## **RESEARCH ARTICLE**

# **Circulating Tumor Cells are Associated with Bone Metastasis** of Lung Cancer

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## Abstract

Lung cancer (LC) is the leading cause of cancer mortality worldwide, predominantly due to the difficulty of early diagnosis and its high metastatic potential. Recently, increasing evidence suggests that circulating tumour cells (CTCs) are responsible for cancer metastatic relapse, and CTCs have attracted interest in cancer metastasis detection and quantification. In present study, we collected blood samples from 67 patients with bone metastasis, and 30 patients without such metastasis, and searched for CTCs. Then the association of CTC numbers with bone metastasis and other clinico-pothological variants was analyzed. Results demonstrated that when 5 or 1 was taken as a threshold for the CTC number, there were significantly higher positivity of CTCs in the bone metastasis group than in the non-metastasis group. While the increase in CTC number was not significantly associated with any other clinicopathological factor, including age, gender, pathological type, intrapulmonary metastasis and lymph node metastasis, the CTC number in patients with positivity of the last above mentioned variants was obviously higher than in patients with negativity of the two variants. Taken together, the CTC number appears to be significantly associated with the bone metastasis from lung cancer.

Keywords: Circulating tumor cells - bone metastasis - lung cancer - MRI

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## Introduction

Lung cancer (LC) is the leading cause of mortality worldwide (Jemal et al., 2011), predominantly due to the difficulty of early diagnosis and the highly metastatic potential of the cancer. In most cases, the metastasis has already developed before the diagnosis of lung cancer (Stenbygaard et al., 1999; Herbst et al., 2008; Al et al., 2009). Particularly, the skeleton is the most common site of lung cancer metastasis, and skeletal complica tions from bone metastases present a major challenge in disease management (Brown et al., 2005), approximately 30% of patients with advanced lung cancer will develop bone metastasis in the course of their disease, resulting in a significant poor prognosis (Stenbygaard et al., 1999; Sone et al., 2007; Al et al., 2009). And the progress in improving treatment of advanced cancer has been disappointing, so late diagnosis of lung cancer is a fundamental obstacle to progress in lung cancer outcomes (Chute et al., 1999; Carney et al., 2002). Therefore, the early detection of the metastasis to bones or other organs is clinically vital for the prevention and treatment of lung cancer.

Symptoms of diagnostic value have been reviewed by Hamilton and Sharp (Hamilton et al., 2004) and Shapley et al (Shapley et al., 2010). Reviewers analysed all higher quality evidence of symptoms that predicted LC in an unselected primary subjects with LC, and only few symptoms that might have implications for the diagnosis of LC. Although the spiral computed tomographic (CT) scanning potentially provides a robust tool for the detection of lung cancer. It fails to detect early lung cancer routinely, is costly and lacks direct convince (Mulshine et al., 2002). Magnetic resonance imaging (MRI) plays an important role in detecting LC bone metastases, with a higher specificity and sensitivity in the early detection of LC bone metastases, compared to bone scintigraphy (Schmidt et al., 2009). Moreover, over these years, new tools such as positron emis-sion tomography/ computed tomography (PET/CT), transbronchial needle aspiration (TBNA), endobron-chial ultrasound (EBUS), oesophageal ultrasound and medical thoracoscopy have been introduced in lung cancer diagnostic and staging algorithms (Currie et al., 2009). PET/CT has been a major step forward in detecting local and distant metastasis) and is more accurate than CT. The ultrasound guided transbronchial and transoesophageal biopsy provides a less invasive method of staging the mediastinum, both of which display encouraging results in localized cancer identification. Given the seriousness of high LC metastasis and the high cost of MRI, PET/CT, present progresses in early detection of LC metastasis are still far from satisfaction, in developing countries, i.e. China.

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More recently, increasing evidence suggests that circulating tumour cells (CTCs) are responsible for cancer metastatic relapse (Krebs et al., 2010; Cristofanilli et al., 2014), and CTCs have attracted interest in cancer metastasis detection and quantification of lung cancers (Ma et al., 2012), of prostate cancers, and particularly of breast cancers (Tarhan et al., 2013, Turker et al., 2013). And numerous methods have been developed for the enrichment and detection of CTCs, particularly the epithelial cell adhesion molecule (EpCAM)-based enrichment of CTCs offers the advantage for highthroughput detection (Gorges et al., 2012, Grover et al., 2014). The high CTC numbers were detected in breast cancer patients with bone metastases (Mego et al., 2009), and the CTCs were associated with the FDG-PET/CT results in metastatic breast cancer patients (Ni et al., 2013) Moreover, CTCs have been also detectable and as prognostic factor in lung cancer, breast cancer (Hiltermann et al., 2012; Isobe et al., 2012; Stovold et al., 2012; Wong et al., 2012; Ni et al., 2013) and even in prostate cancer patients (Wang et al., 2011), and CTCs have been shown to be a good indicator for lung cancer metastasis (Hou et al., 2011; O'Flaherty et al., 2012).

In present study, in order to confirm whether an increased level of CTCs is closely related to the MRIdetected metastasis in bone in patients diagnosed with progressive lung cancer. We quantified the CTCs in lung cancer patients, and analyzed the association of CTC numbers with the bone metastasis via MRI imaging and other clinicopathological variants. It was implied that there was a significant association of CTC number with MRI-detected bone metastasis of LC.

## **Materials and Methods**

#### Subjects

Present study included a total of 67 lung cancer patients, having MRI-confirmed bone metastasis, who were admitted from January 2009 to April 2013 at our hospital. And other 30 lung cancder patients without bone metastasis were also involved, as control. Complete Clinico-pathological data, including age, gender, history, physical examination, radiographic studies and other variants were collected. The clinical and pathological stages were determined according to the current tumournode-metastasis (TNM) classification as revised in 2009 (Goldstraw et al., 2007). Our Institutional Review Board approved this study, and written informed consent was obtained from each subject prior to joining the study.

#### CT Examinations and tumor size measurement

CT scans of the chest were performed with a fourrow MDCT scanner (Volume Zoom; Siemens Medical Solutions, Forchheim, Germany) was as follows: patients were scanned in the supine position from the cranial to caudal direction, from the clavicles to the adrenal glands at end-inspiration; 100mL of iopromide (Ultravist 300, 300mg iodine/mL; Bayer HealthCare Pharmaceuticals Inc, Wayne, NJ, USA) was injected intravenously with an automated injector (Medrad, Pittsburgh, PA, USA) at a rate of 2-3mL/second, with a scan delay of 30sec,

unless medically contraindicated. The parameters were as follows: 120kVp, 165mAs, 2.5mm scanning thickness, and 0.5sec exposure time. The axial images were reconstructed using B40f kernel for standard algorithm and B60f kernel for lung algorithm. Chest CT images were reconstructed with the standard algorithm were anonymized and transferred to a workstation with threedimensional medical visualization and analysis software (Vitrea 2, version 4.0, Vital Images, Minnetonka, MN, USA) for analysis. The bidimensional diameters, volume and CT attenuation (Hounsfield unit) of target lung lesions using a commercially available volume analysis software (Vitrea 2). Axial chest CT images of target lesion site with various diameters were selected And the lesion was automatically segmented from the surrounding normal lung and adjacent structures by software using a threedimensional seed growing algorithm (Wang et al., 2008).

#### Whole-body MR imaging

MR imaging (MRI) was performed with a 1.5-Tesla (T) imaging system (Magnetom Sonata<sup>™</sup>, Siemens Medical Solutions, Erlangen, Germany) with an amplitude of 40mT/m and a slew rate of 200mT/m/ms. The MRI examination covered a scan ranging from head to feet. Patients were examined on a fully MR-compatible rolling table platform (BodySURF<sup>™</sup>, MR-Innovation, Essen, Germany), which was mounted on the existing, original table of the MR system, and which was placed on roller bearings for manual movement in Z direction. For every examination, whole-body MRI were obtained in the coronal and sagittal planes with a body coil and a moving table. Different anatomic regions were imaged with surface coil quality in rapid succession without changing the coil positions.

#### Circulating tumour cell counting

Venous blood samples for CTC analysis were collected according to previous report (Cristofanilli et al., 2004). The CellSearch System (Veridex LLC, Raritan, NJ, USA) was used for the isolation and enumeration of CTCs. The Isolated cells were then fluorescently labeled with the nucleic acid dye 4', 6-diamidino-2-phenylindole and labeled monoclone antibodies to leukocytes (CD45allophycocyanin) and epithelial cells (cytokeratin 8, 18, 19-phycoerythrin). CTCs are defined as nucleated cells lacking CD45 and expressing cytokeratin. Identification and enumeration of CTCs was done using the CellSpotter Analyzer (Immunicon Corporation, Huntingdon Valley, PA, USA). For CTCs evaluation, a threshold of 1 CTC/7.5ml or 5 CTCs/7.5ml blood was used to evaluate results. And the results were expressed as a logarithmic CTC number to the base 2.

#### Statistical analysis

Student's unpaired t-test was used for symmetrical data comparison between two groups, and non-parametric tests, Mann-Whitney U-test was used for asymmetrical comparison between two groups. Statistical significance was defined as a P value of <0.05. All statistical analyses were performed using SPSS16.0 software (IBM SPSS, Inc., USA).

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| Table 1. Patients Characteristics (n=97) |                         |           |         |  |  |
|--|-------------------------|-----------|---------|--|--|
| Items                                    | Types                   | Value (%) |         |  |  |
| Age (years)                              | Median range)           | 64        | (38-84) |  |  |
| Sex                                      | Males                   | 64        | -66     |  |  |
|  | Females                 | 33        | -34     |  |  |
| Histological                             | Squamous cell carcinoma | 33        | -34.02  |  |  |
| type                                     | Adenocarcinoma          | 42        | -43.3   |  |  |
|  | Small-cell carcinoma    | 18        | -18.55  |  |  |
|  | Other tumor             | 4         | -4.120  |  |  |
| TNM stage                                | Ia–IIb                  | 3         | -3.09   |  |  |
|  | IIIa                    | 9         | -9.28   |  |  |
|  | IIIb                    | 18        | -18.56  |  |  |
|  | IV                      | 67        | -69.077 |  |  |

Table 2. Association of Clinico-pathological Variants with Bone Metastasis

| Variants                 | Non-meta<br>stasis (n=30) | Metastasis<br>(n=67) | P value 5 |
|--------------------------|---------------------------|----------------------|-----------|
| Age (years)              |                           |                      | 0.647     |
| ≤70                      | 22                        | 52                   | 7         |
| >70                      | 8                         | 15                   | 2         |
| Gender                   |                           |                      | 0.924     |
| Males                    | 20                        | 44                   |           |
| Females                  | 10                        | 23                   |           |
| Pathology                |                           |                      | 0.749     |
| Non-small-cell lung can  | cer 25                    | 54                   |           |
| Small-cell lung cancer   | 5                         | 13                   |           |
| Intrapulmonary metastasi | s                         |                      | 0.039     |
| Yes                      | 14                        | 46                   |           |
| No                       | 16                        | 21                   |           |
| Lymph node metastasis    |                           |                      | 0.792     |
| Yes                      | 24                        | 52                   |           |
| No                       | 6                         | 15                   |           |
| CTC (cells /7.5 mL)      | 0.0016                    |                      |           |
| <1                       | 22                        | 27                   |           |
| ≥1                       | 6                         | 36                   |           |

## **Results**

## patients' characteristics

A total of 67 lung cancer patients with bone metastasis from our hospital were selected in this study, and another 30 cases without bone metastasis were included as control. Totally, the median age of all patients was 64 (range 38-84) years, 64 patients were male, 33 patients were female. The histological classification of the lung cancers was confirmed by two pathologists. There were 42 cases of adenocarcinoma, 33 cases of squamous cell carcinoma, 18 cases of small-cell lung cancer and 4 cases of large cell lung cancer. According to the staging system (TNM Stage Ver. 6), there were three stage Ia-IIb cases, 9 stage IIIa cases, 18 stage IIIb cases, and 67 stage IV cases (Table 1).

Besides the histopothological diagnoses, tissue confirmation by computed tomography of the lung cancer was conducted. Chest and abdominal CT image confirmed the primary tumor sites for all 97 patients. And as shown by the CT imaging, the primary tumor diameter averaged  $43.4\pm31.2$ mm in the bone metastasis group, while the patients without bone metastasis had only 24.5±13.7mm in the primary tumor diameter, there was a significant difference (p < 0.01) (Figure 1 A-C). The chest CT scan also indicated intrapulmonary metastasis in 46 of 67 cases (68.66%) in the bone metastasis group, while in 14 of 30



Cancer and its Intrapulmonary Metastasis. (A). CT imaging of central lung cancer; (B). CT imaging of peripheral **5,0** ung cancer; (C). The intrapulmonary metastasis of central lung cancer under CT sca38.(D). The intrapulmonary metastasis of al lung cancer under CT23.7 h



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Figure 2. Bone Metastasis of Lung Cancer Under MRI Imaging. (A). Singular vertebral metastasis from lung cancer under MRI; (B). Multiple sites of vertebral metastasis from lung cancer under MRI; (C). Ischial metastasis from lung cancer under MRI; (D). Femur metastasis from lung cancer under MRI. The metastatic sites were labeled with square marker

patients (46.67%) without the bone metastasis, as was also shown by the CT imaging (Figure 1 C and D). The bone metastasis was detected by MRI, and there was at least one bone metastatic site for the 67 patients in the bone metastasis group. The targeting metastatic bone included spine, pelvis, femur and so on (Figure 2 A-D). Anong the metastasis group, 18 patients had only one invaded site, 32 patients had two metastatic sites, and the remaining 17 patients were attacked in more than three sites.

### Circulating tumor cells was associated with the bone metastasis in lung cancer patients

Firstly, the association of bone metastasis with the clinico-pathological variants, such as age, gender, pathological type, lymph node metastasis, and intrapulmonary metastasis.

30.0

30.0

30.0

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Table 3. Association of CTC Number with the BoneMetastasis and Other Clinicopathological Variants

| Variants                  | Average Log2 CTC number (n) | P value |
|---------------------------|-----------------------------|---------|
| Age (years)               |                             | 0.463   |
| ≤70                       | 3.12±0.47 (61)              |         |
| >70                       | 2.88±0.63 (19)              |         |
| Gender                    |                             | 0.624   |
| Males                     | 3.17±0.53 (44)              |         |
| Females                   | 2.93±0.44 (36)              |         |
| Pathology                 |                             | 0.128   |
| Non-small-cell lung car   | ncer 3.00±0.37 (65)         |         |
| Small-cell lung cancer    | 3.32±0.65 (15)              |         |
| Intrapulmonary metastasis | 5                           | 0.065   |
| Yes                       | 3.20±0.58 (52)              |         |
| No                        | 2.81±0.56 (28)              |         |
| Lymph node metastasis     |                             | 0.073   |
| Yes                       | 3.15±0.59 (61)              |         |
| No                        | 2.76±0.63 (19)              |         |



Figure 3. Association of Circulating Tumor Cell (CTC) Count with Bone Metastases. (A). Significantly high level of CTC number in the lung cancer patients with bone metastasis (p=0.0045). (B). Difference in CTC number between patients with and without intrapulmonary metastasis (p=0.0567)

And it was shown in table 2 that there was no significant difference between the bone metastasis group and control group in above mentioned variants (p>0.05for each variant), except intrapulmonary metastasis. There were more patients having intrapulmonary metastasis, in the bone metastasis group than in the control group (p<0.05). Then the CTC counting was analyzed in all 91 patients from the two groups, who were consented to the optional blood collection for evaluation of CTCs, including 63 blood samples from 67 lung cancer patients with bone metastasis, and 28 samples from the 30 nonmetastasis patients. CTC were detectable (CTC≥1) in more than 93.65 % of patients with bone metastasis (59/63) and in about 71.43% patients (20/28) without bone metastasis, and the CTC numbers of these samples were not normally distributed, we evealuated CTC numbers via a Logarithmic value to the base two.

As shown in the Figure 3, the average CTCs value was  $3.51\pm0.63$  in the metastatic group,  $1.74\pm0.46$  in the nonmetastatic group. And there was a significant difference between the two groups (p=0.0045). And when 5 was taken as a threshhold for the CTC number, there was 27 samples from the 63 metastasis patients, while only 6 samples from the 28 non-metastasis patients. And we also concluded that the CTC number was significantly higher in the metastasis group than in the non-metastasis group (p=0.0016). The repeatability of CTC measurements was evaluated by samples at three time points from 24 cases of metastatic patients and 15 cases of non-metastatic patients, and there was a high intraclass correlation coefficient (Ri =0.995 and 0.997 respectively) and a high CR value ( $R^2$  =0.990 and 0.993 respectively). Therefore, there was a significant association of CTCs with the bone metastasis of lung cancer.

Association between the CTC number and other clinico-pathological variants was analyzed (Table 3). The increase in the CTC number was higher in the group with intrapulmonary metastasis, or with lymph node metastasis than in the non-intrapulmonary or non-lymph node metastasis group (p=0.065 and 0.073 respectively). While the increase in CTC number was not significantly associated with any other clinicopathological factor, including age (p=0.463), gender (p=0.624), pathological type (p=0.128). Taken together, the CTC number was significantly associated with the bone metastasis from lung cancer.

#### Discussion

Metastasis usually cuts down the treatment effectiveness of lung cancers and portends a poor prognosis for such patients. It is now apparent that tumor cell invasion and distant metastasis can progress via the bloodstream, lymphatic vessels, or transcoelomic spread into the pleural, pericardial, and abdominal cavities (Fidler et al., 1978). The hematogenous system is thought to be the primary and most common route for the formation of distant metastasIs. Disseminating tumor cells can also circulate to and lie dormantly in the bone marrow, potentially for a number of years, and then re-enter the bloodstream enroute to secondary metastatic sites (Riethdorf et al., 2008). Thus, the detection of metastasis, particularly of bone metastasis is necessary before making treatment plan and prognosis assessment.

The uncontroled growth of cancer cells usually invade adjacent normal lung tissues and destroy the intactness of local vascularity. And theoretically, the release of tumor cells into the bloodstream to develop the CTCs happens before the distant metastasis via blood, and the CTC counting has been proved to serve as a sensitive and prospective indicator to the metastasis of colorectal cancers, breast cancers, Prostate Cancers (Pierga et al., 2013; Shiota et al., 2013; Tseng et al., 2014). And CTCs have been also detectable and as prognostic factor in lung cancer (Hiltermann et al., 2012; Ni et al., 2013), and have been shown to be indicative for lung cancer metastasis (Hou et al., 2011; O'Flaherty et al., 2012).

In present study, to evaluate the association of CTC numbers with the bone metastasis, we selected a total of 67 lung cancer patients with and 30 cases without bone metastasis. The diagnosis for all 97 patients was confirmed by histopothological evidences and CT imaging. The primary tumor size in bone metastasis group was larger than in the non-metastasis group, with a significant difference. And the chest and abdominal CT scan also indicated a high intrapulmonary metastasis rate in the bone metastasis group. And the CTC number was higher in patients with larger tumor size or with intrapulmonary metastasis than in the patients with smaller size or without intrapulmonary metastasis. However, the difference was

not large enough to sustained a sinificant association of CTC numbers with the tumor size and intrapulmonary metastasis. In addition, patients in the two groups were neither significantly different in the age, gender, histological classification and lymph node metastasis. It is well documented that the FDG–PET/CT is specific and sensitivie for detection of bone metastases . However, the expensiveness of it limits it wide use for most people in developing country, such as China. In the present study, we show that increased levels of CTC are strictly associated with the presence of bone disease as detected by MRI, indicating a possible relationship between CTC counts and the bone tumor burden.

In summary, we qualified the CTC in lung cancer patients with of without bone metastasis in present study, and we found that the CTC number was significantly associated with the bone metastasis which was revealed by MRI. However, there was no significant association of the CTC number with the intrapulmonary metastasis, lymph node metastasis or other clinico-pathological variants.

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