Clinical Study Protocol

Project Title:

Predictive Value of Serum Histone Succinylation in Malignant Solid Tumors

Leading institutions:

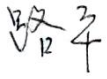
The First Affiliated Hospital of Xinxiang Medical University

School of Life Sciences, East China Normal University

Version: V4.1

Date: 19-Jul-2025

Principal Investigators: Ping Lu, Jiemin Weng

Protocol Approval: 

Compliance Statement

Participants must comply with the Declaration of Helsinki and commit to conducting this study in accordance with this protocol. They must be trained and the study can only be carried out after obtaining written approval from the ethics committee and written informed consent from the subjects. Any revision to the protocol requires re-approval.

一、scenario summary

In recent years, with the in-depth research on post-translational modifications of proteins, scientists have discovered that histone succinylation modification, as a novel protein modification method, is closely related to the occurrence, development and prognosis of tumors. Succinylation modification affects processes such as cell metabolism, proliferation and apoptosis by altering the physicochemical properties and functions of proteins. In tumor cells, the abnormal expression of succinylation modification is closely related to tumor type, stage and prognosis, providing new ideas for the early diagnosis of tumors. This project uses blood serum samples and detects and analyzes the level of histone succinylation modification in the serum through specific histone succinylation modification antibodies. Combined with other clinical information, it aims to achieve early, rapid and accurate diagnosis of pan-tumors, realize the rapid screening of tumors with "one tube of blood", and promote the transformation of precise early diagnosis of tumors from "gene-driven" to "epigenetic metabolism-driven". The implementation of this project aims to explore the feasibility of histone succinylation modification in serum as a tumor marker, providing more effective biomarkers for tumor early screening, therapeutic effect evaluation and prognosis. This will help develop more accurate and convenient blood testing methods in the future, benefiting a wider patient population and having significant social value.

二、introduction

2.1 Research background, project objectives and the foundation of previous research

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, both the number of new cancer cases and deaths in China ranked first in the world. With the rapid development of medical technology, early diagnosis of tumors is of vital importance for improving the survival rate and quality of life of patients. However, existing tumor diagnostic methods, such as imaging examinations, histopathological examinations, and traditional tumor marker detection, still have deficiencies in terms of sensitivity,

specificity, and early detection capabilities. They cannot achieve the detection of multiple tumors with a single method, and may have limitations such as high professional requirements, long time consumption, and high detection costs. Therefore, it is of vital importance to develop a novel pan-tumor screening and diagnosis method that is low-cost, highly sensitive and highly specific.

Free nucleosomes in cells are nucleosomes released into circulating tissues after apoptosis or necrosis of cells, and they retain some specific information of the origin cells and tissues. The histone modification status on the free nucleosomes produced by tumor cells reflects the metabolic state and epigenetic changes of tumor cells. Therefore, histone modification of free nucleosomes in cells is expected to become a new biomarker for non-invasive tumor detection. Although previous studies have found the association between histone succinylation modification and tumors, and free nucleosomes in cells retain specific histone modifications of the origin tumor cells, histone succinylation modification of free nucleosomes in cells has not yet been applied to tumor screening and detection. Therefore, it is urgently necessary to study whether histone succinylation modification of free nucleosomes can be used as a low-cost, highly sensitive and highly specific pan-tumor marker and applied in the early screening, therapeutic effect evaluation and prognosis of tumors.

Succinylation modification, as a widely existing post-translational modification of proteins, has potential biomarker value in malignant solid tumors. Besides tumors, existing studies have shown that some diseases (such as inflammatory diseases, infectious diseases, metabolic diseases, cardiovascular diseases, neuro-psychological diseases, etc.) have certain correlations with non-histone succinylation modification. The role of histone succinylation modification in other diseases is currently lacking in relevant clinical research evidence and awaits further exploration in this study. Therefore, this research focuses on malignant solid tumors, not only filling the knowledge gap in this field but also laying the foundation for future expansion to other diseases.

2.1.2 **project objective**

The core objective 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: Verify the potential of pan-tumor screening: Through large-scale case-control studies, it is clearly confirmed that there are significant differences in histone succinylation levels in serum between patients with malignant solid tumors and healthy individuals, thereby providing a solid evidence-based basis for developing it into a blood-based early screening tool suitable for multiple tumor types.

Objective 2: Exploring clinical application scenarios: The project will conduct an in-depth analysis of the association between serum histone succinylation levels and different types of tumors as well as different tumor stages, and evaluate its ability to distinguish tumor types and reflect disease progression. Meanwhile, analyze its correlation with therapeutic effects (changes in levels before and after surgery, radiotherapy, and systemic treatment) and clinicopathological characteristics, and explore its application prospects in dynamic monitoring of therapeutic effects and prognosis assessment.

2.1.3 Preliminary research foundation

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

Professor Weng Jieming's research group previously prepared histones from human breast cancer, prostate cancer, lung cancer, kidney cancer, and lung cancer tissue samples from mice. The Western blot results (see Figure 1) indicated that the levels of succinylation modifications at histone H4K31 and H3K14 sites in cancer tissues were significantly higher than those in adjacent tissues, such as breast cancer (Figure 1A), prostate cancer (Figure 1B), renal cancer (Figure 1C), and lung cancer (Figures 1D and 1E). It reveals that histone succinylation modification can serve as a potential biomarker for tumorigenesis and development.

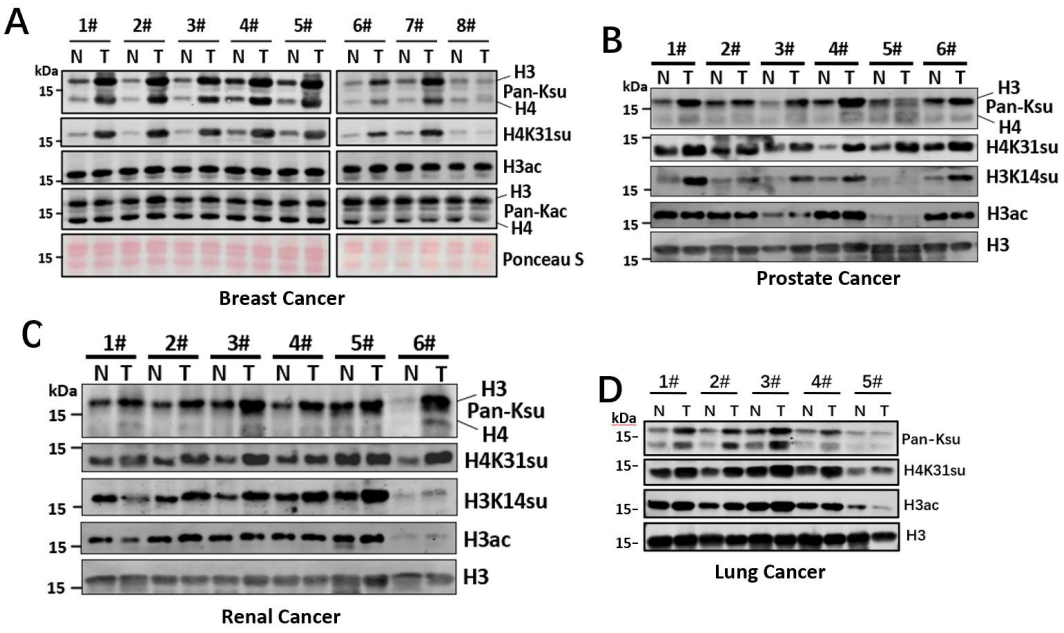


Figure 1: The succinylation modification at the histone H4K31 site in tumor tissues was significantly higher than that in adjacent tissues: (A) Western blot analysis of the levels of succinylation modification and histone acetylation modification at the histone H4K31 site in human breast tumor samples compared with those in adjacent tissues; (B) Western blot was used to analyze the levels of succinylation modification and histone acetylation modification at the histone H4K31 site in human prostate tumor samples compared with those in adjacent tissues. (C) Western blot was used to analyze the levels of succinylation modification and histone acetylation modification at the histone H4K1 site in human renal tumor samples compared with those in adjacent tissues. (D) Western blot was used to analyze the levels of succinylation modification and histone acetylation modification at the histone H4K31 site in human lung tumor samples compared with those in adjacent tissues.

Based on the findings in solid tumors, the level of histone succinylation modification in tumor tissues was significantly higher than that in adjacent tissues. Therefore, the research group, in collaboration with hospitals, collected blood samples from patients with different types of tumors and found that the succinylation modification at the H3K23 site of free nucleosome histones in the blood of tumor patients was significantly higher than that of normal people. However, the number of serum

samples from cancer patients still needs to be further expanded.

The research group previously isolated free nucleosomes from human colon cancer, gastric cancer, breast cancer, lung cancer, pancreatic cancer, multiple myeloma, liver cancer, cervical cancer, prostate cancer, ovarian cancer, esophageal cancer, cholangiocarcinoma and normal people's blood for Western blot. The results and gray-scale analysis statistics (see Figure 2) indicate that the succinylation modification of free nucleosome histone H3K23 sites in the blood of patients with different types of tumors (for specific information on different tumor types, see Figure 3) is significantly higher than that of normal people, revealing that the succinylation modification of free nucleosome histone in the blood of tumor patients may serve as a biomarker for the early diagnosis of tumors. However, at present, due to the small sample size, it is necessary to carry out this project to further expand the sample size and conduct in-depth research.

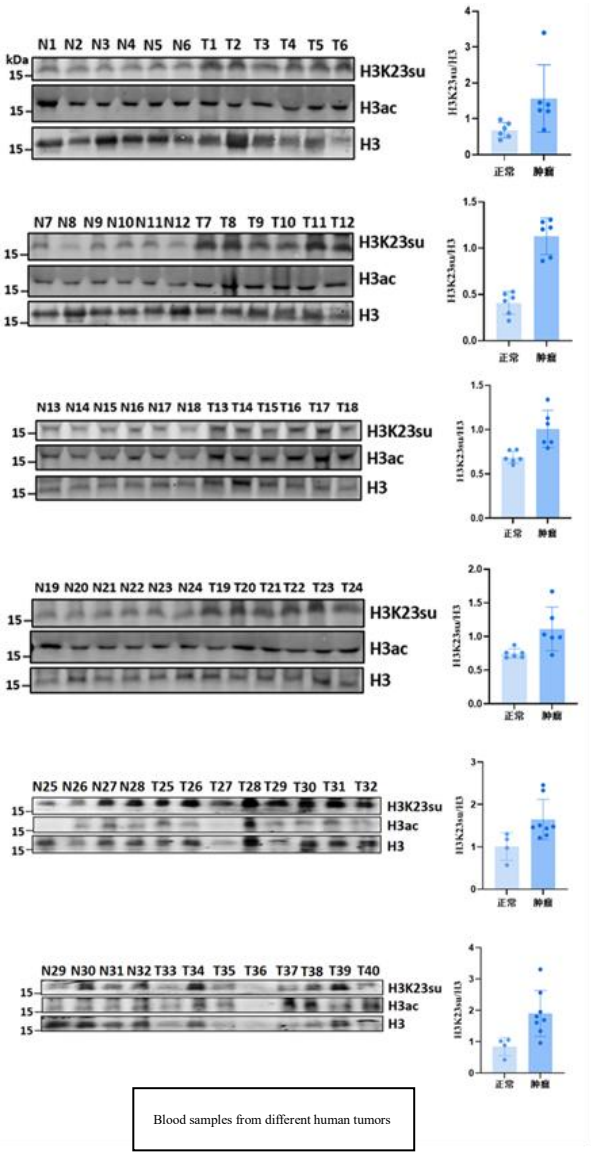


Figure 2 shows that the levels of free nucleosome histone succinylation modification in the blood of patients with different types of tumors are significantly higher than those of normal people: Enrich the free nucleosomes in the blood of tumor patients. Use histone H3K23 specific site succinylation modification and histone H3 acetylation modification antibodies to analyze the levels of histone succinylation modification and acetylation modification of free nucleosomes in the blood of different types of tumor patients by Western blot. And conduct gray-scale analysis and statistics on the Western blot results. Among them, N: normal human serum; T: Serum of cancer patients. Specific information on different types of tumors is shown in Figure 3.

Sample	Cancer patients	Sample	Controls
T1	Colon Cancer	**N1**	Healthy
T2	Stomach Cancer	**N2**	Healthy
T3	Colon Cancer	**N3**	Healthy
T4	Breast Cancer	**N4**	Healthy
T5	Breast Cancer	**N5**	Healthy
T6	Colon Cancer	**N6**	Healthy
T7	Colon Cancer	**N7**	Healthy
T8	Lung Cancer	**N8**	Healthy
T9	Lung Cancer	**N9**	Healthy
T10	Stomach Cancer	**N10**	Healthy
T11	Pancreatic Cancer	**N11**	Healthy
T12	Multiple myeloma	**N12**	Healthy
T13	Multiple myeloma	**N13**	Healthy
T14	Liver Cancer	**N14**	Healthy
T15	Cervical Cancer	**N15**	Healthy
T16	Lung Cancer	**N16**	Healthy
T17	Lung Cancer	**N17**	Healthy
T18	Prostate Cancer	**N18**	Healthy
T19	Ovarian Cancer	**N19**	Healthy
T20	Breast Cancer	**N20**	Healthy
T21	Ovarian Cancer	**N21**	Healthy
T22	Stomach Cancer	**N22**	Healthy
T23	Stomach Cancer	**N23**	Healthy
T24	Lung Cancer	**N24**	Healthy
T25	Stomach Cancer	**N25**	Healthy
T26	Stomach Cancer	**N26**	Healthy
T27	Stomach Cancer	**N27**	Healthy
T28	Pancreatic Cancer	**N28**	Healthy
T29	Pancreatic Cancer	**N29**	Healthy
T30	Pancreatic Cancer	**N30**	Healthy
T31	Pancreatic Cancer	**N31**	Healthy
T32	Esophageal Cancer	**N32**	Healthy
T33	Liver Cancer		
T34	Liver Cancer		
T35	Bile Duct Cancer		
T36	Bile Duct Cancer		
T37	Bile Duct Cancer		
T38	Ovarian Cancer		
T39	Ovarian Cancer		
T40	Cervical Cancer		

Figure 3. Information of different types of tumor samples and information of normal people

2.2 Risk/Benefit assessment

risk assessment：

This study was an observational one, and the core intervention was only a single venous blood sample collection of 3mL (approximately 1ml of serum). This operation

is a routine and mature medical practice in clinical practice, with extremely low and controllable risks.

Benefit evaluation：

Cancer patients: They will not receive immediate individualized diagnosis or treatment recommendations from this study. However, the data it contributes will drive the development of better tumor markers, which may benefit its future disease management (such as more precise follow-up monitoring).

Healthy control subjects: Contribute to establishing baseline standard values for healthy populations and promote public health. After the research is completed and the method is mature, the baseline data in which it participated may provide a reference background for its own future similar screening.

三、Research objectives and endpoints

3.1 objective

3.1.1 main purpose

To evaluate the differences in serum histone succinylation between malignant solid tumors and healthy individuals, in order to verify its early screening value.

3.1.2 secondary objective

To clarify the correlation between histone succinylation modification in serum and different types of tumors and tumor stages, and to statistically analyze the correlation between histone succinylation in serum and therapeutic effects (including surgical treatment, radiotherapy, and systemic therapy) as well as clinicopathology.

3.2 evaluation index

The evaluation indicators are: the sensitivity, specificity, positive predictive value, negative predictive value of the serum histone succinylation screening method, and the predictive value of the Youden index (early diagnosis, therapeutic effect evaluation and prognosis).

四、study population

4.1 inclusion criteria

case group

1）≥18 years old, gender not limited;

2）Malignant solid tumors diagnosed by cytology or histology require subsequent anti-tumor treatment (systemic therapy, radical concurrent chemoradiotherapy/radiotherapy, or surgical treatment).

control group

1) ≥18 years old, gender not limited;

2) Non-tumor patients undergoing health check-ups;

4.2 exclusion criteria

1) Pregnant women 2) Those with severe mental disorders or language communication disorders; 3) Other circumstances where the researcher deems it unsuitable to participate in this study.

The control group was the same as the case group.

五、study design

5.1 overall design

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

5.2 Research design process

5.2.1 Study the specific implementation process

1. Phase One: Screening and enrolling the population

i. Conduct ethical reviews and clinical research protocol reviews for the project;

ii. It is planned to select 500 patients with malignant solid tumors, collect their basic information and sign the "Informed Consent Form".

iii: It is planned to select 500 healthy subjects and collect basic information.

2. Phase Two: Collect blood samples for serum histone succinylation detection

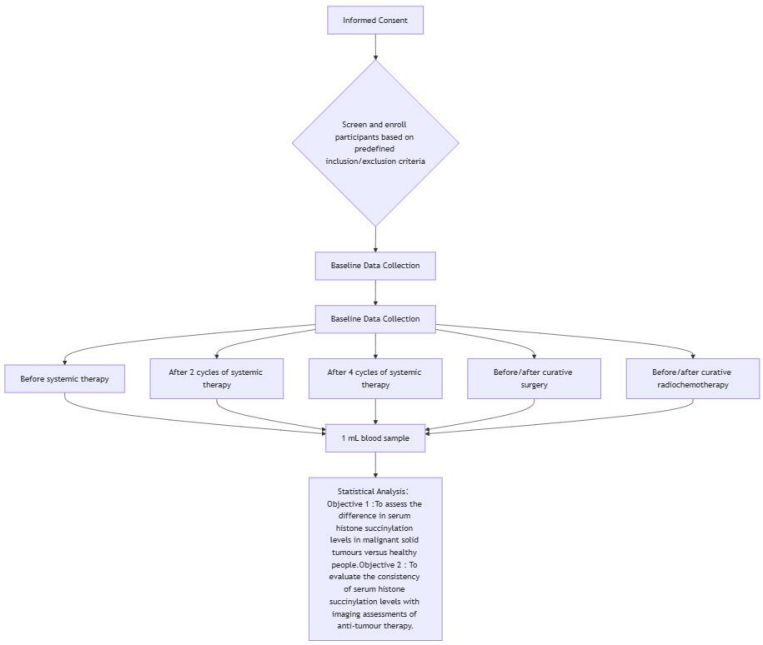
Blood samples were collected from all 500 enrolled individuals and 500 healthy subjects who met the requirements for serum histone succinylation detection.

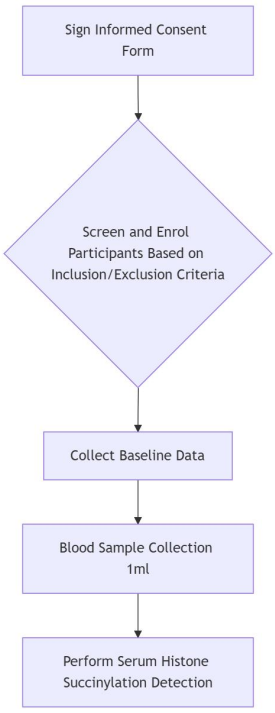
3. Phase Three: Statistical analysis of Screening Results

i. Western blot was used to evaluate the differences in serum histone succinylation between malignant solid tumors and healthy individuals to verify its early screening value.

ii. Statistically analyze the correlation between serum histone succinylation and tumor efficacy (including surgical treatment, radiotherapy, and systemic therapy) as well as clinicopathology. The therapeutic effect was evaluated using RECIST1.1 criteria.

5.2.2 Research flowchart





5.2.3 Blood collection process table

<table>
<tr><td>grouping</td><td colspan="8"></td></tr>
<tr><td></td><td>Screening period</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>healthy person</td><td>D-7—D0</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td rowspan="3">Cancer patients are receiving systemic treatment</td><td></td><td colspan="6">Chemotherapy cycle</td><td></td></tr>
<tr><td></td><td>C1D1</td><td>C2</td><td>C3</td><td>C4</td><td>C5</td><td>C6</td><td></td></tr>
<tr><td>D-7—D0</td><td></td><td></td><td>D1±3</td><td></td><td>D1±3</td><td></td><td></td></tr>
<tr><td rowspan="2">Cancer patients who receive radical chemoradiotherapy/radiotherapy</td><td rowspan="2">D-7—D0</td><td colspan="2">After the completion of radiotherapy/chemoradiotherapy</td><td colspan="3">Four to six weeks after the completion of radiotherapy/chemoradiotherapy</td><td></td><td></td></tr>
<tr><td colspan="2">$D_{end}D_{end}$+3</td><td colspan="3">On the day of follow-up</td><td></td><td></td></tr>
<tr><td rowspan="2">A tumor patient undergoing surgery</td><td rowspan="2">D-7—D0</td><td>postoperation</td><td colspan="3">Postoperative discharge day/the day before discharge</td><td></td><td></td><td></td></tr>
<tr><td>D1—D3</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

5.3 statistic analysis

This study adopted a mismatched case-control study (500 cases in the case group and 500 cases in the healthy control group). The sample size was determined based on the effect size of the previous pre-experiment (Cohen's d=1.2) and considering a 10% dropout rate. All statistical analyses were performed using R, with a test level of α=0.05. Main analysis 1) Comparison of histone succinylation levels between groups - After Shapiro-Wilk normality and Levene homogeneity of variance tests, independent sample t-tests (conforming to normal distribution) or Mann-Whitney U tests (non-normal data) were selected. Report mean difference (MD)/median difference and 95% confidence interval (CI), Cohen's d effect size; 2) Diagnostic

efficacy evaluation - Calculate the area under the curve (AUC) and its Bootstrap 95% CI through the ROC curve, and determine the optimal cut-off value by maximizing the Youden index. And calculate the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and their exact Clopper-Pearson 95% CI. Secondary analysis: 1) Subgroup correlation - One-way analysis of variance (ANOVA) or Kruskal-Wallis test was used to compare the level differences among different tumor types (Bonferroni-adjusted post hoc test), and Cuzick's trend test was used to analyze the ordered association of TNM stages; 2) Therapeutic effect evaluation - The level changes before and after treatment were evaluated using paired t-test /Wilcoxon signed-rank test; 3) Prognostic value - The baseline succinylation level (median grouping) was included in the Cox proportional hazards model. After adjusting for age, stage, and treatment regimen, the hazard ratio (HR) and 95% confidence interval (CI) were calculated. The survival curve was plotted using the Kaplan-Meier method (Log-rank test).Multivariate analysis: A Logistic regression model was constructed (including succinylation level and covariates such as age and gender). AUC was compared by DeLong test, goodness-of-fit was evaluated by Hosmer-Lemeshow calibration curve, and clinical net benefit was analyzed by decision curve (DCA). If multi-site modifications are detected, LASSO regression is used to screen biomarker combinations. Quality control: Grubbs test /Winsorize for outliers, multiple imputation (MICE) for filling <5% of missing covariates. Sensitivity analysis: Robustness was verified by excluding stage I tumors, and the 70%-30% dataset was split for cutoff value training - validation. All multiple comparisons were controlled for the false discovery rate (FDR) using the Benjamini-Hochberg method.

六、Ethical requirements

Clinical research will comply with relevant regulations such as the Declaration of Helsinki of the World Medical Congress. The research can only be implemented after it has been approved by the ethics committee before it commences.

Before each subject is enrolled in this study, the researcher is responsible for providing the subject or their agent with a complete and comprehensive introduction

to the purpose, procedures and possible risks of this study, and informing the subjects that they can refuse to participate or withdraw from this study at any stage without discrimination or retaliation, and that their medical treatment and rights will not be affected. After fully understanding and agreeing, the subjects signed the written informed consent form, which was retained as a clinical research document for future reference. During the research process, researchers will protect the personal privacy and data confidentiality of the subjects. In this study, the informed consent form was electronically signed. The doctor/working group coordinator communicated with the subjects on-site. Before the subjects were enrolled in the study, the doctor would inquire and record the subjects' medical history. If the subject is a qualified inclusion and voluntarily participates, they will be asked to sign an informed consent form. All electronic informed consent forms will be filed.

七、References

[1] BRAY F, LAVERSANNE M, SUNG H Y A, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries [J]. Ca-a Cancer Journal for Clinicians, 2024, 74(3): 229-263.

[2] ZHENG R S, CHEN R, HAN B F, et al. Cancer incidence and mortality in China, 2022 [J]. Zhonghua zhong liu za zhi [Chinese journal of oncology], 2024, 46(3): 221-231.

[3] LONE S N, NISAR S, MASOODI T, et al. Liquid biopsy: a step closer to transform diagnosis, prognosis and future of cancer treatments [J]. Molecular Cancer, 2022, 21(1): 79.

[4] XIE Z Y, DAI J B A, DAI L Z, et al. Lysine Succinylation and Lysine Malonylation in Histones [J]. Molecular & Cellular Proteomics, 2012, 11(5): 100-107.

[5] TONG Y Y, GUO D, YAN D, et al. KAT2A succinyltransferase activity-mediated 14-3-3ζ upregulation promotes β-catenin stabilization-dependent glycolysis and proliferation of pancreatic carcinoma cells [J]. Cancer Letters, 2020, 469: 1-10.

[6] WANG Y G, GUO Y R, LIU K, et al. KAT2A coupled with the α-KGDH complex acts as a histone H3 succinyltransferase [J]. Nature, 2017, 552(7684): 273-277.

[7] WANG H W, WANG Y, ZHANG D J, et al. Circulating nucleosomes as potential biomarkers for cancer diagnosis and treatment monitoring [J]. International Journal of Biological Macromolecules, 2024, 262(Pt1): 130005.

[8] FEDYUK V, EREZ N, FURTH N, et al. Multiplexed, single-molecule, epigenetic analysis of plasma-isolated nucleosomes for cancer diagnostics [J]. Nature Biotechnology, 2023, 41(2): 212-221.

[9] SADEH R, SHARKIA I, FIALKOFF G, et al. ChIP-seq of plasma cell-free nucleosomes identifies gene expression programs of the cells of origin [J]. Nature Biotechnology, 2021, 39(5): 586-598.

[10] SNYDER M W, KIRCHER M, HILL A J, et al. Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin [J]. Cell, 2016, 164(1-2): 57-68.