Observational clinical research protocol initiated by researchers

Project Name: Patient Derived Organoids (PDOs) to Observe the Clinical Consistency of Personalized Neoadjuvant Therapy for Resectable Esophageal Squamous Cell Carcinoma

Research Institution: Zhongshan Hospital affiliated to Fudan University

Principal Investigator: Lijie Tan

Sponsor: Zhongshan Hospital affiliated to Fudan University

Version Number: V1.3

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Investigator's Statement

I will conscientiously fulfill my responsibilities as a researcher by personally participating in or directly supervising this clinical study. We have read and confirmed this protocol, agreeing with its scientific and ethical aspects. We will adhere to relevant responsibilities as prescribed by Chinese laws and regulations, the Helsinki Declaration, Chinese GCP, and this study protocol, and it can only be implemented after approval by the academic committee and ethics committee, unless measures must be taken to protect the safety, rights, and interests of the subjects. We will keep this study protocol confidential.

Research Institution: Zhongshan Hospital affiliated to Fudan University

Principal Investigator: Lijie Tan

Summary of the Plan

Project Name	Patient Derived Organoids (PDOs) to Observe the Clinical Consistency of Personalized Neoadjuvant Therapy for Resectable Esophageal Squamous Cell Carcinoma
Research background	Esophageal squamous cell carcinoma is one of the common malignant digestive tract tumors in China. In recent years, neoadjuvant therapy for esophageal

	squamous cell carcinoma has developed rapidly, but the efficacy varies significantly among individuals. To further improve the benefit rate of neoadjuvant therapy, it is necessary to explore more efficient efficacy prediction models. Human tumor organoid models (patient derived organoids, PDO) are biological models obtained by culturing patient tumor samples, which can better retain the original tumor characteristics and interpatient variability. Existing studies have confirmed the feasibility of PDO immune co-culture models and their potential for predicting the efficacy of neoadjuvant personalized therapy.
Research Objective	Establish a human tumor organoid (PDO) model for ESCC patients, explore the consistency between patient-derived PDO models and the clinical efficacy of neoadjuvant personalized therapy for resectable esophageal cancer, and evaluate their clinical application value.
Study Endpoints	Primary Endpoint: Culture formation indicators and pathological identification indicators of esophageal squamous cell carcinoma; interpretation of drug sensitivity results. Secondary Endpoint: Clinical efficacy evaluation (reference RECIST1.1)
Research Design	This is a single-center, prospective, observational study that uses patient-derived human tumor organoid models (PDO) for drug sensitivity tests and compares them with actual clinical efficacy to evaluate consistency and potential clinical application value.
Sample size estimation	A preliminary plan to enroll 30 patients
Inclusion criteria/Exclusion criteria/Withdrawal criteria during the study	See the plan
Expected research duration	2025-2 to 2025-12
Statistical analysis plan	See the plan

Research background

(1) Research significance

Esophageal cancer is one of the common malignant digestive tract tumors in China. According to the data from the World Health Organization, the incidence and mortality rates of esophageal cancer in China in 2022 were 224,000 and 187,000 cases,

respectively, ranking 7th and 5th among all cancers [1]. The esophageal cancer patients in China are mainly esophageal squamous cell carcinoma (ESCC) [2], and the recurrence rate is high when treated with radical surgery alone. Therefore, comprehensive treatment strategies are usually used in the treatment of ESCC to prolong the recurrence-free survival and overall survival of patients.

Neoadjuvant therapy refers to the comprehensive treatment before tumor resection, mainly including neoadjuvant radiotherapy, chemotherapy, and immunotherapy. For most resectable esophageal cancers, neoadjuvant therapy can reduce tumor stage, increase the rate of R0 resection, and prolong patient survival and improve quality of life without increasing mortality. Neoadjuvant chemoradiotherapy has been proven to significantly prolong patient survival and is widely used nationwide. However, the response to neoadjuvant therapy varies greatly among different patients, and about 40% of patients experience recurrence [3]. Therefore, how to improve neoadjuvant therapy and predict the clinical response of patients to individual chemotherapy have become urgent clinical problems. In recent years, tumor immunotherapy, which activates immune responses, has developed rapidly, and immunotherapy such as immune checkpoint inhibitors has been approved as first-line treatment for various solid tumors (such as melanoma and non-small cell lung cancer) [4]. However, the response to neoadjuvant immunotherapy varies significantly among different ESCC patients, and only tumors with rich lymphocytes and high PD-L1 expression can benefit significantly from the treatment, accounting for only about 30% of all ESCC patients [5]. Currently, the main method to determine whether patients can benefit from neoadjuvant therapy is gene sequencing or immunohistochemistry, which has relatively low predictive accuracy. To further improve the benefit rate of neoadjuvant therapy, we urgently need to explore more efficient efficacy prediction models.

Organoids are 3D stem cell-derived tissue cultures that can self-organize into isolated "mini-organs." Human tumor organoid models (patient derived organoids, PDO) are biological models obtained by culturing patient tumor samples, which can better retain the original tumor characteristics and interpatient variability, with structural and genetic integrity, and can be long-cultured and form biological sample banks [6]. Studies have shown that PDOs greatly exceed traditional cancer cell lines in accurately reproducing 3D tumor structure, heterogeneity, and drug response [7,8]. Based on this property, in the future era of precision medicine, PDOs will have broad application prospects, such as individualized prediction of drug efficacy, investigation of drug resistance mechanisms, and potential translational research.

(2) Current research status at home and abroad

PDO models and human tumor xenograft models (patient derived xenografts, PDX) are currently the most cutting-edge tools for predicting the efficacy of tumor drugs. PDX largely maintain the original histological and genetic characteristics of patient tumor cells, offering potential for detecting biomarkers and predicting drug efficacy. However, PDX also has certain disadvantages; on one hand, its tumors are mixed with some mouse-derived cells, leading to differences between PDX and the primary tumor in terms of genomics and gene expression profiles; on the other hand, PDX modeling is time-consuming, has a low success rate, and is costly, which limits its application in clinical efficacy prediction [9]. In contrast, PDO not only retains the characteristics of human tumor cells but can also be co-cultured with the patient's autologous immune

cells, thereby simulating the interaction between immune cells and tumor cells in vivo [6], more accurately predicting the clinical efficacy of drugs.

Currently, there are two main types of immune co-culture models supported by technology. The first is to culture tumors together with the in situ immune cells within the tumor. The advantage of this method is that it retains all immune cells, including exhausted T cells. However, since the culture medium required for immune cells and PDO is different, immune cells cannot maintain their activity for long periods, so this model is more suitable for investigating treatment strategies that take effect in the short term [10]. The second method involves culturing PDO and peripheral blood immune cells separately. The advantage of this method is that it can maintain the activity of immune cells for longer periods and can continuously amplify and add activated autologous immune cells outside the PDO [10]. Evidence suggests that this model can obtain tumor-reactive CD8+ T cells after co-culture for 14 days [11].

Currently, there are multiple studies that have successfully established such models [12–14], and some research has already validated that the PDO and peripheral blood immune cell co-culture model can well predict the efficacy of neoadjuvant immune combined with chemotherapy regimens in diseases such as gastric cancer [15], colorectal cancer [16], and skin cancer [17]. However, there are currently no similar studies in esophageal cancer, which may be due to the relatively greater difficulty in culturing squamous epithelial cells, and this is one of the innovations of this study.

The main purpose of this study is to establish PDO models of tumors from ESCC patients and explore the consistency between the PDO model and the clinical efficacy of patient's neoadjuvant personalized therapy, as well as to use the PDO model to explore possible drug mechanisms and resistance mechanisms, providing precise guidance for efficacy prediction and personalized medication in future neoadjuvant immunotherapy and chemotherapy.

Research Objective

1. Main research objectives

Establish human-derived tumor organoids (PDO) models for ESCC patients, comparing the pathological and genetic features of PDOs with corresponding tumor tissues for consistency.

2. Secondary research objectives

Construct an immune co-culture model for esophageal squamous cell carcinoma PDOs, by comparing the drug sensitivity results of different chemotherapy immune combination regimens and their consistency with clinical efficacy, to evaluate the feasibility of using the esophageal squamous cell carcinoma organoid immune co-culture model to guide the selection of clinical chemotherapy immune combination regimens.

Exploratory research objectives

Investigating the possible resistance mechanisms of esophageal cancer to neoadjuvant personalized therapy using patient PDO and immune co-culture models.

Research overview

This study is a single-center, prospective, observational study that uses patient-derived human tumor organoid models (PDO) for drug sensitivity tests and calculates

consistency by comparing actual efficacy, thereby evaluating its clinical application value.

Enrolled patients should be confirmed by endoscopic biopsy histology and evaluated for tumor resectability by CT or PET/CT scans. Enrolled patients should have an ECOG performance score ≤1 and no significant organ dysfunction.

The tumor biopsy samples collected by endoscopy will be used for PDO modeling, and blood samples will be used to construct an immune co-culture model and perform drug sensitivity experiments. Patients will be grouped for data analysis based on drug sensitivity test results and compared with the final disease remission status of the patients.

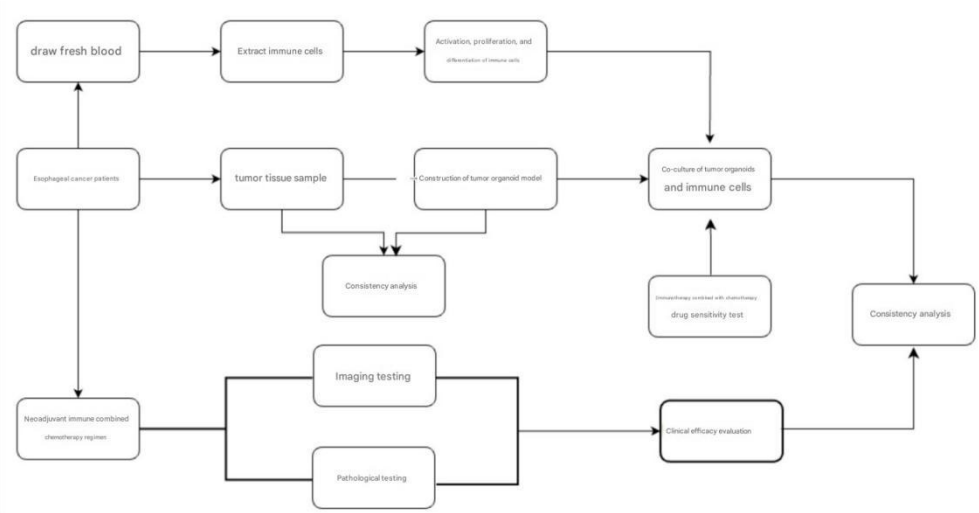
Enrolled patients will receive neoadjuvant therapy, and the specific treatment plan will be determined by the treating physician. This study does not interfere with clinical treatment plans.

Patients will receive the following follow-up:

(1) Safety follow-up: Adverse reactions related to the treatment drug within 90 days after the last dose of medication, followed up every 30 days, including survival status, adverse reactions, concomitant medications, and concomitant treatment information.

(2) Survival follow-up: Every 3 months, collect survival information and data on antitumor treatment and disease progression time after the study ends, until the participant dies, is lost to follow-up, or the study is terminated.

Design roadmap



Research population

1. Data source

All patients enrolled in this study were from Zhongshan Hospital, histologically confirmed esophageal squamous cell carcinoma patients who were reviewed and considered resectable by two experienced thoracic surgeons.

2. Inclusion/Exclusion/Withdrawal criteria

Inclusion criteria

Patients with squamous cell carcinoma confirmed by gastroscopic biopsy pathology, and who have gastric endoscopy tissue and blood samples collected at this center before treatment;

The primary tumor is located in the chest, and the primary site of esophageal cancer is determined by the center of the mass in the esophagus (upper esophageal segment: from the thoracic inlet to the lower margin of the aortic arch level, endoscopic examination distance from the incisor is 20 cm to <25 cm; middle esophageal segment: from the lower margin of the aortic arch to the level of the inferior pulmonary vein, endoscopic examination distance from the incisor is 25 cm to <30 cm; lower esophageal segment: from the level of the inferior pulmonary vein to the stomach, endoscopic examination distance from the incisor is 30 cm to <40 cm)

Based on the above examinations, preoperative clinical assessment can be performed for esophageal cancer patients who can undergo surgical resection (Whether the tumor had obvious external invasion, mediastinal lymph node enlargement, and distant organ metastasis were evaluated. If the primary tumor is suspected to be T4b, multiple mediastinal lymph node metastases, or distant metastases, whole-body PET-CT and endoscopic ultrasonography (EUS) , or other optional examinations to further confirm the clinical stage, should be performed.);

Age ≥ 18 years, ≤ 75 years, ECOG performance status 0-1 (see Appendix 1), expected survival ≥ 12 months;

Subjects must not have major organ dysfunction, and basic normality in complete blood count, lung, liver, kidney function, and cardiac function. Laboratory test values must meet the following requirements: Blood: White blood cells > 4.0×10^9/L, absolute neutrophil count (ANC) ≥ 2.0×10^9/L, platelet count > 100×10^9/L, hemoglobin > 90g/L; Lung function: FEV1 ≥ 1.2L, FEV1% ≥ 50% and DLCO ≥ 50%. Note: FEV1 (L): Forced vital capacity (FVC) measured value (liters). FEV1%: FVC measured value/estimated value (%). DLCO%: Carbon monoxide single-breath diffusion capacity measured value/estimated value (%); Liver function: Serum bilirubin less than 1.5 times the maximum normal value; ALT and AST less than 1.5 times the maximum normal value. Kidney function: Serum creatinine (SCr) ≤ 120μmol/L, creatinine clearance (CCr) ≥ 60ml/min

Have legal capacity, able to understand the situation of this study and sign an informed consent form.

2.2 Exclusion criteria

Exclusion criteria related to cancer:

Patients with clinical stage T4b who are not resectable (determined by senior thoracic surgeons), have multiple lymph node enlargements (estimated lymph node metastasis ≥ 6), multiple lymph node stations (estimated number of lymph node metastasis stations ≥ 3), or distant metastasis (M1) as determined by imaging studies such as contrast-enhanced CT of the chest and abdomen, neck lymph node ultrasound, whole-body PET-CT (optional), or EBUS (optional) (AJCC/UICC 8th Edition);

Patients who have already received or are currently receiving other chemotherapy, radiotherapy, or targeted therapy;

Patients with non-squamous cell carcinoma on endoscopic examination pathology;

A history of other tumors (previous cervical in situ carcinoma or local basal cell carcinoma of the skin that has been cured is excluded);

Other exclusion criteria:

History of autoimmune diseases;

Use of hormones or immunosuppressants recently or currently;

Has previously received immunotherapy;

Has a history of severe hypersensitivity to antibody drugs;

Has a history of or is currently suffering from chronic or recurrent autoimmune diseases;

Interstitial lung disease, pulmonary fibrosis, diverticulitis, or systemic ulcerative gastrointestinal inflammation;

A confirmed history of congestive heart failure; angina with poor medication control; transmural myocardial infarction confirmed by electrocardiogram (ECG); poorly controlled hypertension; clinically significant valvular heart disease; or uncontrolled high-risk arrhythmia;

Severe uncontrolled systemic intermittent diseases, such as active infection or poorly controlled diabetes; coagulation disorders, with a tendency to bleed or receiving thrombolytic or anticoagulant therapy;

Women who are serum pregnancy test positive or breastfeeding, as well as fertile men and women who are unwilling to use adequate contraception during the study of drug treatment;

Known active infection with human immunodeficiency virus (HIV), hepatitis B virus (HBV), or hepatitis C virus (HCV), or known HIV seropositivity, including positive surface antigen for HBV or HCV (RNA);

Known allergy to any research drug;

History of organ transplantation (including autologous bone marrow transplantation and peripheral stem cell transplantation);

Presence of peripheral neuropathy or history of significant psychiatric or central nervous system disorders;

Concomitant use of antineoplastic drugs outside the research protocol;

Based on the researcher's judgment, there are other factors that may lead to the forced termination of the study, such as having other serious illnesses (including mental illness) requiring combined treatment, alcoholism, drug abuse, family or social factors;

2.3 Withdrawal criteria (if applicable)

Participants can withdraw informed consent at any time for any reason. If an adverse event occurs, the researcher can decide to terminate the study treatment for the participant. Additionally, if the participant does not meet the inclusion and exclusion criteria, violates the trial plan, or due to management and/or other safety reasons, the researcher can terminate the treatment and disqualify the participant.

Terminating study treatment does not mean the patient has withdrawn from this trial, as long as the participant has not withdrawn informed consent to refuse further follow-up, the participant still needs to continue to complete the remaining study visits according to the protocol requirements after termination of treatment. The main reasons for the participant's termination of treatment are recorded in the CRF.

If any of the following situations occur, the subject must terminate treatment but continue follow-up:

The subject withdraws informed consent or refuses to continue receiving the original drug regimen treatment;

The investigator judges that the subject's clinical symptoms worsen or physical condition declines;

Imaging disease progression/recurrence or metastasis, unless the trial group subject meets the criteria for continued treatment after disease progression and agrees to continue treatment;

Occurrence of intolerable adverse events, or other events affecting the subject's safety, including but not limited to the occurrence of any clinical AE, laboratory abnormalities, or other medical conditions;

Significant protocol deviation;

Other situations deemed necessary to terminate research drug treatment by the investigator;

Subject's request;

Pregnancy;

Initiation of other systemic antitumor therapy;

Subjects will withdraw from the study if any of the following occur:

Subject withdraws informed consent, or refuses further follow-up;

Lost to follow-up;

Death;

Study termination;

Other reasons considered by the investigator as necessary to withdraw from the study;

When an eligible subject is found to be 不合格 after enrollment, the investigator must discuss with the principal investigator (sponsor) whether the subject should continue or withdraw from the study.

Study Grouping

This study is observational, and no patient grouping or intervention will be implemented during the treatment phase. Grouping will be conducted during the data analysis phase, primarily based on the drug sensitivity test results of PDO for data grouping and statistical analysis.

Study Endpoints

Primary research endpoint

Culture formation indicators and pathological identification indicators of esophageal squamous cell carcinoma; interpretation of drug sensitivity results: the maximum inhibition rate of the measured drug.

Secondary research endpoint

Clinical efficacy evaluation: (to be performed according to the RECIST 1.1 version for solid tumor response evaluation criteria)

Efficacy evaluation	Efficacy evaluation
Clinically effective	CR, PR, SD
Clinically Ineffective	PD

Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 version:

Complete Response (CR): All target lesions disappeared;

Partial Response (PR): Sum of the longest diameters of baseline target lesions decreased by ≥30%;

PD: Sum of baseline lesion longest diameters increased by ≥20% or new lesions appeared

SD: Sum of baseline lesion longest diameters decreased but not reaching PR or increased but not reaching PD

Safety endpoint

Adverse events: Any adverse medical event experienced by a patient or clinical trial subject after receiving a drug, which may not necessarily have a causal relationship with the treatment. The incidence and severity (including serious adverse events and immune-related adverse events) are determined according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) 5.0 standard (see Appendix 2).

Research Steps

1. Screening Phase:

After the patient is admitted, medical history collection, physical examination, and relevant ancillary examinations (see inclusion/exclusion criteria) must be completed within 2 weeks to determine if the patient meets the inclusion criteria. For patients who meet the inclusion criteria, the doctor must introduce the details of the trial, and after the patient consents, they must sign the "Informed Consent Form". After completing all screening items, the researcher must review the results/data, and only after passing the review can the subject be enrolled.

1) Record the patient's basic information, current medical history, past medical history, physical examination, vital signs, and BMI index;

2) Record pre-treatment complete blood count, liver function, renal function, blood biochemistry, tumor markers (CEA, CA19-9, CA125, CYFRA21-1, and SCC, the same below), cardiopulmonary status, enhanced CT of the chest and abdomen, ultrasound of cervical lymph nodes, PET-CT, EUS, etc.;

3) Collect pre-treatment gastroscopic biopsy samples, and possibly 1 blood sample (10ml) depending on the organoid culture conditions and the patient's condition, for PDO culture and other exploratory research.

4) Collect post-treatment pathological tissue samples for exploratory research.

2. Organoid modeling:

PDGF culture method

Collect surgical tissue, digest, wash, and resuspend in organoid culture medium;

Add matrix gel, mix until non-segregated, then inoculate into culture dishes and place in a 37℃ constant temperature incubator for fixation;

After fixation, add organoid culture medium and place in a 37℃ constant temperature incubator.

Change the culture medium every 2-3 days until ESCC organoids are grown.

Tumor organoid model identification

Collect appropriate organoid samples and perform consistency identification with the source tissue;

Screen appropriate organoids for paraffin embedding, sectioning, HE staining, and immunohistochemical staining, and compare with the pathology and immunohistochemistry of the source tissue.

2) Culture of Tumor-Invading Lymphocytes (TIL)

Collect tumor tissue, take about 0.1g, wash and then cut the tissue into small pieces of about 1mm^3;

The small tissue pieces are transferred separately to different wells of a 24-well plate and immune cell induction medium is added;

After observing the amplification of immune cells, filter or sort using a sieve, enrich the immune cells, and continue the culture;

After increasing to a sufficient number of immune cells, freeze for the next experiment.

3) Culture of peripheral blood lymphocytes (PBMC)

Collect blood samples, use EasySepTM Direct Human PBMC Isolation Kit to isolate PBMCs;

First perform organoid culture;

The organoids after cultivation are digested into small clusters using TrypLE, and the organoids are mixed with PBMCs at a certain ratio for co-culture.

4) Drug sensitivity test

According to the experimental design, co-culture of immune cells and the addition of immune combination chemotherapy drugs are performed.

Through microscopy and immunohistochemical staining, the sensitivity of the co-culture model to immune combination chemotherapy drugs is detected.

In the organoid drug sensitivity test, one or more of the following drugs are planned to be used:

Paclitaxel Injection

Albumin-bound Paclitaxel Injection

Cisplatin Injection

Cisplatin Injection

Nivolumab

Pembrolizumab

Trastuzumab

Tislelizumab

Camrelizumab

The specific drug and its combination to be used should refer to the clinical treatment plan that the patient may adopt. The final plan will be decided jointly by the principal investigator and the responsible physician.

3. Follow-up period:

Safety Follow-up: Adverse reactions related to the treatment drug within 90 days after the last dose of medication, followed up every 30 days, including survival status, adverse reactions, concomitant medications, and concomitant treatment information.

Survival Follow-up: The first follow-up 30 days after surgery, and then every 3 months, collecting survival information and data on antitumor treatment and disease progression time after the study ends, until the participant dies, is lost to follow-up, or the study is terminated.

Data Governance/Data Management Plan

All cases must complete the case report form (CRF) or EDC.

1. Filling and Handing Over of Case Report Forms (CRFs):

All relevant information for the cases is recorded by the treating physician in the CRF according to the trial protocol, ensuring that the data is recorded promptly, complete, accurate, and truthful. The CRF should generally not be altered; if there is a definite need for correction, the person making the change must sign and date it. Completed CRFs must be reviewed and signed by the clinical researcher at each center, and then reviewed by the monitor dispatched by the sponsor. If there are any questions, the researcher must decide whether to make changes, and once confirmed, no further modifications can be made on the CRF. The CRF is in triplicate; after the trial ends, one copy is retained by the trial hospital, one is given to the clinical lead hospital, and one is given to the data administrator for data entry management. After data management and statistical analysis are completed, it is returned to the sponsor. All processes must be documented.

2. Data Entry and Modification:

The designated data administrator is specifically responsible for this process. A dedicated database system for this trial is established. After training the data entry personnel, data entry is performed using the double-entry method, with two individuals completing it independently.

3. The emergence and resolution of questions:

When the data manager conducts a comprehensive check, data entry, or data review of the CRF, and has questions, they must fill out a question form and return it to the monitor. The investigator is required to provide written answers to the questions in the question form and sign it, before returning it to the data manager to correct the data. The data manager is not authorized to modify the original data. The question form should be properly stored.

4. Data review:

The data manager should report on the data management process and general situation, the enrollment and completion of cases (including a list of dropouts), the items involved when determining the statistical analysis population, and the issues to be discussed and resolved (such as inclusion/exclusion criteria review, completeness check, logical consistency check, outlier data check, time window check, concomitant medication check, adverse event check, etc.), and it should be confirmed by the principal investigator, sponsor, and statistician.

5. Database Locking:

After the data has been reviewed and confirmed to be correct, the database will be locked by the principal investigator, sponsor, and statistician. Once the database is locked, it should not be modified in principle. If there is indeed a problem, corrections can only be made in the statistical analysis program after confirmation by the leading unit. All modifications must be documented and explained. Data security must be ensured, and unauthorized personnel should not have access to or modify the data. The data must be backed up.

6. Privacy Concerns:

To ensure strict protection of patient privacy, all research procedures will exclusively utilize unique research identifiers (Research ID) to replace any personally identifiable information. Upon screening and eligibility confirmation, each participant will be assigned a sequential, alphanumeric Research ID (e.g., ESCC-PDO-001), which will serve as the sole identifier throughout the study and be recorded on all documents, biological sample containers, and imaging records, with no patient names or contact information linked to data unless required by regulatory authorities. All clinical data, including tumor stage, treatment response, and adverse events, will be documented under the Research ID, while biological samples like tissue biopsies and blood samples will be stored in containers marked with the ID, with sample tracking systems using the ID to link samples to clinical data without referencing identities. The informed consent form will include the patient’s name for legal purposes but will be cross-referenced with the Research ID in a secure database, stored separately from study data.

For analysis and reporting, all statistical analyses will use the Research ID to aggregate results, with group comparisons based on ID-linked data to prevent individual identification, and research findings will be reported in aggregate form without

disclosing case-specific data that could reveal identities. Privacy controls will include access restrictions for authorized personnel with confidentiality agreements, physical and digital data storage security measures, and the conversion of data shared with external collaborators to anonymous datasets. The research team will also conduct annual audits to ensure compliance with privacy regulations, verifying no personal information is included in study materials. This approach ensures all research activities remain anonymous, with the Research ID serving as a non-identifying link between data points to uphold patient confidentiality while maintaining scientific rigor.

Consideration of Bias

1. Selection Bias: The potential correlation between modeling success rate and patient response to treatment may cause selection bias. Some studies have shown that patients with poor treatment response have tumors with relatively stronger stress resistance, making it more likely that PDO modeling will be successful [18]. This means that the patients whose PDO cultures ultimately succeed and are included in the analysis have been screened, which may introduce selection bias. We will statistically analyze the correlation between modeling success rate and tumor responsiveness and discuss its impact in the results.

2. Confounding Bias: Uneven distribution of characteristics such as age, gender, and tumor stage among included patients may lead to confounding bias. We will conduct statistical tests for confounding factors on the sensitive and insensitive groups predicted by the PDO drug sensitivity test, aiming to minimize the impact of confounding factors.

Statistical analysis plan

1. Sample Size Estimation

The main objective of this study is to explore the predictive accuracy of the PDO model for drug sensitivity experiments on PD-1 antibody immunotherapy, specifically investigating whether the model has statistical significance.

This research project is primarily aimed at subsequent modeling and scientific exploration, therefore the sample needs to meet the requirements for modeling and testing. Based on the budget support and the duration of the research, this study plans to successfully culture 20 patient-derived organoids for subsequent consistency analysis. Assuming the PDO predicts sensitivity and specificity to be both 80%, according to the research, the clinical response rate of ESCC patients to PD-1 antibody combined with chemotherapy is approximately 58.3% [19. Based on the above data, calculate the sample size. Perform a four-fold table exact Fisher test:

		Clinical Efficacy		Total
		Effective	Ineffective	
PDO Prediction Group	Sensitive	10	2	12
	Insensitive	2	6	8
Total		12	8	20

P value and statistical significance			
Test	Fisher's exact test		
P value	0.0194		
P value summary	*		
One- or two-sided	Two-sided		
Statistically significant (P < 0.05)?	Yes		
Data analyzed	efficient	invalid	**Total**
sensitive	10	2	12
insensitive	2	6	8
Total	12	8	20
Percentage of row total	efficient	invalid	
sensitive	83.33%	16.67%	
insensitive	25.00%	75.00%	
Percentage of column total	efficient	invalid	
sensitive	83.33%	25.00%	
insensitive	16.67%	75.00%	
Percentage of grand total	efficient	invalid	
sensitive	50.00%	10.00%	
insensitive	10.00%	30.00%	

Fisher's exact test results: bilateral P=0.0194, which can validate the statistical significance of the PDO prediction results.

Since the modeling success rate for esophageal squamous cell carcinoma is approximately 71.4%18, a preliminary plan is to include 30 patients. If the modeling success rate of PDO, the drug sensitivity test success rate, and the expected results do

not match during the subsequent research process, the researcher needs to appropriately adjust the expected number of patients to be enrolled.

2. Dataset Definition

The safety dataset includes all enrolled patients, including those who completed the experiment and those who withdrew due to reaching safety endpoints or 中途退出, and did not complete the experiment. The effectiveness dataset only includes patients whose PDO culture was successful and who successfully underwent drug sensitivity testing.

3. Handling of Missing Data

For patients with missing data, assess whether the missing data include the primary endpoint. If the missing data do not include the primary endpoint or the missing data do not significantly affect the main analysis, the patient should not be excluded. If the missing data significantly affect the main analysis, the patient should be excluded.

4. Descriptive Analysis

Descriptive analysis primarily focuses on the "sensitive" and "insensitive" groups of the PDO drug sensitivity test, statistically analyzing confounding factors such as age, gender, stage, etc., in both groups, and performing statistical tests; it also statistically analyzes and tests the primary and secondary endpoints between the two groups.

Primary Analysis

Statistical analysis of drug sensitivity results of organoids and clinical gold standard in the form of a contingency table, calculate clinical sensitivity, clinical specificity.

		Clinical Efficacy Imaging Interpretation (Gold Standard)	Total
		Valid Invalid	
Organoid drug sensitivity result interpretation	Sensitive	a b	a+b (R1)
	Insensitive	c d	c plus d (R2)
Total		a plus c (C1) b plus d (C2)	a plus b plus c plus d (N)

Clinical sensitivity (%) = [a / (a + c)] × 100%

Clinical specificity (%) = [d / (b + d)] × 100%

Consistency (%) = [(a + d) / (a + b + c + d)] × 100%

Kappa value analysis: $kappa = \frac{N(a+d) - (RC+RC)}{N^2 - (RC+RC)}$

All statistical tests are two-tailed. A P value less than 0.05 will be considered statistically significant. The confidence interval (CI) is 95%.

Quality Control

1. Laboratory quality control measures: The laboratory establishes standard operating procedures and quality control programs. Special examination items must be handled by

dedicated personnel;

2. Researchers participating in clinical trials must have professional expertise, qualifications, and capabilities in clinical trials, undergo qualification review, and require relatively fixed personnel;

3. Trial participants receive training on the trial protocol before the trial starts, enabling trial personnel to have a full understanding and recognition of the clinical trial protocol and its specific connotations of each indicator.

Research-related ethics

1. Ethics committee review

This protocol and written informed consent forms, as well as materials directly related to the subjects, must be submitted to the ethics committee and may only be formally initiated upon written approval from the ethics committee. The researcher must submit a continuing review report one month before the expiration of the ethics approval letter to apply for an extension of the approval. Upon termination and/or completion of the study, the researcher must notify the ethics committee in writing; the researcher must promptly report all changes that occur in the research work to the ethics committee (such as revisions to the protocol and/or informed consent forms) and may not implement these changes until they receive approval from the ethics committee, except for changes made to eliminate obvious and direct risks to the subjects. In such cases, the ethics committee will be notified.

2. Informed consent and the process of obtaining informed consent

Researchers must provide an easily understandable informed consent form approved by the ethics committee to the participant or their legal representative, and give the participant or their legal representative sufficient time to consider this research. No participant may be enrolled before obtaining a signed written informed consent form from the participant or their legal representative. During the participant's involvement, all updated versions of the informed consent form and written information will be provided to the participant. The informed consent form should be kept as an important document of the clinical trial for reference.

Confidentiality Measures

The results of this project research may be published in medical journals, but we will keep the personal information of patients confidential in accordance with legal and regulatory requirements, unless required by relevant laws, the personal information of patients will not be disclosed. When necessary, government administrative departments, hospital ethics committees, and relevant personnel may review patient information in accordance with regulations.

Expected Schedule and Completion Date of the Research Project

2025/02 – 2025/09 Completion of patient enrollment and sample collection, model construction and testing

2025/09 – 2025/12 Completion of data analysis and reporting

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