Fluctuation of circulating tumor cells in patients with lung cancer by real-time fluorescent quantitative-PCR approach before and after radiotherapy

ABSTRACT
Background and Aims: The failure to reduce the mortality of patients with solid tumours is mainly a result of the early dissemination of cancer cells to secondary site, which is usually missed by conventional diagnostic procedures used for tumour staging. The possibility to use easily accessible body fluids as a source for circulating tumour cells (CTCs) detection enables longitudinal observations of the disease. In the study, we evaluated the CTCs in lung cancer following locoregional radiation therapy. Methods: Samples of 5ml peripheral blood was taken from each lung cancer patients (n=15) both before and after the radiotherapy course. Meanwhile tumour size was determined by chest X-ray or computed tomography. Using cytokeratin 19(CK19) as marker, the blood samples were subjected to real time RT-PCR assay. All patients with lung cancer were treated with primary definitive and mediastinal radiotherapy. Results Compare to that of pre-treatment, the value of CK19 mRNA in peripheral blood after therapy decreased dramatically(5.0932±1.0628 vs. 4.2493±0.8323, t=3.192, P=0.007). The change of CK19 mRNA level before and after radiotherapy was closely related to the type (NSCLC vs. SCLC, 0.5389±0.9030 vs. 1.6826±0.9467, t=2.1465, P=0.051). Meanwhile, there appeared to be a close link between the grade (Well/Mod vs. Poor) and the change of CK19 mRNA (0.5024 vs. 1.5271, t=2.017, P=0.065). The change of CK19 mRNA level was related to variation of tumour burden during radiotherapy(r=0.0575, P=0.025). Of the 15 cases studied, 12 cases were positive before radiotherapy (12/15, 80%). The positive rate was 53%(8/15) after radiotherapy, meaning that four patients converted into negative after radiotherapy. Conclusions: The disseminated circulating cancer cells can be affected by radiotherapy; meanwhile further more systemic adjuvant treatment should be conducted. Due to concordance between molecular response and radiological remission, assessment of the therapeutic response might be possible by serial quantitative of CTCs.

Key words: Lung neoplasm, Blood, Polymerase chain reaction, Cytokeratin, Messenger RNA, Radiotherapy

INTRODUCTION
The failure to reduce the mortality of patients with solid tumour is mainly a result of early dissemination of cancer cells to secondary site, which is usually missed by conventional diagnostic procedures used for tumour staging [1]. Due to the intrinsic fault in the current tumour-nodes-metastasis (TNM) staging system, it is an urgent agenda to absorb some cellular or molecular factors into the system [2]. The detection of the earliest manifestation 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 ten 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 [3]. The possibility to use easily accessible body fluids as a source for minimal residual circulating cell detection enables longitudinal observation of the disease [4]. Theoretically, radiotherapy is a localized strategy and if it imposes any effect on disseminated lung cancer cells is unknown. In the study, we evaluated the real time RT-PCR of cytokeratin 19(CK19) target for its feasibility to estimate the change of circulating tumour cells (CTCs) in patients with lung cancer following locoregional radiation therapy and correlated our observation to clinical finding.

MATERIALS AND METHODS
Clinical Data
After obtaining informed consent, a sample of 5 ml of peripheral blood was taken from each lung cancer patient (n=15) before and after the radia-
tion therapy course. Pre-therapy blood samples were collected from the patients studied at least one day before radiotherapy. Post-radiotherapy blood samples were obtained from the same patients immediately after therapy. The tumour size was determined by chest X-ray or computed tomography before and after radiotherapy.

All patients with lung cancer were treated with primary definitive and mediastinal radiotherapy. No other treatment (e.g. operation or chemotherapy) was carried out during radiotherapy. The median radiation dose applied was 60 Gy (range: 30 ~ 72) and the median treatment time was 48 days (range: 10 ~ 56).

The study population consisted of 15 patients with histologically documented lung cancer. Staging procedures included chest radiograph, bronchoscope, thoracic computed tomography, sonograph and bone scintigraph. As negative controls for FQ-RT-PCR, 20 peripheral blood samples were collected from healthy subjects. The characteristics of the lung cancer patients is summarized in Table 1.

**PROCEDURE**

**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.

**cDNA Synthesis**

Total RNA was denatured at 70°C for 5 min. Reverse transcriptase reaction was carried out in 18µl 1x reverse transcriptase buffer [50mM Tris-HCL (pH8.3), 75 mM KCL and 3mM MgCl2] with 0.5 mM deoxynucleotide triphosphates, 1µl of RNasin, and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega). CDNA were synthesis 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 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.

**Taqman PCR Reaction**

PCR was conducted in 43µl 1x PCR buffer [10mM Tris-HCL (pH8.4), 50mM KCL, and 1.5mM MgCl2] with 0.2 mM deoxynucleotide triphosphates, 5 µl of CDNA, 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°C, followed by 35 cycles of 95°C for 30 seconds, 62°C for 20 seconds, and 72°C for 20 seconds, and a final extension at 72°C 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

<table>
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Abbreviation: F, female; M, male; ADC, adenocarcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung cancer; Poor, poor differentiated; Well/mod, well or moderate differentiated. *The values of results were expressed as log₁₀ CK19mRNA copies per millilitre serum.
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 \( \log_{10} \text{CK}_{19} \text{mRNA} \) 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 (CCD) camera collects the emission from each sample and the data are analysed automatically. The software accompanying the 7000 system calculates \( \text{Ct} \) 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

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**Figure 1:** The receiver operator characteristic (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-5 cell line dilutions. The graph shows the \( \text{Ct} \) value versus the log of the number of \text{CK}_{19} \text{mRNA}, measured in triplicate. The standard curve shows 4 orders of linear dynamic range

**Figure 3:** Typical amplification plot. The graph of the increment of fluorescence reporter signal (\( \Delta Rn \)) versus cycle number during PCR shows three stages: baseline, exponential phase, and plateau. The \( \text{Ct} \) 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 (\( \text{Rn} \)). \( \Delta Rn \) represents the normalized reporter signal (\( \text{Rn} \)) minus the baseline signal.

**Figure 4:** The change of \text{CK}_{19} \text{mRNA} in lung squamous cell cancer/adenocarcinoma or small cell lung cancer before and after radiotherapy.

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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 FQ-RT-PCR for construction of the calibration curves and ROC curves. Each sample was measured in triplicate.

**Statistical Analysis**
The results were analysed with the statistical software package SPSS 10.0 for windows. To compare the change in level of CK19 mRNA during radiotherapy, paired samples t test was used. To assess any correlations between the level of CK19 mRNA in PB and clinocopathological data, either bivariate correlate or paired samples t test was applied, with \(P < 0.05\) indicating significance.

**RESULTS**

**Evaluation of the CK19 Diagnostic Test**
To rigorously define the value of the real time RT-PCR analysis for the detection of circulating tumour cells (CTCs), we performed a receiver operator characteristic (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%CI: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. If \(\log_{10}\) CK19 >4.120 (cut-off value), the case would be classified as positive. Of the 15 cases studied, 12 cases were positive before radiotherapy (12/15, 80%). The positive rate was 53% (8/15) after radiotherapy, indicating that 4 patients converted into negative after radiotherapy.

**Comparison of CK19 mRNA in Peripheral Blood before and after Radiotherapy**
Compare to that of pre-treatment, the value of CK19 mRNA in peripheral blood after therapy decreased dramatically (5.0932±1.0628 vs. 4.2493±0.8323, \(t=3.192, P=0.007\), paired samples t test).

**The Relationship between CK19 Transcriptant and Classic Clinicopathological Parameter**
The change of CK19 mRNA level before and after radiotherapy was related to the type (NSCLC vs. SCLC, 0.5389±0.9030 vs. 1.6826±0.9467, \(t=2.1465, P=0.051\), Independent Samples T Test, Figure 4). Meanwhile, there was a close link between the grade (Well/Mod vs. Poor) and the change of CK19 mRNA, although it was not confirmed by statistical analysis (0.5024 vs. 1.5271, \(t=2.017, P=0.065\), Independent Samples T Test, Figure 5). There were no correlations regarding stage, gender or age etc.

**The Variation of Tumour Burden and CK19 mRNA During Radiotherapy**
The change of CK19 mRNA level in PB was related to variation of tumour burden during radiotherapy (\(r=0.0575, P=0.025\), Bivariate Correlate).

**The Positive Rate of CK19 mRNA before and after Radiotherapy**
The upper value of the confidence interval (CI) of the median of the healthy volunteer group was considered as the “cut-off” value. If \(\log_{10}\) CK19 >4.120 (cut-off value), the case would be classified as positive. Of the 15 cases studied, 12 cases were positive before radiotherapy (12/15, 80%). The positive rate was 53% (8/15) after radiotherapy, indicating that 4 patients converted into negative after radiotherapy.

**DISCUSSION**
Traditionally clinical response criteria was defined according to the change of the mass during therapy, e.g. complete response is defined as complete disappearance of all measurable and available clinical evidence of cancer. Currently, 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 [5]. Therefore, the detection of CTCs is important for assessing treatment response, evaluating the prognosis and tailing therapeutic strategy. According to the reported studies of
Pachmann et al. and Rolle et al., the patients with a continuous increasing of circulating epithelial cell after complete resection of lung cancer are at an increased risk of early relapse. Other studies also demonstrated that detection of disseminated tumour cells is important for evaluating the prognosis.

Since Traweek et al. described the high sensitivity of RT-PCR in the detection of cytokeratin-19 (CK19) gene transcripts present in MCF-7 breast cancer cells, being able to identify one tumour cell among 10^6 HL-60 cells, several authors elaborated a similar approach to detect breast cancer cells in haematopoietic harvests by identifying target gene transcripts from cytokeratins, indicating the presence of CK19 mRNA in the peripheral blood may be a potential marker of disseminated tumour cells for breast cancer. The RT-PCR technique is a tool capable of detecting minute quantities of circulating tumour cell-derived transcripts. 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 promising tool for therapy monitoring.

In the present study, we developed a quantitative real-time RT-PCR method for measuring cytokeratin 19 mRNA in peripheral blood of lung cancer patients. The analytic sensitivity of our method, i.e., the smallest amount of CK19 mRNA tested and reliably quantified, is 1000 copies/reaction mixture. In practise, however, we have detected up to 4.2x10^5 copies/ml, because the linearity of our standard curve permits us to interpolate values located below the lower limit of the dynamic range (1x10^5 copies/ml). On the other hand, the system can quantify the CK19 RNA in samples with more than 10^6 copies/reaction mixtures. This degree of sensitivity along with a considerably wide dynamic range allows the use of a single method for the detection of the wide range of loads found with CK19.

The permanent access to CTCs enables longitudinal studies to monitor therapy outcome. This aspect is substantially improved by recent quantitative method (e.g. real-time PCR) allowing the identification of circulating tumour cells accurately. The study shown there appeared to be certain relationship between variation of CK19 mRNA in peripheral blood and histological type of the lung cancer (P=0.051) or grade (P=0.065), although it was not verified by statistical analysis. It is speculated that, due to different radiotherapeutic sensitivity of varied type or grade lung cancer, the number of shedding tumour cells might be varied during therapy. On the other hand, there were quite a few patients whose level of CK19 mRNA still exceed cut-off value even if radiotherapy was performed, suggesting further more systemic adjuvant treatment (e.g. chemotherapy or immunotherapy) should be conducted. Theoretically, radiotherapy is a localized strategy and imposes little effect on disseminated cancer cells. We speculated that the reduced number of CTCs during radiotherapy should attribute to the falling tumour burden that releases less tumour cells into the circulation than before.

A traditional evolutionary criterion of curative effect depends on the variation of tumour size during treatment. In this study, concordance between radiological remission and molecular response was observed in the majority of patients after radiotherapy, suggesting that early assessment of the therapeutic response may, therefore, be possible by serial quantitation of CTCs. 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 carried out for prognostication of disease outcome, therapy monitoring or drug targeting. This aspect is substantially improved by recent quantitative methods allowing the identification of change in the quality and number of tumour cells. Therapy influenced the number of disseminated cells enabling the monitoring of its efficiency.

In therapeutic studies, long-term observations are still required to establish whether the therapy-associated reduction in individual disseminated cells is associated with improved prognosis. Perspective clinical studies are needed to determine whether the assay will be useful in assessing prognosis, tailoring therapy, or developing new strategies for ex vivo purging.

A series of studies provided compelling evidences that sequential quantification of circulating tumor cells in many solid tumor such as nasopharyngeal carcinoma, follicular lymphoma, rectal cancer, prostate cancer, hepatocellular carcinoma as well as cancer of the head and neck receiving radiotherapy, surgical resection or chemotherapy could be useful for assessing the efficacy of therapy and predicting clinical metastasis or recurrence. The study suggested that detection of disseminated lung cancer cells may be valuable for evaluating the efficacy of radiotherapy and could thus play an important role in deciding on systemic therapy, as measured by circulating cytokeratin 19 mRNA.

In conclusion, we believe that there is an increasing body of evidence demonstrating that detection of tumour cells disseminated in peripheral blood can provide clinically important data that are of value for tumour staging and maybe for prognostication, and that can identify surrogate markers for early assessment of the effectiveness of adjuvant
therapy. Thus, these data would have a substantial influence on future oncology diagnosis and treatment. At the very least, examination for occult metastases should be incorporated into future clinical trials to evaluate cancer treatments. In the future, adjuvant therapy, specifically tailored to the disease in subgroups of patients or individual patients with residual disease, may represent a substantial advance [87]. Additionally long-term follow-up will have to show whether patients with high expression of CK19 mRNA actually have an increased risk or recurrence and whether the method is suitable as well to detect progression.

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REFERENCES