Organoid in colorectal cancer: progress and challenges

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Abstract

Patient-derived tumor organoids (PDOs) currently represent important modeling tools in pre-clinical investigation of malignancies. Organoid cultures conserve the genetic and phenotypic characteristics of the original tumor and maintain its heterogeneity, allowing their application in many research fields. PDOs derived from colorectal cancer (CRC) have been used for genetic modeling to investigate the function of driver genes. Some researchers have been exploring the value of CRC PDOs in chemotherapy, targeted therapy, and radiotherapy response prediction. The successful generation of PDOs derived from CRC could deepen our understanding of CRC biology and provide novel tools for cancer modeling, for realizing precision medicine by assessing specimens from individual patients *ex vivo*. The present review discusses recently reported advances in CRC PDOs and the challenges they face as pre-clinical models in CRC research.

Keywords: Colorectal cancer; Patient-derived tumor organoids; Pre-clinical model

Introduction

Colorectal cancer (CRC) represents a major malignancy with an incidence increasing year by year in China, and ranks third in terms of cancer mortality.[1,2] The clinical outcomes and treatment responses of patients with CRC vary greatly,[3] yet remain unsatisfactory. The so-called standard treatment is limited in current clinical practice.[4,5] Meanwhile, a multitude of drugs demonstrating effectiveness in cancer models eventually failed in clinical trials, indicating a barrier between *in vivo* and *in vitro* model systems and clinical practice. Therefore, models should be developed, which can accurately reflect the genetic diversity and specificity of tumors, facilitating the understanding of specific changes in tumorigenesis, tumor maintenance, and therapeutic sensitivity.

Fluoropyrimidines remain the backbone of chemotherapeutic agents for metastatic/recurrent CRC.[6-9] Their treatment outcome has been improved greatly from 12 months to over 30 months in terms of overall survival.[10] With the inclusion of biologically targeted agents, such as monoclonal antibodies targeting epidermal growth factor receptor (EGFR) and anti-angiogenic agents regulating vascular endothelial growth factor signaling,[11] patient survival has been increased, especially after stratification by Ras/Raf mutation[12] and sidedness.[13] Studies have shown that instability-high CRC can benefit from programmed cell death 1 blockade.[14-16] Despite a step forward toward personalized treatment, it still is impossible to identify which patient might be responsive or not. The treatment is essentially experimental, and results are uncertain. Some patients may respond well and others not, or even be refractory after the initial treatment.[17]

Cancer, including CRC, involves multiple and complex events, with a series of genetic changes.[18] Tumor heterogeneity is particularly evident in CRC, caused by chromosome (CIN) or microsatellite instability,[19] which has an important impact on targeted therapy[3] and response prediction.[20]

Common cancer models, derived from primary tumors and mimicking tumor development, include malignant cell lines and xenografts in experimental animals. Human malignant cell lines are commonly cultured under two-dimensional culture conditions *in vitro*. In addition, only rare clones derived from primary patient tumors can be extended and maintained for many generations. Thus, such cell lines might have undergone tremendous genetic changes, resulting in the absence of genetic heterogeneity of the corresponding original tumors. Although animal

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cancer models are important in cancer research, their establishment takes time. In addition, animal models often fail to faithfully reflect the pathogenesis of patients since human cancer is histologically complex and genetically heterogeneous. Primary patient-derived tumor xenografts (PDTXs) are obtained via transplantation of freshly collected human tumors into immunodeficient mice. PDTXs enable *in vivo* assessment but are time-consuming and require large amounts of resources.

Recently, three-dimensional (3D) culture technology has led to the development of novel cancer models. When gut stem cells expressing leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) are supplemented with R-spondin-based culture conditions and maintained in a 3D extracellular matrix, they are capable of generating a steadily growing, self-organizing epithelial structure physiologically comparable to the non-diseased intestinal tissue. The newly developed culture system was termed organoid culture, and is eventually applicable for generating CRC organoids.[21] Subsequently, R-spondin-based culture conditions have been developed for noncancerous and cancer tissues of the pancreas,[22,23] stomach,[24] prostate,[25] and liver.[26] Compared with PDTXs, organoids from patients can be easily expanded for a long time. In this review, we discuss important advances in patients with CRC-derived tumor organoids [Table 1] and the challenges they face as pre-clinical models in CRC research.

Organoid Culture: An *in Vitro* Model Mimicking the Primary Tumor

Human tumor organoids have been generated from colon,[21,33] pancreas,[23] prostate,[38] breast,[39] gastric,[40] lung,[41] esophageal,[42] bladder,[43] ovarian,[44] kidney,[45] and liver[46] tumor tissues. Many studies have demonstrated that cultured organoids closely reproduce the genetic and morphologic heterogeneity of malignant cells in the initial cancer tissue.[33,47,48] It was shown that primary tumors produce a large number of different organoids in the culture process, which suggests that the heterogeneity of primary tumors is largely conservative.[33] Transcriptome analysis also confirmed the heterogeneity of these cultures, which produce different expression profiles. A high degree of consistency in the morphologic and mutational characteristics of tumor organoids and matched patients' tumors was demonstrated by histologic analysis and DNA sequencing.

Maintaining cancer cell heterogeneity in culture is critical in targeted drug development.[49,50] Gene expression assessment in solid tumor samples and respective cell lines has revealed substantial differences.[51] In addition, cell lines usually do not include all gene profiles of tumors. It is known that tumor tissues and cell lines retain common mutations rather than scarce mutations.[52] This could partially account for the low value of data generated in cell lines in translational research.[51,53] Of high importance, early and late-passage organoids display mostly identical mutations.[47,54]

Characteristics of Organoids for Clinical Application

Tumor organoids closely mimic the clinical phenotypes of human malignancy as well as cancer progression

CRC represents a heterogeneous malignancy with varying clinical features and prognoses. Organoids could routinely be obtained from cancer tissues at different clinical stages. For early stage malignancies, patient-derived tumor organoids (PDOs) could be employed for identifying molecular alterations potentially serving as biomarkers and targets for prevention. Fujii *et al* demonstrated that it is feasible to grow different types of precancerous colon neoplasia in culture. Organoids in all disease phases were independent of Wnt/R-spondin due to the activation of Wnt signaling while dependence on the remaining niche growth factors subsides during the adenoma to carcinoma transformation.[27]

Genetic cancer modeling in organoid cultures

Organoids can routinely be derived from normal human epithelia, enabling *in vitro* modeling of mutations in all cancer phases. Organoids can undergo subcloning following clustered regularly interspersed short palindromic repeat (CRISPR) modifications, allowing a comprehensive analysis of gene functions in cancer. Matano *et al*[28] used CRISPR-CRISPR-associated protein 9 (Cas9) for introducing many such mutations in organoids obtained from patients' noncancerous gut epithelial specimens. Through culture condition changes to mimic the gut environment, the authors identified isogenic organoids mutated for tumor suppressor genes such as *APC*, *SMAD4*, and *TP53*, as well as for oncogenes including *KRAS* and *PIK3CA*. Organoids designed to harbor these five mutations showed normal growth without niche factors in culture. The mutated organoids generated tumors upon implantation, but could not form colonies in the liver. Meanwhile, organoids from chromosome-instable human adenoma specimens generated macrometastatic colonies. These findings indicate that mutation of driver pathways helps stem cells to survive a hostile tumor microenvironment, with further molecular alterations needed for invasiveness.

Another report by Drost *et al*[29] had a similar strategy. After *KRAS*, *APC*, *P53*, and *SMAD4* mutation, human intestinal organoids can grow without stem-cell-niche molecules and with the P53 stabilizer nutlin-3 in culture, and as tumor tissues showing invasive cancer properties *in vivo*. In addition, they found that the engineered CRC organoids showed substantial CIN as well as aneuploidy. In a follow-on study, Fumagalli *et al*[30] orthotopically transplanted human colon organoids harboring various CRC mutation combinations, and demonstrated that sequential accumulation of oncogenic mutations in *Wnt*, *EGFR*, *P53*, and transforming growth factor-β(TGF-β) pathway genes promotes cancer cell growth, migration, and metastasis. Reconstituting specific niche signals could restore metastasis in cancer cells devoid of one of these oncogenic mutations, which implies that the capability of metastasizing, e.g., invading distant sites, directly results from loss of dependency upon particular niche factors.

Table 1: Examples of studies of CRC using human organoid models.

Tumor location	Source of tumor tissues	Sample collection method	Study intervention	Findings	References
Adenoma CRC	Primary tumor, metastases	Surgical resection, endoscopy biopsy	Established a CRC organoid library and performed gene expression microarray analysis	Established a CRC organoid library with different stages and subtypes, CRC organoids could closely mimic the clinical phenotypes of patient tumors	27
Adenoma, CRC normal epithelia	Primary tumor	Surgical resection, endoscopy biopsy	Genetic modeling CRC organoids using CRISPR-Cas9	Human organoids could be used as a new experimental tool for cancer modeling, PDOs that were introduced a series of mutations to perform genome editing displayed different metastatic capabilities, and introducing *KRAS* mutation induced a change in targeted drug response	28–32
Colon cancer	Primary tumor	Surgical resection	Drug sensitivity assay 83 compounds including drugs for clinical use, agents assessed in clinical trials and experimental compounds	The organoids could be used to assay the relationship between genes and drugs	33
mCRC, mGOC, metastatic cholangiocarcinoma	Metastases	Ultrasound, computer-tomography-guided or endoscopic biopsies	Drug sensitivity assay 55 agents under assessment in phase I-III studies or clinically applied, the PDO assay results were compared to the response of the patients in clinical trials	The PDO assay has 100% sensitivity, 93% specificity, and 88% and 100% positive and negative predictive values, respectively, in predicting the therapeutic responses	34
mCRC	Metastases	Obtained by 18G core needle biopsies	A prospective clinical study named TUMOROID to predict sensitivity to stand-of-care chemotherapeutic regimen in metastatic CRC	The PDO assay accurately predicted treatment response in >80% of individuals administered irinotecan-containing products	35
Colon cancer	Primary tumor	Surgical resection	Targeted therapies sensitivity assay to evaluate RAS pathway inhibitors and drug combinations using a panel of CRC organoids with wild-type or mutant *RAS*, as well as normal organoids and tumor organoids	RAS mutation was associated with resistance to the targeted therapies. PDO organoid libraries could be used to assess targeted anti-cancer suppressors and combinatory drugs pre-clinically	32
Rectal cancer	Primary, metastatic, or recurrentpre- and post-treatment samples	Surgical resection, endoscopy biopsy	To assess the response to chemoradiation using PDO, PDOs were treated with 5-FU and FOLFOX or radiation	PDOs showed a heterogeneous response to chemo/radiotherapy that correlates with clinical response.	36
Rectal cancer	Primary tumor, pre-treatment biopsies	Endoscopy biopsy	Co-clinical trial to assess the ability of PDOs to predict responses to chemoradiation, PDOs were treated with irradiation, 5-fluorouracil, and irinotecan, TRG represents the reference in assessing LARC patient response	Patient responses to chemoradiation were reflected by PDOs (accuracy, 84.43%; sensitivity, 78.01%; specificity, 91.97%)	37

Cas9: CRISPR-associated protein 9; CRC: Colorectal cancer; CRISPR: Clustered regularly interspersed short palindromic repeat; FOLFOX: Folinic acid (or leucovorin), fluorouracil, and oxaliplatin; LARC: Locally advanced rectal cancer; mCRC: Metastatic colorectal cancer; mGOC: Metastatic gastroesophageal cancers; PDO: Patient-derived tumor organoid; TRG: Tumor regression grade.

Drost *et al*[31] employed CRISPR-Cas9 for deleting major DNA repair genes in colon organoids from humans, revealing that mutation accumulation in organoids not expressing the mismatch repair gene *MLH1* was due to replication errors, which closely reflects the mutation patterns reported for mismatch repair-deficient CRC.

Verissimo *et al*[32] introduced an oncogenic *KRAS* mutation via CRISPR/Cas9-mediated homologous recombination in a patient-derived CRC organoid which was a wild type for the whole downstream EGFR pathway, and observed a marked change on targeted drug response.

Organoid culture can detect the genotype-to-drug association

Studies confirmed that organoid culture can detect the genotype-to-drug association. Van de Wetering *et al*[33] established tumor organoid cultures from 20 CRC cases enrolled consecutively. The organoids were obtained from surgically resected tissues from previously untreated patients with CRC. The most frequently altered CRC genes were also largely found in organoid cultures. Inhibiting changes to tumor-suppressive factors (*APC*, *TP53*, *FBXW7*, and *SMAD4*) and inducing alterations in *KRAS* (codons 12 and 146) and *PIK3CA* (codons 545 and 1047) were noted. Inducing *BRAF* and *TGFBR1/2* gene alterations were found in organoids with hypermutation. Gene expression assessment revealed that the main CRC molecular subtypes were represented. These authors used a customized library of 83 compounds to screen drug sensitivity in 3D organoid cultures, including drugs for clinical use, chemotherapy, and agents previously assessed in or currently under clinical trials, as well as experimental molecules for different cancer targets. The relationship between genes and drugs could be detected by high-throughput drug screening. For instance, a particular organoid culture showing high sensitivity to Wnt secretion suppressors was mutated for ring finger protein 43 (*RNF43*), a negative feedback modulator of Wnt signaling, with no *APC* mutation.

Application in Colorectal Cancer

Organoid culture predicts sensitivity to chemotherapeutic agents in metastatic CRC

Chemotherapy markedly improves the overall survival in tumor patients. However, cancer cell chemosensitivity is highly heterogeneous, and individuals might undergo unnecessary therapy and be needlessly exposed to toxic products. It was reported that organoid cultures could be successfully initiated from metastatic biopsy samples in CRC, encompassing genetic features of the original metastatic lesion. Indeed, 90% of somatic mutations are present in both organoid and biopsy specimens from a given individual.[48]

Vlachogiannis *et al*[34] contemplated the possibility of employing PDOs from metastatic gastrointestinal cancer (CRC and gastroesophageal cancer) as drug screening tools. They ran 3D screening assays with 55 agents under assessment in phase I–III studies or clinically applied. The authors reported 100% sensitivity, 93% specificity, and 88% and 100% positive and negative predictive values, respectively, in predicting the therapeutic responses in patients.

Our team also established a CRC organoid model with robust growth over 25 days, which could be employed to screen drugs and perform individualized treatments.[55]

Subclonal populations of tumors were demonstrated to be resolved within the spheroid cultures,[56] which indicates that organotypic cultures could be a new tool for investigating the evolution of clonal architecture over time in response to therapies. In the above study, optical metabolic imaging was used to interrogate the metabolic activity at the single-cell level. Such single-cell analysis can dynamically quantify heterogeneous drug response, potentially identifying refractory sub-populations.

Recently, Ooft *et al*[35] performed a prospective clinical study based on PDOs from CRC metastatic tumors for identifying non-responders to the standard-of-care chemotherapeutic regimen. The study, named TUMOROID, involved multiple centers and assessed common treatment options applied in CRC, for example, a chemotherapeutic regimen based on 5-fluorouracil (5-FU) alone or with oxaliplatin, or irinotecan or irinotecan alone. Their PDO assay accurately predicted treatment response in >80% of individuals administered irinotecan-containing products without misclassification of cases who could have treatment benefits. Such correlation was specific to irinotecan-containing regimens, but could not reflect treatment outcome in patients administered the 5-FU-oxaliplatin combination. These findings indicate that PDOs could recapitulate patient response, with potential application in individualized treatment.

Organoid culture could help evaluate and optimize targeted anti-cancer therapies

Recently, treatments targeting the mitogen-activated protein kinase pathway have been applied in metastatic CRC. However, certain mutations confer resistance to such treatments, for example, in the *KRAS* and *BRAF* genes.[57-60] Recently, multiple reports have demonstrated that it is possible to overcome such resistance by simultaneously blocking the mutated molecules.[61] It is, therefore, necessary to identify suitable targeted anti-cancer inhibitors and drug combinations for patients. Verissimo *et al*[32] have assessed clinically employed KRAS-signaling suppressors and drug combinations against noncancerous colon and CRC organoids. *RAS* mutation was tightly associated with chemoresistance, in both malignant and noncancerous organoids. In addition, simultaneously inhibiting the EGFR and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathways in organoids mutated for RAS transiently caused cell-cycle arrest but not cell death. Finally, EGFR pathway inhibition strongly sensitized for cell death induction in *RAS*-mutant CRCs by minimal addition of navitoclax, a known B-cell lymphoma-2/B-cell lymphoma

XL suppressor. These results indicate a strong potential for patient-derived CRC organoid in assessing targeted anti-cancer suppressors and combinatory drugs pre-clinically.

Organoid culture predicts sensitivity to chemoradiotherapy in rectal cancer patients

Neoadjuvant chemoradiation (nCRT) and subsequent total mesorectal excision is routinely employed for treating locally advanced rectal cancer (LARC). The sensitivity of patients to radiotherapy presents a great heterogeneity. Some individuals show complete response to nCRT alone, with no need for operation,[62] unlike others who undergo radical surgery after nCRT.[63] Many efforts have been made to develop a method for predicting sensitivity to chemoradiotherapy to identify individuals sensitive to chemoradiation for decision making after nCRT.

The Memorial Sloan Kettering Cancer Center group[36] produced rectal cancer organoids (RCOs) from primary, metastatic, and recurrent cancer cases. They demonstrated that RCOs could help assess the responses of individuals to chemoradiation. RC tumoroids are heterogeneous in their responses to physiologic 5-FU and folinic acid (or leucovorin), FU, and oxaliplatin (FOLFOX) levels. These authors then employed areas under the dose-response curves (AUCs) to reflect *ex vivo* effects and found that AUCs for both 5-FU and FOLFOX in *ex vivo* assays were associated with progression-free survival of respective patients (Spearman $r = 0.86$, $P = 0.024$). They also exposed tumoroids to irradiation *ex vivo*, and a heterogeneous response was obtained. The association of *ex vivo* and patient-radiation sensitivities was further examined by comparing endoscopic tumors right pre- and post-radiation and assessing radiotherapy response. Interestingly, RC tumoroids displayed different sensitivities to radiation, corroborating patient response.

More recently, co-clinical trial data from Fudan University Shanghai Cancer Center indicated that PDOs could predict rectal cancer response clinically, and might constitute a tool for diagnosing rectal cancer. In this phase III study, 80 primary rectal cancer biopsies were collected from LARC cases. Pathologic tumor regression grade following total mesorectal excision represents the reference in assessing LARC patient response following nCRT. Cases had good treatment response with tumor organoids showing sensitivity to one or more of the three therapeutic components, including irradiation, 5-FU, and irinotecan (CPT-11). Patient responses to chemoradiation significantly reflected those of RCOs (accuracy, 84.43%; sensitivity, 78.01%; specificity, 91.97%).[37]

The data given earlier indicate that *ex vivo* chemoradiation systems could help assess clinical patient response prior to radiation and chemotherapy administration, which could avoid toxic effects of irradiation in resistant cases. On the contrary, in patients failing standard treatments or experiencing disease progression, these platforms might provide a possibility to investigate novel treatment options *ex vivo* using their own tumor tissues.

Current Challenges and Perspectives

Multiple anti-cancer compounds assessed clinically eventually fail to obtain approval because of inadequate safety and/or efficacy, suggesting that current pre-clinical predictive models are limited. The accuracy of *in vitro* high-throughput drug screening, therefore, depends on further optimization of drug screening platforms mirroring patient disease. Even though drug screening based on two-dimensional tumor cell lines provides important insights for predicting therapeutic response, poor reproducibility of the original cancer tissue may be the reason behind the frequent failure of novel drug in clinical studies. Tumor organoids derived from patients could better reproduce the primary tumor and may be an ideal model for identifying and testing new anti-cancer drugs. The development of high-throughput drug screening methods is just a start in PDOs

As demonstrated above, organoids have a great potential in CRC research, and their assessment could help clinical decision making and improve patient survival with CRC treatment. However, several challenges for the organoid system need to be addressed. One limitation is that organoids do not have multiple cell constituents of the tumor microenvironment, for example, stromal, vascular endothelial, and immune cells, which limits its ability to completely simulate the primary tumor. Although this limitation could be overcome by combining the PDO model with fibroblasts, and immune and endothelial cells. Many researchers are trying to solve this problem, but it remains a challenge for researchers to enhance mimicry of the tumor microenvironment.[64]

Although the success rate of PDO establishment is high, its efficiency needs to be improved. The time and costs of organoid generation also need to be reduced. The main issue hampering organoid cultures is insufficient fresh tissue with live cancer cells. CRC organoids are derived from biopsies of metastatic cancer or treatment-naive rectal cancer cases, but sometimes low amounts of live cancer cells are found in biopsies. The success rate of cultures could be further improved by obtaining many core biopsies and direct evaluation by pathologists.

To ease model establishment, multiple current protocols employed for CRC PDO culture should be optimized and standardized. The culture components are complex, which affects PDO growth and consequently the test results. In addition, standardized characterization by whole-exome sequencing, copy number assessment, RNA sequencing, and drug sensitivity tests, is required for achieving reproducibility.

Conclusions

CRC organoid cultures conserve the genetic and phenotypic heterogeneities of primary cancers. They could help detect gene-drug associations and perform high-throughput drug screening. CRC organoids thus constitute a model system comparable to genetically engineered mouse models, cell lines, and PDTXs. Additionally, they are expanded relatively faster, and can be cryopreserved and genetically altered. CRC PDOs can be used to generate

living tumor organoid biobanks, and provide a platform for high-throughput drug screening, not only chemotherapeutic agents and targeted treatments, but also radiotherapy. The utility of organoids in CRC still warrants further evidence, especially those in clinical trials.

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Conflicts of interest

None.

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