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Research Paper

Prognostic Impact of Circulating Tumor Cell Detected Using a Novel Fluidic Cell Microarray Chip System in Patients with Breast Cancer

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ABSTRACT

Various types of circulating tumor cell (CTC) detection systems have recently been developed that show a high CTC detection rate. However, it is a big challenge to find a system that can provide better prognostic value than CellSearch in head-to-head comparison. We have developed a novel semi-automated CTC enumeration system (fluidic cell microarray chip system, FCMC) that captures CTC independently of tumor-specific markers or physical properties. Here, we compared the CTC detection sensitivity and the prognostic value of FCMC with CellSearch in breast cancer patients. FCMC was validated in preclinical studies using spike-in samples and in blood samples from 20 healthy donors and 22 breast cancer patients in this study. Using spike-in samples, a statistically higher detection rate ($p = 0.010$) of MDA-MB-231 cells and an equivalent detection rate ($p = 0.497$) of MCF-7 cells were obtained with FCMC in comparison with CellSearch. The number of CTC detected in samples from patients that was above a threshold value as determined from healthy donors was evaluated. The CTC number detected using FCMC was significantly higher than that using CellSearch ($p = 0.00037$). CTC numbers obtained using either FCMC or CellSearch had prognostic value, as assessed by progression free survival. The hazard ratio between CTC+ and CTC− was 4.229 in CellSearch (95% CI, 1.31 to 13.66; $p = 0.01591$); in contrast, it was 11.31 in FCMC (95% CI, 2.245 to 57.0; $p = 0.000244$). CTC detected using FCMC, like the CTC detected using CellSearch, have the potential to be a strong prognostic factor for cancer patients.

1. Introduction

Circulating tumor cells (CTC) are cancer cells that are present in the blood stream among 5×10^6/mL of leukocytes and 5×10^9/mL of red blood cells (Allard et al., 2004). CTC are considered to be an important clue for estimation of the possibility of metastasis formation (Fidler, 2003) and are expected to be a prognostic marker of cancer patients (Cristofanilli et al., 2005). Therefore numerous technologies for analysis of CTC have been developed in the past decade (Joosse et al., 2014; Haber and Velculescu, 2014; Ignatiadis et al., 2015; Ferreira et al., 2016). One such technology, the CellSearch system, has been used in a number of prospective clinical trials and is the only CTC detection system approved by the FDA. These clinical trials indicated that the number of CTCs detected using CellSearch had prognostic value in patients with breast, colon, prostate, non-small cell lung, small cell lung and gastric cancer (Cristofanilli et al., 2004; Cohen et al., 2008; de Bono et al., 2008; Krebs et al., 2011; Naito et al., 2012; Matsusaka et al., 2010). In patients with breast cancer in particular, CellSearch detection of just one CTC in the early stage had prognostic value (Lucci et al., 2012). Thus, CellSearch is thought of as a firmly established system that can indicate strong prognostic value in breast cancer.

Abbreviations: CTC, circulating tumor cell; FCMC, fluidic cell microarray chip; CM, cell microarray; NCCH, National Cancer Center Hospital; CICK, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital; CK, cytokeratin; PFS, progression free survival; PR, partial response; SD, stable disease; PD, disease progression; CT, chemotherapy; HT, hormonotherapy; DGC, density gradient centrifugation; EMT, epithelial mesenchymal-transition.

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However, most of the methods for CTC enumeration, including the CellSearch system, can potentially lose CTCs, which might affect the sensitivity of CTC detection. Because of the low abundance of CTC in blood, almost all methods of CTC detection require enrichment of CTC from blood cells using label-dependent or physical property-based selection (Joosse et al., 2014). These enrichment processes may possibly reduce CTC detection sensitivity. Therefore, an enrichment process with minimal CTC loss that is independent of protein expression or a physical property is needed.

To overcome such problems, we previously developed the cell microarray chip (CM chip) that enables high sensitivity detection of rare cells in blood such as malaria-infected erythrocytes or spiked-in cancer cells (Yatsushiro et al., 2010; Yamamura et al., 2012). The CM chip enables rare cell detection independent of cell surface protein expression with few enrichment steps. In order to increase the detection sensitivity and robustness of the CM chip, we developed a fluidic cell microarray chip (FCMC) device and a semi-automated FCMC system based on the CM chip, which aimed to eliminate the possibilities of target cell loss.

In this article, we show the performance of this FCMC system in preclinical studies and the results of head-to-head comparisons of the CTC detection rate of the FCMC system with that of the CellSearch system in patients with breast cancer. Importantly, we also compare the prognostic impact of the FCMC system with the CellSearch system in this study.

2. Materials and Methods

2.1. Study Participants

All patients and healthy donors in the present studies below provided informed consent and their participation in the studies was approved by the institutional review committee of Konica Minolta, Inc., the National Cancer Center Hospital (NCCH; Tokyo, Japan) and the Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital (CICK; Tokyo, Japan). Patients who were pathologically diagnosed with breast cancer, and healthy donors who did not have any cancer history were recruited. Patients who had double cancers or who had any prior cancer history were not eligible for the present studies. This work was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Fluidic Cell-Microarray-Chip Device (FCMC Device)

We developed the FCMC device based on the CM chip as shown in Fig. 1-A. About 18,000 microchambers were contained in the redesigned CM chip; each microchamber was 120 μm in diameter at the top, 90 μm in diameter at the bottom, and 50 μm deep (Fig. 1-B). The proximal and distal distance of microchambers from each other was 200 μm and 300 μm, respectively (Fig. 1-C). We coated BSA on the chip surface on the outside of the microchambers after UV-ozone exposure. A BSA coating prevents nonspecific adsorption of cells onto the surface. This coating supported the movement of untrapped cells into the microchambers. We formed a flow channel by bonding a cover plate to the new CM chip using black double-sided adhesion film. The bonding process formed a flow channel (15 mm wide, 50 mm long and 100 μm deep (Fig. 1-B, D). Each microchamber can hold approximately 50 cells inside as a tight monolayer in a state in which the fluorescent intensity of every cell can be easily analyzed (Fig. 1-E, F). Therefore, one FCMC device can analyze up to 9×10^5 cells. The use of multiple FCMC devices enabled CTC enumeration in this study.

2.3. Blood Processing for the FCMC System

Peripheral blood samples (2 mL, anticoagulated with EDTA) for CTC analysis were collected after withdrawal of the first several milliliters of blood for clinical use to avoid potential skin cell contamination from the venipuncture. Two milliliters of blood sample were processed using Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK). Precipitated cells were fixed with 4% formaldehyde, and were then suspended into 340 μL PBS (Supplementary Fig. S1). The workflow of sample processing is shown in Fig. 2-A. Based on preliminary experiments (Supplementary Fig. S2), the samples were processed within three hours after blood collection, and were then analyzed with the FCMC device within three days.

2.4. Formation of Cell Monolayers in Microchambers Using the FCMC Device

The FCMC device was filled with PBS before loading the cell suspension. Cells were trapped in the microchambers as a monolayer by the following steps (Fig. 2-B and Supplementary Fig. S3). One fifth of the cell suspension (68 μL) was loaded from the reservoir tank into the flow channel by suction. The cells had completely settled down on the chip surface within 1 min. At this time, many excess cells remained on the chip surface. We subsequently applied two automated suction methods to improve cell capture efficiency, termed “Suction for Trapping” and “Suction for Monolayer”. “Suction for Trapping” involves 10 cycles of a brief suction (suction rate, 0.1 mL/min; total volume of suction, 0.3 μL) and incubation (10 s). This step moves untrapped cells gradually towards downstream microchambers. “Suction for Monolayer” is a long suction (suction rate, 0.1 mL/min; total volume of suction, 41 μL) followed by an incubation (10 s). This step moves overlapped cells out of microchambers. Cells that are present as monolayers at the bottom of the microchambers can maintain their position during the time of “Suction for Monolayer” because the height of the cell monolayer at the bottom of the microchamber (10 μm) means that these cells are little affected by suction flow as the in-silico simulation shows (Fig. 3-A). After ten repetitions of “Suction for Trapping” and “Suction for Monolayer”, all cells are completely trapped as a monolayer (Fig. 3-B, C).

2.5. Immunostaining for Discriminating CTCs From Leukocytes

The first immunostaining solution, which included 1:2 diluted anti-cytokeratin (CK) mAb CAM5.2 (Becton, Dickinson and Company, San Jose, CA) and 1:50 diluted anti-human CD45 mAb HI30 (BioLegend, San Diego, CA) in PBS solution containing 1% Tween 20 (Calbiochem, San Diego, CA) and 3% BSA (Thermo Fisher Scientific, Lafayette, CO), was loaded onto the FCMC device. After incubation for 30 min at room temperature and washing with PBS, the second immunostaining solution was then loaded. The second immunostaining solution included 1:500 diluted Alexa Fluor 488 Goat Anti-Mouse IgG1 (γ1) (Life Technologies, Grand Island, NY), 1:500 diluted Alexa Fluor 647 Goat Anti-Mouse IgG2a (γ2a) (Life technologies) and 1:1000 diluted Hoechst 33342 10 mg/mL solution (Life technologies) in PBS solution containing 1% Tween 20 and 3% BSA. After incubation for 30 min at room temperature, unbound mAbs were washed out with PBS.

2.6. Detection of CTCs in Fluorescent Microscopic Images

Immunofluorescent stained cells in the FCMC system were analyzed using an Axio Imager M2 fluorescence microscope equipped with standard filter sets (49, 38HE, 50 for Hoechst 33342, Alexa488, and Alexa 647 respectively), a monochrome CCD camera, (AxioCam MRm), a 5× objective (EC Plan-NEO FLUAR) and ZEN2012 blue edition software (Carl Zeiss, Jena, Germany). Approximately 400 views were captured to cover the entire area of the microchamber. The fluorescence images were analyzed using in-house software that identifies candidate CTCs. First, the software identified cells that were CK^+ and Hoechst 33342^+. If these cells displayed a strong CD45 signal, the software excluded them as CTC candidates. Next, multiple skilled inspectors examined the list of candidates and manually excluded $CD45^{weak}$ cells from the list of CTC candidates. Each candidate was shown with its

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