Journal of Depression and Anxiety
ISSN: 2167-1044

Chen et al., J Depress Anxiety 2017, S11
DOI: 10.4172/2167-1044.S11-004

Research Article Open Access

Anti-Cancer Drug Assay for Micro-Ellipse Filters after Sensitively Capturing Circulating Tumor Cells

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Abstract

Circulating tumor cells (CTCs) carrying significant information from primary tumor rendering precise enumeration valuable. Extremely rare number detected in the patient sample belongs to early stage of cancer. Detection, characterization and further molecular analysis determine prognosis and diagnosis. In this work, we performed Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) to precisely enumerate number of tumor cells captured on the highly-sensitive gradual narrowed micro-ellipse filters. Clinical assay was performed on Ellipse filters to assess further validity in non-small-cell-lung cancer (NSCLC) patient blood with CTCs detected positive. Anti-drug cancer assays were performed with micro-ellipse filters after cells captured on the chip treated with baicalein (BAE) of different concentrations. Highly-sensitive micro-ellipse filters validate efficiency of clinical enumeration of metastatic patient samples, anti-cancer drug responses after improvement of co-culture design and mutational biological molecular analysis.

Keywords: Circulating Tumor Cells (CTCs); Epithelial Cell Adhension Molecule (EpCAM); Epithelial-to-Mesenchymal Transition (EMT)

Introduction

Circulating tumor cells (CTCs) are tumor cells shed from the primary tumor, circulating in the blood system and lymphatic circulating system [1]. Those tumor cells carry significant information of primary tumor. Thus, they have significance for molecular genetic analysis and followed diagnosis. When they found supportive environment at another distant site, they would grow as regeneration tumor. This procedure is called metastasis. 90% cancer patients are dead for metastasis [2,3]. Before generation of second tumor, this stage could be called early stage of cancer. When we detect CTCs in the patient blood, the patient has cancer and possible metastasis, but not definitely for metastasis. Early stage of cancer has significance for diagnosis of patients. Doctors still could come out timely therapy. However, there is only 1-100 CTCs in 7.5 ml patient blood and millions of White Blood Cells (WBCs) and billions of Red Blood Cell (RBCs) in 1 ml whole blood [4]. Robust and reproducible enumeration is critical significant to reflect real number of CTCs in patient blood. The rare number of CTCs could be detected indicates both doctors and patients have more time to fight with the cancer.

Molecular analysis such as genetic mutational analysis could be carried out on the microfluidic chip with Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) after CTCs precisely captured on the microellipse filters. Many filters with high purity have been expored, microfluidic funnel rachets [5], microfluidic rachets [6] and a multi-obstacle architecture (MOA) filter [7]. Microposts are in the shape of square [8], rectangular [9] or weir-style [10]. Gap spacing are usually 8 μm [11], 5 μm [12] or 4 μm [13]. Viable CTCs benefit to perform anti-cancer drug assays on chip. Thus, circular spiral structure microposts are suitable to be taken to reduce any shear stress force, friction and puncture [12]. In order to avoid residue of Red Blood Cells (RBC), Red blood cells lysis (RBCL) could be considered to be replaced by a preprocessing circular spiral microfluidic chip with high throughput. Simultaneously, genetic mutational analysis, biological analysis and anti-cancer drug assays are keys to diagnosis of patients. In this work, we performed Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) to precisely enumerate number of tumor cells captured on the highly-sensitive micro-ellipse filters [13]. Clinical assay was performed on Ellipse filters to assess. Anti-drug cancer assays were carried out with micro-ellipse filters after MCF-7 cells captured on the chip.

Materials and Methods

Fabrication

The size of the microfluidic chip is less than a glass microscope. The structure of the microfluidic chip is created using lithography technology. Patterns of the microstructure are drawn to produce a high resolution transparency optical photomasks. Inverse versions of the microstructures are fabricated on a silicon wafer through the following. The silicon wafer is spin-coating with a 7 μm thick AR-N 4450-10 (ALLRESIST GmbH, Germany), soft baking, UV light exposure and then post exposure baking. After developing, a silicon master pattern with the micro-structure was generated. Through casting a liquid Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) in a monomer to curing agent ratio of 10:1, against the master and baking in an oven for 40 min at 80°C, PDMS structure was fabricated with inlet and outlet (1.0 mm) punched. Treated with a High Frequency Generator (Electro-Technic Products, Inc, Chicago, IL), PDMS structure was bonded to a glass slide after thoroughly ultrasonic cleaning.

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Received April 24, 2017; **Accepted** May 08, 2017; **Published** May 11, 2017

Citation: Chen H, Cao B, Chen H, Yang H, Liu P, et al. (2017) Anti-Cancer Drug Assay for Micro-Ellipse Filters after Sensitively Capturing Circulating Tumor Cells. J Depress Anxiety S11: 004. doi:10.4172/2167-1044.S11-004

Cell cultures

HeLa cells were offered by Suzhou Institute of Systems Medicine and HepG2 cells (hepatocellular carcinoma) were provided by Tianjin Medical University Cancer Institute and Hospital. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, USA) medium supplemented with 10% fetal bovine serum (FBS) (GIBCO, USA) and 1% penicillin-streptomycin (Ying Reliable biotechnology, China) and incubated in a humidified atmosphere at 37°C with 5% CO_2 atmosphere. When cell lines were grown as adherent monolayers to 95% confluence, they were detached from the culture dishes with 0.25% Trypsin solution for two minutes.

Staining

For cells captured on the chip, Hoechst (Life, USA), Cytokeratin-FITC (BD Biosciences) and CD45-PE (BD Biosciences) were used for on-chip staining. Clinical patient sample were processed through the chip followed by washing with PBS, fixing and permeabilization. Anti-Cytokeratin and anti-CD45 and Hoechst applying in 1% bovine serum albumin were utilized for all samples.

Instrument setup

A syringe pump was used to load cell suspension into the wave chip. The syringe attached to the pump was connected to the inlet of the wave chip through plastic tubing. The system operated under a flow rate of 1ml hr^{-1} for 1 hour. An inverted phase contrast fluorescence microscope (Nikon, Japan) equipped with a high speed CCD camera (Nikon, Japan) was used to monitor the chip and to visually count the number of CTCs captured on the biochip.

Cell re-culture and clinical sample

Metastatic patient sample of non-small cell lung cancer patient blood was offered by Longhua Hospital Affiliated to Shanghai Medial University. HeLa cells propagate against micropost side wall freely. Treated with baicalein (BAE) of different concentration, cell apoptosis appear and is ready for microscopic imaging. For distinct time, after staining and washing, Live/Dead assay applied.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

RNA from flushing the microfluidic chip with ellipse microstructures was extracted using the TRIZOL RNA Isolation Protocol (Invitrogen). RNA was used for cDNA synthesized using the Reverse Transcriptase M-MLV (Takara) with oligo dT primer. The resultant cDNA were diluted 20X. Real-time PCR was carried out in a 20 µL reaction mixture containing 1X SYBR Green, Universal PCR Master Mix (Life Technologies), 500 nM of each Forward and Reverse primers, and 2 µL of DNA template. Primes sequences that were used for qPCR are AFP homo Forward Primer, TCCATAAGGATCTGTGCCAA, and AFP homo Reverse primer, CTGCAATGACAGCCTCAAGT. Thermal cycling was performed on an ABI 7500 (Life Technologies) system with the following conditions, 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 1 min.

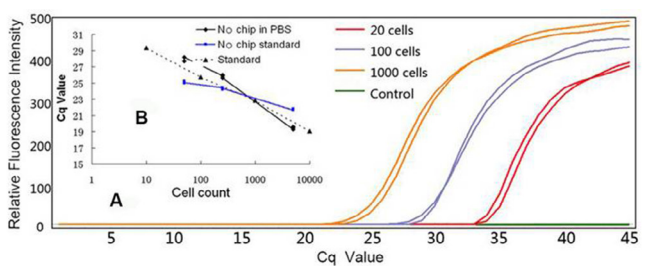

Figure 1: qRT-PCR experiments were performed to detect liver biomarker AFP starting from 0 (N=3), 20 (N=2), 100 (N=2), 1000 (N=2) HepG2 cells, after spiked in PBS and captured inside the microfluidic chip with a micro-Ellipse construction. (A) qRT-PCR curves (Cq) obtained for different HepG2 cell counts and negative control. (B) (inset). Cq values obtained for standard curve (HepG2 cells not flown through the chip), HepG2 cells spiked in PBS.

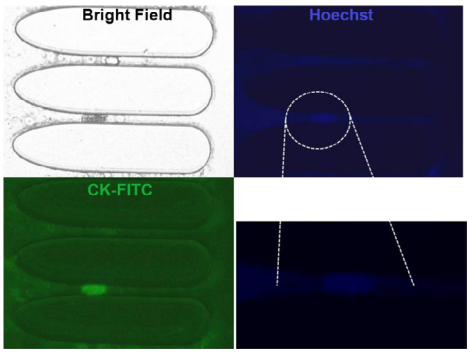

Figure 2: Clinical performance of micro-ellipse filters (Bright Field, Hoechst and CK-FITC).

Results and Discussion

In-situ analysis and biomarker AFP detection

CTCs enumeration is mainly clinical usage to predict prognosis and monitor therapy effect. However, clinical treatment decisions may be more effective if they are based on molecular characteristics of CTCs. Therefore, identifying presence or absence of CTCs and specific molecular characterization are an extremely important diagnostic procedure. Utilizing qRT-PCR approach results in an assay that is both highly sensitive and specific.

HePG2 cells were spiked in PBS and captured inside the chip. Amplification curves and standard curves of different samples are shown in Figure 1. As few as 20 HePG2 can be detected of AFP level with Cq 33 ± 0.5 after micro-Ellipse matrix construction capturing. Cq values of 33 ± 0.5, 29.8 ± 0.2, 25 ± 0.2 corresponds to 20, 100, 1000 HepG2 cells spiked into the chip, respectively. In this work, we explored AFP detection by Syber Green I method which is the most widespread implementation. Recent report shows using more sensitive Taqman probe could identify single mutation in CTC DNA sample [14].

In addition, the ability to examine multiple genes at once from a very small initial sample volume could be offered by this technique, which has significant advantage. For future task, qRT-PCR could also be used to examine a multiple of hepatocellular carcinoma prognostic markers including, but not limited to, GPC3, IGFII, ALF and albumin.

Clinical performance of micro-ellipse filters

For clinically evaluating micro-ellipse filters, a non-small cell lung cancer patient blood is utilized to perform the assay. CTCs were tested positive for the 2-3 ml blood samples with the Ellipse filter. A CTC detected is illustrated in Figure 2, CTC is Hoechst+/CK+ characterized with blue and green fluorescence.

Anti-cancer drug assay of baicalein (BAE)

Anti-cancer drug assay of baicalein (BAE) were performed on captured tumor cells after propagating freely on ellipse filters. After

captured on Ellipse filters and cultured 1-2 days, tumors cells appear obvious apoptosis. Figure 3 showed that morphological changes become obviously after 12 h. Within 2-3 days, many tumor cells show apoptosis phenonmenon. From this assay and above, it could be seen that Ellipse filters are suitable to perform anti-cancer assay after tumor cells captured. This has some significance for doctors to come out some treatment especially after redesigning this chip with another 2-3 different cells co culture layer added.

Treated with baicalein (BAE) with distinct concentrations of 10 μg/ml and 50 μg/ml for certain time, we observe obvious apoptosis of tumor cells. This phenomenon indicates that baicalein (BAE) could inhibit growth of tumor cells. Due to different number of cells captured on these two Ellipse filters, apostasies would last differently.

Live/dead assay for tumor cells treated with baicalein (BAE) after captured on ellipse filters

Anti-cancer drug assays of baicalein (BAE) with concentration of 10 μg/ml and 50 μg/ml were carried out, respectively. After captured on Ellipse filters and cultured 1-2 days, tumors cells appear obvious apoptosis. At 36 h treated with BAE of 10 μg/ml and 48 h of 50 μg/ml, respectively, Live/Dead assay was performed on the chip (Figure 4). Apoptosis rate for 10 μg/ml was 42.2% and 48.8% for 50 μg/ml, respectively, for a specific chosen region around arrays.

Discussion

Gradual Micro-ellipse filters are effective in precise enumeration of tumor cells and CTCs particularly shown in qRT-PCR, which determines this filters could be applied to clinical usage. After RBCL and for simple tests of normal checking, residues of RBCs would produce interference disturbing distinguishing. Since RBCs without nucleus, Purity appeared false impression and seemed to be poor. To enhance purity and get rid of disturbance, an extra layer of micropores could be built on the top to perform advance filters. To release captured CTCs, another micropore filter could be replaced underneath. This micropore filter could accurately orient right position of captured CTCs. With a single-fine needle, captured CTCs could be extracted to perform next biological genetic analysis. To further develop co culture assay, reversely release trapped CTCs to following co culture layer, 2-3 cells propagate freely in this layer and anti-cancer assay could be performed.

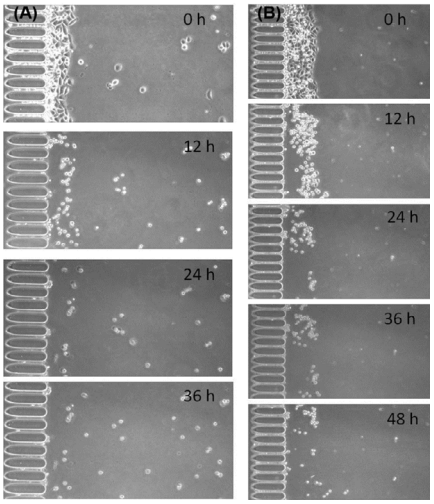

Figure 3: Anti-cancer drug assay of BAE with concentration of (A) 10 μg/ml and (B) 50 μg/ml, respectively.

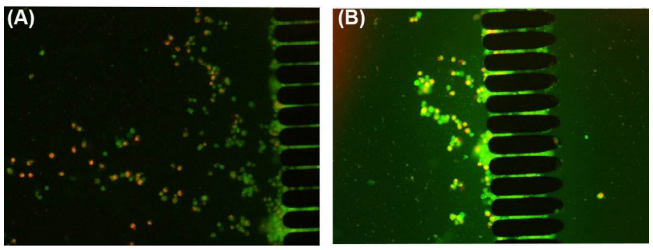

Figure 4: Dive/dead assay for MCF-7 cells treated with BAE with different concentrations after captured on micro-ellipse filters (A) 10 μg/ml and (B) 50 μg/ml, respectively.

Strength and Limitations

Capture efficiency for micro-ellipse filters is robust and reproducible from qRT-PCR and the clinical experiment fully proving micro-ellipse filters is suitable for clinical tests. The limitation lies in simple structure could not avoid residues of RBCs although RBCs without nucleus have no effect on distinguishing. This makes visual field faint, not clear especially in the front region. To refrain from disturbance, improvements such as micropores microfilters could be displaced on the top and below to form a three layer microfluidic chip conveniently for RBCs residues depletion and extraction of captured CTCs. The bottom micropore microfilters also could be replaced by a 2-3 cells co culture chip to carry on-chip anti-cancer drug assays.

Conclusion

We have demonstrated validity of Ellipse filter for sensitively capturing tumor cells with RT-PCR and CTCs for a clinical sample. Anti-cancer drug assays were performed with BAE of different concentrations for certain time. Obviously, tumor cells could propagate freely after captured on Ellipse filters and tumor cells appeared to be apoptosis after treated with BAE indicating BAE could inhibit growth of tumor cells. We could anticipate Ellipse filter as a clinical enumeration and anti-cancer therapy tools after improvement as our next step.

Acknowledgements

This research work was supported by the Major State Basic Research Development Program of China (973 Program) (Grant No. 2011CB933102), National Natural Science Foundation of China (Key Program, Grant No. 61335010), Key Research Program of Chinese Academy of Sciences (Grant No. KJZD-EW-TZ-L03-6) and Postdoctoral Research Funding (Grant No.2014M550794). We deeply appreciate Prof. Yu-Sheng Lin from Sun Yat-Sen University helped with editing and revising the manuscript. Prof. Zhang Ning from Medical University of Tianjin, Prof. Siwang Yu from Peking University, Prof. Fan from Florida University, Prof. Chen from École Normale Supérieure (ENS) and Prof. Richard Stimets from University of Massachusetts Lowell offered deep discussion. Assistant Prof. Biyi Xu from Nanjing University contributed revising and deep discussion. Zhaoxin Geng, Graduate student Xiaoqing Lv, Wenting Liu from Chinese Academy of Sciences helped with fabricating the chips and culture cells. Barbara Costello offered help for revising the manuscript.

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