# Research plan

Study name
Study on the Identification of New Subtypes of Liver Cancer Based on 29 Genes and the Correlation with the Efficacy of Immunotherapy
Research Unit
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Protocol Version Number
V1.0
Protocol Version Date
July 13, 2025

Study on the Identification of New Subtypes of Liver Cancer Based on 29 Genes and the Correlation with the Efficacy of Immunotherapy

## I. Research Background

Liver cancer is one of the leading causes of cancer - related deaths worldwide. Its significant heterogeneity makes it difficult for traditional diagnosis and treatment models to meet the needs of individualized precision treatment. The application of immune checkpoint inhibitors (ICI) has brought breakthrough progress to liver cancer treatment, but there are still severe challenges in clinical practice. Although the dual - drug combination immunotherapy regimen has been widely used clinically, about 60% of patients will still experience primary or secondary drug resistance, resulting in significant differences in patients' treatment benefits.

There are two key bottlenecks in the current field that urgently need to be broken through, as follows:

(1) Lack of precise typing tools: The existing clinical and molecular typing systems cannot stably distinguish subtypes sensitive to immunotherapy, and it is difficult to provide reliable references for clinical decision - making, making it difficult to achieve precise application of immunotherapy;

(2) Lag in clinical translation process: Although a variety of immune checkpoint inhibitors have been clinically promoted and used, differentiated treatment strategies for different subtypes have not been established, and there is a lack of typing - guiding schemes verified by multiple centers.

To solve the above problems, this study intends to systematically develop a liver cancer typing system based on gene sets. The specific process is as follows: First, collect tumor and adjacent non - tumor tissue samples from patients with hepatocellular carcinoma, and perform whole - transcriptome sequencing to obtain transcriptome data; calculate the gene expression level of each patient sample based on the sequencing results, and then divide hepatocellular carcinoma into several subtypes through cluster analysis; map

these subtypes to the TCGA HCC cluster respectively, and screen out the subtypes that cannot be completely matched as new subtypes; further analyze the differential genes between the new subtypes and other subtypes. The screening criteria are: the ratio of gene expression between the new subtype and other subtypes > 4, the false discovery rate (FDR) < 0.01, and the gene expression in the new subtype > 1 and < 1 in other subtypes. Through this method, this study finally obtained a set of differential genes containing 29 genes.

Based on this, this study plans to use this 29 - gene set as a standard, obtain frozen surgical tissues from liver cancer patients for transcriptome sequencing, type the patients, and compare the differences in immunotherapy response between the new subtype and other subtypes at the large - sample level, aiming to provide a basis for precise immunotherapy of liver cancer.

II. Research Objectives

Evaluate the differences in immunotherapy response (including indicators such as objective response rate, disease control rate, and progression - free survival) between the new subtype defined by the 29 - gene set system and other subtypes, and confirm that patients with the new subtype have a higher response rate to immunotherapy.

III. Research Design and Methods

3.1 Research Objects

This study intends to include patients diagnosed with primary hepatocellular carcinoma who are treated in multiple centers including Sir Run Run Shaw Hospital, Zhejiang University School of Medicine (the leading unit). The relevant inclusion and exclusion criteria are as follows.

3.1.1 Inclusion Criteria

① ⩾ 18 years old.

② Patients who are treated in multiple centers including Sir Run Run Shaw Hospital, Zhejiang University School of Medicine (the leading unit) and diagnosed with primary hepatocellular carcinoma by surgical pathological histology.

③ They have received targeted drug combined with immune checkpoint inhibitor treatment after the specimen collection time point (post - surgery or post - puncture) (that is: local treatments such as TACE, ablation, etc. are allowed before the start of targeted - immunotherapy combination treatment, but an interval of ⩾ 4 weeks from the targeted - immunotherapy treatment is required).

④ Have measurable tumor lesions at the observation start point (according to RECIST 1.1 criteria), and can judge the tumor response status (including complete response CR, partial response PR, stable disease SD, or progressive disease PD) through imaging examination during the observation (such as post - treatment evaluation).

⑤ Have accessible frozen tumor tissue samples from surgical resection (sample volume ⩾ 3g); or tumor tissue samples obtained by puncture (puncture samples need to meet the pathological diagnosis requirements, and the sample quality can support subsequent transcriptome sequencing and analysis).

⑥ Voluntarily participate in this study and sign the informed consent form. If the subject

is unable to read and sign the informed consent form due to incapacity and other reasons, their guardian shall act as the agent for the informed process and sign the informed consent form. If the subject is unable to read the informed consent form (such as illiterate subjects), a witness shall witness the informed process and sign the informed consent form.

3.1.2 Exclusion Criteria

① Received any local treatment during the targeted - immunotherapy combination treatment (including during treatment and within 4 weeks after treatment interruption), including:

Transcatheter arterial chemoembolization (TACE), transcatheter arterial embolization (TAE), drug - eluting bead transcatheter arterial chemoembolization (DEB - TACE) and other vascular interventional therapies;

Radiofrequency ablation, microwave ablation, cryoablation, laser ablation and other ablation therapies;

Stereotactic body radiation therapy (SBRT), three - dimensional conformal radiation therapy, intensity - modulated radiation therapy and other local radiation therapies;

Intratumoral injection therapy, high - intensity focused ultrasound (HIFU) and other local physical/chemical therapies.

② Local treatment was initiated before the start of targeted - immunotherapy combination treatment, and the local treatment continued into the targeted - immunotherapy treatment period (that is, there was an overlap between local treatment and targeted - immunotherapy treatment or the interval was < 4 weeks).

③ Complicated with other active malignant tumors (except for those with cured basal cell carcinoma of the skin, carcinoma in situ of the cervix, and papillary thyroid carcinoma with no recurrence for more than 5 years after surgery).

④ Severe impairment of important organ functions:

Liver function: Child - Pugh class C, or total bilirubin > 3 × ULN;

Renal function: Serum creatinine > 1.5 × ULN and estimated glomerular filtration rate (eGFR) < 50 ml/min;

Cardiac function: New York Heart Association (NYHA) cardiac function class Ⅲ - Ⅳ, or left ventricular ejection fraction (LVEF) < 50%.

⑤ Have uncontrolled active infections, including:

Hepatitis B virus DNA > 2000 IU/ml (without receiving antiviral treatment);

Hepatitis C virus RNA positive and without receiving antiviral treatment;

Active tuberculosis infection, septicemia, etc.

⑥ Have a history of autoimmune diseases and currently still need to use systemic glucocorticoids (prednisone > 10 mg/day) or other immunosuppressive therapies.

⑦ Pregnant or lactating women, or patients planning to become pregnant during the study.

⑧ Have a definite history of allergy to the targeted drugs or immune checkpoint inhibitors involved in the study.

⑨ The researcher believes that there are other factors that may affect the judgment of treatment response (such as uncontrolled hypertension, coagulation dysfunction, simultaneous use of other antineoplastic drugs during targeted - immunotherapy

treatment, etc.).

3.1.3 Withdrawal Criteria

① The subject voluntarily requests to withdraw from the study and signs a written withdrawal statement.

② Severe adverse events occur during the study (such as grade 3 or above immune - related adverse reactions, severe infections, etc.), and after the researcher's evaluation, it is considered that continued participation in the study may endanger the subject's safety.

③ The subject does not comply with the research protocol, including but not limited to: failure to follow up at the specified time, refusal to complete necessary examinations, unauthorized acceptance of other antineoplastic treatments, etc.

④ It is found during the study that the subject does not meet the inclusion criteria of this study (such as the previous treatment history does not meet the requirements found in the baseline data verification).

⑤ The subject is unable to continue participating in the study due to disease progression, death, and other reasons.

⑥ The study is terminated in advance (such as protocol revision, ethical committee requiring termination, etc.).

3.2 Research Content

3.2.1 Data Acquisition System

3.2.1.1 Molecular Feature Collection

(1) Sample Source: Obtain frozen tumor tissues (≥ 3g) from patients through surgical resection or tumor tissue samples obtained by puncture (puncture samples need to meet the pathological diagnosis requirements, and the sample quality can support subsequent transcriptome sequencing and analysis). Use the Illumina HiSeq platform for RNA - seq sequencing. The raw data undergoes quality control (FastQC), alignment (HISAT2), quantification (FeatureCounts), and differential expression analysis (DESeq2) to screen for differential genes with FDR < 0.01 and expression ratio > 4. This part of the testing is outsourced to Hangzhou 迪安 Medical Laboratory Center Co., Ltd. for testing.

(2) Typing Algorithm: Perform hierarchical cluster analysis based on the 29 - gene set, and divide the subjects into a new typing cohort (unable to map to the TCGA HCC cluster) and other typing cohorts.

3.2.1.2 Clinical Data Integration

Structured extraction of subjects' baseline data (age, gender, Child - Pugh class, etc.), treatment information (targeted - immunotherapy combination regimen, dosage, and cycle, etc.), imaging evaluation (enhanced CT/MRI reports, etc.), and survival status (last follow - up date or death time, etc.) from the electronic medical record system. Simultaneously collect AFP (electrochemiluminescence method), PIVKA - II (enzyme - linked immunosorbent assay), and liver function biochemical data (total bilirubin, albumin, INR, etc.). The detection frequency matches the imaging evaluation cycle (that is, equivalent to clinical routine).

3.2.2 Immunotherapy Response Evaluation System

3.2.2.1 Primary Observation Indicators

(1) Time to Progression (TTP)

Definition: The time from the start of targeted - immunotherapy combination treatment to

the first time meeting the mRECIST progression criteria.
Evaluation Criteria:
Target lesions: The sum of the diameters of arterial - phase enhancement increases by ⩾ 20% compared to the baseline and the absolute value ⩾ 5mm;
Non - target lesions: New lesions appear or existing non - target lesions have definite progression;
Distant metastasis: New occurrences of lung, bone, or lymph node metastases.
(2) Objective Response Rate (ORR)
Definition: The proportion of patients achieving complete response (CR, disappearance of arterial - phase enhancement in all target lesions) and partial response (PR, the sum of the diameters of target lesions is reduced by ⩾ 30%).
Evaluation Time: For each line of targeted - immunotherapy combination treatment regimen received by the patient, the determination is made simultaneously at Week 8, Week 16, Week 24 of the regimen treatment, and at disease progression (or regimen termination). If the patient changes the targeted - immunotherapy treatment regimen due to poor efficacy, the treatment cycle of the new regimen needs to be recorded separately (Weeks 8, 16, 24 are recalculated from the start time of the new regimen), and the CR/PR status is evaluated at the corresponding time points. In the final ORR calculation, the CR/PR status achieved by the patient in a certain regimen only corresponds to the treatment cycle and evaluation time of that regimen, that is, it is clearly associated with the efficacy data of the specific targeted - immunotherapy combination regimen (including the first regimen or the subsequent new regimens).
3.2.2.2 Secondary Observation Indicators
(1) Core Indicators of Immunotherapy Response
① Progression - Free Survival (PFS)
Definition: The time from the start of targeted - immunotherapy combination treatment to the first disease progression (determined according to mRECIST) or death from any cause, whichever occurs first.
Evaluation Method: Combine imaging examinations (once every 8 weeks) and survival status follow - up, and use the Kaplan - Meier method to perform Log - rank test to compare the differences between groups.
② Overall Survival (OS)
Definition: The time from the start of targeted - immunotherapy combination treatment to the patient's death from any cause; for lost - to - follow - up patients, the last follow - up date is used as the censored data.
Evaluation Method: Record the survival status through regular telephone/outpatient follow - up, draw survival curves, and analyze the differences between groups.
Dynamic changes of tumor markers.
(2) Dynamic Monitoring Related to Tumors
Dynamic changes of tumor markers
Monitoring Indicators: Alpha - fetoprotein (AFP), protein induced by vitamin K absence or antagonist - II (PIVKA - II), detected once at baseline and every 4 weeks.
Judgment Criteria: An increase of ⩾ 20% compared to the baseline and lasting for more than 4 weeks, combined with imaging results to assist in predicting the progression

risk (such as the early warning value of marker increase before imaging progression).

(3) Evaluation of Treatment - Related Status

① Correlation with Liver Function Status

Evaluation Nodes: Collect liver function data (Child - Pugh class, total bilirubin, albumin, INR) simultaneously when disease progresses.

Analysis Logic: Compare with baseline liver function, exclude interfering factors such as drug - induced liver injury, and evaluate the direct impact of tumor progression on liver function injury.

② Survival Analysis after Progression

Record Content: Treatment adjustment plans after disease progression (changing targeted drugs, adding local treatments, etc.).

Analysis Indicators: Post - progression survival (PPS, the time from disease progression to death or the end of the study), and analyze the impact on survival by combining differences in treatment plans.

3.2.3 Statistical Analysis

3.2.3.1 Data Description and Comparison between Groups

Measurement data (such as age, AFP level, liver function indicators, etc.): Normally distributed data are expressed as mean ± standard deviation, and independent sample t - test (for two groups) or analysis of variance (for multiple groups) is used for comparison between groups; non - normally distributed data are expressed as median (interquartile range), and Mann - Whitney U test (for two groups) or Kruskal - Wallis test (for multiple groups) is used.

Count data (such as immunotherapy response type, typing results, etc.): Expressed as frequency (percentage), and chi - square test is used for comparison between groups; if the frequency of a cell < 5, Fisher's exact probability method is used instead.

3.2.3.2 Survival Analysis

Use the Kaplan - Meier method to draw survival curves of time to progression (TTP) and post - progression survival (PPS), and compare the survival differences between the new typing cohort and other typing cohorts through Log - rank test; use the Cox proportional hazards regression model to analyze the impact of 29 - gene set typing and clinical factors (such as Child - Pugh class, changes in tumor markers) on the progression risk.

3.2.3.3 Immunotherapy Response Analysis

The differences between groups in objective response rate (ORR) and disease control rate (DCR) are verified by chi - square test or Fisher's exact probability method, and the efficacy judgment follows the mRECIST criteria.

3.2.3.4 Analysis Tools

Use GraphPad Prism 10 for data visualization and basic statistics, and R language (survival, ggplot2 packages) for survival analysis and regression modeling. A P - value < 0.05 is used as the criterion for judging that the difference is statistically significant.

3.2.4 Protection Measures for Vulnerable Groups

The vulnerable groups that may be involved in this study include the mentally ill/cognitively impaired/critically ill patients/elderly/illiterate, etc. This study will take the following measures to protect their interests: 1) Ensure that the subjects are fully informed. If the subject is unable to read and sign the informed consent form due to

incapacity, their guardian shall act as the agent for the informed process and sign the informed consent form. If the subject is unable to read the informed consent form (such as illiterate subjects), a witness shall witness the informed process and sign the informed consent form. 2) If it is a critically ill patient, the researcher will closely monitor the vital signs of such subjects, and report in a timely manner once there is a situation.

IV. Sample Size Calculation

This study plans to include subjects from 10 top - three hospitals nationwide. Calculate the new typing score of HCC for each sample through the 29 - gene signature. It is estimated that there will be 100 cases in the new typing cohort and 250 cases in other typing cohorts.

Therefore, the final planned total sample size of this study is 350 cases.

V. Data Management and Confidentiality

All records related to the subject's identity will be kept confidential, and these materials will not be disclosed outside the scope permitted by relevant laws and/or regulations.

VI. Informed Consent

For the prospective observational study part involved in this study, before the subjects are included in this study, the researcher responsible for the informed consent discussion should introduce the purpose, nature, procedure, and possible benefits and risks of this study to them in written form completely and comprehensively. The subjects should be informed that they have the right to withdraw from the study at any time. Before inclusion, each subject should be fully informed and have sufficient time to consider whether to participate. Only after the subject voluntarily participates and signs the informed consent form can they be included in this study.

For the retrospective study part involved in this study, if it only involves the use of previously saved clinical data and biological specimens, does not interfere with the clinical diagnosis and treatment process, and will not have an adverse impact on the health of the subjects. During statistical analysis, the identity of the subjects is represented by codes, and there is no disclosure of the subjects' privacy and personal information. This part of the study does not involve commercial interests and can apply for exemption from informed consent.

VII. Adverse Events and Related Handling Measures

For the prospective observational study part involved in this study, only clinical data of the subjects are collected, no additional biological samples are collected, and the clinical diagnosis and treatment process is not interfered with, which will not have an adverse impact on the health of the subjects.

# Informed Consent Form

Protocol Name
Study on the Identification of New Subtypes of Liver Cancer Based on 29 Genes and the Correlation with the Efficacy of Immunotherapy
Version Number
V1.0
Version Date
July 13, 2025

Dear Subject,

We invite you to participate in the "Study on the Identification of New Subtypes of Liver Cancer Based on 29 Genes and the Correlation with the Efficacy of Immunotherapy" which has been reviewed and approved by the Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang University School of Medicine.

This informed consent form will provide you with some information to help you decide whether to participate in this clinical study. Your participation in this study is completely voluntary, and your decision will not affect your normal medical treatment rights and benefits in our hospital. If you choose to participate in this study, our research team will do our best to ensure your safety and rights during the research process.

Please read it carefully. If you have any questions, please raise them to the researcher in charge of this study.

## I. Research Background, Significance and Research Objectives

### 1.1 Background and Significance

Liver cancer is one of the leading causes of cancer - related deaths worldwide. Its significant heterogeneity makes it difficult for traditional diagnosis and treatment models to meet the needs of individualized precision treatment. The application of immune checkpoint inhibitors (ICI) has brought breakthrough progress to liver cancer treatment, but there are still severe challenges in clinical practice. Although the dual - drug combination immunotherapy regimen has been widely used clinically, about 60% of patients will still experience primary or secondary drug resistance, resulting in significant differences in patients' treatment benefits.

There are two key bottlenecks in the current field that urgently need to be broken through, as follows:

(1) Lack of precise typing tools: The existing clinical and molecular typing systems cannot stably distinguish subtypes sensitive to immunotherapy, and it is difficult to provide reliable references for clinical decision - making, making it difficult to achieve precise application of immunotherapy;

(2) Lag in clinical translation process: Although a variety of immune checkpoint inhibitors have been clinically promoted and used, differentiated treatment strategies for different

subtypes have not been established, and there is a lack of typing - guiding schemes verified by multiple centers.

To solve the above problems, this study intends to systematically develop a liver cancer typing system based on gene sets. The specific process is as follows: First, collect tumor and adjacent non - tumor tissue samples from patients with hepatocellular carcinoma, and perform whole - transcriptome sequencing to obtain transcriptome data; calculate the gene expression level of each patient sample based on the sequencing results, and then divide hepatocellular carcinoma into several subtypes through cluster analysis; map these subtypes to the TCGA HCC cluster respectively, and screen out the subtypes that cannot be completely matched as new subtypes; further analyze the differential genes between the new subtypes and other subtypes. The screening criteria are: the ratio of gene expression between the new subtype and other subtypes > 4, the false discovery rate (FDR) < 0.01, and the gene expression in the new subtype > 1 and < 1 in other subtypes. Through this method, this study finally obtained a set of differential genes containing 29 genes.

Based on this, this study plans to use this 29 - gene set as a standard, obtain frozen surgical tissues from liver cancer patients for transcriptome sequencing, type the patients, and compare the differences in immunotherapy response between the new subtype and other subtypes at the large - sample level, aiming to provide a basis for precise immunotherapy of liver cancer.

1.2 Research Objectives

Evaluate the differences in immunotherapy response (including indicators such as objective response rate, disease control rate, and progression - free survival) between the new subtype defined by the 29 - gene set system and other subtypes, and confirm that patients with the new subtype have a higher response rate to immunotherapy.

II. Research Process

2.1 Basic Research Situation

This study is a prospective observational clinical study. The expected number of subjects participating in this center is 100. If you agree to participate in this study, you need to sign this informed consent form. After that, we will judge the inclusion and exclusion criteria. If you meet the inclusion criteria and do not meet the exclusion criteria, you will be included in this study.

2.2 Observation Indicators

This study will structurally extract the following data of yours from the electronic medical record system. The detection frequency matches the imaging evaluation cycle (that is, equivalent to clinical routine).

(1) Baseline data: age, gender, Child - Pugh class, etc.;

(2) Treatment information: targeted - immunotherapy combination regimen, dosage, and cycle, etc.;

(3) Blood indicators: AFP (electrochemiluminescence method), PIVKA - II (enzyme - linked immunosorbent assay), and liver function biochemical data (total bilirubin, albumin, INR, etc.);

(4) Imaging evaluation information: enhanced CT/MRI reports;
(5) Survival status: your last follow - up date, etc.

III. Possible Risks and Corresponding Measures

The prospective observational study part involved in this study only collects the clinical data of subjects, without the need to collect additional biological samples, and does not interfere with the clinical diagnosis and treatment process, which will not have an adverse impact on the health of subjects. Therefore, there are no research - related adverse events involved.

IV. Expected Benefits

You may not directly benefit from this study. However, the data you provide will provide useful information for the research of this disease, help researchers further understand the pathogenesis of the disease and further explore the treatment plan related to the disease, and may ultimately bring benefits to your future diagnosis and treatment and to patients with the same disease.

V. Alternative Treatments

This study is a prospective observational study, only collecting the clinical data of subjects, so it does not involve treatment.

VI. Research Costs

All inspection and evaluation items in this study are routine clinical inspection and evaluation items, so the relevant costs need to be borne by you yourself. Therefore, participating in this study will not additionally increase your routine medical treatment costs.

In addition, this study does not provide subsidies.

VII. Compensation and Handling of Injuries

In case of damage, our research team will provide you with timely treatment. If, as judged by the researcher, the damage is related to this study, this study will bear the corresponding treatment costs, compensation.

VIII. Confidentiality

If you decide to participate in this study, your participation in the trial and your personal information and related materials in the trial are confidential. Your research - related materials will be identified by research number digits instead of your name. Information that can identify your identity will not be disclosed to members outside the research group unless your permission is obtained. All research members are required to keep your identity and information confidential. Your files will be kept in a locked file cabinet and can only be accessed by researchers. To ensure that the research is carried out in accordance with regulations, if necessary, members of government management departments or ethics review committees can access your personal information in the research unit in accordance with regulations.

When the results of this study are published, no personal information of yours will be disclosed.

IX. Voluntariness

You can choose not to participate in this study, or you can notify the researcher at any

time to request to withdraw from the study. Your data will not be included in the research results, and your medical treatment and rights will not be affected.

If you need other treatments, or you do not comply with the research plan, or if research - related injuries occur, or for any other reasons, the research physician can terminate your continued participation in this study.

X. Subject's Obligations

As a subject, you have the following responsibilities: truthfully provide the true information about your own medical history and current physical condition; inform the research doctor of any discomfort you experience during this study; tell the research doctor whether you have participated in other studies recently or are currently participating in other studies.

XI. Contact Information

You can learn about the information and research progress related to this study at any time. If you have questions related to this study, or if you experience any discomfort or injury during the research process, you can contact the research personnel at any time (Dr. Xu Junjie, 13656688716, 4th Floor, Building 7, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, 3 Qingchun East Road, Hangzhou, Zhejiang).

If you have any questions about the rights of a subject participating in the research, please contact the Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang University School of Medicine (0571 - 86006811).

Informed Consent Form - Signature Page

I have read this informed consent form.

I had the opportunity to ask questions and all questions have been answered.

I understand that participation in this study is voluntary.

I can choose not to participate in this study, or withdraw by notifying the researcher at any time without being discriminated against or retaliated against, and my medical treatment and rights will not be affected.

If I need other treatments, or I do not comply with the research plan, or if research - related injuries occur or for any other reasons, the research physician can terminate my continued participation in this study.

I will receive a signed copy of the “Informed Consent Form”.

Subject's Name (in block letters): _____ Subject's Signature: _____ Contact Information: _____ Date: _____ Year _____Month _____Day

If the subject is unable to read and sign the informed consent due to incapacity or other

reasons, or if the subject is a minor, the guardian will act as the agent for the informed process and sign.

Guardian's Name (in block letters): _____ Guardian's Signature: _____ Relationship with Subject: _____ Contact Information: _____ Date: _____ Year _____Month _____Day

If the subject is unable to read the informed consent form (such as an illiterate subject), a witness will witness the informed process and sign.

Witness's Name (in block letters): _____ Witness's Signature: _____ Contact Information: _____ Date: _____ Year _____Month _____Day

I have accurately informed the subject of this document, he/she has accurately read this informed consent form, and it is proved that the subject had the opportunity to ask questions. I prove that he/she voluntarily consented.

Researcher's Name (in block letters): _____ Researcher's Signature: _____ Contact Information: _____ Date: _____ Year _____Month _____Day