RESEARCH ARTICLE

Colorectal cancer on a dish: exploring the 3D-sphere culture of primary colorectal cancer cells from an Indonesian perspective [version 1; peer review: 1 not approved]

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Abstract

Background : Colorectal Cancer (CRC) is one of the deadliest types of cancer and has emerged as one of Indonesia's most devastating diseases. The growing number of colorectal cancer cases is frequently undiagnosed until the disease has progressed to a metastatic stage. This issue has lasted for years, limiting therapy options and resulting in a bad prognosis for the majority of patients. Thus, the purpose of this work is to develop a CRC detection method for Indonesia and other low-middle income nations that integrates in vitro 3D culture, molecular analysis, and in silico analysis.

Methods : Colorectal cancer biopsies were transported to the lab and underwent mechanical disaggregation and centrifuged at 300 x g for five minutes. Approximately 10,000 cells were seeded in each Nunc-Sphera 96-well plate (u-bottom) for the following 7 days in standard culture medium. The 3D-sphere was harvested and RNA was extracted afterwards. Molecular analysis was performed using qPCR and the Human Cancer Pathway Profiler. Protein interaction and pathway analysis were conducted using STRING and Reactome online tools.

Results : Following initial seeding, primary CRC 3D-spheres were grown for 14–16 days. Gene profiling and in silico analyses suggest that CDC20, AURKA, and ACLY are expressed at lower levels than the positive control in the 3D-sphere. These markers have been implicated in metastasis, CRC proliferation, and as a drug target ligand.

Conclusion : A combination of 3D culture, gene profiling, and in silico analysis is feasible to detect CRC for Indonesia and other low- and middle-income countries. A future possibility is to use minicolorectal cancer in a dish for ex vivo cancer modeling and pharmacological testing.

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1

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view

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Any reports and responses or comments on the article can be found at the end of the article.

Keywords

Colorectal cancer, 3D-sphere, primary cell culture, in silico analysis, CRC detection.

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

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Introduction

Colorectal cancer has been found as one of the deadliest cancers worldwide and may be affected by various causes, including lifestyles, smoking habits, and western food diets. Based on the Global Cancer Observatory (GLOBOCAN), colorectal cancer is the third most common cancer with 9,000 deaths and 15,000 new cases in 2018 worldwide (Bray *et al.*, 2018). So far, cancers have been commonly treated using anticancer, targeted chemotherapy, or particular molecular markers that are overexpressed in cancer patients (Perry & Weitzman, 2005; Kumar *et al.*, 2017). Such progress was mainly affected by the successful breakthrough of cancer modelling, which then led to a greater connection between laboratory invention and clinical implications (Thomas *et al.*, 2016). Therefore, cancer modelling is one of the keys to the development of therapeutic agents, while identification and validation of molecular markers are needed, respectively (Perry & Weitzman, 2005; Thomas *et al.*, 2016).

One of the most common cancer models, in particular preclinical models, is cancer cell lines, which are pure cancer populations that are derived from cancer patients. This model has been widely used in cancer biology and also the development of anticancer drugs worldwide (Ferreira, 2013; Mirabelli, Coppola & Salvatore, 2019). Cancer cell lines were mostly well characterized in many various levels, from genetics to expression systems, including transcriptomic and proteomics characterizations (Mirabelli, Coppola & Salvatore, 2019). However, since tumors were constructed with various types of cells, cancer cell lines were lack of tumor heterogeneity such as stromal cells, and do not represent the original tumors (Ferreira, 2013; Strickaert *et al.*, 2017). On the other hand, ex-vivo models are models that are derived from tumor tissue which may be given to animals as in vivo models or grown as in vitro models. These methods represent tumor characteristics and in vivo heterogeneity such as tumor microenvironment (Meijer *et al.*, 2017). Moreover, ex-vivo models are not only potential for drug testing, but also models to predict patient response towards particular drugs (Meijer *et al.*, 2017). Therefore, focusing on developing ex-vivo models is potential in many ways.

Recently, there have been several types of ex-vivo models, from in vitro to in vivo models. in vivo models may represent the tumors, for instance, using Patient-Derived Xenograft (PDX) models to predict drug responses. However, ethical issues may arise while considering using animal models in cancer research (Festing & Wilkinson, 2007; Meijer *et al.*, 2017). On the other hand, in vitro models such as cancer cell lines do not always require ethical clearance for each experiment. Nevertheless, in vitro models lacked tumor heterogeneity, which may have later related to an unfamiliar response to the patients (Ferreira, 2013; Strickaert *et al.*, 2017). Respectively, primary cancer cell cultures derived from patients have been acknowledged to mimic tumors in vivo and as potential models (Miserocchi *et al.*, 2017).

Primary ex vivo models may become suitable (Miserocchi *et al.*, 2017). However, isolation and establishment of primary cell cultures from cancer patients, in particular colorectal cancer, is found to be challenging (Failli *et al.*, 2009). Thus, in this study, we were shown fast and simple methods to dissociate tumor biopsies for primary culture. We also identified similar expressions of three genes: ACLY, Aurora Kinase A (AURKA), and CDC20 in both ex-vivo primary cell culture models. These potential tools predict genes that are expressed while the cells grow in an in vitro environment.

Methods

Ethics statement

The study has been 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 biopsies 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.

Primary cell isolations using mechanical disaggregation

Biopsies were cleaned with 0.1 percent Povidone Iodine in a complete primary culture medium (DMEM) including 10% FBS and 5% antibiotic-antimycotic solutions at Biosafety Cabinet Level 2. This procedure proved crucial for successfully diagnosing primary colorectal cancer and preventing contamination by washing it with solutions containing high concentrations of antibiotics and antimycotics. Following the washing process, the biopsy was rinsed in full medium 150 mm Petri plates (Biologix, Germany) to get fragments with a diameter of 1-3 mm³. Then, fragments were pinched with sterile tweezers and scraped with a cell scraper to detach the cells from their associated tissue. After that, 5 ml complete medium was added to the fragment-containing plate and numerous times resuspended with 10 ml serological pipette tips before being passed to pre-wet 100 m cell strainers on 50 ml centrifuge tubes. Finally, the leftover pieces on the cell strain were mashed using a 12 ml syringe plunger. The medium was added to the cell top to allow the cells to flow through. After centrifuging the suspension at 300 × g for 5 minutes, the pellets were resuspended in complete mediums.

RNA extraction

3D-sphere primary CRC was harvested on the day of 7. Approximately 10,000 cells were obtained by pipping up and down to a single cell suspension. The suspension was transferred to 1.5 ml of centrifuge tubes and spun at 300 × g for five

minutes afterward. After removing the supernatant, the cells were resuspended in 100 μ l of DNA/RNA shield (ZymoResearch, USA). The RNA was extracted using RNA Miniprep Plus (ZymoResearch, USA) according to the manufacturer's protocols. The RNA purity and concentrations were measured using nano spectrophotometers.

cDNA synthesis using RT2 First-strand kit

RNA was reverse transcribed to cDNA using the RT2 First-strand kit (Qiagen, Germany #Cat#330401) according to the manufacturer's protocols. The cDNA concentration and purity were measured afterward using nano spectrophotometers.

Gene profiling using Human Cancer PathwayFinder™

Samples cDNA were processed to qPCR assay according to manufacturer's instructions of RT2 Profilers (Qiagen, Hildenburg, Germany, PN.330231, Cat#PAHS-033ZC-6). qPCR was conducted using standard PCR conditions as follows: 1 cycle of 10 min, 95°C HotStart DNA Taq Polymerase is activated by this heating step, continued by 40 cycles of fluorescence data collection in 15 s in 95°C and 1 minute in 60°C. The qPCR assay was performed in ABI 7500FAST machine (Applied Biosystem, USA). Lists of housekeeping genes were already included on the kit and the primers were already designed by Qiagen.

Protein-Protein Interaction (PPI) analysis

Protein-protein from positively expressed genes was analyzed using the String-8 Platform using multiple protein list, interactive was set to experiment, gene fusion and co-expression and highest confidence (0.900) developed by [Jensen et al. \(2009\)](#). The PPI was enriched one time by clicking "more" and represented as images.

Results

Patients profiles

Primary cultures were developed from two patients who had undergone surgical resections. Patients' profiles were then monitored from the Electronic Health Record of dr. Cipto Mangunkusumo Hospital, Jakarta. The CRC 001 was developed in a 57-year-old female. The tumor locations were rectal, the stadium was IV, the tumor size was T4b, N1 nodules, liver metastasis, and a well-differentiated adenocarcinoma. The CRC 002 was developed in a 45-year old male. The tumor location was the rectum. The stadium was II, N0 nodules, no metastasis, and also a well differentiated adenocarcinoma. The sample clinicopathology profiles are presented in [Table 1](#).

Tissues extractions and primary cultures

Endoscopic and resected tissues were sampled, and the samples were transferred into a transport medium and delivered within 24 hours at room temperature. For resected tissues, since the samples were large, after mechanical disaggregation, viability using a trypan blue exclusion assay should be conducted to determine appropriate cell numbers that should be seeded into the well. Therefore, we seeded the samples with 10,000 cells per well to demonstrate cell morphology. Cell morphology was evaluated by visualizing the samples using an Axio Carl Zeiss inverted microscope in Axiovision Zen Blue 2.1 edition.

We grew the cells in standard media in an ultra-low u-shaped NuncSphera plate (Nunc, Thermoscientific, USA). Three–seven days after initial seeding, 3D cultures were created and captured using Carl Zeiss Microscopy, Zen Blue Editions software at 5 $\times$ magnification. The majority of cells were cultured in aggregates. However, the cells were not developed into circular shapes in a regular manner. The heterogeneity of cells produced from single biopsies and their ability to develop aggregated were proven to be dependent on the biopsies' intracellular composition. Additionally, our models demonstrated significant patient characteristics, as illustrated in [Figures 1 and 2](#).

ACLY, AURKA and CDC20 were positively expressed in ex-vivo models

A pathway finder was used to investigate human cancer pathways, while positive expressing cells were analyzed. The CT was positioned above the signal in accordance with protocol specifications. Amplification of samples utilizing RT-Profilers using this pathway resulted in the expression of three genes, namely ACLY, AURKA, and CDC20, as

Table 1. Sample clinicopathology profiles.

No.	Age/Sex	Clinical feature					
		Location	T	N	M	Stadium	Differentiation
CRC 001	57/Female	Rectum	T4b	N1	M1	IV	Well
CRC 002	45/Male	Rectum	T4	N0	M0	II	Well

Figure 1. Heterogeneity in primary cultures of CRC 001. Primary cultures are 3 days after initial seeding. Cells were captured using Carl Zeiss inverted microscopy at 20 × magnification. All of the samples were derived from adenocarcinoma patients, yet distinct morphology and growth patterns were observed.

Figure 2. The morphology of primary cultures of CRC002. After 14 days, primary cultures cells were captured using Carl Zeiss inverted microscopy at 10 × magnification. All of the samples were derived from adenocarcinoma patients, yet distinct morphology and growth patterns were observed.

indicated in [Table 2](#). On CRC 001, the genes were largely expressed earlier than on CRC 002. However, because the data were not standardized for undiscovered housekeeping gene levels in both samples, such values were not evaluated, despite the presence of positive and reverse transcript controls. Those undetected may be due to the assay using insufficient numbers of cells (10,000 cells), which may be implied by undetected expressions. On the other hand, detected samples may be associated with elevated levels of gene expression, resulting in the detection of the genes.

Table 2. Relative CT value comparison to control.

Genes		CT value	
		CRC 001	CRC 002
ACLY	Metabolism	32.803	35.184
AURKA	Cell cycles	35.736	37.336
CDC20	Cell cycles	34.221	39.439
Positive control	None	15.7182	20.596
Reverse transcript control	None	27.094	30.649

Figure 3. Protein-Protein Interaction analysis using STRING. CDC20 and AURKA was strongly connected (combined score = 0.987) while ACLY was not related to CDC20 and AURKA.

Protein-Protein Interaction analysis using STRING

There were two important pathways at this point, one involving metabolism-related genes, ACLY, and another involving cell cycle-related genes, AURKA and CDC20, respectively. As a result, this may be studied individually based on the intricacy of one pathway versus another. In **Figure 3** we show that CDC20 and AURKA that has been enriched with other proteins from the STRING database, with strict criteria (highest confidence = 0.900), experiments, gene fusion, and co-expression. The co-expression score between CDC20 and AURKA was 0.808, while the experimental score was 0.487, and the combined score was 0.987 (Jensen *et al.*, 2009). Biologically, the expression of both proteins may result in the breakdown of mitotic proteins, hence influencing chromatid segregation and mitotic protein degradation.

Discussion

Ex-vivo models can be created from patients in a variety of ways, including primary culture, xenografts, and organoids (Meijer *et al.*, 2017). Primary cell culture was certainly a supply of cells for a variety of applications, including preclinical models and personalized cancer therapy (Meijer *et al.*, 2017; Miserocchi *et al.*, 2017). According to our study, one of the most suitable methods for application in clinical settings was primary cell culture from patients, which may be collected concurrently with biopsies. We used samples from two distinct types of colorectal cancer biopsy in this study: endoscopic biopsies and resection tissue acquired during surgery.

One point to emphasize prior to sample collection is the use of a complete medium containing high concentrations of antibiotic and antimycotic solutions, which we did by supplementing DMEM with 10% FBS and 5% Antibiotic Antimycotic solutions. Supplemented mediums with FBS were thought to reduce cell death in these conditions, but ABAM solutions avoided contamination. Because colon tissues may have a high concentration of gut microbiotas, we recommended a beginning concentration of 5%, consistent with earlier research (Jeppesen *et al.*, 2017; Failli *et al.*, 2009). Using antibiotics and antimycotics at high concentrations may limit cell development, whilst using them at low concentrations below 2%, for example, 1%, increases the susceptibility of samples to be contaminated by microbial organisms.

Primary culture development can be used in a variety of ways, including two-dimensional or three-dimensional culture systems (Miserocchi *et al.*, 2017). We employed 3D rather than 2D in our investigation. When compared to two-dimensional systems, primary culture in three dimensions results in a more physiological environment because the cells are aggregated and have three-dimensional structures that may facilitate tumor core formation and more closely imitate *in vivo* systems (Miserocchi *et al.*, 2017; Riffle & Hegde, 2017). According to Riffle *et al.*, three-dimensional tumor models were representative. In our instance, three-dimensional media will allow us to cultivate spheroids for a minimum of three to seven days without medium modifications.

We proved that growing primary cultures from resected tissue was not difficult by considering the optimal transport medium previously indicated to minimize such contamination. Samples may be extracted in around two hours using our procedures. Appropriate seeding with 10,000 cells may be employed for 3–7 days. In Figures 1 and 2, we demonstrate successful formation from two different patients (Table 1). Following that, samples can be harvested and seeded with 1,000 cells per well, as illustrated in figure 7A. However, no statistically significant variations in cell growth capacity were identified (Figure 7b), but morphological differences were observed. In the future, our simple methods may be employed to establish primary cell culture in three-dimensional (3D) systems. Additional pharmacological testing or the development of preclinical models employing 3D culture systems may be pursued in the future.

We next used the Cancer Pathway Profiler to analyze the cancer pathways that were activated in both samples utilizing this spheroid culture. The results indicated that Aurora kinase A/AURKA (NM 003600), cell division cycle protein 20 homolog/CDC20 (NM 001255), and ATP-Citrate Lyase/ACLY (NM 001096) were all expressed positively. However, housekeeping genes such as Beta actin/ACTB (NM 001101) and Glyceraldehyde-3-phosphate dehydrogenase/GAPDH (NM 002046) were not found, although positive controls and reverse transcriptase were. One possibility is that the amount of cDNA included in the samples was insufficient, resulting in the absence of multiple genes, including the housekeeping genes. On the other side, the presence of AURKA, CDC20, and ACLY may imply that these genes were expressed at a higher rate than housekeeping genes.

AURKA and CDC20 are important in cell cycle regulation in biological systems, whereas ACLY is a metabolic enzyme. AURKA is a serine-threonine kinase that catalyzes the activation of numerous proteins during mitosis (Koh *et al.*, 2017). AURKA was a gene that induced anaphase during complex biological processes. These include the activation of spindle assembly checkpoints (SAC) and the reading of bipolar chromosome movements (Courthéoux *et al.*, 2018). Increased AURKA expression in cancer cells may result in cells evading SAC, resulting in aneuploidy, and so inhibiting AURKA may be beneficial in future cancer treatment (Courthéoux *et al.*, 2018). Increased AURKA expression was associated with a poor outcome in colorectal cancer liver metastases and may become a useful biomarker for prognosis prediction (Goos *et al.*, 2013). Additionally, AURKA exhibited the potential to serve as a novel prognostic marker in colorectal cancer (Koh *et al.*, 2017). Through co-regulation of Myc-driven oncogenes with TPX2, AURKA was demonstrated to increase carcinogenesis in colorectal tumors (Takahashi *et al.*, 2015). Simultaneously, we generated *ex vivo* models from colorectal adenocarcinoma. Positive expression of AURKA may be essential for cells to develop *in vitro*.

Ex vivo models also expressed CDC20 similarly in our findings. These proteins are crucial for initiating anaphase by interacting with Anaphase-Promoting Complexes (APC/C). Due to the maintained CDC20 level, immature anaphase might be prevented (Mondal *et al.*, 2007). In oral tumors, overexpression of CDC20 impairs aneuploidy (Mondal *et al.*, 2007). TP53 and TP21 interactions negatively controlled CDC20 expression via CDH-CHE motifs in CDC20 promoters. Kidokoro *et al.* demonstrated that silencing CDC20 mRNA results in G2/M arrest and cell growth retardation (Kidokoro *et al.*, 2008, p. 20). Thus, positive expression of CDC20 in our *ex vivo* models may demonstrate that CDC20 is required for tumor growth to continue. CDC20 and AURKA were both shown to be associated with cell cycle progression and may be related. Reactome study revealed that both genes may interact more closely during the G1-G1/S phases of the cell's growth. As a result, inhibiting AURKA and CDC20 may hold promise as a cancer treatment.

Furthermore, proteins involved in the cell cycle, such as CDC20 and AURKA we found that both cells expressed ACLY positively. ACLY genes encoded ATP-Citrate lyase, which converts citrate to acetyl-CoA and oxaloacetate (Zaidi, Swinnen, & Smans, 2012). The enzyme is required for lipogenesis and its expression was shown to be increased in colorectal cancer compared to normal mucosa. Additionally, ACLY has been implicated in medication resistance (Zhou *et al.*, 2013). ACLY

expression has been shown to decrease cytosolic citrate while increasing glycolysis. Additionally, it was observed that ACLY activates the oncogenic PI3K/MAPK kinases, leading to the Warburg effect. Additionally, Warburg may promote ACLY expression within cells via feedback regulation (Icard *et al.*, 2020). ACLY activity generates acetyl-CoA, a precursor for fatty acid synthesis, and the Mevalonate pathway, which is required for the generation of substrates for protein prenylation, such as geranylgeranyl-pyrophosphate and farnesyl-pyrophosphate. These changes facilitate malignant transformation, invasion, and metastasis (Zaidi, Swinnen, & Smans, 2012). Additionally, by stabilizing CTNNB1, ACLY displayed greater migration and invasion. As a result, ACLY was proposed as a primary therapeutic target for both treatment and prevention (Khawairakpam *et al.*, 2014). To summarize, employing primary ex-vivo models may help to understand how cancers grow in vitro and may be useful for predicting either the tumor's genetic profile or its application as precision medicine.

Conclusions

Using a mix of mechanical disaggregation and three-dimensional culture techniques, we developed preclinical ex vivo models. These techniques were convenient and straightforward to employ, allowing for the rapid production of preclinical ex vivo models in 3–7 days. In ex vivo models, the cells produced ACLY, AURKA, and CDC20, which may be linked with key genes required for cell proliferation.

Data availability

Open Science Framework: Colorectal Cancer on a Dish: Exploring the 3D-sphere culture of primary colorectal cancer cells from an Indonesian perspective <https://doi.org/10.17605/OSF.IO/DPYWQ> (Abdullah, 2021).

This project contains the following underlying data:

- Amplification Plot_CRC001.jpg
- Amplification Plot_CRC002.jpg
- CRC 001.tif
- CT results - human cancer pathway finder.xlsx
- disaggregation result - CRC 001.tif
- Figure 1. tif (CRC 001 morphology)
- Figure 2. tif (CRC 002 morphology)
- Figure 3. png (Protein-Protein Interaction/PPI interaction of co-expressed genes)
- Melt Curve_CRC001.jpg
- Melt Curve_CRC002.jpg

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

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Estela Solanas

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This article aims to develop a method for the detection of colorectal cancer using an in vitro model of 3D spheres formed of cells from patient biopsies. For this purpose, the authors disaggregate tumour samples from 2 patients and seed and culture them on Nunc-Sphera 96-well plate for 7 days. On the spheres formed, the profile of genes involved in human cancer and the pathways involved were studied by qPCR, while protein-protein interactions from positively expressed genes was analysed using the String-8 Platform. The results support the expression of CDC20, AURKA, and ACLY in these tumour models. These markers have been implicated in metastasis, CRC proliferation, and as a drug target ligand. They conclude that the presented model may allow CRC detection in developing countries.

Some important aspects are not well explained in the text and are not clear. One of them is the aim of the study. The authors want to develop a CRC screening method to use in resource-poor countries, but the proposed method seems to be more costly and complicated than a pathological analysis of biopsies. Therefore, it would be more understandable that the aim would be to develop a model for personalised CRC screening and drug testing. It is also unclear at what time/s the different analyses are performed, whether at 7 or 14 days post culture, and the number of samples used in each patient.

As for the methodology used, the way in which the spheres are obtained is explained in the results section instead of in the methodology section, where it should be. In this regard, the exact time of culture is not detailed, nor when exactly the analyses are carried out (in some places it is said that they are cultured for 7 days and in others that they are analysed after 14 days of culture...).

As for the statistical analysis, it is not detailed or mentioned in the methodology section, although the existence of significant differences between samples is mentioned in the discussion. Therefore, it would be necessary to detail the type of analysis, number of samples per patient, etc., since if samples from each patient are analysed and compared, a specific statistical analysis is required.

A very important methodological aspect to take into account is that the genetic profile of the patient's biopsies is not studied or not detailed, which is fundamental to know if the profile found in the spheres is the same as that of the original tumour from which they originate and therefore if the proposed model or method is valid. Also in this sense, not finding expression for housekeeping genes and not being able to normalise the expression of the rest of the genes does not allow us to compare the expression of the different samples and much less to compare the profiles of different patients, invalidating to a large extent the conclusions obtained. It would be necessary to study the genetic profile of the original tumours of the patients and see if it corresponds to that found in the spheres in culture. Nor is there a study of whether the genes found are characteristic of CRC or whether they are associated with one stage or another of the tumour?

On the other hand, the images of the spheres obtained from the different patients are taken with different lenses (10x and 20x), so visual comparison is not possible.

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?

Partly

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

No

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

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: tissue culture methods, in vitro models, gastrointestinal diseases, liver regeneration

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 29 Aug 2022

Murdani Abdullah

"This article aims to develop a method for the detection of colorectal cancer using an in vitro model of 3D spheres formed of cells from patient biopsies. For this purpose, the authors disaggregate tumour samples from 2 patients and seed and culture them on Nunc-Sphera 96-well plate for 7 days. On the spheres formed, the profile of genes involved in human cancer and the pathways involved were studied by qPCR, while protein-protein interactions from positively expressed genes was analysed using the String-8 Platform. The results support the expression of CDC20, AURKA, and ACLY in these tumour models. These markers have been implicated in metastasis, CRC proliferation, and as a drug target ligand. They conclude that the presented model may allow CRC detection in developing countries.

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Indeed the aim in particular understanding genes that may related to development of CRC in Indonesia. for molecular analysis, cultures was harvested on day 7. In this paper, we want to share our experience for developing CRC in a dish using low plates and aggregation methods.

"As for the methodology used, the way in which the spheres are obtained is explained in the results section instead of in the methodology section, where it should be. In this regard, the exact time of culture is not detailed, nor when exactly the analyses are carried out (in some places it is said that they are cultured for 7 days and in others that they are analysed after 14 days of culture...)."

The exact times was approximately 7 days, and during the pandemic times and limited time to enter the lab. some was taken on day 14.

"As for the statistical analysis, it is not detailed or mentioned in the methodology section, although the existence of significant differences between samples is mentioned in the discussion. Therefore, it would be necessary to detail the type of analysis, number of samples per patient, etc., since if samples from each patient are analysed and compared, a specific statistical analysis is required."

Statistical would be very good when we have sample size, however the study was conducted during pandemic times, while patients were very limited and enter the lab in a "normal schedule" would be very difficult.

"A very important methodological aspect to take into account is that the genetic profile of the patient's biopsies is not studied or not detailed, which is fundamental to know if the profile found in the spheres is the same as that of the original tumour from which they originate and therefore if the proposed model or method is valid. Also in this sense, not finding expression for housekeeping genes and not being able to normalise the expression of the rest of the genes does

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In a future study, correspond genetics or markers would be very interesting. however in this study we want to simply observed what genes are expressed during the growth of these culture. and not goes to more detailed comparative questions such as genetic alterations and so on.

"On the other hand, the images of the spheres obtained from the different patients are taken with different lenses (10x and 20x), so visual comparison is not possible."

Yes, indeed. Figure 1 gave presentations of heterogeneity of "tumor core" while figure 2 just shown morphology. The main idea was because in figure 2 the tumor core are difficult to observed or specifically may not form heterogeneity morphology as it shown in Figure 1.

Competing Interests: No competing interests were disclosed.

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