Determination of Circulating Tumor Cells in Peripheral Blood By Flow Cytometry

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Objective: Circulating tumor cells (CTCs) have been interest of subject in the past few decades in terms of prognosis and response to the therapy in several cancers and have potential as a "liquid biopsy" in the diagnosis and treatment of cancer. The aim of our study was to improve a convenient method for the strategies of CTC enrichment and detection.

Methods: In this modified method, 7.5 mL peripheral blood samples were firstly collected and peripheral blood mononuclear cells (PBMCs) were isolated from the fresh blood samples by ficoll gradient separation. Next, the leukocytes in PBMCs were removed by magnetic microbeads conjugated with CD45 for a negative selection. Finally, the retained cells were labeled with anti-epithelial cell adhesion molecule (anti-EpCAM), anti-cytokeratins (anti-CKs) and the leukocyte-specific marker as anti-CD45. All samples were analyzed by a flow cytometry.

Results: CTCs were detected in blood samples of cancer patients such as breast cancer, lung cancer, colorectal cancer, bladder cancer, and laryngeal cancer using with our modified convenient method for the strategies of CTC enrichment and detection. For instance, our results showed that CTCs were not detected in the volunteer sample while 9 CTCs were detected with the use of EpCAM-based gating strategy but the number of detected CTCs was 7 when gated on CK14,15,16,19 in the breast cancer sample.

Conclusion: CTCs can be detected with our modified method including highly sensitive assays that provide us monitoring the process of cancer treatment and knowing some important information about cancer prognosis.

Keywords: Cancer, circulating tumor cells (CTCs), flow cytometry

Introduction

The clinical importance of circulating tumor cells (CTC) as a new target for early cancer detection, diagnosis, prognosis and prediction has been widely investigated in recent years (1). The analysis of CTC presents the opportunity for a minimally invasive ‘liquid biopsy’ that provides the evaluation of disease response and targeting therapy for patients (2). Thus, CTCs can be used for determining metastases and prognosis of cancer patients. CTCs in the peripheral blood of cancer patients are promising research area in the field of biomarker development and novel treatment for targeting in today’s cancer research (3). CTCs are only found 1 in 10^5-10^8 numbers of leukocytes in the peripheral blood of cancer patients (4). For this reason, highly sensitive assays with techniques to enrich and detect for CTCs are required (2).

In general, four approaches are mainly used for the strategies of CTC enrichment that are size-based, density-based, immunomagnetic separation and microfluidic-based. For the detection of CTCs, two approaches include nucleic acid-based techniques and/or protein-based techniques (3, 4) (Table 1, Figure 1).

The aim of our study was to improve a convenient method for the strategies of CTC enrichment and detection. We preferred to use a density-based (ficoll) and an immunomagnetic separation (CD45 negative selection) for the enrichment and detection of CTCs. CTCs were analyzed by a flow cytometry.

Materials and Methods

Material
- Phosphate buffered saline (PBS) (Gibco, Life Tech, USA)
- Biocoll Separation Solution (Ficoll) (Merck Millipore, Darmstadt, Germany)
- EDTA disodium salt dihydrate (Genaxxon, Ulm, Germany)
- Bovine Serum Albumin (BSA) (Albumin fraction V, Biovest, Nuaille, France)
- CD45 Microbeads (Miltenyi Biotec, Bergisch-
Table 1. The strategies of CTC enrichment and detection.

<table>
<thead>
<tr>
<th>Approaches</th>
<th>Explanation</th>
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<tbody>
<tr>
<td><strong>Enrichment strategies</strong></td>
<td></td>
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<tr>
<td>Size-based</td>
<td>A filtration device with specific sized pores</td>
</tr>
<tr>
<td>Density-based</td>
<td>Ficoll gradient separation</td>
</tr>
<tr>
<td>Immunomagnetic separation</td>
<td>Iron-conjugated antibodies targeted toward CTCs (e.g., EpCAM; positive selection) or contaminating blood cells (e.g., CD45; negative selection) and incubation in a magnetic field.</td>
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<tr>
<td><strong>Detection strategies</strong></td>
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<tr>
<td>Microfluidic-based</td>
<td>CTC Chip and iChip</td>
</tr>
<tr>
<td>Protein-based</td>
<td>Immunofluorescence or flow cytometry (using antibody-mediated detection)</td>
</tr>
<tr>
<td>Nucleic acid-based</td>
<td>RT-PCR or RT-qPCR</td>
</tr>
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Gladbach, Germany)
- MACs Starting Kit (MSK) (LD column, magnet, separator) (Miltenyi Biotec, Bergisch-Gladbach, Germany)
- Cellwash (BD Biosciences, San Jose, California)
- Cytofix Fixation Buffer (CFB) (BD Biosciences, San Jose, California)
- Mouse Anti-Human CD45-APC-H7 (BD Biosciences, San Jose, California)
- Anti-EpCAM-PE (BD Biosciences, San Jose, California)
- Flow Cytometry Staining Buffer (with Fetal Bovine Serum (FBS)) (FCSB) (BD Biosciences, San Jose, California)
- Perm/wash Buffer (PWB) (BD Biosciences, San Jose, California)
- Permbuffer III (PIII) (BD Biosciences, San Jose, California)
- Mouse Anti-Human Cytokeratin 14-15-16-19-Alexa 647 (BD Biosciences, San Jose, California)
- Anti-Cytokeratin-FITC (BD Biosciences, San Jose, California)

Method
The peripheral blood samples (7.5 mL) from cancer patients and healthy volunteers were collected in EDTA-coated tubes and they were stored at room temperature.

The Strategy of CTC enrichment

a. Density gradient separation
This step consists of the isolation of mononuclear cells by ficoll gradient centrifugation.
1. Peripheral blood sample (7.5 mL) is diluted to a final volume of 22.5 mL with PBS.
2. This suspension is carefully layered onto 22.5 mL ficoll in a 50 mL conical tube.
3. Centrifuge at 300xg for 25 minutes at 20°C.
4. After centrifugation, PBMCs layer is removed carefully by us-

![Figure 1. The enrichment and detection of CTC (4).](image-url)
ing a 3 mL pasteur pipette in a new 50 mL conical tube. This layer contains lymphocytes, monocytes and thrombocytes.

5. Cells are washed in 10 mL PBS and centrifuge at 300xg for 10 minutes at 20°C.

6. The supernatant is completely removed and the pellet is broken by flicking the bottom of the tube.

7. Repeat the step 5 and 6.

8. 10 mL PBS is added.

9. 10 µL cell suspension is counted with thoma chamber (Isoleab).

b. Magnetic separation

This step includes the CD45 negative selection by using a magnetic separation.

1. Centrifuge at 300xg for 10 minutes at 20°C. Then, the supernatant is removed.

2. Prepare MACs Buffer containing PBS with 0.5% BSA and 2 mM EDTA disodium salt dihydrate (pH 7.2) and keep it cold (4-8°C).

3. Add 80 µL MACs buffer per 10^7 total cells.

4. Add 20 µL CD45 Microbeads per 10^7 total cells and mix well.

5. Incubate the sample for 15 minutes at 4-8°C.

6. Wash cells by adding 1 mL of MACs buffer per 10^7 cells.

7. Centrifuge at 300xg for 10 minutes at 4-8°C. Remove the supernatant completely.

8. For the depletion step using with LD columns, resuspend the cell pellet in 500 µL of buffer up to 1.25x10^8 cells.

9. Place LD column in the magnetic field of a suitable MACs separator.

10. Prepare the column by rinsing with 2 mL of MACs buffer and collect the total effluent into 15 mL conical tube.

11. A new conical tube is placed under the LD column and apply cell suspension onto the column.
12. Collect unlabeled cells passing through and wash the column with 1 mL of MACs buffer twice. Collect the total effluent containing CTCs.

13. 10 µL unlabeled cell fraction is counted with a thoma chamber.

The Strategy of CTC Detection

The unlabelled cell fractions are separated as a negative control tube and a test tube. After the magnetic separation step, transfer 50 µL unlabelled cells suspension into a negative control tube for defining a gating strategy and transfer the rest of unlabelled cell fractions into a test tube.

The Negative Control Tube

1. Add 100 µL Cell wash into the negative control tube.
2. Centrifuge at 350xg for 5 minutes at 4°C and remove the supernatant.
3. Add 500 µL Cellfix and incubate in the dark for overnight at 4°C.
4. Centrifuge at 350xg for 5 minutes at 4°C and remove the supernatant.

5. Add 500 µL Flow Cytometry Staining Buffer and vortex it gently.
6. Incubate in the dark at 4°C until it is analyzed by flow cytometry.

The Test Tube

1. Centrifuge the test tube at 350xg for 5 minutes at 4°C and remove the supernatant.
2. Add 100 µL Cellwash per 10⁶ cells.
3. Add a fluorochrome-labeled antibody for the detection of surface antigens (e.g. EpcAM, CD45) and incubate in the dark for 30 minutes at 4°C.
4. After the incubation, add 2 mL Flow Cytometry Staining Buffer.
5. Centrifuge at 350xg for 5 minutes at 4°C and remove the supernatant.
6. Add 500 µL Cytofix fixation buffer and incubate in the dark for overnight at 4-8°C.
7. Centrifuge at 350xg for 5 minutes at 4°C and remove the supernatant.
8. Prepare a 1X Permeabilization Buffer Solution (Perm/wash Buffer).
9. Add 2 mL Perm/wash buffer into the test tube and vortex it carefully.

10. Centrifuge at 350xg for 5 minutes at 4°C and remove the supernatant.

11. Repeat the step 9 and 10.

12. Add 100 µL Permbuffer III gently while vortexing.

13. Incubate in the dark for 30 minutes at 4-8°C.

14. Repeat the step 9 and 10.

15. Add 100 µL Flow Cytometry Staining Buffer.

16. Add a fluorochrome-labeled antibody for the detection of intracellular antigens (e.g. cytokeratins).

17. Incubate in the dark for 30 minutes at 4-8°C.

18. Repeat the step 9 and 10.

19. Add 500 µL Flow Cytometry Staining Buffer and vortex it carefully.

The negative control and test tubes are analyzed by flow cytometry (The BD FACS Aria™ III Cell Sorter).

Conclusion

CTCs have been shown that they are an important tool for diagnosis, prognosis, observation of disease, the effect of the treatment and the evaluation of the recurrence in cancer patients (3). They let us to gain a better understanding about the expansion mechanism of tumor cells and shows a great potential on controlling and hindering metastasis disease (5).

As outlined in this study, we modified to improve the technique for enumerating CTCs in 7.5 mL peripheral blood samples. Our study was approved by the local human ethics committee (Ankara Ataturk Training and Research Hospital, Ankara/Turkey, 135). The patient group was composed of breast cancer patients who were newly diagnosed, untreated with identified stage while the control group consisted of healthy volunteer carrying no suspicion of cancer in this study. On the first step of our study, PBMCs were isolated from the whole blood samples by using ficoll gradient separation for patient and control groups (Figure 2). Next, PBMCs were passed through with magnetic microbeads conjugated with CD45 and were eliminated from leukocytes. On the second step of our study, the retain cells were labelled with Anti-CK (14, 15, 16, 19)*, Anti-CK (7, 8)*, Anti-EpCAM* and Anti-CD45* phenotype which were described as CTCs. The analyse was confirmed by a flow cytometry.
In this study, we preferred to use EpCAM-based and CK14, 15, 16, 19-based gating strategies for the detection of CTC’s. The results showed that CTCs were not detected in the blood sample of a healthy volunteer (Figure 3), but 9 CTCs were captured with EpCAM-based gating strategy and 7 CTCs were detected with CK14, 15, 16, 19-based gating strategy in the blood sample of a breast cancer patient (Figure 4). It is accepted that the cut off value is 5 CTCs for breast cancer (6) and CTC is negative if it is below this value or CTC is considered as a positive, if it is equal to or above this value, which might be an indication for poor prognosis.

As a result, CTCs were detectable in blood sample of cancer patients such as breast cancer, lung cancer, colorectal cancer, bladder cancer, and laryngeal cancer using with our modified convenient method for the strategies of CTC enrichment and detection.

Acknowledgement
The authors declared that this study was supported by Yildirim Beyazit University Research Foundation Project 1676.

Peer-review: Externally peer-reviewed.


Conflict of Interest: No conflict of interest was declared by the authors.

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