2.32	3.44	3.57
27 #	M/61	SCC	II	3.6	2.9	3.65	3.85
28 #	M/63	SCLC	II	11.9	2.9	5.41	3.30
29 #	M/47	SCLC	II	17.5	6.3	4.55	3.29
30 #	M/66	ADC	IV	13.2	11.3	4.44	3.70
31 #	M/61	ADC	IV	8.4	8.7	6.10	6.05
32 #	M/60	ADC	IV	9.6	5.1	3.86	3.73

Abbreviation: F, female; M, male; ADC, adenocarcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung cancer.
* The values of results were expressed as $\log_{10}$CK19 mRNA copies per millilitre serum.

sess efficacy of the treatmental strategy?

Clinical cancer care and study depends on accurately staging the extent of disease to assess prognosis and determine efficacy of treatment regimens. Staging criteria for most solid tumours with which general surgeons areconcerned are based on the tumour node metastasis (TNM) system. But current approaches are limited in adequately identifying individual patients who are at high risk for future relapse and have the need for a more aggressive therapy. This is mainly due to inaccuracy in the identification of early stage metastasis. Furthermore the diagnosis tools for therapy monitoring are restricted to imaging techniques and the measurement of protein serum markers. Whereas imaging techniques suffer in general from their resolution of about 0.5 cm, the clinical usefulness of tumour markers is questionable, leading to a

progressive reduction of their use in clinical practice [1-6].

Since even small tumours already show dissemination of cells, their disclosure has been considered as a tool to improve early cancer detection. In addition to the 'tumour marker' function of CTCs, these cells remain in the lymphatic/systemic circulation after surgical removal of the primary tumour and are responsible for the later recurrence of the disease. Consequently this undetected cell dissemination by conventional measures is of relevance for cancer progression and has been already demonstrated to be of prognostic impact in several malignancies [7-12].

CTCs are referred to the tumour cells that disseminated from the primary tumour and survive in circulating during the proceeding of tumour growth. There are no symptoms, and current clinical procedures (i.e. radiography and routine pathological examination) often fail to identify the foci. Generally most of these cells are dormant (i.e. no proliferation or a balance between cell growth and death). If the host suffers from trauma or immunological impairment, dormant tumour cells will proliferate continually and growth into a mass focus eventually. Therefore, the detection of CTCs is important for evaluating the prognosis and tailoring therapeutic strategy [13].

As surgical treatment evolves and local control has improved, the failure of cancer treatment has largely remained the result of systemic metastasis. There is therefore much reason to develop effective adjuvant systemic therapy. Identification of patients most likely to benefit from adjuvant strategies remains problematic. With the aim of aiding the development of a new standard of curative effect, this study was designed to track the number of CTCs in patients with lung cancer during chemotherapy.

2. MATERIAL AND METHODS

2.1. Clinical Data

After obtaining informed consent, samples of 5ml peripheral blood(PB) were taken from each lung cancer patient (n=32) on the day before chemotherapy as well as the third week after the chemotherapy cycle. Meanwhile the tumour size (cm^2) was determined by chest X-ray or computed tomography. The control population consisted of healthy individuals who had no any evidence of solid tumours or haematological malignancy (n=20).

All the cases were diagnosed for the first time and received the first cycle of chemotherapy. They had never received any other treatment (e.g. surgery or radiotherapy). Each patient with SCLC included in this study received the first cycle of chemotherapy consisting of etoposide 100mg/m^2 per day on day(s) 1 to 3, cisplatin 50 mg/m^2 per day on day1. For patients with NSCLC, the first cycle of chemotherapeutic strategy was composed by mitomycin 10mg/m^2 per day on day 1 and 8, vindesine

(VDS) 3mg/m^2 per day on day 1 and cisplatin 80mg/m^2 per day on day1.

The study population consisted of 32 patients with histologically documented lung cancer. Staging procedures included chest radiograph, bronchoscope, thoracic computed tomography, sonography, bone scintigraphy and biopsy of distant lesion (e.g. lymph node). The clinicopathological features of the lung cancer patients were summarized in **Table1**.

2.2. Procedure

2.2.1. RNA Isolation
The peripheral blood mononuclear cells (PBMNs) were separated with Ficoll-Paque solution. As outlined by manufacturer, the RNA was Isolated 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 by gel electrophoresis.

2.2.2. CDNA Synthesis
Total RNA was denatured at 70℃ for 5min. Reverse transcriptase reaction was carried out in 18μl 1×reverse transcriptase buffer [50mM Tris-HCL(PH8.3), 75 mM KCL and 3mM MgCL$_2$] with 0.5 mM deoxynucleotide triphosphates, 1μl of RNasin, and 200 units of Moloney murine leukaemia virus reverse transcriptase(Promega). CDNA were synthesis at 37℃ for 30min.

2.2.3. 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 follow: 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 518nm) and at the 3' end of the probe was additionally phosphorylated 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.

2.2.4. Taqman PCR Reaction
PCR was conducted in 43μl 1× PCR buffer [10mM Tris-HCL (PH8.4), 50mM KCL, and 1.5mM MgCL$_2$] with 0.2 mM deoxynucleotide triphosphates, 5 μl of cDNAs, 2 units of Taq DNA polymerase (Promega), 0.4 μM of sense and antisense primers for CK19 respectively. The optimised thermal profile was initiated with 5 min denaturation at 95℃, followed by 35 cycles of 95℃ for 30seconds, 62℃ for 20 seconds, and 72℃ for 20 seconds, and a final extension at 72℃ for 10 min. LC-5

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 $\log_{10}CK_{19}$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 7000 system has a built-in thermal cycler and a laser directed via fiber optical cables to each of the 96 sample wells. A charge-coupled device camera collects the emission from each sample and the data are analysed automatically. The software accompanying the 7000 system calculates cycle threshold (Ct) and determines the starting copy number in the samples.

2.2.5. 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 diehypyrocarbonate-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. 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 fqRT-PCR for construction of the calibration curves and receiver operator characteristic (ROC) curves. Each sample was measured in triplicate.

2.3. Statistical Analysis

The results were analysed with statistical software SPSS10.0 for Windows. The statistical assays included paired samples t test, independent samples t test and bivariate correlate analysis, with $P<0.05$ indicating significance. The method used to calculate the appropriate sample size in the study was similar to that used to paired measurement data. Suppose if Type Ⅰ error is 0.05,the sample sizes for both pre- and post-chemotherapeutical group were thirty, respectively. The weighted sample size i.e. thirty-two, calculated by the proposed method, preserved the power (1-β) above 0.9.

3. RESULTS

3.1. Evaluation of the CK19 Diagnostic Test

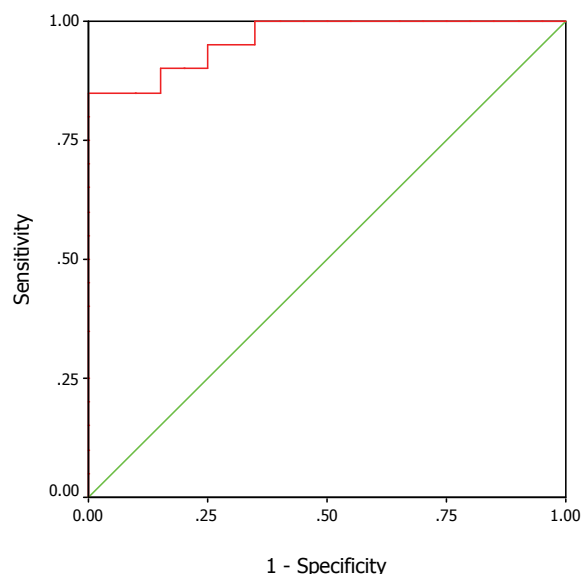To rigorously define the value of the real time RT-PCR

Figure 1. ROC curve of CK19 diagnostic test. ROC curve analysis is based on a plot of sensitivity as a function of 1-specificity. The area under the curve in this study was 0.965, indicating high accuracy. In this study, the upper value of the confidence interval 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%.
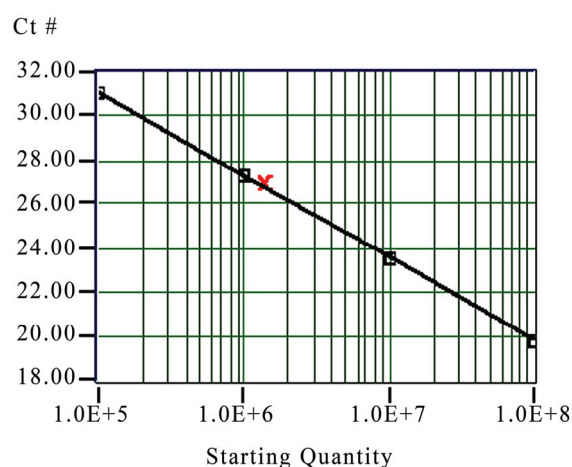

Figure 2. Standard curve for LC-5cell line dilutions. The graph shows the Ct value versus the log of the number of CK19 mRNA, measured in triplicate. The standard curve shows 4 orders of linear dynamic range.

analysis for the detection of CTCs, we performed a ROC curve analysis using SPSS 10.0 for windows software. ROC curve analysis is based on a plot of sensitivity as a function of 1−specificity. The area under the ROC curve in this study was 0.965 (95% confidence interval:0.916～1.014) (**Figure 1**).

In order to define the criteria to identify true positivity,the upper value of the confidence interval of the median of the volunteer group was considered as the cut-off value,

　　　　　　　　　　Openly accessible at http://www.scirp.org/journal/HEALTH/

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 ($10^5 \sim 10^8$) of the standard curve in different assays and within an assay: the intraassay and interassay CVs of the threshold cycle were 2.2 and 6.5%, respectively, on average.

Standard curve and amplification plots are showed in **Figure 2** and **Figure 3**, respectively.

3.2. Comparison of CK19 Mrna Level in PB Before and after Chemotherapy

Compared to that of pre-chemotherapy, the expression level of CK19 in peripheral blood significantly declined post-chemotherapeutically (4.7323±0.9698 vs. 3.9864 ± 0.8309, t=4.659, *P*=0.000. Paired Samples T Test).

3.3. The Relationship Between CK19 MRNA Level in PB and Classic Clinocopathological Features

The level of CK19 mRNA in patients with SCLC was higher than that of patients with NSCLC (5.2853±0.4880 vs. 4.5480±1.0263, t=1.944, *P*=0.061, Independent Samples T Test, **Figure 4**). Nevertheless, there was no link between value and other clinicopathological parameters such as stage, gender and age etc. (*P*>0.05).

The difference of CK19 mRNA level correlated well with the type during the treatment. Relatively the disparity of SCLC is more obvious (1.8924 vs. 0.3637, t=6.073, *P*=0.000, Independent Samples T Test). However, there was no relationship between the difference of CK19 mRNA level and other parameters such as stage, gender and age etc. (*P*>0.05). The variation of CK19 mRNA level before and after chemotherapy was positively related to the disparity of tumour burden (r=0.593, *P*=0.000, Bivariate Correlation Analysis). There was also a significant association between the type (NSCLC vs. SCLC) and the difference of tumour size (3.0525 vs. 14.6875, t=3.686, *P*=0.001, Independent Samples T Test). **Figure 5** showed the difference of tumour volumes of different types of tumour before and after treatment.

3.4. The Positive Rate of CK19 Gene Expressive Level before and after Chemotherapy

The upper value (4.120) of confidence interval of the median (3.6647) of the healthy volunteer group was considered as the cut-off value. If lgCK19>4.120, the case would be classified as a positive one. The positive rate before chemotherapy was 71.9% (23/32), while that after chemotherapy was 37.5% (12/32), indicating that 11 patients converted into negative after chemotherapy. Of the 16 patients who were in Ⅳ-stage,11 cases were positive(11/16,68.8%). Surprisingly, of the remaining 16 patients who were Ⅱ/Ⅲ stage,12 cases were regarded as

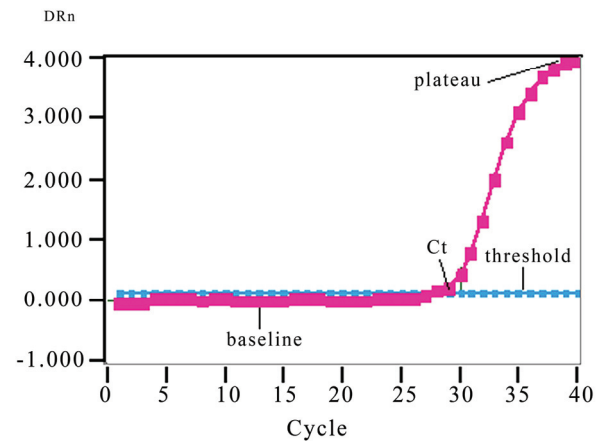**Figure 3.** Typical amplification plot. The graph of the increment of fluorescence reporter signal($\triangle$Rn) versus cycle number during PCR shows three stages: baseline, exponential phase, and plateau. The Ct value is calculated by determining the point at which the fluorescence exceeds an arbitrary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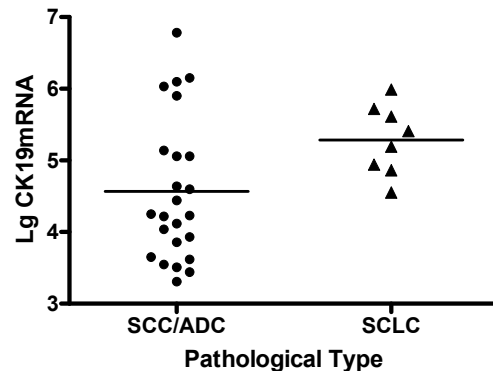

Figure 4. The comparison of CK19 mRNA in PB of patients with SCLC and NSCLC before chemotherapy.

positive according to the criteria (12/16,75%).

4. DISCUSSION

Since traditional RT-PCR technology is at best semi-quantitative, it has been difficult to differentiate between baseline level of gene expression in normal tissues and increased level of gene expression associated with cancer, rising the concerning 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.
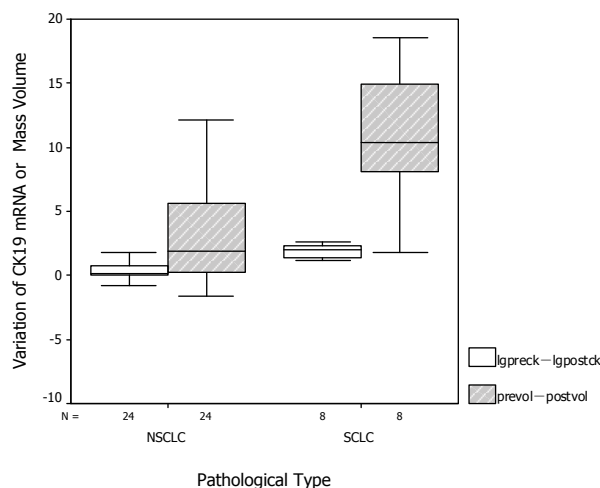
Figure 5. The comparison of CK19 mRNA in PB and tumour volumes of patients with SCLC and NSCLC before and after treatment.

In this study, we could detect low level of CK19 expression in healthy volunteers, suggesting low expression in non-malignant cells in peripheral blood. In order to define criteria to identify true positivity, we have taken this observation into account in the calculation of the median and 95% CI of the median (i.e. the upper limit for normal expression) of the volunteer group. In order to define criteria to identify true positivity, the upper value of the confidence interval of the median of the volunteer group was considered as the "cut-off" value. Therefore, the specificity of CK19 analysis could be restored by the use of a quantitative assay with subtraction of background transcription. The possibility of obtaining quantitative information represents a milestone in the identification of subtle changes in the expression profiles and numbers of circulating tumour cells. The real-time PCR method offers several advantages over other current quantitative PCR methods [14].

The study demonstrated that, comparing with that of pre-chemotherapy, the expression level of CK19 in PB of patients with lung cancer declined dramatically after chemotherapy (P=0.000). We believed that, due to anti-cancer drugs, the number of cancer cells shedding from primary tumour decrease greatly. Furthermore, these drugs could directly kill cancer cells in the circulation. The level of CK19 mRNA in PB of patients with SCLC was higher than that of NSCLC (P=0.061). After one cycle of chemotherapy, the dropping of CK19 mRNA level in SCLC was much more obvious than that of NSCLC (P=0.000). The phenomena could be due to two group's different sensitivity to chemotherapy, coordinating with previous studies. The traditional standard for evaluating the curative effect of solid tumour depends on the variation of the mass size. It belongs to magnificent criterion. In clinical practise, in order to develop more

accurate standard, other factors such as cytology and molecular ones should be taken into account. To improve the current staging and effect of tumours, oncologists are striving for new norm. This study showed that the difference in expression level of CK19 in PB was correlated well with the variation of tumour size during chemotherapy, indicating that it could be considered as a brand new criterion regarding assessing the result of therapy. Blood can be sampled throughout the course of the disease and its collection is a minimally invasive procedure. The permanent access to CTCs enables follow up studies to be done to forecast prognostication of disease outcome, therapy monitoring or drug targeting. For example, for postoperative patients whose mass no longer exits, it could be exploited to evaluate the animated therapeutic results.

It was interesting that we found there was no correlation between the existence of CTCs and the traditional TNM staging system. Why were only 11 patients not all who were in Ⅳ stage positive in this study? We insisted that this phenomenon could be due to intermittent shedding of tumour cells in the circulation. This problem should be overcome by sequential sampling, defined as the analysis of multiple blood samples at different time points. False-negative results could also be due to down-regulation of the target gene by therapy (e.g. hormonal treatment) or to the presence of poorly differentiated subclones that do not express the tissue-specific marker being tested. For example, PSA mRNA expression was shown to be decreased by antiandrogen therapy [15] and in poorly differentiated prostatic carcinoma [16]. The use of multiple markers will help diminish these types of false negative. On the other hand, it was surprising that, of the 16 patients who were on Ⅱ/Ⅲ stage, 12 cases were positive! We believe that many cancers have systemic spread at an early stage of their development. The presence of CTCs in PB should be incorporated in the UICC staging nomenclature by including isolated tumour cells (Mi) in the M-stage 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 [17].

The permanent access to CTCs enables longitudinal studies to monitor therapy outcome. This aspect is substantially improved by recent quantitative methods (e.g. FQ-PCR) allowing the detection of changes in the quality and number of tumour cells [18]. In contrast to solid metastatic tumours, micrometastatic tumour cells are an appropriate target for intravenously applied agents because macromolecular and immunocompetent effectors cells should have access to the tumour cells. Because the majority of micrometastatic tumour cells may be non-proliferative (G_0 phase), standard cytotoxic chemotherapies aimed at proliferating cells may be less effective,

which might explain, in part, the failure of chemotherapy. Thus, adjuvant therapies that are aimed at dividing and quiescent cells, such as antibody-based therapies, are of considerable interest [19].

Our study showed that quantitation of CTCs is possible during the course of therapy. Because cancer cells express different levels of CK19 mRNA, patients with higher CK19 mRNA level do not necessarily have more CTCs in peripheral blood than do patients with lower one. Nevertheless, the real-time fluorescent quantitative-PCR approach is useful for measuring the relative number of CTCs in a patients' peripheral blood to monitor the effectiveness of treatment. In a limited number of patients, we demonstrated that the relative number of CTCs correlated well with the tumour burden and therapeutic response. This method highlights an alternative approach to rapidly access the treatment response of patients. The treatment response can be immediately assessed by serial quantitation of CTCs after chemotherapy. This method may, therefore, help to design more comprehensive and reasonable therapeutic regimen at an earlier date for patients [20]. A large prospective study is needed to determine whether prognostic significance can be derived from the presence of CTCs and whether this group of patients might benefit from adjuvant treatment.

5. ACKNOWLEDGEMENTS

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