T.C.
DOKUZ EYLÜL UNIVERSITY
IZMIR INTERNATIONAL
BIOMEDICINE AND GENOME
INSTITUTE

CHARACTERIZATION OF PATIENT-DERIVED COLORECTAL CANCER ORGANOIDS

MELİS KANIK

MOLECULAR BIOLOGY AND GENETICS MASTER'S PROGRAM

MASTER OF SCIENCE THESIS

IZMIR-2022

THESIS CODE: DEU.IBG.MSc.2019850021

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Supervising Faculty Member: Prof. Dr. Ş. Esra ERDAL

This research was supported by the TUBITAK-TEYDEB KOBI R&D Startup Support Program-1507 with a project number of 7201148.

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ABBREVIATIONS

2D: Two Dimensional
3D: Three Dimensional
AD: Alzheimer's disease
APC: Adenomatous polyposis coli
ASCs: Adult Stem Cells
ATM: ATM serine/threonine kinase
CAFs: Cancer-Associated Fibroblasts
CDX2: Caudal Type Homeobox 2
CF: Cystic fibrosis
CIMP: CpG island methylator phenotype
CIN: Chromosomal instability
CIViC: Clinical Interpretation of Variants in Cancer
CK7: Cytokeratin 7
CRC: Colorectal Cancer
ECM: Extracellular Matrix
EGF: Epidermal Growth Factor
eHEPO: Endoderm-Derived Hepatic Organoids
EM: Expansion Medium
ESCs: Embriyonic Stem Cells
HCMI: Human Cancer Models Initiative
H&E: Hematoxylin and Eosin Stain
iPSCs: Induced Pluripotent Stem Cells
ISC: Intestinal Stem Cells
KRAS: KRAS proto-oncogene
LGR5: Leucine-Rich "Repeat-Containing G-protein coupled receptors 5
NRAS: NRAS proto-oncogene
PDO: Patient Derived Organoids
PSCs: Pluripotent Stem Cells
PDTO: Patient Derived Tumor Organoids
PDTX: Patient Derived Tumor Xenografts
PDX: Patient Derived Xenografts
PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
SCNA: Somatic Copy Number Alterations
SMAD4: SMAD family member 4
SNV: Single Nucleotide Variants
TME: Tumor Microenvironment
TML: Tumor Mutational Load
TP53: Tumor protein p53
WES: Whole-Exome Sequencing

Acknowledgments

First of all, I would like to thank my thesis advisor, Prof. Dr. Esra ERDAL for giving me this chance to work with her, for believing in me, and for her inspiration, passion, and support during the M.Sc. study and research.

I would like to express my sincere thanks to my friend Berna Bıçak, who I have been working with since the beginning of this project. I am especially grateful for any support given by all my lab members during my studies. I am particularly grateful for the assistance given by Soheil Akbari and Mustafa Karabiçici during the experiments helping me on both theoretical and experimental sides.

I would like to thank Nevin Ersoy for her help in completing my histopathology experiments. I would like to express my sincere thanks to Melek ÜÇÜNCÜ, a member of the "IBG-İZMİR optical imaging core facility", for her assistance.

I am sincerely honored to have been selected for the scholarship of 2210-A National MSc/MA. Therefore, I would like to thank TUBITAK-BIDEB for giving me this chance.

And last but not least, my lovely thanks go to my family Leziz Kanık, Ertan Kanık, Zeynep Kanık, and my friend Yusuf Kaan Poyraz for continuous tenderness, confidence, and believing in me.

CHARACTERIZATION OF PATIENT-DERIVED COLORECTAL CANCER ORGANOIDS

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ABSTRACT

Precision oncology aims to find effective treatments for each patient individually. Preclinical models, such as patient-derived tumor xenografts (PDTXs), present a lot of technical issues with regard to predicting therapy responses. Patient-derived organoids (PDOs) offer promise for personalized medicine. PDOs are structures that can be obtained from individual cancer patients and can recapitulate morphological and genetic features of the original tumor among the 3D culture systems. However, non-standardized and ill-defined culture procedures are a lack of in cancer organoid research. This study aims to establish the organoids of colorectal cancer (CRC) patients by using an in-house protocol and to demonstrate histological and genetic similarities to the original tissue. After obtaining patient consent, fresh tissue resection samples (tumor and non-cancerous adjacent counterparts) were acquired from colorectal cancer patients (n=17) over the age of 18. Cells were counted and embedded in matrigel after mechanical and chemical dissociation of tissues. As a result, we were able to generate organoids from tumor biopsies and healthy tissues with over %90 successful rates. According to an independent pathology consultant, organoids have characteristics of CRC such as cribriform structure as in their original tumor samples. Finally, bioinformatics analysis demonstrated that a patient and matching disease model share similar somatic mutation profiles, particularly for mutations pertaining to well-recognized mutated genes of the tumor. Therefore, our in-house optimizations have provided us with a robust and efficient protocol for PDO establishment and made it possible to use them as a tool in personalized treatment approaches in a proper way.

Keywords: Patient-derived organoid (PDO), colorectal cancer (CRC)

HASTA KAYNAKLI KOLEREKTAL KANSER ORGANOİDLERİNİN KARAKTERİZASYONU

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ÖZET

Hassas onkoloji, her hasta için ayrı ve etkili tedaviler bulmayı amaçlamaktadır. Hastadan türetilen tümör ksenogreftleri (PDTX'ler) gibi klinik öncesi modeller, tedavi yanıtlarını tahmin etme konusunda birçok teknik sorun sunmaktadır. Hasta kaynaklı organoidler (PDO'lar) ise kişiselleştirilmiş tıp için umut vaat etmektedir. PDO'lar, bireysel kanser hastalarından elde edilebilen ve orijinal tümörün morfolojik ve genetik özelliklerini 3D kültür sistemleri arasında özetleyebilen yapılardır. Bununla birlikte, standartlaştırılmamış ve kötü tanımlanmış kültür prosedürleri, kanser organoid araştırmalarında eksikliktir. Bu çalışma, kurum içi bir protokol kullanarak kolorektal kanser hastalarının organoidlerini oluşturmayı ve orijinal dokuya histolojik ve genetik benzerlikleri göstermeyi amaçlamaktadır. Hasta onamı alındıktan sonra, 18 yaş üstü kolorektal kanser hastalarından (n=17) taze doku rezeksiyonu örnekleri (tümör ve kanserli olmayan komşu örnekler) alınmıştır. Dokuların mekanik ve kimyasal parçalanmasından sonra, hücreler sayılarak matrigel içine gömülmüştür. Sonuç olarak, tümör biyopsilerinden ve sağlıklı dokulardan %90'ın üzerinde başarılı oranlarda organoidler üretebildik. Bağımsız bir patoloji danışmanına göre, organoidler orijinal tümör örneklerinde olduğu gibi kribriform yapı gibi CRC özelliklerine sahipti. Son olarak, biyoinformatik analiz, hasta ve eşleşen hastalık modelinin, özellikle tümörün iyi tanınan mutasyona uğramış genleriyle ilgili mutasyonlar için benzer somatik mutasyon profillerini paylaştığını göstermiştir. Bu nedenle, kurum içi optimizasyonlarımız bize PDO kurulumu için sağlam ve verimli bir protokol sunmuş ve bunları kişiselleştirilmiş tedavi yaklaşımlarında bir araç olarak uygun bir şekilde kullanmayı mümkün kılmıştır.

Anahtar Kelimeler: Hasta kaynaklı organoid, kolorektal kanser

1. INTRODUCTION AND PURPOSE

1.1. Statement and Importance of the Problem

In terms of incidence and death, the 3rd most common malignancy in the world and the second most prevalent cause of death from cancer is colorectal cancer (CRC). (Y.-H. Lo et al., 2020). The most significant current limitation in CRC treatment is insufficient patient-specific treatment prediction even though there have been significant advances in molecular and genetic understanding of CRC in recent decades. As a result, some patients experience adverse side effects while waiting for effective treatment. Xenografted or transgenic animal models and in vitro immortalized two-dimensional cancer cell lines are being used for preclinical cancer biology investigations. However, these methods have some disadvantages. For example, prolongedly passaged cell lines do not truly represent the biology and pathophysiology of the original parent tumors, and animal models are time-consuming and costly to produce and implement (Y. H. Lo et al., 2020) Patient-derived colorectal cancer (CRC) organoids are well-known and becoming useful tools for predicting treatment response. However, protocols for PDO establishment and quality controls are not globally standardized. Also, the diversity between individuals and protocols causes variations in outcomes from group to group. Therefore, a standardized, improved clinical tool is required to predict treatment sensitivity for individual patients.

1.2. Aim of the Study

In this study, we aimed to obtain the organoids from CRC patients with an in-house protocol and to demonstrate the similarity of the morphological and genetic landscape of the tumor organoids to the original primary tissue.

1.3. The hypothesis of the Study

This PDO model can be used as a personalized test platform for a variety of approaches, including high-throughput drug screening, targeted therapy testing, and also for the investigation of the tumor microenvironment and biobank studies.

2. GENERAL INFORMATION

2.1. Organoid Technology

2.1.1. Organoid Definition

Organoids are 3D cell culture systems that are designed to imitate the structural and functional properties of the organ being studied. Intestinal stem cells were used in 2009 to create an early model of an organoid. Thus, using the presence of stem cells in the lower part of the intestinal crypt, 3D culture models, namely organoids, were developed (Jung et al., 2011; Sato et al., 2011). This led to the creation of organoid cultures from a variety of tissues (Barbáchano et al., 2021).

Currently, an organoid is described as a *"self-organizing structure that is created from stem cells and mimics the in vivo architecture and multi-lineage differentiation of the original tissue."* (Figure 2.1) (Lancaster & Knoblich, 2014). Organoids have a more complex and ordered design that mimics an organ's natural form, which is why they are called *"mini-organs."* Organoids can remain stable, reflecting the in vivo features of the type of tissue after several passaging without substantial amendments of genetic and phenotypic characteristics, and also can be cryopreserved, and genetically modified once formed. (Akbari, Arslan, et al., 2019).

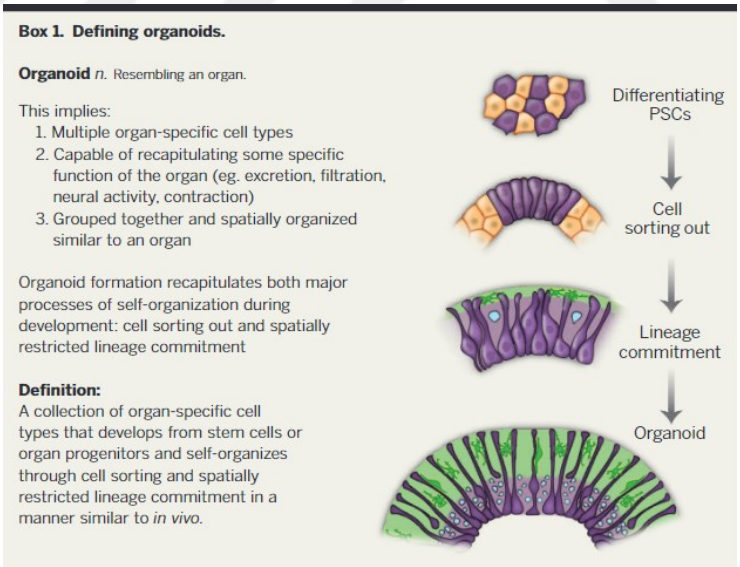

Figure 2.1: Definition of organoids (Lancaster & Knoblich, 2014).

Organoids are developed from pluripotent stem cells (PSCs) or adult stem cells (ASCs) (Clevers, 2016). Through guided differentiation of PSCs, which includes the initial stage of germ-layer determination, organoids generated from PSCs are produced. After that, the requested organ's particular cell types are acquired via culture using certain growth and

signaling molecules. PSC-derived organoids might have cells from distinct germ layers to closely match the in vivo counterpart. Organoid cultures generated from adult stem cells (AdSC) need the extraction of tissue-specific stem cells, which will then be encapsulated in an extracellular matrix (ECM). (Kim et al., 2020). Matrigel is mostly used as ECM material in organoid culture. It is produced from mouse sarcomas (Engelbreth-Holm-Swarm). With the help of Matrigel, cellular signals and cross-talking between the cells become fact in functional organoids. To enable organoid formation, stem cells embedded Matrigel dots are covered with expansion media including a specific concoction of growth factors. Adult stem cells in particular were thought to be unable to proliferate in vitro. However, it has been observed that these cells can also be grown as organoids by incorporating growth factor combinations into the organoid media that simulate different organ stem cell environments. (Clevers, 2016; Y.-H. Lo et al., 2020). These growth factors and small molecules represent specific stem cell signaling pathways in the organoid culture. As a result, they control the production of organoids and are essential for development. AdSC-derived organoids, as demonstrated in Figure 2.2., are epithelial in origin and do not have a mesenchymal or immunological component unless it is introduced subsequently (Kim et al., 2020). Organoids generated from adult stem cells are a useful model for studying tissue homeostasis and regeneration. Pluripotent stem cell-derived organoids are more suited for researching organ formation since they can contain a variety of cell types. (Driehuis et al., 2020).

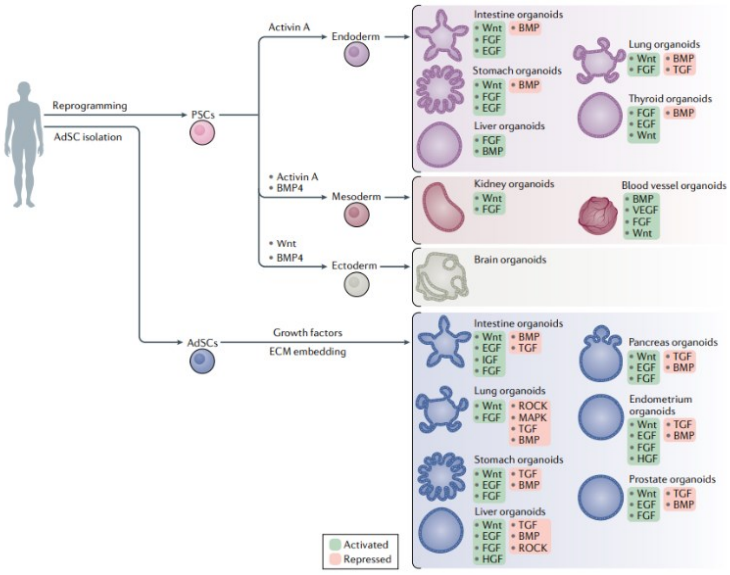

Figure 2. 2: The procedure for establishing PSC-derived and AdSC-derived organoids

Signaling components crucial for directed differentiation and niche function are demonstrated; active signaling pathways are represented in green, while suppressed signaling pathways are displayed in red (Kim et al., 2020).

2.1.2. Patient-Derived Organoids

After obtaining ASCs-derived organoids, patient-derived organoids (PDOs) have been effectively generated for a variety of human tissues by tissue biopsies, surgical resections, circulating tumor cells, and ascitic fluids. PDOs can effectively mimic the histopathological, genetic, and behavioral properties of original tumors according to multiple research. Furthermore, following recurrent passaging of PDOs, tumor heterogeneity were retained, showing that they are robust biologically and have a wide range of therapeutic applications. Also, biological variability inside and across tumors is due to the unique cellular and environmental aspects of each patient's malignancy. Organoids grown directly from human tumor samples have been proven to correctly reflect this biological variability (Corrò et al., 2020; LeSavage et al., 2022; Y.-H. Lo et al., 2020).

Sato et al. (2009)effectively developed organoids from colorectal cancer (CRC) patient tumor tissue, which is the earliest use of cancer organoid culture. As a result of this groundbreaking discovery, organoids have been properly generated from a variety of primary types of tumors, and it has been demonstrated that they actually more reflect the characteristics of the original tumor than classical cell lines. PDOs have usually had greater success rates than normal 2D culture and PDX e.g.success rate of >70%. Although no conventional definition has yet been created, a functional cancer organoid derivation is frequently described by the capacity to grow, passage, and cryopreserve cells that maintain genetic and histological properties of the original tumor (LeSavage et al., 2022).

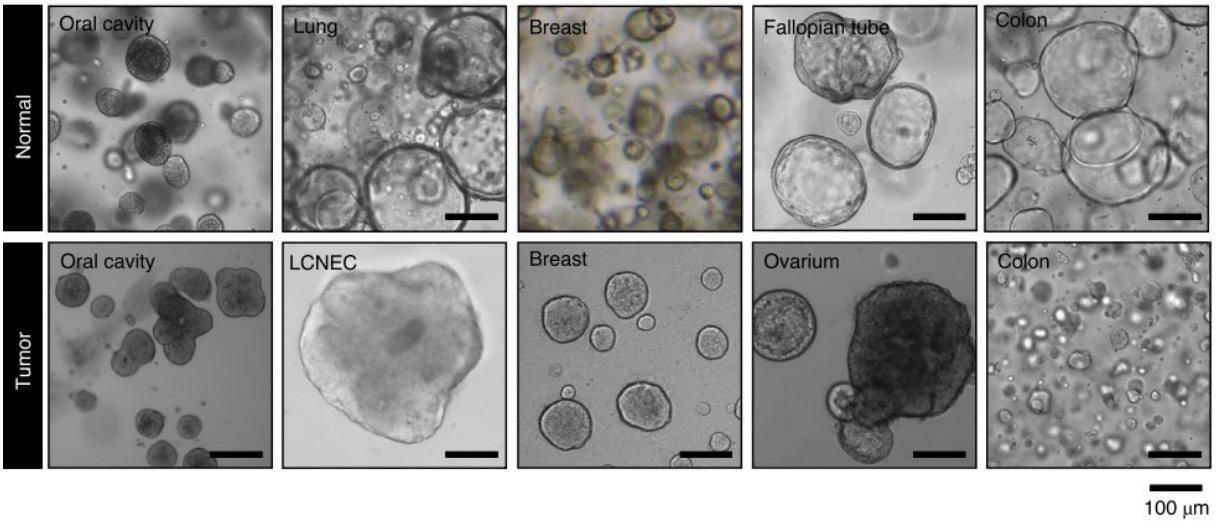

Figure 2. 3: Brightfield images of normal and tumour organoids developed from different epithelial tissues (LeSavage et al., 2022).

2.2. Applications of Organoids in Biomedical Research

Organoids are increasingly being used in biomedical research as a cell culture technique. Organoids' wide range of tissue types, potential for long-term growth, and physiological 3D shape make them suitable for both basic/laboratory and clinical applications.

2.2.1 Basic/Laboratory Applications

Basic laboratory applications include modeling embryonic organogenesis, tumor initiation and metastasis, and intra-tumor heterogeneity. In addition, disease modeling and accordingly drug screening tests and drug toxicity studies for anticancer drugs can be performed (R. Nguyen et al., 2020).

For genetic disease modeling, intestinal organoids are used for modeling of Cystic fibrosis (CF). For instance, Dekkers et al. (2013)produced the first intestinal organoids obtained from actual CF patients who carried the F508del CFTR mutation to mimic the disease. Similarly, Akbari and Sevinç (2019) modeled citrullinemia disease with Endoderm-derived hepatic organoids called *'eHEPOs'*. The ammonia aggregation phenotype associated with the illness was reversible in eHEPOs by overexpression of the wild-type ASS1 gene. This demonstrated that this model can be genetically modified (Akbari, Sevinç, et al., 2019). Organoids of the brain are also useful models for neurodegenerative illnesses like Alzheimer's disease (AD), the most prevalent form of the dementia (Raja et al., 2016).

In order to research host-pathogen relations involving viruses, bacteria, and protozoan parasites, 3D organoid technology provides ideal models. Recently, for instance, the processes causing the microcephaly brought on by the Zika virus have been studied using cerebral organoids. When compared to controls, Zika virus-infected brain organoids had reduced sizes (Woods et al., 2005).

Organoids generated from various mice or human tumors are currently commonly used in the research of various cancers. It may also be produced by employing gene-editing techniques like CRISPR-Cas9 or RNA interference approaches to introduce pathogenic mutations into wild-type organoids (Corrò et al., 2020).

2.2.2 Clinical Applications

Biobank investigations were made possible by long-term cultivation and the freezing and thawing ability of organoids. Thus, biobanks may be utilized for therapeutic applications like drug screening for targeted therapy and bioinformatics research for cancer categorization. In recent years, live organoid biobanks have been created from a range of tumors. As a result, it is possible to define patient-specific mutations for personalized medicine (Corrò et al., 2020). Also, regeneration investigations were made possible by organoids which could be options to organ transplantation in therapy. For example, intestinal organoids generated from human PSCs have been implanted into the mouse and shown intestinal structure, showing the possibility for translating this research into the treatment of short bowel syndrome. (Watson et al., 2014).

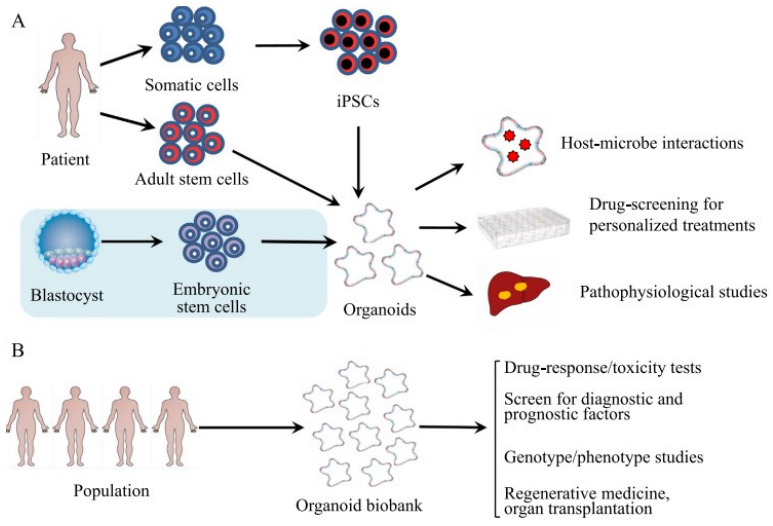

Figure 2. 4*:* Organoid applications in biomedical research (R. Nguyen et al., 2020).

2.3 Cancer Modelling via Organoid Technology

Treatment of cancer patients has progressed over the last decade from being focused on the kind of tumor to now being based on the molecular features of a tumor. Numerous patients with advanced tumors now have better prognoses thanks to precision medicine. Despite the fact that cancer is a hereditary illness, it is required to fully understand the malignant processes and develop more effective treatments. Clearly, there is a high demand for a biological system that allows detailed analysis of tumor biology. (Veninga & Voest, 2021).

Because they were generally more affordable and readily available than other in vitro models, cell lines were the standard method for oncology research for a long time. At the same time, immortalized cells frequently undergo clonal selection, often passaged, genetically manipulated. Following that, patient-derived xenografts (PDXs) were created more accurately mimic the tumor tissue. Even while PDXs are physiological, they are extremely costly(Corrò et al., 2020; LeSavage et al., 2022).

Genetically engineered models, on the other hand, allow for novel analyses of tumor genesis and development. Unfortunately, their total genetic manipulation is still restricted, and adding fresh mutations repeatedly is a time-consuming procedure. Organoid technology has provided an innovative technique for modeling human malignancies in vitro in recent years (Corrò et al., 2020; LeSavage et al., 2022).

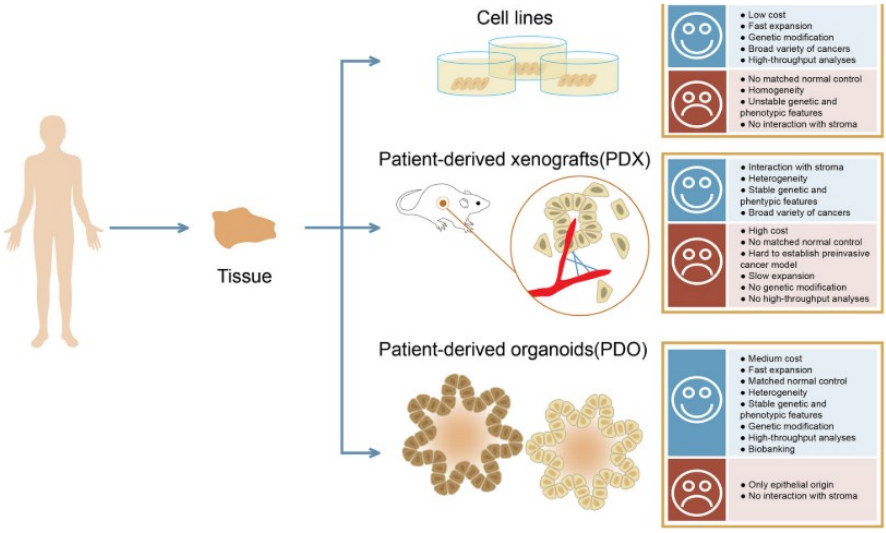

Figure 2. 5: Comparison of cell culture methods (Y. Li et al., 2020).

2.3.1. Cancer organoid modeling via healthy organoids

Human embryonic stem cells or patient-specific induced pluripotent stem cells can also be used to create organoids representing specific cancer subtypes. Primary wild-type organoids in which genetic modification is done via CRISPR-Cas9, or RNA interference methods allow for an opportunity to figure out how cancer progresses and how different mutation patterns influence cellular responses to anticancer drugs (LeSavage et al., 2022). Oncogenic conversion of human gastric, pancreatic, esophageal, breast, and liver tissues has been achieved using CRISPR–Cas9 oncogene editing of wild-type human organoids. For example, to generate invasive colorectal carcinoma after transplantation under the kidney subcapsule in mice, driver mutations such as KRAS, APC, SMAD4, TP53, and PIK3CA were introduced into healthy wild-type organoids (Matano et al., 2015). Deletion of CDH1 in human stomach organoids had been resulted in transformation, with a diffuse gastric cancer phenotype (Nanki et al., 2018). Barrett's esophagus-associated neoplasia developed rapidly in normal esophageal organoids lacking APC (Liu et al., 2018). Engineered breast organoids with mutations in four tumor-suppressor genes, TP53, PTEN, RB1, and NF1, also reproduced breast carcinogenesis (Dekkers et al., 2019).

However, there are several disadvantages to utilizing iPSCs for cancer modeling, such as their embryonic background, which can make adult cancer research difficult (Y.-H. Lo et al., 2020).

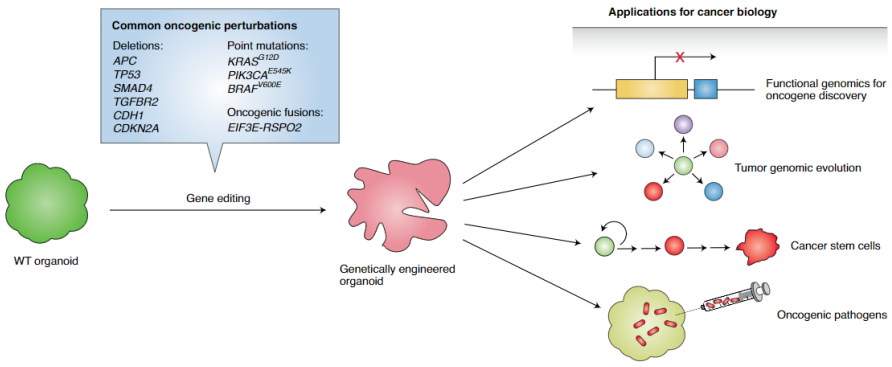

Figure 2. 6: Modeling cancer in wild-type organoids

It is possible to construct wild-type tissue organoids with tumorigenic alterations to reflect the cancer biology. (Y.-H. Lo et al., 2020).

2.3.2. Cancer modelling via PDOs

Organoids are now widely regarded as a superior system for studying cancer biology (Barbáchano et al., 2021). A great number of protocols for creating organoids from various tissues have been developed(Dye et al., 2015; Schutgens et al., 2019). There have been 12 different tumor organoids developed with promising outcomes. New PDO models will be biobank as part of the Human Cancer Models Initiative (HCMI), which will include genetic sequencing and clinical annotations. PDOs were shown to be phenotypically and genotypically similar to the original tumors (Foo et al., 2022).

System	Cancer Type	Success Rate of PDTOs
Digestive	Pancreatic Cancer	62% (52/83)
		75% (103/138)
		85% (17/20)
	Colorectal Cancer	100%
		~ 90% (22/27)
	Hepatocellular Carcinoma	26% (10/38)
		100% (13/17)
	Gastric Carcinoma	50%
		71% (10/14)
	Metastatic Gastrointestinal Carcinoma	70% (> 100)
		76% (13/17)
	Esophageal Carcinoma	31% (10/32)
	Appendiceal Carcinoma	75% (9/12)
Respiratory	Lung Carcinoma	88% (n = 16)
	Non-Small Cell Lung Cancer	71.43% (10/14)
	(Primary & Metastatic)	100% (3/3)
		28% (n = 18)
	Mesothelioma	100% (2/2)
Urinary	Prostate Cancer (Primary & Metastatic)	16% (4/25)
		18% (6/32)
	Bladder Carcinoma	70% (12/17)
	Renal Cell Carcinoma	74% (25/35)
Reproductive	Breast carcinoma	~ 80% (> 155)
	Endometrial Carcinoma	100% (15/15)
	Ovarian Cancer	65% (n = 32)
Nervous	Glioblastoma	91.4% overall
		66.7% (IDH1 mutant)
		75% (recurrent)

Figure 2. 7: A list of produced PDOs and their success rates are included in the figure above (Foo et al., 2022).

The main causes of resistance development in conventional therapy are inter and intra-patient heterogeneity. Because of this, not all patients will benefit equally from conventional chemotherapy. PDOs enable for the study of specific tumors while preserving the features of the original tumor. Consequently, it has been suggested to employ tumor organoids as a prototype platform for precision medicine (Veninga & Voest, 2021). Precision medicine is a new technique for selecting the best treatment options for each individual. When it comes to cancer therapy, precision medicine refers to the discovery of successful therapeutic approaches that are specific to every patient by utilizing tailored medicines that are less intrusive and harmful than conventional treatment options. (Foo et al., 2022).

Researchers are also looking at whether or if drug-screening techniques can be applied with these cultures. Such displays are frequently used to simulate clinically administered therapy regimens. To test these PDOs' ability to predict outcomes, patient responses were compared to corresponding patient-derived organoid responses. Results thus far show that the PDOs generally reflect the therapy response of the patient. (Driehuis et al., 2019; Girirajan et al., 2011; Sachs et al., 2018; Vlachogiannis et al., 2018; Yao et al., 2020). As a result, individualized medicine is now a possibility because to organoid technology's capacity to forecast patient responses.

PDOs are also very easy to work with. For instance, lentiviral transduction and CRISPR-based gene editing methods can be applied to the organoids, and also PDOs can be implanted into the mouse as xenografting. Therefore, PDOs may be more useful than patient-derived xenografts. Moreover, coculture of non-neoplastic cell types, such as cancer-associated fibroblasts (CAFs), allows in vitro cancer organoid platforms to replicate the tumor microenvironment. (Corrò et al., 2020; Y.-H. Lo et al., 2020).

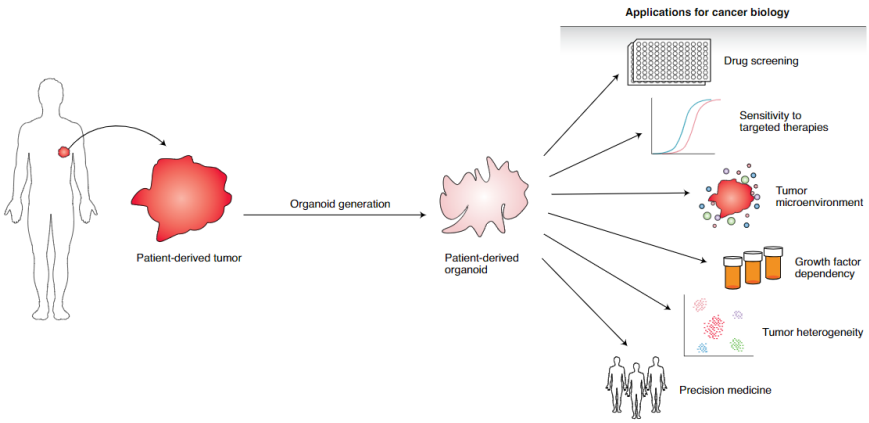

Figure 2. 8: Cancer modeling in patient-derived tumor organoids

There are several applications for patient-derived tumor organoids in translational cancer research. (Y.-H. Lo et al., 2020).

2.4. Colorectal Cancer (CRC) and Modeling of CRC via Patient-derived Organoids

2.4.1. Colorectal Cancer

Due to the existing standard therapy, the incidence of colorectal cancer is rising (Dehaan et al., 2020).

Colorectal cancer (CRC) is caused by several genetic and epigenetic abnormalities inside cells. There are three main mechanisms that lead to the development of colorectal cancer as shown in Figure 2.9. The conventional chromosomal instability (CIN) mechanism is followed by the majority of colorectal cancer (CRC) patients (70–80%). APC mutations are the first to appear, followed by mutations in KRAS, PIK3CA, and SMAD4, loss of heterozygosity of chromosome 18 (LOH 18q), and TP53 mutations. The serrated route is responsible for around 20–30% of CRC cases. Serrated cancers are commonly related with MGMT, CDKN2A, or MLH1 silencing. The MSI pathway is a third mechanism of CRC formation induced by malfunction of DNA mismatch repair genes such as those producing MLH proteins or MSH proteins, in addition to the CIN and CIMP pathways (Schmitt & Greten, 2021).

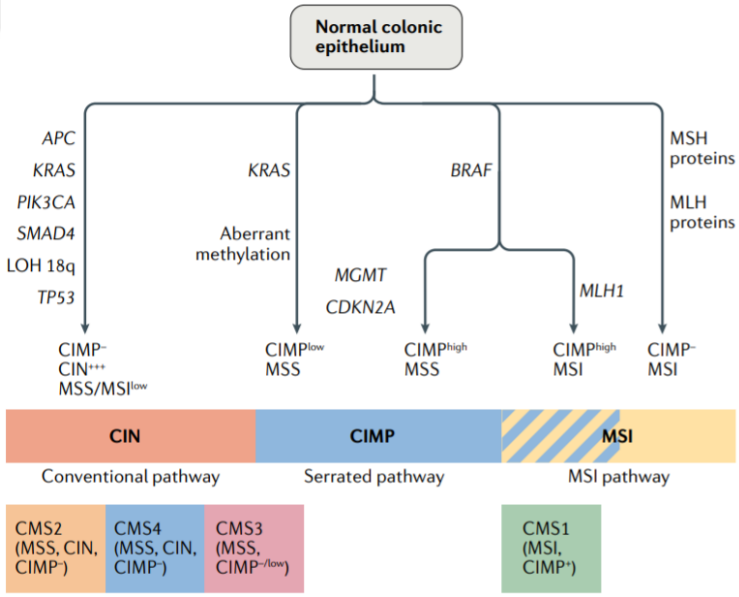

Figure 2. 9: Colorectal cancer has three primary molecular mechanisms (Schmitt & Greten, 2021).

2.3.3. CRC Organoids

Colorectal cancer tumor organoids are created by the direct culture of colorectal cancer cells in the ECM structures (Driehuis et al., 2020). A unique 3D human intestinal stem cells (ISC) culture system has been produced based on a better knowledge of stem cell niche parameters (Sato et al., 2009). Using this technology, single ISCs were able to form structures resembling intestinal epithelial crypts known as organoids. A laminin-rich extracellular matrix (Matrigel) and the niche factors epithelial growth factor (EGF), Noggin, R-spondin1, and Wnt3A, as well as a TGF-b inhibitor (A83-01) and a p38 inhibitor (SB202190), are used in human colonic organoid culture. However, not all of these important growth factors need to be used in patient-derived CRC organoids (Jung et al., 2011). Over 94 percent of CRCs have mutually unique mutations in genes encoding important components of the WNT/ β-catenin pathway. This eliminates the need for Wnt3A-conditioned media in the cultivation of CRC PDOs. Surprisingly, the lack of WNT prevents non-tumor cells from growing in PDO conditions. Nonetheless, CRC PDO establishment effectiveness is typically about 70%, indicating that the medium is still not optimized (X. Li et al., 2020; van de Wetering et al., 2015). Sato's team improved the culture medium by mixing the presence or absence of Wnt3A, SB202190, and oxygen concentrations, which improved tumor organoid formation efficiency by up to 100% (Fujii et al., 2016). Because of the accumulation of changes during the adenoma-carcinoma development, some culture medium variables may become redundant for some PDOs (Fujii et al., 2016; Matano et al., 2015; Sato et al., 2011).

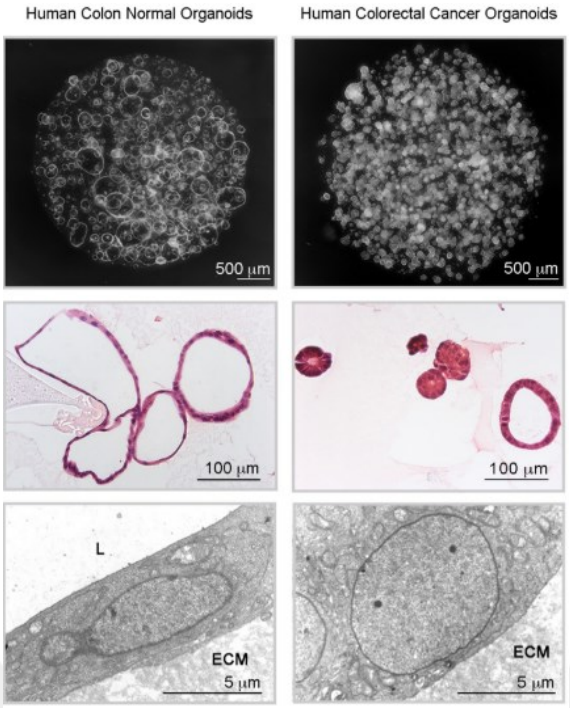

Figure 2. 10: Normal and tumor patient-derived organoids from the human colon. Light microscopy photos in the upper panels. Hematoxylin and eosin (H&E) staining in the middle panels. Lower panels: electron microscope images of cells in both types of organoids with the same undifferentiated phenotype (Barbáchano et al., 2021).

Also, it is demonstrated that different types of precancerous colon neoplasia can be grown as an organoid (Fujii et al., 2016).

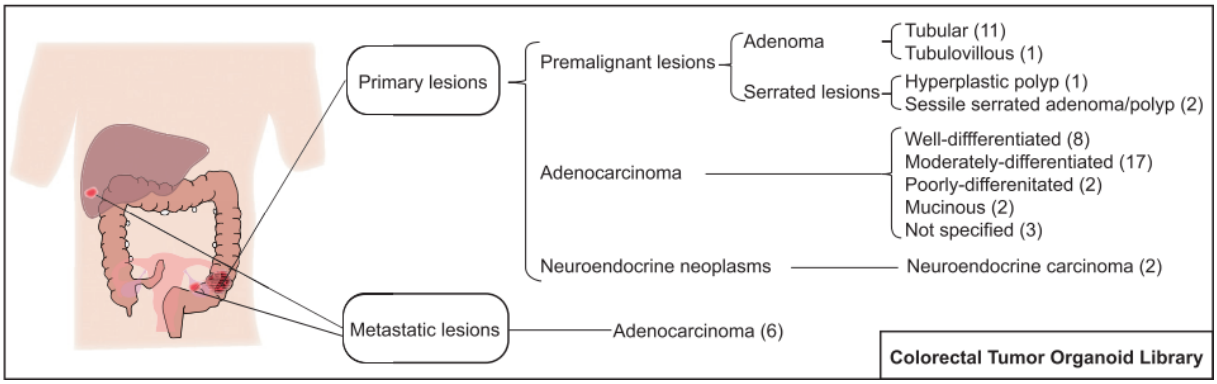

Figure 2. 11: Examples of different types of precancerous colon neoplasia used in CRC PDOs (Fujii et al., 2016).

Metastasis, recurrence, and drug resistance are all linked to tumor heterogeneity in CRC as in other cancer types.

Organoids derived from metastatic colorectal cancer and metastatic gastroesophageal cancer have a lot of similarities with the biopsies in terms of morphology, mutational spectrum, altered copy number genes, and expression patterns of common clinical diagnosis markers like CDX2 and CK7 (Xu et al., 2022). Therefore, PDOs may better mimic primary tumors, making them more accurate models for drug screening and development. The Clevers lab used their CRC organoid biobank to perform the first medium-scale drug screen. Organoid cultures were plated on 384-well plates and treated with an 83-compound library that addressed a variety of cancer targets (Seidlitz & Stange, 2021; van de Wetering et al., 2015).

CRC PDOs produced from patient tumor material can be used to improve our understanding of cancer at the molecular level. PDO sequencing can reveal low-frequency subclonal alterations that were previously undetectable in primary tumor tissues (Seidlitz & Stange, 2021). A collection of 20 individual CRC PDOs was used to create the first cancer organoid biobank. PDOs matched the somatic copy number alterations (SCNA) and mutation spectra seen in CRC, according to in-depth DNA sequencing and RNA expression analyses. Activating mutations in KRAS, PIK3CA, BRAF, and TGFBR1/2 were identified, as well as inactivating mutations in the tumor suppressor genes APC, TP53, FBXW7, and SMAD4 (van de Wetering et al., 2015).

Proteomic analysis were done on a subset of these PDOs in a separate study, proving that proteomic techniques in PDOs are possible. At the proteomic level, distinct organoid profiles were observed between PDOs and healthy counterparts, as well as between patients, highlighting the importance of personalized profiling of each individual sample (Cristobal et al., 2017).

Also in line with the studies (Fujii et al., 2016) created an organoid biobank containing 55 CRC PDOs of various histological subtypes and clinical states as mentioned above. Ganesh K and colleagues also created a biobank of 65 rectal cancer PDOs derived from patients with primary, metastatic, or recurrent rectal cancer, with an overall success rate of 77%. Rectal cancer organoids faithfully mimic the genomic and histological characteristics of matched primary tumors on a consistent basis. Moreover, tumoroids have a 92 percent concordance with the mutational fingerprint of the matched primary tumors. Thus, Colorectal tumoroids,

accurately replicate native tumors and make a mechanistic study on this cancer type easier.(Ganesh et al., 2019; Xu et al., 2022)

3. MATERIALS AND METHODS

3.1. Type of Research

This study used an experimental research method.

3.2. Time and Place of the Research

It was found ethically appropriate by the Dokuz Eylul University Ethics Committee with the decision no 2020-040 on 25/09/2020. This project was supported by TÜBİTAK-TEYDEB with the number 7201148 within the scope of 1507 projects. Experimental procedures were carried out at ORGANO-ID Biotech Company Laboratory between March 2021 and June 2022.

3.3. Research population, sampling, and experimental groups

Human tissue samples were obtained from patients over 18 years of age, either male or female, who were diagnosed with colorectal cancer, after obtaining a patient consent form.

3.4. Research Materials

3.4.1. Human Tissue Samples

Tumor tissue (20 mm x 20 mm x 10 mm) and at least 1 surrounding normal tissue (20 mm x 20 mm x 10 mm) were collected from each patient. For surrounding normal tissue, tissue samples were taken more than 5-10 cm from the tumors. Patient tissues were taken from İzmir Kent Hospital Pathology Department during the project period (March 2021- June 2022). The pathologist placed tissues in a sterile tube containing transfer medium under a fume hood macroscopically. All samples were transferred to the laboratory at +2-8 °C conditions within 24 hours.

3.4.2. Organoid culture buffers/media

Table 3. 1: Organoid culture buffers/media

Buffer/Media	**Components**
1X PBS	50ml 10X PBS (Gibco), 450 ml autoclaved ddH2O
Basal medium (BM)/adDMEM+++, 500 ml	485 ml Advanced DMEM/F12 (Invitrogen), 5 ml HEPES (Gibco), 5 ml Penicillin/Streptomycin (Gibco), 5 ml Glutamax (Gibco)
Transfer Medium, 100 ml	100 ml adDMEM+++ , Y-2763 (Tocris), Normocin (Invivogen), Amphotericin B (Gibco), Primocin (Invivogen)
Wash Buffer, 100 ml	97 ml 1X PBS (Gibco), %1 Penicillin/Streptomycin (Gibco), HEPES (Gibco), Normocin (Invivogen), Primocin (Invivogen), Amphotericin B (Gibco)
Dissociation medium, 100 ml	96 ml adDMEM+++, Collagenase XI (Sigma), Dispase II (Sigma), Hyaluronidase (StemCell), Y-2763 (Tocris), DNAse1 (Sigma)
Expansion medium (EM)	Advanced DMEM/F12 +++ (Invitrogen) supplemented with, B27 (Gibco), N2 (Invitrogen), N-acetyl-L-cysteine (Sigma Aldrich), Gastrin (Sigma Aldrich), R-spo1 conditioned medium (home-made), Wnt Surrogate-Fc Fusion Protein (ImmunoPrecise), Human Recombinant Noggin Protein (Peprotech), Prostaglandin E2 (Tocris), hEGF (Peprotech), SB202190 (Sigma Aldrich), Y-27632 (Tocris), Nicotinamide (Sigma Aldrich), A83-01 (Tocris)

3.4.3. Laboratory Equipments

Table 3. 2: Laboratory Equipments

Device Name	**Vendor**	**Catalog#**
Centrifuge	Nuve	NF 800R
EVOS™ XL Core Imaging System	Thermo Fisher Scientific	AMEX1000
Cryostat Microtome	Leica	CM1950
Semi-motorized Rotary Microtome	Leica	RM2245
Autostainer XL	Leica	ST5010

3.5. Research Variables

Patient-derived organoid culture is the dependent variable. Growth factors added to the organoid media during organoid formation are independent variables.

3.6. Data Collection Tools

3.6.1. Establishment of CRC and healthy-counterpart PDOs

3.6.1.1. Biopsy tissue processing and dissociation of tissue

After the surgery, patient tissues are transfered to the laboratory within 24 hours at +4°C with the transfer medium whose content is specified in Table 3.1. Tissues were washed at least 2-3 times with a wash buffer. Then, tissues were transferred to the 100 mm sterile dish, and necrotic yellowish parts of the tissue were removed by using a scalpel. It is critical to get tissue fragments from live (tumor) tissue, which is often pink in color, rather than necrotic tissue, which may seem yellowish, to produce organoids efficiently. Tissues were cut into small pieces with the help of a scalpel and scissors. Subsequently, small minced tissues were digested with a digestion medium for 50 min using an orbital shaker at 37°C.

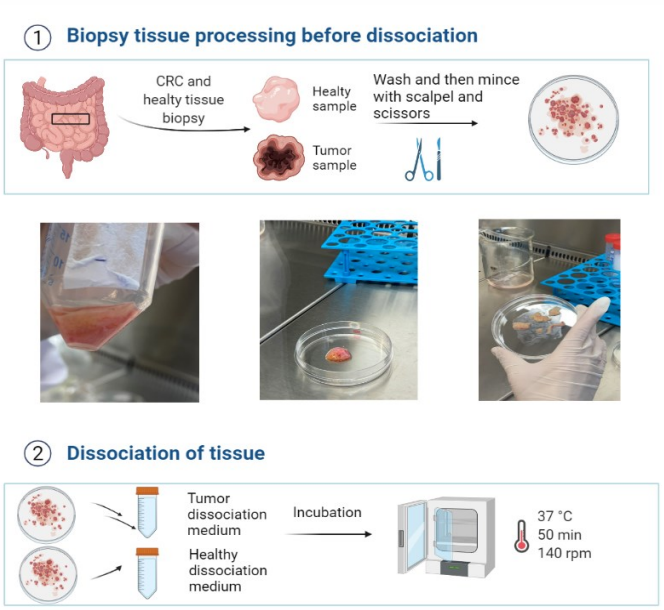

Figure 3. 1: Biopsy tissue processing and dissociation of the tissue.

3.6.1.2. Patient-derived organoid culture

When the mixture becomes cloudy and the remaining tissue fragments were disrupted, the mixture was washed once by topping up with adDMEM+++ and centrifuged at 600xg for 10 min at 4°C. Then the supernatant was aspirated and the pellet was resuspended again in adDMEM+++. After resuspending, the mixture was filtered using a 100 µm cell strainer. Then the same process was performed with a 40 µm strainer to remove the tissue pieces from the cells. Filtered cells were centrifuged at 450xg for 5 min at 4°C. Then the supernatant was aspirated. If the red blood cells were present (observed as a dark red pellet), the cell pellet was resuspended in a 3 ml red blood cell lysis buffer and was incubated for 5 minutes at room temperature. Afterward, the washing and centrifugation process was applied as mentioned above. In the final step, the pellet was resuspended in BME or Matrigel. The volume of ECM (Extracellular matrix) depends on the cell numbers. Drops of 30-40 ul were added to each well of a pre-heated 6-well plate. The plate was kept for 2 min in the cabinet before placing it into the incubator. Then, the plate was flipped and placed in the 37°C CO^2 incubator for 20 min. When dots were solidified, 2 ml of expansion medium supplemented with 100 µg/ml Primocin were added to each well. The plate was placed in the incubator and the medium was changed every 2-3 days with a pre-heated expansion medium until the dots become dense and crowded.

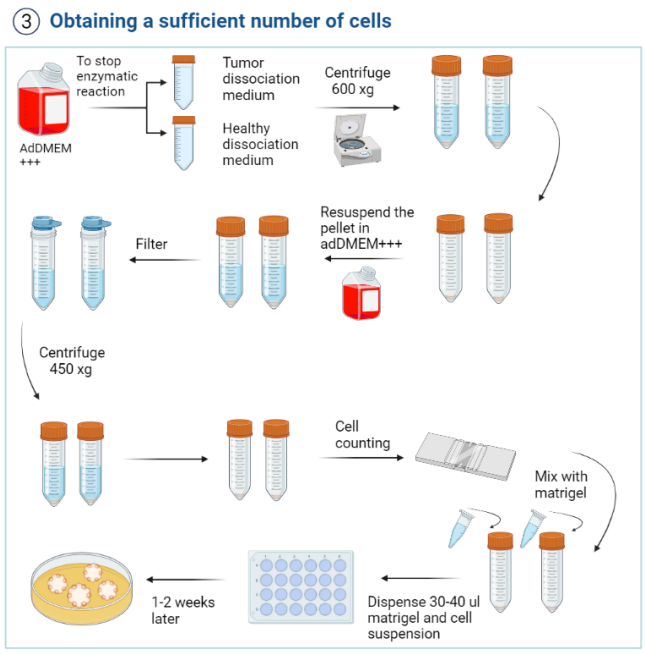

Figure 3. 2: Patient-derived organoid culture.

3.6.1.3. Passaging the PDOs

1 ml ice-cold basal medium was added to the wells. Organoids and matrigel were disrupted mechanically using a P1000 pipette and samples were transferred into a 15 ml conical tube. The plate was washed with an additional 1 ml of basal medium. Then 6 ml of basal culture medium were added to the tube and centrifuged at 400xg, 4°C for 5 min to the disrupt organoids. The supernatant was discarded and the cell pellet was resuspended with Matrigel in the range of 30-40 ul. Matrigel should be >80 (vol/vol). Preheated 6 well plates were used and 5 dots can be added to each well. The plate was flipped after waiting 2 minutes carefully and allowed matrigel for 10 min to polymerize at 37°C. Finally, 2 ml of culture medium was overlayed on each well. The entire medium should be changed every 3 days and the organoids should be passaged at the rate of 1:2 when they confluent at 80%.

3.6.2. Histological analysis of CRC PDO/Tissue

In order to maintain their 3D structure, PDOs were collected gently with cold +++AdDMEM, pelleted (300xg, 4 min, 4 °C), and fixed overnight in %10 Formalin. Following fixation, the fixative was discarded and PDOs were washed with 5 ml of PBS. After each wash should have waited at least 10 minutes for the organoids to settle to the bottom. 0.5% Eosin solution can be added to better visualize organoids and should be waited

for 30 minutes. After removal of Eosin, PDOs were washed once with 70% ethanol and then twice with 100% ethanol. For each wash, PDOs were incubated for 30 minutes. Following dehydration, PDOs were kept in a mixture containing xylene:100% ethanol at a ratio of 1:1 for 20 minutes. Then organoids were transferred into the metal block containing xylene. This step was repeated 2 times with a waiting time of 30 minutes. Finally, the xylene in the cassette was removed and paraffin was added to slowly. 4 μm sections of organoids were taken and then subjected to routine hematoxylin and eosin (H&E) staining. (van de Wetering et al., 2015). PDO H&E staining was conducted using the Autostainer XL ST5010 Leica platform (Izmir Biomedicine and Genome Center).

Immunohistochemistry staining of primary tumor tissue and their matched tumor organoids for CDX2 and CK20 was performed by a qualified pathologist at Kent Hospital Pathology Laboratory.

3.6.3.1. Whole Exome Sequencing (WES) Analysis

The tumor and healthy tissues and the patient-derived organoids were sent to Epigenetiks Biotech Company to perform the whole-exome sequencing. Initially, FASTQC was used to assess the quality of each FASTQ file, and the results were summarized via multiQC. Next, trimming and adapter removal were performed using trimmomatic. The processed FASTQ files were then mapped to the human reference genome (hg38) using bwa. The resulting SAM file was then pre-processed, including cleaning the SAM file, sorting SAM by coordinate and converting to BAM, fixing mate information, and marking PCR duplicates (all via Picard). For samples that were sequenced in multiple lanes, data for all lanes were combined at this step. Finally, base quality score recalibration (GATK) was performed, yielding analysis-ready BAM files for the respective samples.

For detecting germline variants (single nucleotide variants (SNVs) and short insertion/deletions (indels)) GATK–HaplotypeCaller was used. For detecting somatic SNV/indels, GATK–MuTect2 was used. Both germline and somatic SNV/indels were annotated using GATK–Funcotator. For detecting somatic copy number alterations (SCNAs), ExomeCNV was used. Annotations of gene-level SCNAs and cytoband annotations were performed via an in-house script.

3.6.3.2. Bioinformatic statistical analysis

All remaining analyses and visualizations were performed using R (4.2.0).

3.7. Research plan and timeline

Table 3. 3: Research timeline.

Work Package (WP) Title/Definition	MONTHS																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Patient-derived CRC Organoid Culture	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
Histological Characterization of CRC Organoids							x	x	x	x	x	x	x	x	x	x		
Molecular Characterization of CRC Organoids							x	x	x	x	x	x	x	x	x	x		
Writing Thesis															x	x		

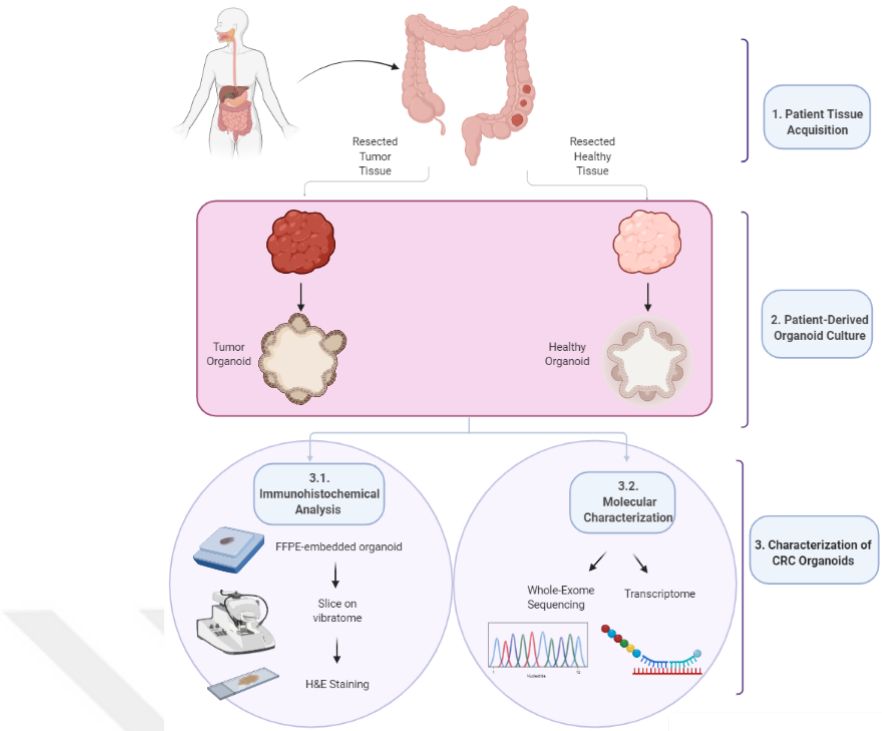

Figure 3. 3: Workflow of the study.

3.8. Limitations of the Research

The limitations of this study are that an insufficient number of cells are removed during the operation of the patient's surgical material, the efficiency of organoid formation varies from patient to patient, and the expensive matrigel chemical used as ECM in organoid culture.

3.9. Ethical committee approval

The ethics committee reviewed our study with protocol number 2020-040 on September 25, 2020, and found it to be ethically suitable.

4. RESULTS

4.1. Characteristics of Patients

The surgical resection materials total of 15 patients diagnosed with colorectal cancer was obtained for this research study between May 2021 and May 2022. Organoid setup could not be established from the samples of five patients during protocol optimization. The remaining 10 patients' organoids were successfully established. The table below summarizes the information about the patients (Table 4.1). Adenocarcinoma is the diagnosis for all of

these patients. Four patients' tumors were found in the sigmoid, and two patients' tumors were found in the right colon.

Table 4. 1: Clinical data from the patients diagnosed as colonic adenocarcinoma.

Patient No (Lab Code)	Sex	Age	Tumor Location	Lymph Node Metastases	Tumor Stage
P1 (KH-FĞ-091221)	Female	73	Right colon	No	T3N0
P2 (KH-ŞR-140122)	Female	58	Sigmoid colon	Yes (11/15)	T4bN2b
P3 (KH-MAK-240222)	Male	60	Sigmoid colon	Yes (22/27)	T3N2b
P4 (KH-SR-210322)	Female		Sigmoid colon	No	T4aN0
P5 (KH-TZ-110322)	Male	72	Right colon	No	T3N0
P6 (KH-AM-211221)	Female	63	Sigmoid colon	Yes (3/11)	T3N1b

4.2. Establishment and Passaging of PDOs

4.2.1. Establishment of PDOs

In the protocol of patient-derived tumor and healthy organoid establishment, the article by van de Wetering et al. (2015) was taken as reference. However, at first, there were challenges with various issues due to the lack of an optimized protocol. For example, few cells obtained from patient tissue, insufficient content of the medium used in the organoid culture medium, and the effect of dot size on cell growth affected organoid culture efficiency. Then, the method was optimized over the period. In order to optimize the method in the first 6 months, the following experiments were carried out.

4.2.1.1. Optimization of Dissociation Medium

To increase the number of cells obtained from tumor and healthy primary tissue, two different dissociation medium contents and different incubation times were tested as shown in Table 4.2 The contents of two different dissociation mediums are shown below.

Table 4. 2: Tested different digestion medium ingredients.

Dissociation medium 1	adDMEM+++, Dispase, Hyaluronidase, Collagenase, Y27632, DNAse1
Dissociation medium 2	TrypLE, Y27632

Since the enzymatic process was more vigorous in the first dissociation medium, more cells were obtained from the tissues. Therefore, further trials were continued with the dissociation medium 1. In addition, during the digestion stage, when the tissues are large, a mucosal structure is formed. This is a situation that makes it difficult to obtain enough cells. To prevent this, DNase 1 was added to the digestion medium. With all these optimizations, an increase in the number of cells was obtained. When we examined the dissociation time, the tissues could not dissociate well in less than 50 minutes. However, this time can be increased as the primary tissue size increases.

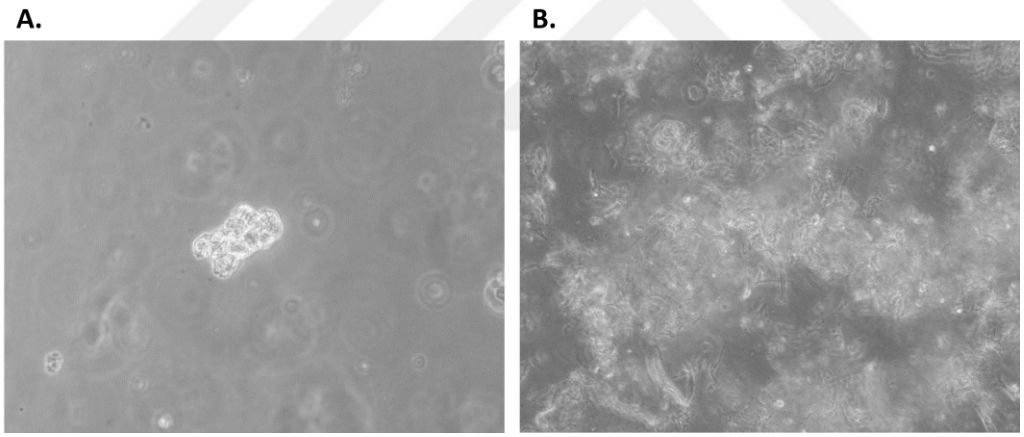

Figure 4. 1: One of the first patient-derived organoid trials from the same patient.

A. Dissociation medium 1 was used for tumor organoid culture. Organoid formation efficiency was higher.

B. Dissociation medium 2 was used for tumor organoid culture. Tissue fragments could not be removed because of the Dissociation medium 2. As a result, the efficiency of organoid formation was reduced due to a lack of sufficient cells.

4.2.1.2. Optimization of Expansion Medium (EM)

Activation of the Wnt pathway is essential for organoid culture expansion. Wnt proteins regulate cell proliferation, differentiation, and self-renewal of stem cells via promoting β--catenin-dependent signaling through the Wnt receptor frizzled (FZD) and the co-receptors LRP5 and LRP6 (Janda et al., 2017). To activate the Wnt signaling pathway, it is possible to use two different Wnt sources. These are Wnt3A conditioned media and Wnt recombinant protein. Wnt3A conditioned medium is a less expensive supply of Wnt ligands, however, it produces a lower degree of Wnt activation and can be inconsistent from batch to batch (Pleguezuelos-Manzano et al., 2020) . On the other way, recombinant Wnt protein has inherent instability due to its high hydrophobicity which is caused by post-translational modifications (Barbáchano et al., 2021). Also as another recombinant protein developed recently, Wnt surrogate Fc fusion protein is a Wnt agonist. It's a FZD–LRP5/LRP6 heterodimer with FZD5/FZD8-specific and generally FZD reactive binding domains that are water-soluble. These Wnt agonists, like WNT3A, elicit a distinct β-catenin signaling response and promote the development of a wide spectrum of primary human organoid cultures (Janda et al., 2017). For this reason, Wnt3a Condition Medium, which we used in the Expansion medium, was thought to affect organoid development since its activity period in culture was low, and Wnt surrogate Fc fusion protein was used in the later stages. Figure 4.1 shows that organoids grow with better efficiency when recombinant protein is used in the expansion medium.

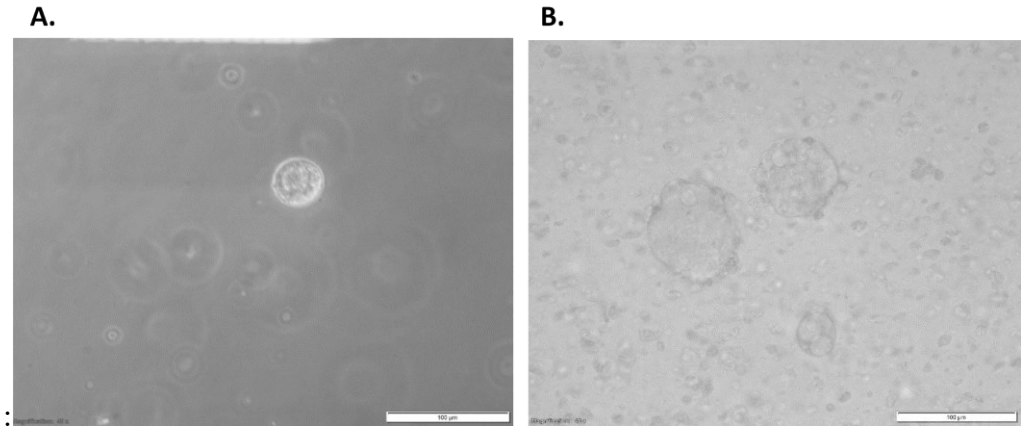

Figure 4. 2: Patient-derived tumor organoids bright-field images with 40X objectives from different donors.

A. Tumor organoid images in medium containing Wnt3a Condition Medium on day 6. The organoid formation efficiency is very low and their diameter is small.

B. Tumor organoid images in medium containing Wnt surrogate Fc fusion protein on day 6. Organoid formation efficiency and diameters have increased.

4.2.1.3. Matrigel Volume Optimization

The volume of Matrigel is determined by the size of the pellet. Cells plated at the right density have enhanced the efficiency of organoid culture establishment. When cells are seeded too densely, they may begin to die in the middle of the Matrigel dot. In order to optimize the Matrigel volume and cell number, the organoid formation was examined by seeding different numbers of cells into dots of different volumes. For instance, When 7000 cells were seeded in a 20µl Matrigel dot, the density was low and a low number of organoid formations was observed. It was observed that when 60,000 cells were seeded in 30-40µl dots, the cells were healthier and the organoid number and diameter were larger as shown in Figure 4.3.

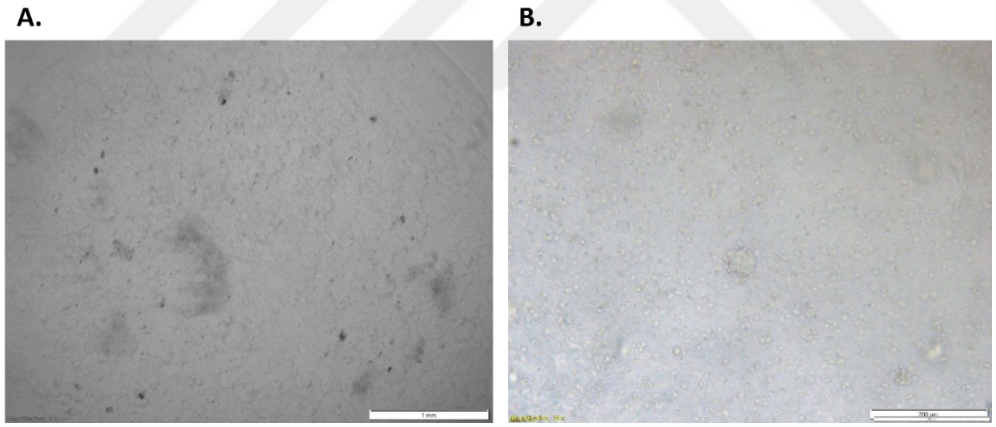

Figure 4. 3: Matrigel volume and number of cell optimization of patient-derived tumor organoids bright-field images with 4X objectives from different donors.

A. Images of the PDTO culture of the patient. 7000 tumor cells were plated in a 20µl Matrigel dot. Organoid efficiency is low.

B. Images of the PDTO culture of the other patient. 60,000 tumor cells were plated in a 30µl Matrigel dot. Organoid efficiency is high.

With the above-mentioned optimizations, patient-derived organoids were produced from different patients (Figure 4.3).

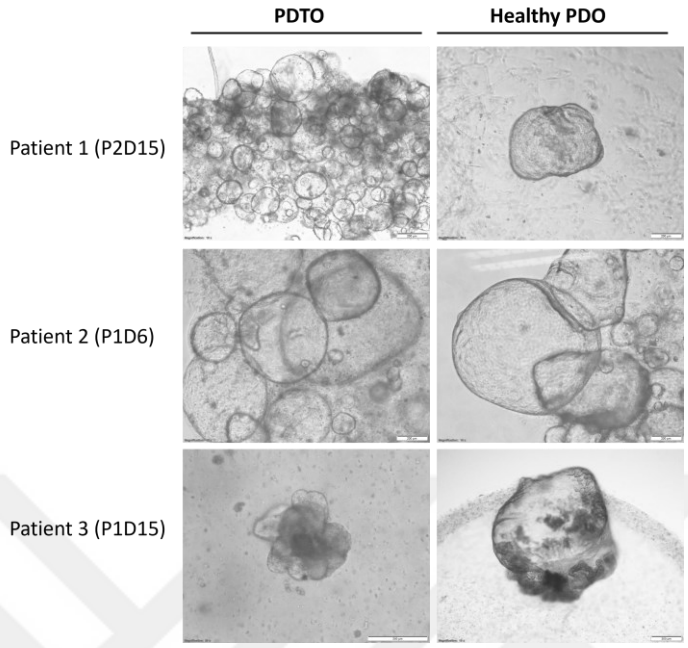

Figure 4. 4: Tumor and healthy counterpart organoid images from 3 different patients (P:Passage number, D: Day).

PDOs can have a variety of morphological structures. Some patients' organoids are filled, dark, and dense, while others grow in the form of empty rings due to tumor heterogeneity. This variation may occur between patients or even within the same patient (Figure 4.5).

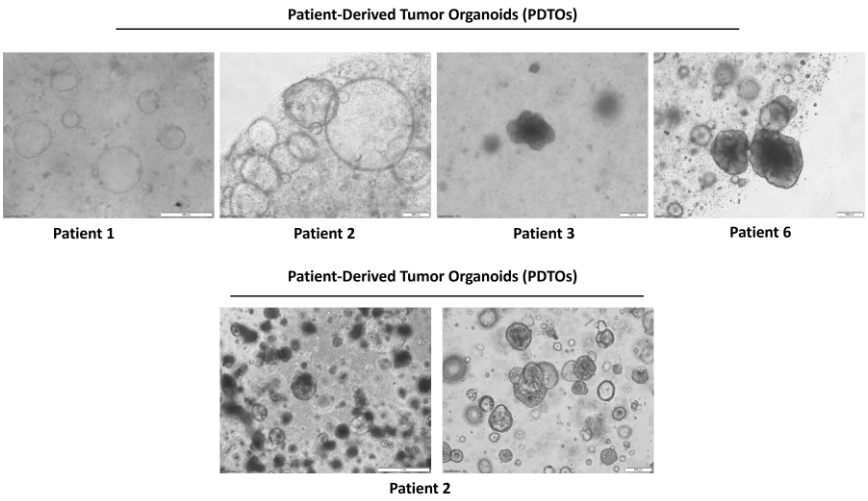

Figure 4. 5: Image of PDTOs having different morphologies in different patients and also different morphologies in the same patient.

4.2.2. Passaging of PDOs

PDOs also need to be passaged like 2D cells. On average, PDOs were passaged every 2 weeks. However, this varies between different patient organoids. PDOs from some patients grow quite fast, whereas those from others grow much more slowly. For example, Patient 2 PDTOs are growing faster than Patient 6 PDTOs (Figure 4.6).

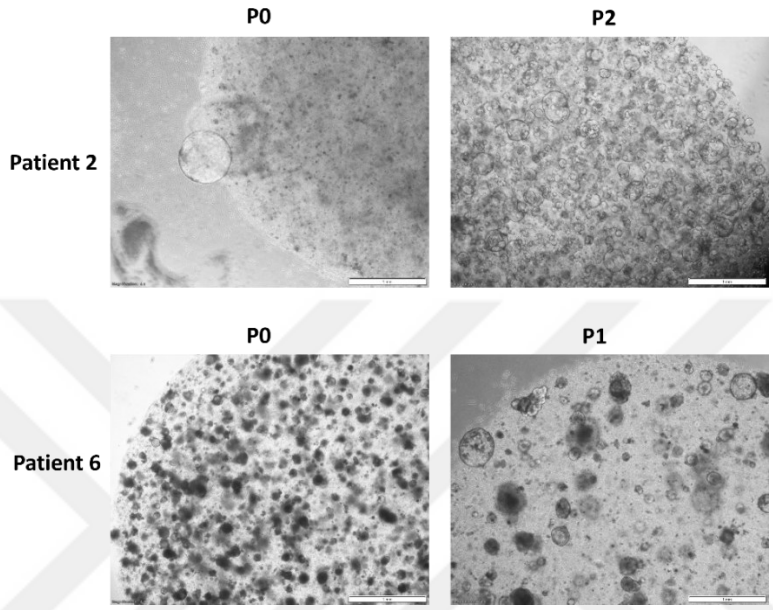

Figure 4. 6: Image of PDTOs having different growth rates in different patients (P:Passage number).

If some organoids are very dense, they must be passaged at least once a week. When structures grow too big, they have a tendency to emerge from the matrigel dome. As the organoids grew in size (bigger than 200 μm), they were passaged using the enzymatic method. Mechanical fragmentation method such as pipetting alone was not sufficient. It was observed that the organoid formation efficiency increased when trypsin was used. It should also be noted that the growth rate of the organoids slowed as the number of passages increased and some PDO morphology can be changed during serial passage. (Figure 4.7). Therefore, we were able to establish organoids from human tumors and healthy tissues with a 90% success rate as a result of all of these optimization steps and passaging efforts.

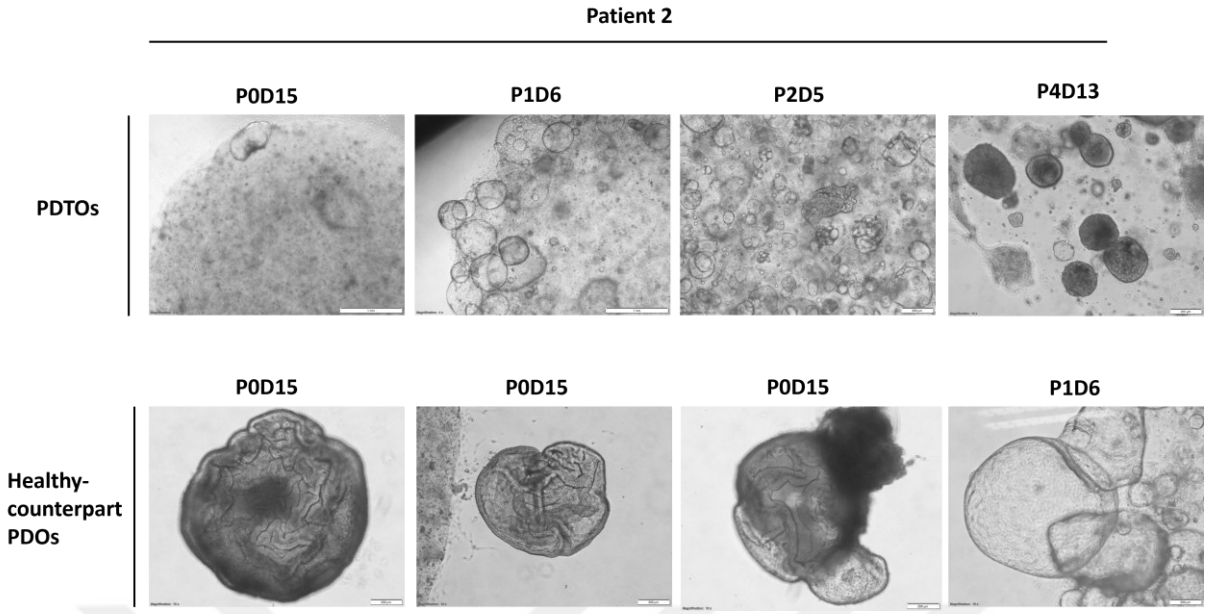

Figure 4. 7: Image of PDTOs and their healthy-counterpart PDOs (P:Passage number, D: Day).

4.3. Histological Characterization of Patient-Derived CRC Organoids

Using the organoid culture system we optimized, we successfully established an organoid culture panel from tumor and healthy surgical resection specimens of patients with the adenocarcinoma CRC type as mentioned in Table 4.1. Firstly, to determine whether patient-derived colorectal tumor organoids (PDTOs) are robust models for modeling primary tumor properties, the biological properties of primary tumors and their corresponding PDTOs were compared first. Therefore, various histological characterization was performed on the organoids from Patient 1, Patient 2 and Patient 4. A qualified pathologist examined the histology of three primary tumors and their matched PDTOs from different patients.

4.3.1. Immunohistochemistry Characterization

In Hematoxylin and eosin (H&E) staining, eosin stains the cytoplasm, and hematoxylin stains the cell nuclei, showing the general histological structure of the organoid. According to the above-mentioned and obtained results, CRC PDTOs exhibited a wide range of growth rates and morphology. H&E staining also demonstrated that tumor-derived organoids reflected PDTO morphologies ranging from thin-walled cystic structures to solid/compact structures (Figure 4.8).

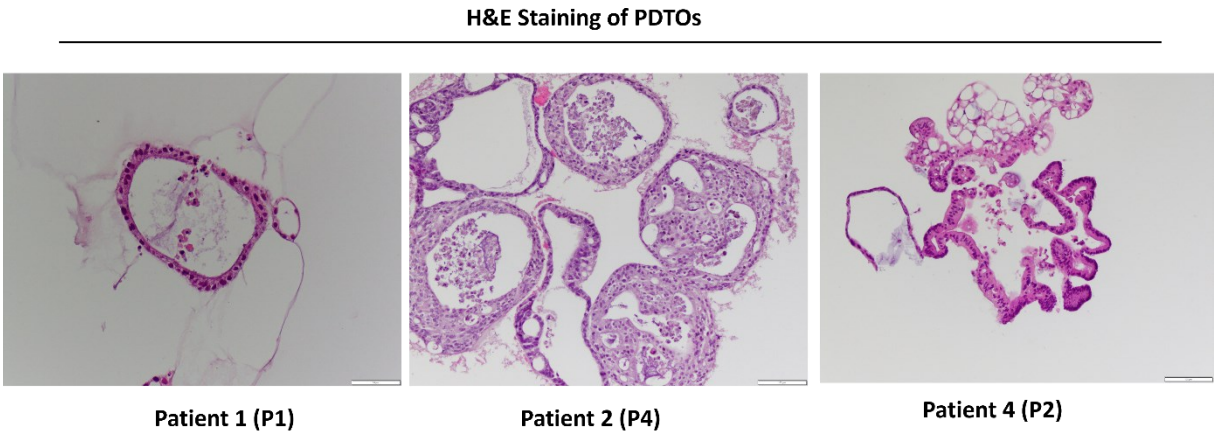

Figure 4. 8: H&E Staining of PDTOs from different patients. Tumor organoids had a wide range of morphologies, from thin-walled cystic forms to compact structures (P:Passage number).

While PDTOs typically have a more cuboidal appearance, the histological pattern between the primary tumor and their PDTOs was generally well maintained as shown in Figure 4.9. Tissue staining showed the resection materials had irregular adenoid and cribriform structures consisting of large, atypical cells with pleomorphic nuclei and extensive eosinophilic cytoplasm. Similar morphology was also observed in the organoids section (Figure 4.9).

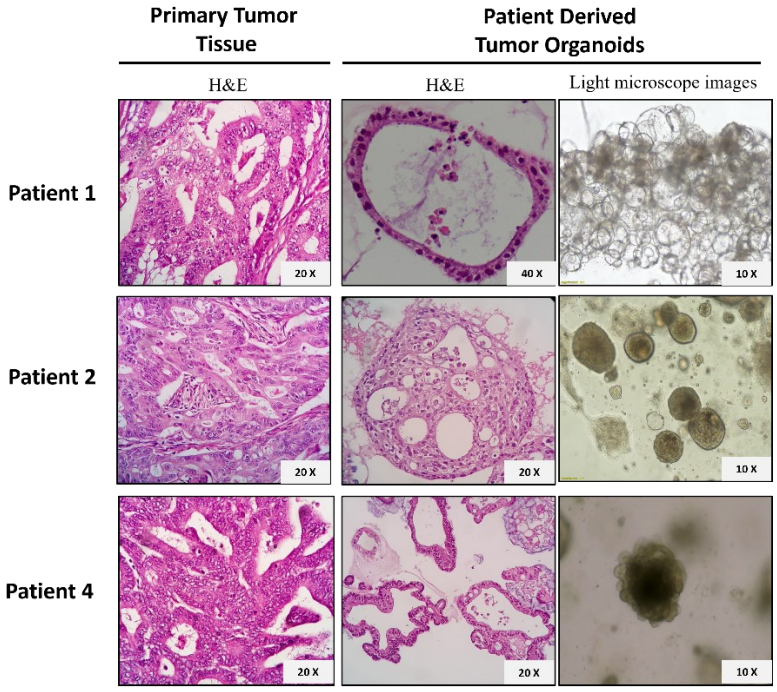

Figure 4. 9: H&E staining of primary tumor tissues and their tumor organoids from different patients.

Also, immunohistochemical staining was performed with CDX2 and CK20 markers. Patient 2's and Patient 4's primary tumor tissue and PDTOs were compared. Diffuse strong positivity was detected in all samples. The positivity of these two markers, which are used as immune markers for adenocarcinomas, in tumor organoids was significant (Figure 4.10).

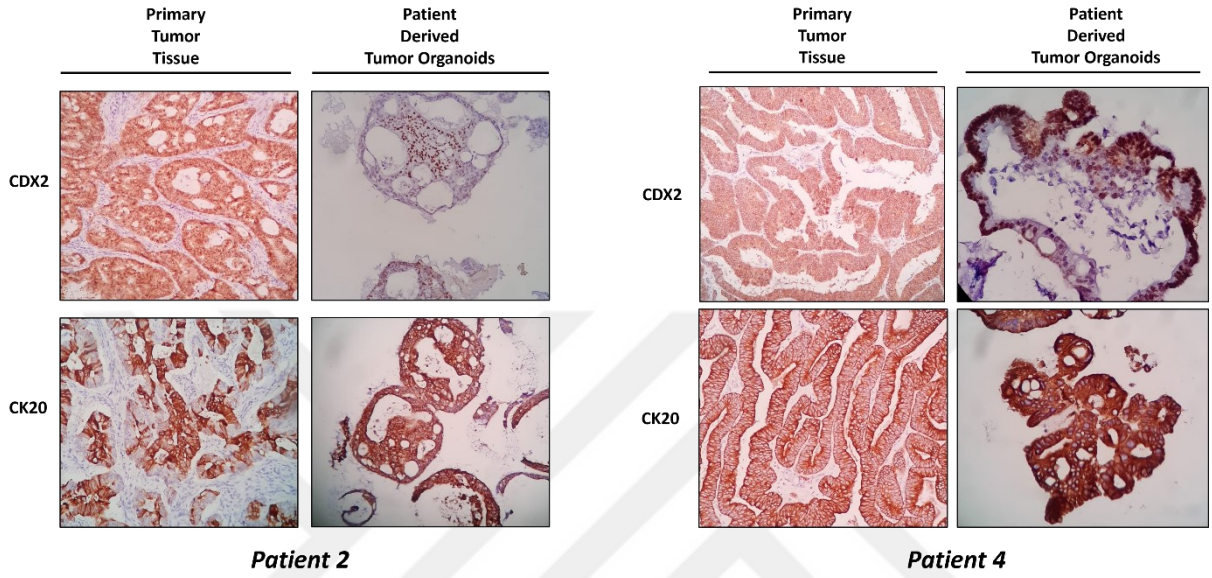

Figure 4. 10: CDX2 and CK20 IHC staining of Patient 2 and Patient 4's primary tumor tissue and their matched PDTOs.

4.4. Molecular Characterization

4.4.1. Whole-Exome Sequencing (WES)

To characterize the molecular features of the organoids, we performed WES in CRC PDTO, healthy-counterpart PDO, and their corresponding surgical material. Based on bioinformatical analyses, tumor mutational load in between PDO pairs (as 11.49 mutation/Mb) was higher in between CRC and healthy-counterpart tissue (as 8.41 mutation/Mb) (Fig 4.11).

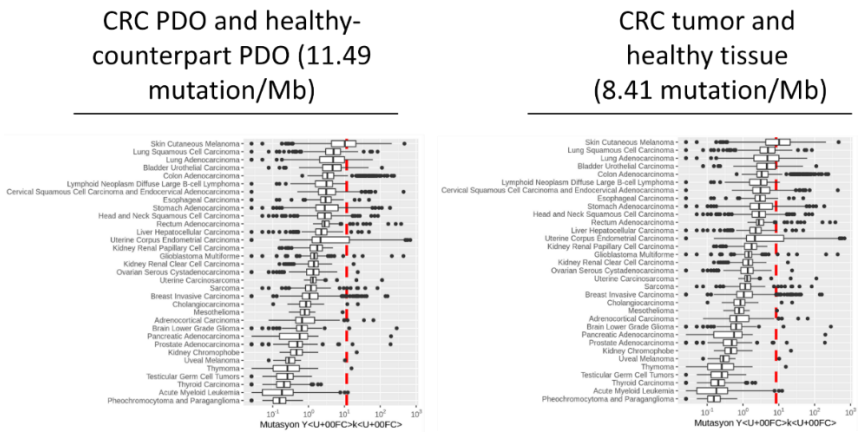

Figure 4. 11: Tumor mutational load (TML) rates in CRC patient-derived organoids and their clinical samples from TCGA profiles.

Similarly, somatic copy number alterations also were higher in PDO pairs in comparison to original tissue pairs (Fig 4.12).

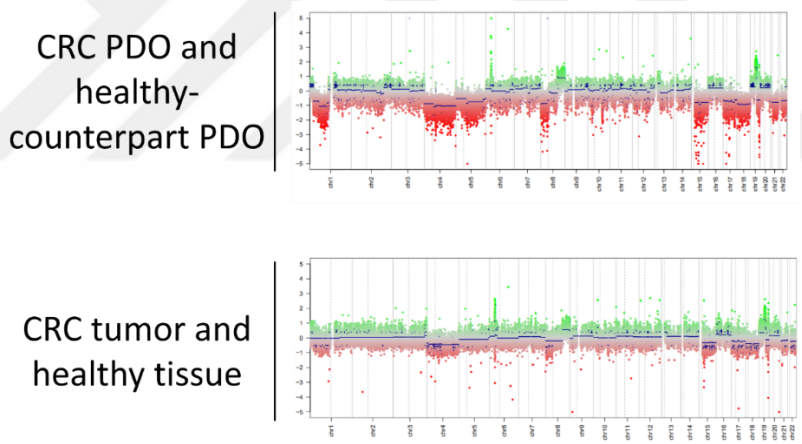

Figure 4. 12: Somatic Copy Number Alterations (SCNA) Graph in CRC patient-derived organoids and their original sample pairs.

Moreover, healthy tissue and its PDO were significanly similar in mutations at the variant-level and the gene-level as %88,8 and %97, respectively (Fig 4.13).

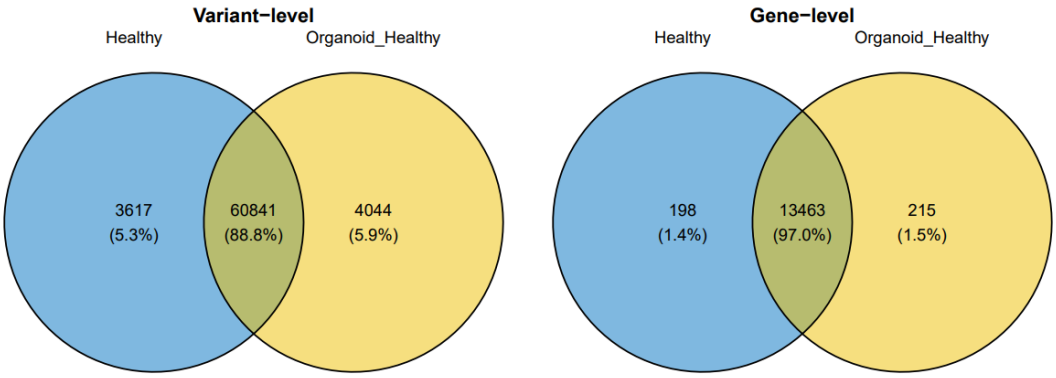

Figure 4. 13: Venn Diagram showing Variant-level and Gene-level similarity between healthy PDO and healthy tissue of the Patient 2.

When we compare the most frequently mutated genes (n=22) in CRC as shown in Table 4.3, 18 genes with no alterations and 4 genes (ATM, NRAS, SMAD4, and TP53) with missense and truncated mutations were detected in both CRC PDO and its original tumor sample (Fig 4.14).

Table 4. 3: Most frequently mutated genes in CRC.

Most frequently mutated genes in CRC

APC	TP53	KRAS	PIK3CA	FBXW7	SMAD4	TCF7L2	BRAF	NRAS	ARID1A	EGFR
ERBB4	ARID2	ERBB3	AXIN2	SMAD2	CTNNB1	POLE	MSH3	ATM	MAP4K1	ROS1

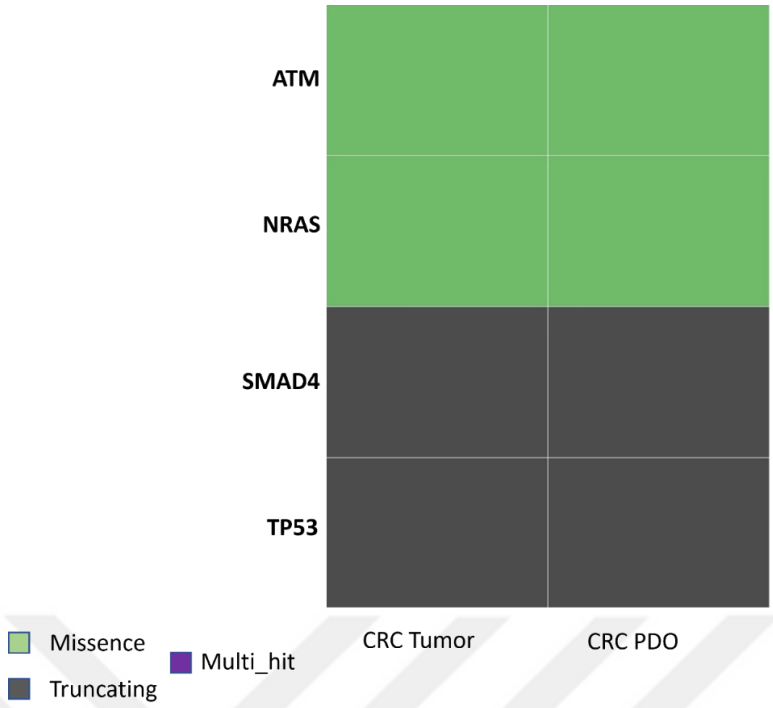

Figure 4. 14: Comparative somatic mutation analysis between CRC Tumor and CRC PDO of genes frequently mutated in CRC. As a result, tumor-specific mutational signature matched in both PDO and its original tumor.

Furthermore, to determine drug and biomarker variants, patient-derived organoids and their clinical samples were compared using Cancer Genome Interpreter (CGI) and CGI and/or Clinical Interpretation of Variants in Cancer (CIViC)) sequentially. %100 matched significant somatic variants were detected between the two groups (Figure 4.15 and Figure 4.16).

Drug Target Variants

PDOs (patient-derived tumoroid and normal PDO)

Gene	Variants	Genome	Protein	VAF
NRAS	Missense_Mutation	g.chr1:114713908T>A	p.Q61L	0.498
NRAS	Missense_Mutation	g.chr1:114713908T>A	p.Q61L	0.499
ALK	Missense_Mutation	g.chr2:29920489G>T	p.D57E	0.527

Clinical samples (primary tumor and normal tissue

Gene	Variants	Genome	Protein	VAF
NRAS	Missense_Mutation	g.chr1:114713908T>A	p.Q61L	0.184
NRAS	Missense_Mutation	g.chr1:114713908T>A	p.Q61L	0.185
ALK	Missense_Mutation	g.chr2:29920489G>T	p.D57E	0.217

100% matched between PDOs and their primary tissue

Figure 4. 15: Clinically significant somatic variant genes for drug target variants in CRC patient-derived organoids and their clinical samples from Cancer Genome Interpreter (CGI).

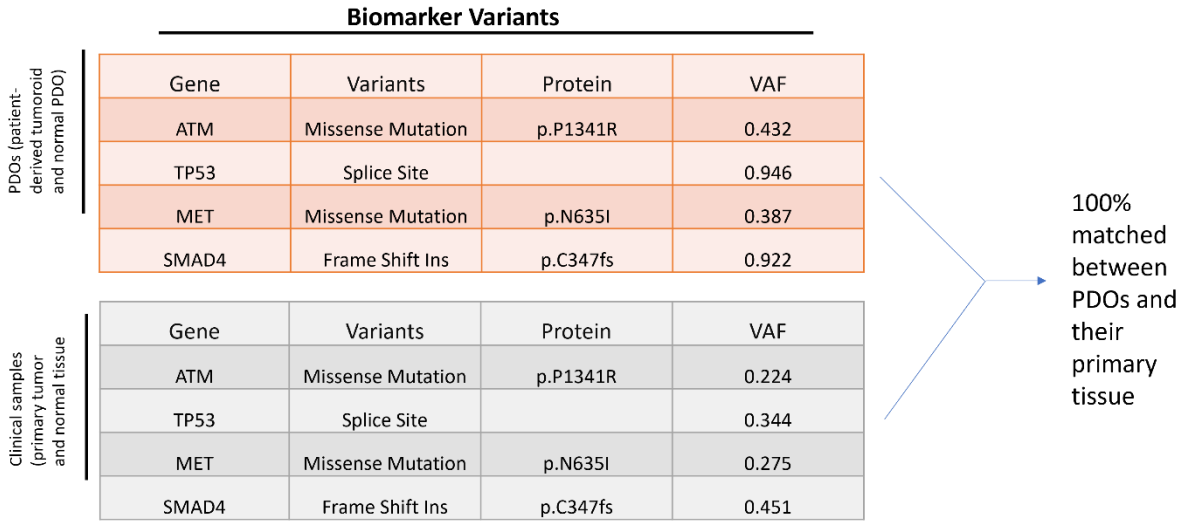

Figure 4. 16: Clinically significant somatic variant genes for biomarker variants in CRC patient-derived organoids and their clinical samples from CGI and/or Clinical Interpretation of Variants in Cancer (CIViC)).

5. DISCUSSION

Many improvements in early detection and treatment method have been developed for Colorectal cancer (CRC) in recent years but it is still the third most common cancer worldwide (H. T. Nguyen & Duong, 2018). Clinical trials for many drugs that show promise in cancer models have failed. This indicates that there is a barrier between in vivo and in vitro model systems and clinical applications (Ji & Wu, 2020). The substantial differences between many patients in responsiveness to antineoplastic therapies emphasize the need for more accurate treatment selection. One of the goals of a personalized therapy platform is to predict treatment responses in individual patients. Numerous attempts have been made for this, but currently, no method has gained clinical acceptance. (Weeber et al., 2015).

For many years, cancer cell lines have been a versatile model in cancer research. Barely, cell lines can not be modeled for each patient. The cells that were derived from primary patient tumors might have undergone genetic changes, resulting in the absence of genetic heterogeneity of the corresponding original tumors(Ji & Wu, 2020). Patient-derived tumor xenograft models have a higher uptake rate and early signs of predictive value but at least the 6-8 months required to obtain adequate material may inhibit their clinical decision-making tool (Hidalgo et al., 2015). There is a need for models that can more accurately

represent the genetic diversity of tumors so that we can better understand specific changes in tumorigenesis and therapeutic susceptibility (Ji & Wu, 2020).

Recently, new cancer models have been developed using 3D culture technology. The new culture system has been called an organoid. Patient-derived organoids (PDO) derived from patients hold a lot of promise for personalized cancer treatment (Benson et al., 2018). PDOs can reflect the histopathological features of the original tumor, its genetic profile, mutational environment, and even its response to therapy, as demonstrated in many studies (Xu et al., 2022). This 3D organoid culture system was also adapted for human CRC cells. Two independent groups reported that healthy and tumor organoids can be derived from surgical resections at >90 percent success rates (Sasaki & Clevers, 2018; van de Wetering et al., 2015).

The methods of organoid derivation have changed dramatically during the previous decade, including the choice of tumor tissue source and downstream processing. While each organoid derivation approach allows for the testing of distinct hypotheses about cancer biology and therapy, current implementation methods are not standardized, compromising their usefulness in clinical trials (LeSavage et al., 2022).

The overall aim of this project is to generate PDOs from patient tissues with in-house protocol and to demonstrate histological and genetical similarities to the original tissue. There are different protocols for establishing organoids from primary tissue, depending on the type of epithelial tissue and from where it was obtained (normal or tumor). For instance, the digestion of primary tissue protocols applied by the different research groups is defined (Driehuis et al., 2020). In our study, tumor organoids from tumor tissues of colorectal cancer patients and healthy organoids from healthy tissue of the same patient were produced with a 90% success rate by making various optimizations in the digestion stage, organoid medium content, and dot sizes.

Patient-derived tumor organoids show differences in growth rate and morphology also they can present patient-specific heterogeneous morphologies (thin-walled cystic structures or solid/compact structures) as demonstrated by H&E staining in studies (van de Wetering et al., 2015; Yao et al., 2020). Our produced CRC PDTOs also showed different morphologies such as empty rings or filled, dark, and dense structures in different patients and also in the same patients at different passage numbers. H&E staining also showed that adenoid and cribriform

structures were detected both in tumor tissue and their PDTOs. All in all, these studies show that organoids recapitulated the histological features of the original tumor tissues.

Organoids derived from cancer patients have been shown to mimic the genomic profiles of corresponding tumors in previous studies. They retained the mutational spectrum observed in paired tumors in the most frequently mutated colorectal cancer genes with a %94.4 overlap (van de Wetering et al., 2015; Vlachogiannis et al., 2018; Yao et al., 2020). To characterize the molecular features of the PDOs, tumor organoids established from tumor colon epithelium and their matched healthy organoids established from healthy colon epithelium were subjected to WES analysis. Tumor mutational load was compared between PDO pairs (tumor organoid and healthy-matched organoid) and tissue pairs (tumor and healthy tissue) on the same Patient 2. PDO pairs (as 11.49 mutation/Mb) had a higher mutation load rate than tissue pairs (as 8.41 mutation/Mb). Likewise, somatic copy number alterations also were higher in PDO pairs in comparison to original tissue pairs.

Somatic variants in the coding regions in healthy organoid cultures had a highly concordant similarity ratio with the corresponding tissue sample. For instance, healthy tissue and its PDO mutations were compared at the variant level and the gene level. %88,8 and %97 similar mutations were detected respectively. For the comparison of tumor organoids and their tissue somatic mutation profiles, the genes known to be mutated the most in 22 CRCs were selected. 18 genes with no alterations and 2 genes (ATM and NRAS) with missense and 2 genes (SMAD4 and TP53) with truncating mutations were detected in both CRC PDO and its original tumor sample.

Besides that, to evaluate drug and biomarker variants, patient-derived organoids and their clinical samples were compared. %100 paired substantial somatic variants were detected between both the two groups.

As a result, these data show that organoid cultures from colorectal cancer can be created with the protocol we optimized. WES data analysis showed that CRC PDO recapitulates the original tumor in the context of caring tumor type mutations while healthy PDOs were exactly similar in genetic makeup with regard to all somatic mutations in original counterparts. However, in tumor PDOs, there is still a need to improve culture conditions to mimic the tumor microenvironment (such as the immune cell compartment of the tumor) to use them in drug response.

6. CONCLUSION AND FUTURE ASPECTS

In conclusion, our research shows that basic response parameters may be determined within weeks of the derivation of PDOs and can be used as a tool for treatment response modeling. Testing the candidate drugs to be selected as a result of PDO bioinformatic analyzes or the drugs that the clinician wants to try according to the mutation profile of the patient, will help to obtain more specific results in the future studies.

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8. CURRICULUM VITAE (CV)

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RESEARCH FIELDS

Molecular Biology and Genetics, Cancer Research, Organoid Technology

İZMİR BİYOTIP VE GENOM MERKEZİ
GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU (İBG-GOEK)
KARARI

Toplantı Tarihi	: 25/09/2020	**Toplantı Günü**	: Cuma
Toplantı Sayısı	: 12	**Toplantı Saati**	: 10:30

Sayın Şerife Esra ERDAL BAĞRIYANIK,

2020-040 Protokol No'lu; sorumlusu olduğunuz "Kolorektal Karsinoma Tümöroidlerinde Kişiye Özel Kemoterapi Etkinlik Testinin Geliştirilmesi" başlıklı araştırmanın uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Dr. Serap ERKEK
Başkan V.

Pandemi süresince online ortamda gerçekleştirilen toplantımızda alınan kararlar tek imzalı olarak düzenlenmektedir. Pandemi sona erdikten sonra ıslak imzalı karar belgesi teslim edilecektir.