Clinical research plan

Project Title: **Predictive Value of Serum Histone Succinylation in Hematological Cancers**

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Declaration of Conformity

Abide by the Declaration of Helsinki and commit to conducting this study in accordance with this protocol. Participants must be trained. The study will be conducted after obtaining written approval from the ethics committee and written informed consent from the subjects. Any amendments to the protocol require re-approval.

I. Summary of the Plan

In recent years, important breakthroughs have been made in the field of protein post-translational modification research. Among them, histone succinylation modification, as a new metabolic-related epigenetic regulatory mechanism, plays a key role in the occurrence and development of tumors. Studies have shown that succinylation modification significantly affects the dynamic structure of chromatin and the gene transcription regulatory network by changing the charge characteristics and spatial conformation of histones, thereby participating in malignant biological behaviors such as metabolic reprogramming, abnormal proliferation and apoptosis escape of tumor cells. In particular, in hematological malignancies (such as leukemia, lymphoma, multiple myeloma, etc.), the abnormal expression pattern of histone succinylation modification is significantly correlated with disease subtype, clinical stage and treatment response, which provides an important theoretical basis for the development of new molecular markers for hematological tumors. This project focuses on hematological malignancies and innovatively uses patient peripheral blood serum as the research object. It uses highly specific histone succinylation modification antibodies combined with high-sensitivity mass spectrometry detection technology to establish a precise quantitative detection system for serum histone succinylation modification. By systematically analyzing the characteristic succinylation profiles of different hematologic malignancies and at various stages of disease progression, combined with multidimensional clinical data integration, the goal is to construct a multi-parameter prediction model based on serum histone succinylation profiles for early diagnosis, therapeutic efficacy assessment, and prognosis prediction of hematologic malignancies. This research has the potential to overcome existing technical bottlenecks, enabling a technological revolution in the non-invasive, single-tube blood test for hematologic malignancies and promoting a

shift in the diagnostic paradigm from traditional "gene-driven" to innovative "epigenetic metabolism-driven" approaches. This project will be the first to systematically evaluate the translational application of serum histone succinylation in the clinical diagnosis of hematologic malignancies. It will not only provide new scientific insights into the epigenetic metabolism regulation mechanisms of hematologic malignancies, but also potentially offer a novel, efficient, minimally invasive strategy for early screening and dynamic monitoring of hematologic malignancies in clinical practice. This project holds significant theoretical significance and promising clinical applications. The findings will open new avenues for the precision diagnosis and treatment of hematologic malignancies and provide valuable insights for epigenetic marker research in other systemic malignancies.

II. Introduction

2.1 Research background , project objectives and preliminary research basis

2.1.1 Research Background

Cancer has become a major social, economic and public health issue in the 21st century and is one of the leading causes of death worldwide. In 2020, China ranked first in the world in both new cancer cases and deaths . With the rapid development of medical science and technology, early diagnosis of tumors is crucial to improving patients' survival rates and quality of life . However, existing tumor diagnostic methods, such as imaging examinations, histopathological examinations and traditional tumor marker tests, still have shortcomings in sensitivity, specificity and early detection capabilities . They cannot detect multiple tumors in one method , and may have limitations such as high professional requirements, long time consumption and high testing costs. Therefore, it is crucial to develop a new pan-tumor screening and diagnostic method with low cost, high sensitivity and high specificity .

Cell-free nucleosomes are nucleosomes released into circulating tissues after apoptosis or necrosis, retaining some specific information about their cell and tissue of origin. The histone modification status on cell-free nucleosomes produced by tumor cells reflects the metabolic state and epigenetic changes of tumor cells.

Therefore, histone modifications on cell-free nucleosomes are expected to become new biomarkers for non-invasive tumor detection. Although studies have found an association between histone succinylation and tumors, and that cell-free nucleosomes retain histone modifications specific to the tumor cell of origin, histone succinylation on cell-free nucleosomes has not yet been applied to tumor screening and detection. Therefore, it is urgent to study whether histone succinylation on cell-free nucleosomes can be used as a low-cost, highly sensitive, and highly specific pan- tumor marker for early cancer screening, efficacy assessment, and prognosis.

2.1.2 Project Objectives

The core goal of the project is to systematically verify and establish the clinical application value of serum histone succinylation modification as a novel, efficient, and non-invasive pan-tumor biomarker.

Objective 1: Validate the potential for pan-tumor screening: Through a large-scale case-control study, clearly confirm that serum histone succinylation levels are significantly different between patients with malignant hematological tumors and healthy people, thereby providing a solid evidence-based basis for developing it as a blood-based early screening tool applicable to multiple tumor types.

Objective 2: Exploring Clinical Applications : This project will conduct in-depth analysis of the association between serum histone succinylation levels and different tumor types and stages, evaluating their ability to distinguish tumor types and reflect disease progression. Furthermore, the project will analyze their correlation with treatment efficacy (e.g., changes in levels before and after surgery, radiotherapy, and systemic treatment) and clinical pathological features, exploring their potential applications in dynamic efficacy monitoring and prognostic assessment.

2.1.3 Preliminary Research Foundation

(1) Professor Weng Jiemin's research group at East China Normal University previously found that histone succinylation modification was significantly higher in various solid tumors than in adjacent tissues. The results are shown in the figure below: Histone H4K31 succinylation modification was significantly higher in various solid tumors than in adjacent tissues.

Professor Weng Jiemin's research group previously prepared histones from human breast, prostate, lung, and kidney cancer tissue samples, as well as mouse lung cancer tissue samples. Western blot results (Figure 1) showed that succinylation of histones at H4K31 and H3K14 sites was significantly elevated in cancer tissues compared to adjacent adjacent tissues, as seen in breast cancer (Figure 1A), prostate cancer (Figure 1B), kidney cancer (Figure 1C), and lung cancer (Figures 1D and 1E). This suggests that histone succinylation could serve as a potential biomarker for tumor development and progression.

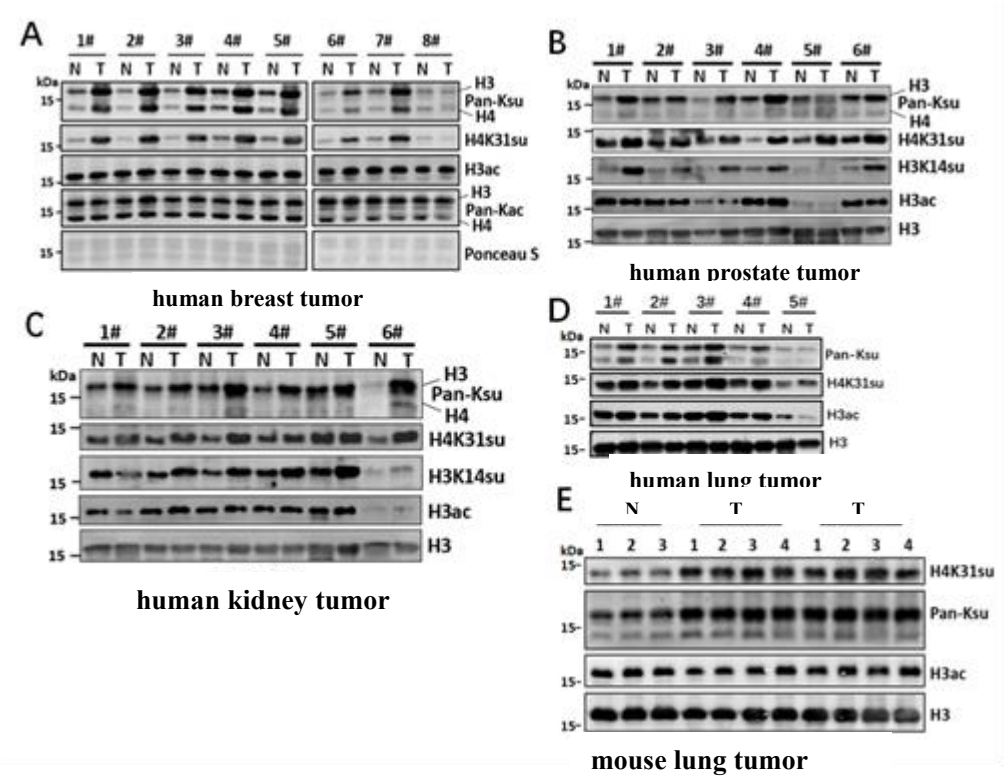

Figure 1. The succinylation modification of histone H4K31 site was significantly higher in tumor tissues than in adjacent tissues: (A) Western blot analysis of the levels of succinylation modification and histone acetylation modification of histone H4K31 site in human breast tumor samples compared with adjacent tissues; (B) Western blot analysis of the levels of succinylation modification and histone acetylation modification of histone H4K31 site in human prostate tumor samples compared with adjacent tissues; (C) Western blot analysis of the levels of succinylation modification and histone acetylation modification of histone H4K31 site in human kidney tumor samples compared with adjacent tissues; (D) Western blot analysis of the levels of succinylation modification and histone acetylation modification of histone H4K31 site in human lung tumor samples compared with adjacent tissues; (E) Western Blot analysis of the levels of histone H4K31 succinylation and histone acetylation in mouse lung tumor samples compared with normal tissues; N: adjacent tissue to the tumor; T: tumor tissue.

(2) Based on the findings in solid tumors, the level of histone succinylation modification in tumor tissue is significantly higher than that in adjacent tissues.

Therefore, the research team cooperated with the hospital to collect blood samples from patients with different types of tumors. It was found that the succinylation modification of the free nucleosome histone H3K23 site in the blood of tumor patients was significantly higher than that of normal people. However, the number of serum samples from tumor patients needs to be further expanded.

2.2 Risk/Benefit Assessment

Risk Assessment:

This study was observational, and the core intervention consisted of a single venous blood sample collection (approximately 1 mL). This procedure is a routine and established clinical practice with extremely low and manageable risks .

Benefit evaluation:

Cancer patients will not receive immediate personalized diagnosis or treatment recommendations from this study. However, the data they contribute will promote the development of better tumor markers and may benefit their future disease management (such as more accurate follow-up monitoring).

Healthy controls: Contribute to establishing baseline values for healthy people and promote public health. Once the study is completed and the methods are mature, the baseline data from their participation may provide a reference background for future similar screening.

3. Study Objectives and Endpoints

3.1 Purpose

3.1.1 Primary Objective

To evaluate the differences in serum histone succinylation levels between patients with hematologic malignancies and healthy individuals, and to confirm whether there is a significant difference in serum histone succinylation levels between these two groups.

3.1.2 Secondary Objectives

To determine the correlation between serum histone succinylation modification levels and different types of tumors as well as tumor stages, and to statistically

analyze the relationship between serum histone succinylation and therapeutic outcomes (including chemotherapy, hematopoietic stem cell transplantation, and cellular immunotherapy) as well as clinical pathological features.

3.2 Evaluation Metrics

The evaluation metrics include: sensitivity, specificity, positive predictive value, negative predictive value, and Youden’s index of the screening method for serum histone succinylation modification levels, assessing their predictive value for early diagnosis, treatment efficacy, and prognosis.

4. Study Population

4.1 Inclusion Criteria

Case group

1) ≥ 18 years old , regardless of gender;
2) Hematological malignancies confirmed by cytology, histology , or molecular biology (such as leukemia, lymphoma, multiple myeloma, etc.), who require subsequent anti-tumor treatment (chemotherapy , hematopoietic stem cell transplantation, radiotherapy, or cellular immunotherapy) ;

control group

1) ≥ 18 years old , regardless of gender;
2) Non-cancer patients undergoing health checkups;

4.2 Exclusion criteria

1) Pregnant women
2) Those with severe mental disorders or language communication barriers
3) Other circumstances that the researcher judges to be unsuitable for participation in this study

The control group was the same as the case group

V. Research Design

5.1 Overall Design

This study was a single-center prospective case-control study.

5.2 Research Design Process

5.2.1 Study the specific implementation process

1. Phase 1: Screening population for enrollment

i. Conduct ethical review and clinical research plan review for the project;

ii. 200 patients with hematological malignancies will be selected , basic information collected , and informed consent signed ;

iii: 200 healthy subjects are planned to be selected and basic information collected;

2. Second stage: Collect blood samples and perform serum histone succinylation test

Blood samples were collected from all 200 eligible participants and 200 healthy subjects for serum histone succinylation testing ;

3. The third stage: statistical analysis of screening results

i. Evaluate the differences in serum histone succinylation between patients with hematological malignancies and healthy controls to validate its value in early screening.

ii. Statistical analysis of the correlation between serum histone succinylation and tumor efficacy (including chemotherapy , hematopoietic stem cell transplantation, cellular immunotherapy) and clinical outcomes . (Efficacy evaluation was performed using RECIST 1.1 criteria)

5.3 Statistical analysis

This study employed an unmatched case-control study (500 cases in the case group and 500 healthy controls). The sample size was determined based on a pre-experimental effect size (Cohen's d = 1.2) and a 10% dropout rate. All statistical analyses were performed using R , with an α level of 0.05. **The following primary analyses were performed: 1) Comparisons of histone succinylation levels between groups were performed using the independent sample t-test (for normal distribution) or the Mann-Whitney** *U test (for non-normal data)* , after

Shapiro-Wilk normality and Levene's homogeneity of variance tests . Mean differences (MD)/median differences with 95% confidence intervals (CIs) and Cohen's d effect sizes were reported. 2) Diagnostic performance was evaluated by calculating the area under the receiver operating characteristic (ROC) curve (AUC) and its bootstrap 95% CI. The optimal cutoff value was determined by maximizing the Youden index. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and their exact Clopper-Pearson 95% CIs were also calculated. **Secondary analyses included** : 1) Subgroup associations: One-way analysis of variance (ANOVA) or the Kruskal-Wallis test (Bonferroni correction post hoc test) was used to compare differences in levels between different tumor types, and Cuzick's trend test was used to analyze ordered associations with TNM stage. 2) Efficacy assessment: Changes in levels before and after treatment were evaluated using paired t-tests/Wilcoxon signed-rank tests. 3) Prognostic value: Baseline succinylation levels (grouped by median) were included in a Cox proportional hazards model. Hazard ratios (HRs) and 95% CIs were calculated after adjusting for age, stage, and treatment regimen. Survival curves were plotted using the Kaplan-Meier method (log-rank test). Multivariate **analysis** : A logistic regression model was constructed (incorporating succinylation levels and covariates such as age and sex). The DeLong test was used to compare area under the curve (AUC). Goodness of fit was assessed using the Hosmer-Lemeshow calibration curve. Net clinical benefit was analyzed using a decision curve analysis (DCA). If multiple marker modifications were detected, LASSO regression was used to select marker combinations. **Quality control** included Grubbs' test/Winsorize to address outliers, and multiple imputation with ice cream (MICE) to impute missing covariates <5%. **Sensitivity analyses** included excluding stage I tumors to validate robustness, and using a 70%-30% dataset split for training-validation cutoffs. All multiple comparisons were performed using the Benjamini-Hochberg method to control the false discovery rate (FDR).

VI . Ethical Requirements

Clinical research will adhere to relevant regulations such as the World Medical

Association's Declaration of Helsinki. Prior to commencement, the study must be approved by the ethics committee.

Before each subject is selected for this study, the researcher is responsible for fully and comprehensively introducing the purpose, procedures and possible risks of the study to the subject or his/her agent, and letting the subject know that they can refuse to participate or withdraw from the study at any time at any stage of the study without being discriminated against or retaliated, and that their medical treatment and rights will not be affected. After the subject fully understands and agrees, he/she will sign a written informed consent form, which will be retained as a clinical research document for future reference. During the study, the researcher will protect the subject's personal privacy and data confidentiality. This study uses electronic signatures for informed consent, and doctors/working group coordinators will communicate with the subjects on-site. Before the subject is selected for the study, the doctor will inquire about and record the subject's medical history. If the subject is an eligible participant and voluntarily participates, the subject will be asked to sign the informed consent form. All electronic informed consent forms will be archived.

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