BRIEF REPORT

Circulating tumor cells isolated from colorectal cancer patients can form spheroids spontaneously: a small study in Ciptomangunkusumo Hospital, Indonesia [version 1; peer review: 1 approved with reservations]

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V1 First published: 21 Jan 2022, 11:75
<https://doi.org/10.12688/f1000research.73414.1>
Latest published: 21 Jan 2022, 11:75
<https://doi.org/10.12688/f1000research.73414.1>

Abstract

Background: Circulating Tumor Cells (CTC) are tumor cells that have been separated from the primary site and enter the blood or lymphatic circulation of cancer patients. CTCs are known to have tumorigenic properties and are thought to have a relationship with metastasis. One of the tests that can be used to assess tumorigenesis is the spheroid formation test. This study was conducted to determine CTCs' ability to form spheroids.

Methods: A 1 ml sample of blood was taken from two patients with metastatic colorectal cancer and placed in separate heparin tubes. The CTCs were then isolated using the DirectCTC enrichment kit according to the manufacturer's procedure. A total of 10,000 cells were grown in nuncsphera u plates in Dulbecco's Modified Eagle Medium (DMEM) for three days under standard culture conditions. Spheroid or aggregation was then evaluated using an inverted microscope.

Results: We identified a CTC culture derived from each of the patients with metastatic colorectal cancer, namely CTC ID001 and CTC 002 respectively. CTC ID001 formed spheroids spontaneously while CTC ID002 did not. CTC ID001 appears to aggregate spontaneously and resembles primary tumor cells in their ability to form spheroids.

Conclusions: We observed that CTCs can mimic primary tumors to form spheroids spontaneously, though some CTCs are not able to form spheroids. We suspected that this was related to the tumorigenic nature of CTC in these patients.

Open Peer Review

Approval Status ?

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21 Jan 2022	view
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1. Arutha Kulasinghe , Queensland	
University of Technology, Brisbane, Australia	
Any reports and responses or comments on the article can be found at the end of the article.	

Keywords

Circulating Tumor Cell (CTC), Colorectal Cancer, Primary Culture, Spheroid Culture

This article is included in the **Oncology** gateway.

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Author roles: **Abdullah M:** Conceptualization, Funding Acquisition, Supervision, Writing – Original Draft Preparation; **Noor DR:** Methodology, Writing – Original Draft Preparation; **Maulahela H:** Funding Acquisition, Writing – Original Draft Preparation; **Aziza Nursyirwan S:** Funding Acquisition, Resources, Writing – Original Draft Preparation

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by Publikasi Terindeks International (PUTI) Q1 Universitas Indonesia grants (NKB-3905/UN2.RST/HKP.05.00/2020) . The grants was assigned to MA, HM, and SA.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Abdullah M, Noor DR, Maulahela H and Aziza Nursyirwan S. **Circulating tumor cells isolated from colorectal cancer patients can form spheroids spontaneously: a small study in Ciptomangunkusumo Hospital, Indonesia [version 1; peer review: 1 approved with reservations]** F1000Research 2022, 11:75 <https://doi.org/10.12688/f1000research.73414.1>

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Introduction

Circulating Tumour Cells (CTCs) are commonly defined as tumour cells that enter circulating systems such as the blood or lymphatic vessels [Grover *et al.*, 2014](#)). CTCs are cells that shed from either primary tumors or from metastatic sites [\(Wang *et al.*, 2018\)](#). Recently, CTCs have been found to initiate tumorigenesis in Patient-Derived Xenograft models [\(Rodrigues and Vanharanta, 2019\)](#). Therefore, CTC might play a key role in understanding such metastatic processes [\(Castro-Giner and Aceto, 2020\)](#).

The tumorigenic capacity of CTC may be demonstrated using xenograft models as previously described [\(Rodrigues and Vanharanta, 2019\)](#) or by tumor spheroid formations in *in vitro* assay [\(Hu *et al.*, 2021\)](#). However, xenograft models and 3D culture using Matrigel cell culture matrix is expensive, in particular for developing countries such as Indonesia. In this study, we report the interesting findings from our pilot study that show CTCs were able to be cultured and that they can form spheroid aggregations spontaneously. Therefore, such methods may be promising and should be further investigated, in particular in developing an economical assay to test the tumorigenesis ability of CTCs. This pilot study is a part of a larger study that aims to assess CTC as a diagnostic tool for colorectal cancer (CRC).

Methods

Ethics statement

This pilot study is a part of a larger study that was approved by the Ethical Committee of Faculty of Medicine, Universitas Indonesia (Protocol ID: 20-04-0643, version 02, June 29th, 2020). Recruited subjects provided their written informed consent for blood donation (biobanking) and for the use and publication of their data for research purposes. All participants signed an informed consent form before participating in the study. In addition, the two patients involved in this pilot study provided written informed consent for participation and publication of their data.

Setting

Patient blood samples were taken in the endoscopy room of the Cipto Mangunkusumo Hospital, Jakarta, Indonesia. Isolation and culture were carried out at the Indonesian Institute of Medical Research and Education, Faculty of Medicine, University of Indonesia. Subjects were recruited (as part of the overarching study) from June 2020 until the culture process was carried out in August/September 2020. Primary culture images were taken separately in August 2021.

Participants and sample collection

A total of 73 patients with colorectal cancer, who were older than 18 years old and who were scheduled for colonoscopy were recruited for the overarching study. After patient consent was obtained, demographic and clinical data were collected from each patient. The demographic data included gender and age, and the clinical data included cancer location, stage, and metastasis. Histological data (cancer type, differentiation) were also noted. Then, 2 ml of patient blood were drawn from a peripheral vein (cubiti vein) and transferred in BD vacutainer (Becton, Dickinson and Company, USA, www.bd.com) heparinized tubes. During the recruitment process for the overarching study, we observed two patients with similar metastatic features and included these patients in the CTC pilot study.

Circulating tumor cell isolations

To obtain the CTCs for this pilot study, two colorectal cancer patients who had similar clinical profiles, namely liver metastases (see [Table 1](#)) were chosen. Approximately 1 ml of blood was taken from each patient and placed in separate heparin tubes. The blood was then transferred from the heparin tubes to separate polypropylene tubes to undergo CTC isolation using [Easysep™ Direct Human CTC Enrichment kit](#) (Catalog #19657, StemCell Technologies, Canada), according to the manufacturer's protocol. The enriched cells were then pooled in 15 ml centrifuge tubes and centrifuged 300 × g for five minutes. Supernatant was removed afterward. Cell pellets were resuspended in 1 ml complete Dulbecco's Modified Eagle medium (Gibco, USA) supplemented with 10% Fetal Bovine Serum (Gibco, USA) and 1% of Antibiotic-antimycotic solution (Gibco, USA).

Spheroid formation assay

The process of spheroid formation was carried out by implanting cells directly on the nuncsphaera u-plates and allowing the cells to immediately aggregate at the bottom of the plate. Approximately 10,000 isolated cells of colorectal

Table 1. Patient demographics and and metastasis feature.

Patient ID	Age/Sex	Primary sites	Metastasis sites
ID 001	51/Female	Rectal adenocarcinoma	Liver
ID 002	57/Female	Rectal adenocarcinoma	Liver

CTC's primary cells were seeded directly in Nunclon™ Sphera™ 96-Well, Nunclon Sphera-Treated, U-Shaped-Bottom Microplate (Catalog number: 174929) (Thermoscientific, USA) and incubated under standard culture conditions (37 °C, 5% CO₂) for three days. For controls, we seeded 5,000 cells of primary breast cancer culture, obtained from the Human Cancer Research Center, Indonesia Medical Education, and Research Institute. This culture was originally collected for optimizations of primary cultures and was obtained from discarded tissues. Written informed consent was provided by the patient for this purpose. All spheroids were examined using Carl Zeiss inverted microscope using 10× magnification in Zen blue 2.1 embedded software (Carl Zeiss, Germany).

Results

In this study, we identified two patients with similar clinical profiles which rectal adenocarcinoma and metastatic cancer in the liver. The demographic information of the two patients is shown in [Table 1](#). At first glance, we wanted to optimize the culture conditions for CTC of the metastatic cancer patients in the short term for approximately 3-7 days using standard culture media. The first question we wished to address was how the CTC culture had grown *in vitro* under standard culture conditions. Our suggestion was to increase the chance of cell-to-cell contact using 3D cultures.

In this study, we were able to identify two cultures from two patients, namely ID 001 and ID 002 after three days of incubation. Surprisingly, we found that ID 001 was able to form aggregation of cells ([Figure 1A](#)) while the CTC 002 did not have aggregation formation ([Figure 1B](#)). The ID 002 displayed clustered grape-like morphology. Therefore, we suspected that the aggregation formation may vary between samples and may depend on intrinsic factors between samples such as homophilic interactions of Cluster of Differentiation (CD)-44/CD44 antigen/CD44 that induced multicellular aggregations.

Since tumors are known for their ability to form aggregations, we chose an aggressive solid type of tumor, namely breast cancer as a control for the study. Breast cancer was a type of solid tumor with a known lobular structure and estimated behold potency to form aggregation in the 3D culture platform. We tried to seed these primary cells directly to the same type of plates used for the CTCs, for the duration of three days. We found that this produced a more rigid structure of spheroid formation (as shown in [Figure 2](#)).

Discussion

In this study, we wanted to know the characteristics of isolated CTC cells by observing the ability of isolates to form spheroids. Based on this study, it was shown that CTC can form spheroids spontaneously. We recommend using an u-plate for the test to allow the aggregation process at the bottom of the plate. After that, it is recommended that the sample be left for three days. Our results showed that, surprisingly, CTC ID 001 had the ability to spontaneously form spheroids within three days, whereas CTC ID 002 did not spontaneously form spheroids ([Figure 1](#)).

The spheroid formation has been found as *in vitro* assay to evaluate the tumorigenic capacity of the cells ([Hu et al., 2021](#)) In this study, we found facts that CTC was able to form spheroids and aggregations. Therefore we suspected that CTCs might have some tumorigenic capacity, which may be displayed by the aggregations. We confirmed these aggregation processes in primary breast cancer cells using the same methods and observed a more dense and darker spheroid

Figure 1. Spheroid culture of circulating tumor cells (CTCs) from patients with similar metastasis status after three days culture. (A) The CTC with ID001 was able to spontaneously aggregate at the bottom of the well, forming a spheroid aggregation. (B) The CTC with ID 002 was unable to form uniform aggregation.

Figure 2. Breast cancer primary cultures derived from biopsy. The primary breast cancer cultures derived from primary tumor were able to form spontaneous spheroid formations after three days culture. This is one of the characteristics of tumor cells.

formation (Figure 2). Thus, the aggregations may also be related to tumorigenesis of the CTCs or at least some similar features, as those primary cells were able to form spheroids.

Previously, the tumorigenicity of CTCs has been reported using Patient-Derived Xenograft (PDX) models (Rodrigues and Vanharanta, 2019). From our perspective, such conditions of spheroid formation may be related to the tumorigenic potential of CTC. At least, these CTC cultures were isolated from two metastatic colorectal cancer patients (shown in Table 1). Our results suggested that the CTCs have the ability to mimic primary tumors in their ability to spontaneously aggregate *in vitro*. The capacity of the CTC to form aggregation may vary depending on intrinsic factors such as expressions of CD44 in the CTC's that could not be identified in this study. A previous study by Liu *et al.* showed that homophilic interactions between CD44 increase the chance of cell to cell contact, leading to multicellular aggregations of the patient-derived breast cancer models (Liu *et al.*, 2019). In the future, we suggest that our findings regarding spheroid aggregation assay may be used as a potential alternative to predict either patient metastasis or tumorigenic capacity of CTC in a larger study.

Data availability

Open Science Framework: Circulating Tumor Cells from CRC CiptoMangunkusumo. <https://doi.org/10.17605/OSF.IO/7CGYA> (Abdullah, 2021).

This project contains the following underlying data:

- Figure 1A. tiff (CTC ID 001 ability to spontaneously form spheroid aggregation *in vitro*)
- Figure 1B. tiff (CTC ID 002 3D culture formations, which could not spontaneously form aggregation)
- Figure 2. tiff (Breast cancer primary cells' ability to form spheroid aggregation *in vitro*)
- Table 1. Patient demographic.xlsx

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

Acknowledgements

We thank Dr. Nadhira Nizam and Dr. Asiyah Nurul Fadilah for their valuable contribution in gathering patients and samples resources. We also thank Dr Erwin Danil from Department of Surgery Oncology CiptoMangunkusumo Hospital and Human Cancer Research Center for supporting the development of breast cancer primary culture.

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Version 1

Reviewer Report 11 April 2022

<https://doi.org/10.5256/f1000research.77062.r126764>

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The study aimed to expand and characterise CTCs through a culture protocol. The authors report on a low culture success, yet there are concerns around what these cells are as they have not been molecularly profiled.

I think the manuscript still needs major amendments – specifically below:

This study recruited 73 patients, but only 2 patient's samples were attempted to be cultured? Can the authors justify this? The sample size and variability is too high. Previous CTC culture studies have shown low culture success - with 2 samples, this is too preliminary. What about the remaining 71 patient samples? Are there reasons as to why the authors think that cultures were not possible?

There is no confirmation that these are indeed CTCs, other than cells clustering, can these be WBCs? Non cancer cells? Molecular confirmation of CTCs by DNA fish of similar should be applied, at least multiplex IF staining of clusters. The authors need to confirm that these are indeed CTCs and not any other cell type selected for by expansion.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Liquid biopsy, CTCs, tumour microenvironment

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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