In vivo flow cytometry visualizes the effects of tumor resection on metastasis by real-time monitoring of rare circulating cancer cells

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Running Title: Monitoring of CTCs by IVFC assesses resection on metastasis

Key words: In vivo flow cytometry, hepatocellular carcinoma, tumor resection, hematogenous metastasis, circulating tumor cells

Financial support:

1) National Basic Research Program of China, 973 Program, 2011CB910400 (X.B. Wei);
2) National Basic Research Program of China, 973 Program, 2012CB966800 (X.B. Wei);
3) China National Key Sci-Tech Special Project, 2008ZX10002-025 (J. Zhou);
4) China National Natural Science Foundation, 30901432 (X.B. Wei);
5) China National Natural Science Foundation, 30972949 (J. Zhou);
6) China National Natural Science Foundation, 20975027 (J. Zhou);
7) Chinese Ministry of Education, 109056 (X.B. Wei);
8) Program for New Century Excellent Talents in University Award, NCET-08-0131 (X.B. Wei);
9) Fudan University 2010 Excellent Ph.D. Research Foundation (Z.C. Fan and J. Yan).
10) Natural Science Foundation of Fujian Province, 2010J01136 (J. Yan).

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**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

The manuscript contains 4,893 words (excluding references) and 5 figures.
Abstract:

The quantification of circulating tumor cells (CTCs) is an emerging tool used to diagnose, stratify and monitor patients with metastatic diseases. In vivo flow cytometry (IVFC) has the capability to measure the dynamics of fluorescently labeled CTCs continuously and non-invasively. In this study, we monitored CTC dynamics in a GFP-transfected orthotopic tumor model of metastatic hepatocellular carcinoma (HCC) using IVFC. Our IVFC approach showed a 1.8-fold higher sensitivity than whole blood analysis by conventional flow cytometry and was able to distinguish CTC changes between orthotopic and subcutaneous tumor models. We also used our model to investigate whether liver resection promotes or restricts hematogenous metastasis in advanced HCC. Both the number of CTCs and early metastases decreased significantly after tumor resection. Resection also prominently restricted hematogenous and distant metastases. Importantly, CTC numbers correlated with tumor growth in the orthotopic tumor model, including the number and size of distant metastases. When combined with orthotopic tumor models, the novel IVFC technique presented here offers the capability to elucidate mechanisms that drive hematogenous metastasis and to monitor the efficacy of cancer therapy.
Introduction

Hepatocellular carcinoma (HCC) is one of the prevalent human cancers worldwide ranking 3rd for mortality and 5th for estimated new cases annually (1). The prognosis of HCC has been significantly improved in recent years due to earlier diagnosis and more effective treatments. However, tumor recurrence and metastasis are still the major obstacles for long-term survival (2, 3).

The metastasis is reported to correlate with the presence of circulating tumor cells (CTCs) in the vasculature as a consequence of either advanced tumor growth and invasion or a therapeutic intervention (4, 5). To monitor CTCs, conventional methods usually isolate and count cells expressing epithelial markers from peripheral blood samples (4-10). However, these methods are restricted by invasiveness, lower sensitivity caused by small blood sample volumes, and difficulty to record the dynamics of CTCs, which in vivo flow cytometry (IVFC) could overcome (11-23). IVFC is optimized to quantify circulating fluorescently labeled cells in live animals, without the need to extract blood samples. In IVFC measurement, when the fluorescent cells in fast flowing blood pass through the laser slit across the artery, the fluorescence signal could be excited and be detected. The technique is capable of monitoring the varieties of target cells in circulation continuously, e.g., cancer cells (15, 18), haematopoietic stem cells (19, 20), lymphocytes (14), and yields quantitative results without affecting the physiology of the subject.

Previously, the intravenous (i.v.) models have been commonly used in the IVFC measurement of CTCs studies (15, 18). In those studies, cancer cells are labeled ex vivo and injected into blood circulation of small animals. Although it reflects some circulating metastatic characteristics or therapy responses of cancer cells, the large number of injected CTCs does not
exist in pathologic conditions (24, 25). Moreover, i.v. tumor models could only reflect the processes that CTCs leave the circulation. They do not provide the information of CTC forming. The subcutaneous (s.c.) metastatic tumor models have been also used in monitoring CTCs by IVFC (15). In these models, the tumor is implanted into subcutaneous tissue, which could not represent the primary tumor site (26, 27). The difference in local environments might lead to different CTC dynamics in comparison to clinically relevant conditions. Clinical observations have suggested that local environment could influence the growth, metastasis and response to therapy of tumors (28-31). In orthotopic tumor models, the tumor is implanted into the normal or usual place, where it originates. For example, orthotopic liver cancer models are made when cancer tissue is implanted into the liver. Therefore, the local environment in these models is very similar to the clinical situation. Thus, compared to i.v. and s.c. models, orthotopic tumor models are better to assess the morphology, growth and development characteristics of clinical disease. They are also more representative of a primary tumor with respect to tumor site and metastasis, especially CTC dynamics.

Here we, for the first time, use an HCC orthotopic metastatic tumor model and perform IVFC measurement to study CTC dynamics under clinically relevant oncology condition and present the difference in s.c. and orthotopic tumor models. In addition, we assess the hematogenous metastatic changes related to the tumor resection, which is usually considered as the first choice for treatment.

**Materials and Methods**

**Cell Culture and Transgenic Procedure**
A human hepatocellular carcinoma cell line HCCLM3 (32) with high metastatic potential was established at the Liver Cancer Institute, Zhongshan Hospital, Fudan University. The HCCLM3 cells were cultured at 37°C and 5% CO₂ in high-glucose DMEM medium (Gibco, Rockville, MD) containing 10% fetal bovine serum (HyClone, South Logan, UT). The HCCLM3 cells were transfected with linearized pEGFP-C1 (BD Clontech, Palo Alto, CA) using Lipofectin reagent (Invitrogen, Carlsbad, CA). Stably transfected populations were obtained by growing the cells in the same media described above supplemented with 600 μg/ml G418 (Invitrogen) at 37°C and 5% CO₂. GFP⁺ cells were isolated by FACS (fluorescence-activated cell sorting) to establish stably transfected HCCLM3-GFP multi-clone cell line. HCCLM3-GFP cells were maintained in the same media supplemented with 600 μg/ml G418 (Invitrogen) at 37°C and 5% CO₂.

**Mouse Models and Resection Procedure**

Animal care and experimental protocols were in accordance with guidelines established by the Shanghai Medical Experimental Animal Care Commission. Balb/c nude mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd and raised under specific pathogen-free conditions. The study was approved by the Ethical Committee of Animal Experiments of Institutes of Biomedical Sciences, Fudan University.

For an s.c. (subcutaneous) metastatic tumor model, 5 × 10⁶ HCCLM3-GFP cells of passage 0 or 1 (P₀ or P₁) were implanted subcutaneously on the back of the mice (male, 6-week-old, 20 ± 2 g). For an orthotopic metastatic tumor model, orthotopic tumor implantation with HCCLM3-GFP tumor was performed under aseptic conditions. The tumor tissue from maternal tumor (s.c. tumor or orthotopic tumor) was cut into small cubes about 1×1×1 mm. The recipient mice (male, 6-week-old, 20 ± 2 g) were anesthetized with pentobarbital sodium salt (1%, 0.1 ml/g mice...
weight). The upper abdomen was prepared with 70% alcohol and betadine scrub. A small left subcostal incision was made. The left lateral lobe of the liver was extracted. Following a small, superficial incision into the liver, the 1×1×1 mm cube of HCCLM3-GFP tumor was implanted. The incision in the liver was closed with a 7-0 suture to avoid possible early peritoneal seeding of tumor cells.

In the resection group, mice with orthotopic metastatic tumor underwent the resection on the day 30 after implantation. Through the left subcostal incision, the left lateral lobe where tumor had been implanted was excised. The tumor size was around 10 mm in diameter and the surgical margin was more than 5 mm.

**In Vivo Flow Cytometry**

To monitoring GFP⁺ CTCs, we set up *in vivo* flow cytometer based on previous experience (11-13, 15, 17-19, 33). Briefly, we use trans-illumination with a 535 ± 15 nm light emitting diode (LED) to visualize the major veins and arteries of the ear microcirculation. An artery of 50-70 μm in diameter is selected for data acquisition. Light from the 488nm laser was focused into a slit by a cylindrical lens and imaged across the selected ear artery. The size of the slit at the focal plane of the sample is approximately 5×72 μm. The depth of focus (i.e. the full width at half maximum of the light slit onto the sample in the axial direction) is approximately 50 μm, a value chosen to match the vessels of interest. The sample is positioned so that the long dimension of the slit traverses the width of the blood vessel; thus, fluorescently labeled cells traverse this slit and are excited one by one as they flow through the chosen artery, producing a burst of fluorescence for each cell. Fluorescence is detected with a photomultiplier tube (PMT) placed directly behind the
mechanical slit and the spectral filtering for GFP, sampled at a rate of 5 kHz with a data acquisition card, and displayed/stored on a computer.

To assess the depletion kinetics of circulating tumor cells, anesthetized mice were positioned onto the stage to detect GFP⁺ CTCs. The IVFC measurement was performed for at least one hour once per week for each mouse starting from tumor implantation till its death. Each group includes at least 6 mice.

To determine the sensitivity and specificity of IVFC, mice without tumor implantation are measured at least once every two hours as blank control. Mice with non-fluorescent tumor implantation are measured at least once every two hours as negative control. Each group includes at least 6 mice. Both control group are used to optimize the analysis algorithm developed in house (40). In IVFC data, the width of signal peaks (with the unit “second”) reflects the time during which the CTCs flow across the detecting laser slit, from the beginning of the cell encountering the slit to the whole cell leaving the slit. Thus, the flow rate can be estimated by time width of signal peak in IVFC results, with the formula: flow rate = (width of laser slit + diameter of the cell) / time width of signal peak. The time width values of more than 100 signal peaks from a number of representative data traces are used to estimate the average flow velocity in our experiments. Moreover, we estimate the flow volume of blood per hour in the arteries we detected with the formula: flow volume per unit time = (π × diameter of artery² / 4) × flow velocity. Diameters of the arteries are obtained from at least 50 ear images by CCD during corresponding IVFC measurement.

Flow Cytometry

To evaluate the sensitivity and specificity of the data acquired from in vivo flow cytometry,
we use conventional flow cytometry analysis as a comparison. The blood samples from mice for conventional flow cytometry analysis are collected immediately after IVFC measurement performed. Whole blood sample was collected from anesthetized mice (at least 6 mice in each group for each time point) by heart extraction in heparin. PBMC (peripheral blood mononuclear cell, including possible present CTCs) were enriched by Ficoll-Paque density centrifugation separation (16). PBMC from non-tumor mouse is used as negative control to exclude the influence of auto-fluorescence and select appropriate gate in conventional flow cytometry. GFP⁺ CTCs were then counted by using the selected gate.

**Tumor Size Measurement by Ultrasound**

To assess tumor growth, in vivo ultrasound images were acquired by VisualSonics Vero 7700™ Imaging System (Visualsonics Inc, Toronto, ON, Canada). Briefly, anesthetized mice were fixated, and ultrasound images were acquired following the standard protocols with the frequency of 17.5 MHz. The tumor size was calculated by the formula used commonly in clinics:

\[
\text{Volume} = \frac{(\text{long diameter} \times \text{short diameter}^2)}{2}.
\]

**Pathology Test**

To assess the metastasis in various organs, lungs, liver, spleen, kidneys and a small number of lymph nodes were harvested from the mice and examined under pathology test. Tissue was fixated by formalin and made to paraffin sections. Tissue sections were stained with H&E using standard protocols.

To evaluate whether GFP⁺ cells in conventional flow cytometry analysis of whole blood sample were indeed tumor cells, GFP⁺ cells were isolated and stained with H&E using standard protocols.
Tissue Metastasis Imaging

In addition to pathology test, we acquired *in vitro* images of tissue metastases by confocal fluorescence microscope (Leica TCS SPE, Leica Microsystems CMS GmbH, Am Friendenplatz, Mannheim, Germany) to assess the level of metastasis. Lungs, liver, spleen, kidneys and a small number of lymph nodes were harvested (at least 6 mice in each group for each time point) and examined under the microscope using a 10× water-immersion objective (NA=0.3) to visualize GFP-expressing tumor metastases. The tissue was immerged with saline in a 3.5-cm-diameter dish, pressed and fixated for imaging.

Whole tissue images were acquired by *in vivo* optical imaging system (NightOWL II LB 983 NC100, Berthold Technologies GmbH & Co., KG, Bad Wildbad, Germany). A 475 ± 20 nm excitation bandpass filter and a 520 ± 10 nm emission bandpass filter were used for GFP imaging. The images of tissues from non-tumor mice were acquired as negative control.

Results

*In Vivo CTCs Counting by IVFC and Validation*

Fast flow rate in artery and sophisticated image-processing algorithms restrict the use of confocal imaging in enumerating CTCs, while *in vivo* flow cytometry is a simple and potentially far more powerful method to enumerate circulating cells(11). CTCs in orthotopic GFP-labeled HCC tumor mice can be readily counted flowing through the ear microcirculation by *in vivo* flow cytometry (Fig. 1a). A typical data trace is shown in Fig. 1b, in which individual peaks correspond to single cells that are excited as they traverse the laser slit of the IVFC. The variations in the intensity of recorded peaks might be due to GFP expression level and deviations from focal plane
of individual cells. The full width at half maximum is associated with the \textit{in vivo} flow velocity of the corresponding cell since it indicates the amount of time required to pass through the excitation slit of light, which is \(\sim 5 \, \mu m\) across.

To assess the specificity, the control traces are acquired from similar arteries of the mice either without tumor implantation (Fig. 1c), or with non-fluorescent HCC tumor implantation (Supplementary Fig. S1). There is no noticeable signal peak present in both control groups. In addition, H&E analysis of GFP\(^+\) cells sorted by fluorescence-activated cell sorting (FACS) in peripheral blood confirm that the cells we considered as CTCs are indeed tumor cells (Fig. 1d).

To assess the sensitivity, we use conventional flow cytometry to analyze GFP\(^+\) CTCs in whole blood at week 4, 6, 8 after HCC tumor implantation, as a comparison. As Fig. 1e shows, when IVFC detects 2.95 \(\pm\) 1.29, 13.50 \(\pm\) 2.56, 35.02 \(\pm\) 4.57 CTCs per hour at 4, 6, 8 weeks, the corresponding CTC counts by conventional flow cytometry are 12.36 \(\pm\) 5.70, 58.13 \(\pm\) 15.11, 157.53 \(\pm\) 26.92 per ml, respectively. We find out that those two groups of data had good linear relationship. Therefore, if IVFC detects one signal peak per hour, it corresponds to 4.36 \(\pm\) 0.66 CTCs per ml blood measured by conventional flow cytometry. Since blood flow velocity of the ear artery in our experiments is 11.9 \(\pm\) 2.3 mm/s calculated by IVFC, the flow volume is 0.128 \(\pm\) 0.021 ml/h, assuming that the cross-section area of the ear artery is \(2.99 \times 10^{-3} \pm 0.49 \times 10^{-3} \, \text{mm}^2\) (diameter of the artery is 61.56 \(\pm\) 5.13 \(\mu m\)). Therefore, if IVFC detects one signal peak per hour, it means that \(\sim 7.81\) CTCs per ml blood are detected (1/0.128). Thus, our IVFC has \(\sim 1.8\)-fold (7.81/4.36) higher sensitivity than whole blood analysis by conventional flow cytometry.

\textbf{The Difference of CTC Dynamics in an s.c. vs an Orthotopic Metastatic Tumor Model}

Clinical observations have suggested that the local environment can influence the growth,
metastasis and therapy response of tumors. Therefore, orthotopic tumor models, which can reflect clinically relevant local environment, are better for studying tumor metastasis. To establish our orthotopic HCC model, HCCLM3 tumor cells are labeled with GFP (97.5% positive in flow cytometry analysis, Supplementary Fig. S2), and implanted into the liver. Lung metastases are found at week 4 after implantation. Massive lung metastases are found at week 8 (Supplementary Fig. S3).

To investigate whether the s.c and orthotopic metastatic HCC tumor models have different CTC dynamics, we perform IVFC measurement on the two groups weekly after tumor implantation. Interestingly, there is a significant difference between the two curves of CTC dynamics (Fig. 2a). For our orthotopic tumor model, CTC dynamics shows similarity to an exponential curve. Therefore we make an exponential fit to the dynamic curve (N = 0.0342*e^{0.9185T}; T: time (weeks); N: number of cell counts per hour; R^2 = 0.9679). In contrast, the CTCs from our s.c. model fluctuate in a low level. In addition, we assess the influence of tumor size on CTC dynamics. We use ultrasound imaging to measure tumor size accordingly (Fig. 2b). The dynamics of CTCs in our orthotopic tumor model correlates with the tumor size. In contrast, there is no correlation between CTC dynamics and tumor size in our s.c. tumor model. The tumor growth is similar between the two groups (Fig. 2c). Therefore, the difference of CTC dynamics between the two groups is not due to the tumor size. To investigate whether local environment contributes to the difference, we have performed pathology test of the tumor (Fig. 3). We find out that the organ/tissue environments between the two groups, especially the blood supply, envelope and boundary of the tumor have remarkable differences. The differences generally could influence the condition for tumor cell entrance into circulation. Furthermore,
compared to s.c. tumor, the better blood supply condition in orthotopic tumor could cause the lower death rate of peripheral tumor cells. The death rate of peripheral tumor cells might be important to CTCs formed. Thus, the differences of CTC dynamics might be due to the organ/tissue environment, rather than tumor growth, emphasizing that orthotopic metastatic tumor models are better for metastasis study.

**CTC Dynamics Change after Liver Resection**

It remains unclear whether liver resection increases hematogenous metastasis in advanced HCC. Therefore, we assess the influence of liver resection to CTC dynamics by IVFC in our orthotopic tumor model. CTC dynamics of two mouse groups are obtained, one group being liver-resection group and the other being control group. As Fig. 4a shows, remarkable difference in CTC dynamics appears between two groups. In the control group, CTC dynamics shows similarity to an exponential increase \( N = 0.0285*e^{0.9528T}, R^2 = 0.9375 \), consistent with what we have reported above. CTC dynamics of the resection group is similar to the control group before the resection. However upon the resection, CTC counts drop down to an undetectable level, followed by another exponential-like curve \( N = 0.0468*e^{1.0397T}, R^2 = 0.9643 \) with a faster increase. Thus, while liver resection decreases the number of CTCs significantly, it might lead to higher hematogenous metastasis potential of residual tumor if the tumors are not removed completely.

To investigate the relationship between CTC dynamics and the tumor size, orthotopic tumor size measurements are performed (Fig. 4b). In the resection group, tumor growth is similar to that of the control group before the resection. With tumors removed, tumor sizes drop to an undetectable level and re-start to increase subsequently. After comparing the data of CTC
dynamics (Fig. 4a) and tumor growth (Fig. 4b), we find out that CTCs increases as the tumor grows in our orthotopic metastatic tumor model, although without a linear relationship (data not shown). In addition, in contrast to CTC dynamics, orthotopic tumor growth does not show faster increase rate after the resection. The observed faster increase of CTC counts after the resection might be induced by advanced metastatic tumors in other organs, as well as by molecular changes. Therefore, in our tumor model, the number of CTCs mainly corresponds to the condition of tumor cell source, not only orthotopic tumor size but also advanced metastatic tumor.

To confirm results of CTC dynamics obtained by IVFC, conventional flow cytometry analyses are performed on whole blood samples of the mice in both the resection and the control groups at week 4, 6, 8, respectively. The results are similar to those obtained by IVFC (Fig 4c): while CTC counts increase continuously in the control group, the CTCs in the resection group decrease after liver resection, and increase again subsequently.

The Curative Effect of the Resection on Metastasis Assessed by *ex vivo* Imaging

To further assess the distant metastases in other organs after the resection, we perform both traditional pathology test (Supplementary Fig. S3) and *ex vivo* fluorescence imaging (Fig. 5a, 5b). Confocal fluorescence imaging has higher sensitivity than pathology test (20), maybe mainly because pathology test could only see one slice of tissue. Lung is the common site of metastasis in advanced HCC. Fig. 5a shows fluorescent images of whole lung tissue. Although still going up after the resection, lung metastases increase remarkably slower in the resection group than those in the control. It confirms curative effect of liver resection on metastasis. Further details of metastases are shown by confocal microscope imaging (Fig. 5b). We find out that the metastases can be classified into two groups according to the size. The metastases with diameter smaller than
100 μm (about 1~5 cells) are considered as early metastases (Fig. 5c), which could be rarely
detected or distinguished by pathology test. The metastases with diameter larger than 100 μm are
considered as advanced metastases (Fig. 5d). The metastases in control group are far more than
those in the resection group regarding both early and advanced metastases. Interestingly, the
number of early metastases in control group is about 17-fold at week 8 than that in the resection
group. In contrast, the number of advanced metastases in control group is about only 4-fold at
week 8 than those in the resection group. Thus, the resection can inhibit early metastases more
than advanced metastases. In addition, the number of early metastases decreases in the resection
group at week 6, consistent with CTC dynamics, while such decrease is not present for advanced
metastases. Therefore we speculate that early metastases might reflect hematogenous metastasis
condition, while advanced metastases might reflect accumulative effects of hematogenous
metastasis. As a consequence, liver resection might decrease the newly produced metastases by
decreasing the CTCs.

Discussion

The major motivation for this work is to monitor the condition of hematogenous metastasis
continuously in vivo in an orthotopic tumor model, and determine whether the resection promotes
or restricts hematogenous metastasis. This issue is important because there has been no research
so far to our knowledge that has monitored CTC dynamics under clinically relevant physiology
condition. Therefore, we take advantage of IVFC technique to monitor CTCs, and assess its
sensitivity and specificity of detection. Furthermore we have discovered significant difference of
hematogenous metastasis condition in an s.c. and an orthotopic tumor model, which demonstrates
that orthotopic tumor models are better to study the metastasis. In addition, we demonstrate that the resection can restrict hematogenous metastasis by decreasing the number of CTCs and distant early metastases.

It has been disputed whether surgical therapy promotes hematogenous metastasis. Tumor resection is usually considered as the mainstay in solid tumor therapy; HCC is no exception for those patients without cirrhosis-induced limited liver function or extent of the tumor. However, previous studies have shown that surgical trauma, such as hepatectomy, may cause the variety of potential growth factors, which mediate liver regeneration after hepatectomy (34-36), enhance neovascularization and tumor cell proliferation (37), and promote tumor metastasis (38, 39). Our study monitors the influence of the resection on hematogenous metastasis in real time. The IVFC results show that tumor resection can decrease CTCs, confirming positive effect of the resection. Imaging experiment also shows that the resection group has less distant metastases than the control group. Furthermore, the number of early distant metastases decreases after the resection. This indicates that forming of neo metastasis is restricted after the resection, which might be due to CTC decrease. Therefore, our study demonstrates that liver resection could decrease hematogenous metastasis in HCC. Nevertheless, we also observe the reappearance of CTCs after the resection. Compared to the control, the rate of CTC re-increase in the resection group is higher. Thus, if the tumors are not removed completely, the resection might lead to higher metastasis potential of residual tumor, consistent with previous studies. The addition of adjuvant therapy (e.g., chemo therapy or radiation therapy) may overcome this problem (40). Further studies are currently undergoing.

It has been more than a century since Paget proposed the seed and soil hypothesis to describe
the mechanism by which cancer spreads or metastasizes throughout the body (41). CTCs might be considered as the seed in recent studies (4-10, 15-18). Nevertheless, there are few studies to monitor CTC dynamics under clinically relevant physiological condition. We have observed some interesting phenomena which could explain the mechanism of CTC dynamics. Firstly, we find out that CTC counts have relationship to the tumor size. Secondly, when the original tumor is removed, the number of CTCs drops to undetectable level (Fig. 4a, data in the resection group at week 5). These imply that CTCs could not maintain in circulation without the supply of solid tumor (also see Supplementary Fig. S4). Furthermore, all the mice with CTC recurrence of after original tumor removed, have metastases observed in both our imaging experiment and pathology test; all the mice without CTC recurrence after original tumor removed, have no metastases observed. Therefore the recurrence of CTCs after resection indicates that the tumor still remained, in the form of either residual tumor or distant metastases. Putting together, we hypothesize that CTCs are in homeostasis as following. On the one hand, cells from original tumor enter circulation to form CTCs. In this stage, CTC increase depends on the size and the properties of original tumor and the micro-environment of host tissue. On the other hand, CTCs depletion might be caused by apoptosis, immune system killing or metastasizing to target organs. Furthermore, in solid tumor cancer, CTCs could not stay long in circulation. Therefore, CTCs just reflect the combined result of these two processes—entrance and depletion. Further studies are needed to test our hypothesis. Research works for understanding molecular mechanism of cancer metastases and therapies by our methodology are promising to improve clinical treatment and are undergoing.

In conclusion, we have determined that our IVFC has higher sensitivity than the currently
used whole blood analysis by conventional flow cytometry. Moreover, this study shows the
significant difference of hematogenous metastasis between an orthotopic model and an s.c. model,
and reveals the dynamics of CTC dependent metastasis under clinically relevant oncology
conditions. In addition, we have used CTC dynamics to demonstrate the significant effectiveness
of surgical resection on cancer hematogenous metastasis, which is important to guide clinical
therapies.

Acknowledgments

We thank Axel Mosig and Chaofeng Wang for assistance in the software development. We
are also grateful for the use of facilities at Institutes of Biomedical Sciences, Fudan University.

Grant Support

This work is supported by the National Basic Research Program of China (973 Program,
2011CB910400 and 2012CB966800; X.B. Wei), China National Key Sci-Tech Special Project
(2008ZX10002-025; J. Zhou), China National Natural Science Foundation (30901432, 30972949
and 20975027; X.B. Wei and J. Zhou), the Chinese Ministry of Education (109056; X.B. Wei),
Program for New Century Excellent Talents in University Award (NCET-08-0131; X.B. Wei), the
Fudan University 2010 Excellent Ph.D. Research Foundation (Z.C. Fan and J. Yan) and the
Natural Science Foundation of Fujian Province (2010J01136; J Yan).

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Figure legends:

Figure 1. CTC measurement by IVFC and pathology validation. A, The trans-illumination image of microcirculation in mouse ear was acquired by a real-time charge coupled device (CCD) camera behind a 40× objective (NA=0.6). A 520 nm-550 nm light emitting diode (LED) was used to visualize the vessels. A laser slit in the IVFC covered across an artery in the mouse ear. The fluorescence signals from the excited cells were recorded. B, Visualization of digitized IVFC signals of HCCLM3-GFP cells using software developed on MATLAB platform. A 10-min recorded data at week 6 after tumor implantation was shown (inset: a single cell trace). C, Visualization of a piece of blank control data. The animals in blank control group (n≥6) did not undergo tumor implantation or injection. D, The H&E staining of GFP+ CTCs isolated by FACS. The nuclei of cells were deeply stained. The nuclei of these GFP+ cells were larger than those of normal cells, thus were confirmed as tumor cells. E, CTC counts comparison of our IVFC analysis vs. whole blood test by conventional flow cytometry. The red, blue and green spots represented the data at week 4, 6 and 8 after tumor implantation, respectively. The data acquired by the two methods had linear relationship. The scale bars of both B and E were 50 μm.

Figure 2. The comparison of CTC dynamics and tumor growth between an s.c. and an orthotopic metastatic tumor model. A, CTC counts of the s.c. and the orthotopic metastatic tumor model measured by IVFC during eight weeks after tumor implantation. The measurement was performed once a week and last at least 1 h each time. B, A representative image of orthotopic tumor at week 4 acquired by ultrasound method. The tumor possesses a long diameter of 10.81 mm and a short diameter of 9.97 mm. C, Tumor growth in the s.c. and the orthotopic metastatic tumor model.
Ultrasound imaging was used to determine the diameters of tumors. The volume (size) was calculated using standard clinical method. The mean values and standard deviations (SD) were presented ($n \geq 6$) for both A and C.

**Figure 3.** The different micro-environments and tumor properties between s.c. and orthotopic tumor. A, s.c. tumor possesses a large amount of cell death, a clear boundary and a connective tissue envelope (white arrow). Rarely present vessels (black arrows) indicate poor blood supply condition in the tumor. B, Orthotopic tumor (deeply stained) immerged in normal liver tissues (light stained). Surrounding the vessel (black arrow), liver tissue forms the blood sinus structure. Thus, the tumor has good blood supply condition. The cell death is rarely observed. In addition, the boundary is not clear with some invasive metastases (white arrows) into normal liver tissue. The scale bar is 100 μm.

**Figure 4.** The surgical treatment depleted CTCs, but might increase metastatic potential of the remained tumor in our orthotopic metastatic tumor model. A, CTC dynamics after tumor implantation in the resection and the control group. Orthotopic tumor resection was performed at week 4 after the IVFC measurement of CTCs. The resection caused a significant decrease in CTC counts. B, The tumor growth in the resection and the control group. The volumes of tumors were determined described above. C, CTC counts per ml in peripheral blood samples measured by conventional flow cytometry, which is similar to IVFC results. The mean values and SD were presented ($n \geq 6$) for A, B and C.
Figure 5. The effects of liver resection on lung metastases. A, The progress of metastasis in the whole lung was assessed by fluorescent images of GFP⁺ metastases acquired by in vivo small animal optical imaging system. The color ruler indicated the photon number per pixel (from 1,000 to 13,000). B, The progress of metastasis in the lung was assessed by fluorescent images of GFP⁺ metastases acquired by confocal fluorescence microscope, which showed the details of micro-metastases. The scale bar was 250 μm. C, The number of early metastases (diameter <100 μm) in the whole lung for both groups at week 4, 6, 8 after tumor implantation, respectively. D, The number of advanced metastases (diameter >100 μm) in the whole lung for both groups at week 4, 6, 8 after tumor implantation, respectively. The number in C and D was counted from the images acquired by confocal fluorescence microscope. The mean values and SD were presented (n≥6) for both C and D.
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Cancer Res  Published OnlineFirst March 26, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-3733

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