Exploratory Research on Constructing a Computational Biological Model Based on Second-generation Sequencing Technology for Monitoring Measurable Residual Disease (MRD) after Breast Cancer Surgery

Applicant:	The Second Affiliated Hospital of Zhejiang University School of Medicine
Team leader unit:	The Second Affiliated Hospital of Zhejiang University School of Medicine
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Version number:	V1.0
Version date:	June 7th, 2023

Confidentiality Statement

The information contained in this document, especially the unpublished materials, is the property of the Second Affiliated Hospital of Zhejiang University School of Medicine and is only provided for review by collaborating researchers, ethics committees, and other relevant collaborating units. Therefore, in addition to explaining this study to the subjects, written consent from the Second Affiliated Hospital of Zhejiang University School of Medicine is required before its content can be published.

Version number V1.0

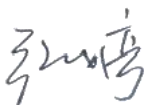
Version time: June 7, 2023

Signature page of the plan

Project name: Exploratory research on constructing a computational biological model based on second-generation sequencing technology for monitoring minimal residual lesions (MRD) after breast cancer surgery

Researcher Statement

I have read this plan and the research will be conducted in accordance with the ethical, moral, and scientific principles outlined in the Helsinki Declaration and the Chinese GCP regulations. I agree to conduct this clinical study in accordance with the design and regulations of this protocol, and comply with all the provisions of this protocol.

Researcher(Signature): 

Date： 2023.06.15

Research Center: Second Affiliated Hospital of Zhejiang University School of Medicine

1.Abstract

project name	Exploratory study on constructing a computational biological model based on second-generation sequencing technology for monitoring minimal residual lesions (MRD) after breast cancer surgery
Study type	Prospective, observational, single center clinical studies
indication	Patients diagnosed with primary breast cancer and undergoing radical breast cancer resection
Plan research cycle	36 months
sample size	80
research objective	Exploring the application value of computational biological model based on second generation sequencing technology in MRD monitoring after breast cancer radical surgery；
Research Design and Planning	This study will include 80 subjects after radical breast cancer resection, collect blood samples of the above subjects, observe and quantify the characteristic changes of cfDNA in the blood of breast cancer subjects after surgery, and establish a computational biological model based on second-generation sequencing technology to monitor breast cancer MRD;
Total number of researchers	80

Inclusion criteria	1. Age ≥ 18 years old, gender not limited; 2. Obtain plasma samples from the subjects during a 3-year follow-up period; 3. The subjects fully understand the study and voluntarily sign the informed consent form; 4. Able to cooperate with a 3-year follow-up visit to the hospital; 5. The physical fitness status score (ZPS) of the ECOG scoring criteria for the subjects must be ≤ 1; 6. Life expectancy ≥ 5 years; 7. Subjects who meet the following criteria: a. Histopathology confirmed primary breast cancer (unlimited molecular type); b. Radical breast cancer resection is expected; c. Adopting postoperative adjuvant therapy or preoperative neoadjuvant therapy;
Exclusion criteria	1. The subject is pregnant or breastfeeding; 2. Serious mental illness or drug abuse; 3. Unable to obtain the subject's plasma during this period; 4. If the subject has a non primary malignant tumor of the breast with a clear pathological diagnosis within 5 years before enrollment: 5. If the subject has suspected non breast malignant tumors (such as B-ultrasound, CT, etc.) on imaging within the past year of enrollment, but without pathological confirmation; 6. Clinical suspicion of distant metastatic lesions; 7. The subject has received any blood product infusion therapy within the past 30 days; 8. Known carriers of pathogenic genetic mutations;

	Participate in other interventional clinical studies within 9.3 months; 10. Subjects with poor compliance or deemed unsuitable by researchers to participate in undergraduate clinical trials;

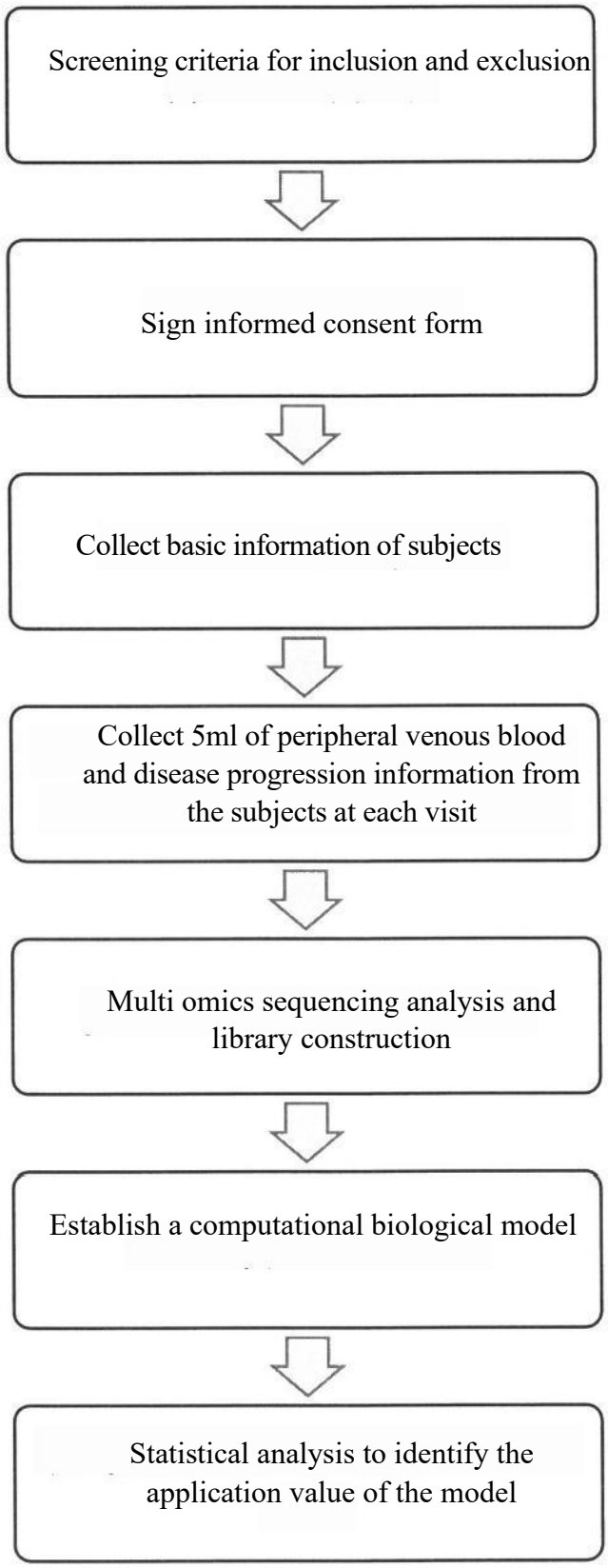
<table>
<tr><td>Evaluating indicator</td><td colspan="2">Main evaluation indicators:

1. Explore the role of computational biological model based on second-generation sequencing technology in monitoring MRD in breast cancer;

2. Disease free survival: The primary endpoint of this experiment is disease free survival (3 years), which will be evaluated using RECIST version 1.1;

Secondary evaluation indicators: establish a clinical cohort of postoperative follow-up for high-risk breast cancer;</td></tr>
<tr><td rowspan="13">Visit arrangement</td><td>screening period</td><td>Visit 1: On the day of subject enrollment</td></tr>
<tr><td rowspan="12">Follow-up period</td><td>Visit 2: One week after adjuvant therapy</td></tr>
<tr><td>Visit 3: 3 months after surgery</td></tr>
<tr><td>Visit 4: 6 months after surgery</td></tr>
<tr><td>Visit 5: 9 months after surgery</td></tr>
<tr><td>Visit 6: 12 months after surgery</td></tr>
<tr><td>Visit 7: 15 months after surgery</td></tr>
<tr><td>Visit 8: 18 months after surgery</td></tr>
<tr><td>Visit 9: 21 months after surgery</td></tr>
<tr><td>Visit 10:24 months after surgery</td></tr>
<tr><td>Visit 11: 30 months after surgery</td></tr>
<tr><td>Visit 12: 36 months after surgery</td></tr>
</table>

2. Research design diagram



Clinical Trial Process Table												
Visits / Project	Screening period	Follow-up period										
	Visits1	Visits2	Visits3	Visits4	Visits5	Visits6	Visits7	Visits8	Visits9	Visits10	Visits11	Visits12
	On the day of enrollment	7 days after chemotherapy	3 months after operation	6 months after operation	9 months after operation	12 months after operatio	15 months after operatio	18 months after operation	21 months after operation	24 months after operation	30 months after operation	36 months after operation
Screening criteria for inclusion and exclusion	X											
Sign informed consent form	X											
Collect basic information of subjects	X											

Collect disease development information from subjects	X	X	X	X	X	X	X	X	X	X	X	X
Collect 5ml peripheral venous blood	X	X	X	X	X	X	X	X	X	X	X	X
AE/SAE records	X	X	X	X	X	X	X	X	X	X	X	X

3. Research background

3.1 Research Basis

According to the World Health Organization, since 2020, breast cancer has become the world's largest cancer, accounting for 11.7% of the total incidence rate of malignant tumors, and ranking first in the number of cases and deaths of female malignant tumors. According to domestic epidemiological data, the incidence rate of breast cancer in China is increasing year by year, accounting for 9.1% of the total number of new cancer cases in China [1], which seriously threatens women's life and health.

Early breast cancer without distant metastasis is a potentially curable disease [2]. Therefore, early detection, early diagnosis and early treatment have become the focus of breast cancer diagnosis and treatment. For those patients who have been diagnosed with breast cancer and need surgical treatment, postoperative recovery and recurrence monitoring have an important impact on the quality of life and productivity recovery of patients after surgery. According to relevant research reports, about 94% of breast cancer patients are in the early stage when they are first diagnosed. However, some of these early patients still have tumor recurrence after receiving radical treatment, which is mainly due to the clinical failure to detect minimal residual lesions (MRD) [3].

MRD (Minimum Residual Disease) refers to the small amount of cancer cells remaining in the body after cancer treatment. The number of residual cancer cells may be small, causing no signs or symptoms, and cannot be detected by imaging methods, but they may lead to cancer recurrence. During the process of cell necrosis or apoptosis, tumor ctDNA is released. At present, second-generation sequencing technology (NGS) can continuously monitor MRD related cfDNA, which has important guiding significance for evaluating tumor treatment efficacy, predicting tumor recurrence, and implementing precise treatment.

Therefore, building a computational biological model that can predict the risk of tumor recurrence after breast cancer surgery has great medical and social value, and provides a new clinical idea for postoperative treatment of breast cancer. At present, in China, scientific research in the field of breast cancer MRD monitoring has not been carried out on a large scale. Therefore, through the research of this project, we expect to build a computational biological model combining the second-generation sequencing technology and computational biology technology to monitor the development of MRD after breast cancer surgery.

Next Generation Sequencing (NGS) is a collection of high-throughput sequencing technologies developed in the past two decades. Compared to the first generation Sanger sequencing method, NGS technology has the ability of multi flux sequencing, with astonishing improvements in data quality and throughput, and significant cost reductions [5], thus achieving commercial applications. Although traditional cfDNA detection techniques have some limitations in terms of sensitivity for postoperative MRD monitoring, relevant literature suggests that tracking a large number of individualized tumor mutations in cfDNA can significantly improve the sensitivity of MRD monitoring, and its sensitivity is positively correlated with the number of trackable tumor mutations [6]. In this study, we intend to use NGS technology to monitor the cfDNA of patients with breast cancer after surgery, in order to establish a database of mutations related to MRD after breast cancer surgery. Computational biology is an interdisciplinary field that combines biological knowledge and artificial intelligence to develop and apply methods of data analysis and theory, mathematical modeling, and computer simulation techniques for the study of biology, behavior, and

social group systems. With the powerful data processing capabilities of artificial intelligence machine learning, it has broad application prospects in the field of medical research involving a large amount of clinical data and bioinformatics data, not limited to sequencing, but. With the help of computational biology techniques, we can develop powerful models and even redefine patient categories. In this study, we plan to use computational biology technology to process the database and build a computational biological model of MRD monitoring after breast cancer surgery.

At present, globally, tumor early screening products available for monitoring minimal residual lesions (MRD) mainly include Guardant Reveal from Guardant Health (USA), Galleri from Grail (USA), and the new liquid biopsy technology demonstrated by Delfi (USA). These products and technologies have shown great potential in monitoring postoperative recurrence of malignant tumors.

The cooperating unit of this project, Aomingcheng (Hangzhou) Biotechnology Co., Ltd., has rich research and development experience in the fields of cancer genomics and tumor evolution. The company's team has developed a large number of technical methods for decoding genome functions, which have been widely used in the Cancer Genome Atlas (TCGA) project. For the first time in the world, they have discovered a large number of key genes and biological mechanisms that drive the occurrence and development of various types of cancer, and have mapped the evolutionary pathways of different tumors. Aomingcheng Company has a globally leading cancer genomics research platform with a profound and cutting-edge understanding of tumor evolution. Based on this, innovative medical products can be developed to improve the existing clinical diagnosis and treatment model of tumors and fill the huge gap in clinical diagnosis and treatment needs of tumors**.**

3.2 Risk/Benefit Assessment

3.2.1 Risk Assessment

This study is an observational, prospective clinical study and will not interfere with the diagnosis and treatment process of patients. Therefore, participating in this study will not lead to the occurrence of risks such as disease progression in patients.

3.2.2 Benefit assessment

This study is a non intervention observational study, and participation in this study may not bring direct medical benefits to the subjects. However, the participation of subjects in this study will help predict the risk of tumor recurrence after radical surgery for breast cancer, improve the accuracy and sensitivity of tumor recurrence monitoring, and benefit patients with similar conditions in the future.

4. Research objectives and endpoints

4.1 Main purpose

To establish a computational biological model based on second generation sequencing technology to monitor MRD, explore the application value of this model in breast cancer MRD monitoring, and conduct multiple cfDNA multigroup liquid biopsies in breast cancer patients during postoperative follow-up;

4.2 Secondary Objectives

To establish a clinical cohort for postoperative follow-up of high-risk breast cancer; It is estimated that 80 patients clinically diagnosed as primary breast cancer will participate in the screening. After radical resection, blood samples will be collected for cfDNA multigroup liquid biopsy before radiotherapy. Follow up will be conducted within 3 years after surgery, including CT/MRI and other imaging tests and blood tests, and multiple cfDNA multigroup liquid biopsies will be performed dynamically, and blood samples will be collected 12 times;

4.3 Study endpoints

Primary endpoint: The number of eligible samples meets the statistical requirements; Secondary endpoint: The total sample size meets the regulatory requirements.

5. Research Design

5.1 Overall Design

This clinical trial adopts a prospective, observational, single center trial design, and selects the Second Affiliated Hospital of Zhejiang University School of Medicine as the research clinical trial center. After all subjects signed the informed consent form, they were screened to be qualified, and their basic information was collected for scientific clinical research to observe the application value of the computational biological model built based on the second generation gene sequencing for post operation MRD monitoring of breast cancer.

Single center: This trial provides data support for subsequent statistical analysis, with a large sample size. Therefore, a single center research-oriented clinical trial is adopted, which can shorten the time and reduce bias of research-oriented clinical trials.

5.1.1 Screening period (on the day of enrollment)

Participants or their legal representatives must sign a written informed consent form on a voluntary basis before participating in screening. Only subjects who meet the inclusion criteria but do not meet the exclusion criteria are eligible to participate in this experiment. On the day of enrollment, the patients' CA15-3, CEA, color ultrasound and other traditional postoperative monitoring results of breast cancer were collected, and 5ml of the subjects' peripheral venous blood was collected.

5.1.2 Follow up period (7 days after adjuvant therapy)

After the subjects were screened into the group and received adjuvant treatment 7 days later, they were interviewed 2 to collect the patients' CA15-3, CEA, color ultrasound and other traditional breast cancer postoperative monitoring results, and collected 5ml of the subjects' peripheral blood.

5.1.3 Follow up period (3 years after surgery)

After the subjects were screened into the group, they were interviewed for 3-12 months at the 3rd, 6th, 9th, 12th, 15th, 18th, 21st, 24th, 30th, and 36th months after the operation, to collect the postoperative monitoring results of traditional breast cancer such as CA15-3, CEA, and color ultrasound, and to collect 5ml of the peripheral blood of the subjects.

5.2 Encoding Rules

After the subjects are enrolled, they are assigned a subject code in order, starting with Y07 and followed by the patient's serial number XXX, such as Y07001, Y07002, Y07003, etc.

After each visit, the blood sample of the subject is given a blood sample code (single collection of multi tube blood is recorded as the same code), starting with the subject code of the subject, followed by - DX, where V is the visit and X is the number of visits in the 12 visits, such as Y07001-V1, Y07003-V7, Y07045-V12, etc. If the subject's scheduled visit date is postponed from the scheduled date, the total number of overtime days X (if the visit is scheduled on May 5, 2023 and the subject arrives on May 8, 2023, then X=3) should be marked after the blood sample code as - XD, such as Y07001-V2-3D Y07002-V4-2D 、 Y07044-V5-12D Wait.

5.3 Termination of Study

It refers to the clinical study that has not yet been completed according to the protocol, and all trials are stopped midway. The main purpose of terminating the trial is

to protect the rights and interests of the subjects, ensure the quality of the trial, and avoid unnecessary economic losses.

1) Researchers have found significant errors in the clinical protocol during the trial, making it difficult to evaluate the trial results, or significant deviations in the implementation of a well-designed protocol, making it difficult to continue evaluating the trial results;

2) The applicant requests termination (such as funding reasons, management reasons, etc.);

3) The National Medical Products Administration has ordered the termination of the trial for some reason.

6. Study population

6.1 Inclusion criteria

1) Age ≥ 18 years old, gender not limited;

2) Obtain plasma samples from the subjects during a 3-year follow-up period;

3) The subjects fully understand the study and voluntarily sign the informed consent form;

4) Can cooperate with a 3-year follow-up visit to the hospital;

5) The physical fitness status score (ZPS) of the ECOG scoring criteria for the subjects must be ≤ 1;

6) Expected lifespan ≥ 5 years;

7) Subjects who meet the following criteria:

a. Histopathology confirmed primary breast cancer (unlimited molecular type);

b. Radical breast cancer resection is expected;

c. Adopting postoperative adjuvant therapy or preoperative neoadjuvant therapy;;

6.2 Exclusion criteria

1) The subject is pregnant or breastfeeding;

2) Serious mental illness or drug abuse

3) Unable to obtain plasma from the subject during this period;

4) The subject had a non primary malignant tumor of the breast with a clear pathological diagnosis within the 5 years prior to enrollment;

5) The subjects have suspected non breast malignant tumors (such as B-ultrasound, CT, etc.) on imaging within the past year of enrollment, but have not been confirmed by pathology;

6) Clinical suspicion of distant metastatic lesions;

7) The subject has received any blood product infusion therapy within the past 30 days;

8) Known carriers of pathogenic genetic mutations;

9) Participate in other interventional clinical studies within 3 months;

10) Subjects with poor compliance or deemed unsuitable by researchers to participate in undergraduate clinical trials;

6.3 Exit criteria

1) The subjects voluntarily withdrew from this study;

2) Participants can choose to withdraw from this study at any time;

6.4 Exclusion criteria

1) Damaged during sample transportation;

2) Subjects who do not meet the inclusion criteria;

3) Subjects who seriously violate the protocol.

7. Subject withdrawal from the study

7.1 Termination of treatment and withdrawal of subjects from the study

Participants may decide to withdraw from the study at any time, or the researcher may arrange for participants to withdraw at any time based on safety, behavior, compliance, and other reasons. But it is expected that this situation is not common.

7.2 Missing Visits

Subjects who have failed to visit the research center for planned visits multiple times and have not been contacted in a timely manner before the end of the study, thus unable to confirm their current status through sufficient information, are considered lost to follow-up.

7.3 Early termination of research

Early termination of a trial refers to the clinical trial not completing the study for all planned sample sizes according to the protocol, and stopping the entire trial or a part of the trial (such as a center) midway. The main purpose of early termination is to protect the rights and interests of the subjects, ensure the quality of the trial, and avoid unnecessary economic losses.

Under normal circumstances, the experiment will not be terminated prematurely. But if any of the following situations occur, the entire experiment (or a certain center) can be terminated in advance

1) The total sample size has met the experimental requirements, but the center has not completed the enrollment as planned in the contract.

2) The researchers at the center are unable to comply with protocol requirements, Good Clinical Practice (GCP) for drug/device trials, etc.

3) Obtaining new information leads to unfavorable risk benefit evaluation of the experimental product, including sufficient evidence indicating a lack of efficacy or unacceptable safety.

4) Due to medical, ethical, or commercial reasons, the applicant believes that continuing the clinical trial is inappropriate.

5) The enrollment progress of the subjects is slow and it is impossible to complete the study within an acceptable time frame.

6) The National Medical Products Administration or Ethics Committee has ordered the termination of the trial for some reason.

8. Biological samples

8.1 Sample collection and transportation

After the subjects sign the informed consent form, professional nurses will strictly follow the guidelines during each visit period

Aseptic operation requires 5ml of patient plasma to be collected (using a cfDNA specific blood collection tube). After blood collection, gently invert 8-10 times, fully fuse the protective agent, and then place the storage tube upright, indicating the subject number and visit period number; Collect blood samples and transfer them to a dedicated blood box.

Professional personnel will seal the transfer box and record the time; Deliver to the central laboratory of Aomingmingcheng (Hangzhou) Biotechnology Co., Ltd. within 4-6 hours.

9. Statistical considerations

9.1 Determination of Sample Size

In this study, 80 patients with breast cancer were admitted to the Second Affiliated Hospital of Zhejiang University School of Medicine.

9.2 Statistical analysis

The data statistical analysis of this experiment was carried out with the participation of professional data management and statistical personnel.

10. Adverse Events and Serious Adverse Events

10.1 Definition

10.1.1 Adverse Events

Adverse events (AEs) are defined as any adverse medical events that occur in a subject and are related in chronological order to drug use, but may not necessarily be causally related to drug use. Therefore, adverse events may be any adverse, unexpected symptoms, signs, abnormal test results (such as laboratory test values, ECG) or diseases (new or aggravated) that are related to the timing of drug use in the study.

10.1.2 Serious Adverse Events

Serious Adverse Event (SAE) refers to any adverse medical event that occurs at any dose and meets the following description:

● Causing death.

● Endangering life. Note: In the definition of "serious" adverse events, "life-threatening" refers to the risk of death for the subject at the time of the event. It does not refer to events that may lead to death in more serious circumstances.

Need for hospitalization or extended hospitalization time.

Note: Generally speaking, hospitalization means that the subject has been left in the hospital or emergency room (usually for at least one night) for observation and/or treatment that is not suitable for being conducted in a doctor's office or clinic.

● Causing disability/disability.

Note: The term 'disability' refers to the severe impairment of an individual's ability to perform daily life functions. This definition does not include events of relatively minor medical significance, such as headache, nausea, vomiting, diarrhea, influenza, accidental trauma (such as ankle sprain) and other events without complications that may interfere with or hinder the function of daily life but without major damage.

Causing congenital abnormalities or birth defects.

Important medical events (which may pose risks to patients or may require medical/surgical intervention to prevent one of the above outcomes from occurring)

The following are not part of SAE:

Hospitalization for elective treatment due to pre-existing conditions that have not worsened compared to baseline.

Hospitalization considering social/convenience factors.

·The planned treatment for the target indications of this study includes hospitalization for infusion therapy or hospitalization due to convenience. 10.2 Record of Adverse Events

All adverse events should be recorded on the CRF, and the description of the adverse event should include its start and end time, whether it constitutes a serious adverse event, measures taken (changes in study treatment, other treatments and related examinations), and the outcome of the adverse event. The researcher should also conduct a correlation evaluation.

10.1.3 Grading of Adverse Events

Researchers need to evaluate the severity of each reported AE and SAE during the study period, and assess and grade adverse events and serious adverse events according to NCI-CTCAE version 5.0.

Toxicity not applicable to NCI-CTCAE grading will be classified according to the following definitions:

·Level 1: Mild; Asymptomatic or mild symptoms; Only clinical or diagnostic observations have been found; No intervention required

treatment.

·Level 2: Moderate; Requires minimal local or non-invasive intervention therapy; Equivalent to age

Limited instrumental activities of daily living (ADL).

·Level 3: Severe or medically significant but not directly life-threatening; Need hospitalization or extension

Length of hospital stay; cause disability; Limited self-care ability in daily life.

Level 4: There are life-threatening consequences; Urgent intervention and treatment are needed· Level 5: Deaths related to adverse events

10.4 Reporting of Serious Adverse Events

Any serious adverse event must be reported to regulatory authorities and IRB/IEC within 24 hours of becoming aware, in accordance with applicable regulatory requirements, by researchers or center personnel. For incomplete reports of serious adverse events, researchers need to conduct thorough investigations to obtain follow-up information.

11. Ethical standards

11.1 Informed Consent

Before each patient is enrolled in this study, the research physician has the responsibility to provide a complete and comprehensive introduction to the purpose and potential risks of this study. Patients should be informed of their rights, risks, and benefits. Prior to enrollment, patients should sign an informed consent form and keep it in the subject folder.

11.2 Ethical norms and policies and regulations

This clinical study must comply with the Helsinki Declaration and relevant clinical research standards and regulations in China. Before the start of the study, the protocol must be approved by the ethics committee. Any modifications made to the research protocol during clinical research should be submitted for ethical review. Execute according to the approved ethical plan.

12. Quality Assurance

In order to ensure that this study can be conducted strictly in accordance with the clinical research protocol, clinical researchers should strictly follow the requirements of the "Investigator Initiated Research Management Measures" throughout the entire clinical research process, and must ensure standardized research procedures, accurate research data, and reliable research conclusions.

13. Data processing and data storage

13.1 Case Report Form (CRF)

The case report form should be filled out by the researcher, and the CRF form should be filled out in a timely manner to ensure accurate content and timely summary. CRF forms should generally not be altered, and if there are errors that need to be corrected, a signature and date should be signed at the modification location. 13.2 Data Preservation

Researchers should keep the data intact. According to the GCP principle in China, data should be preserved for 10 years after the completion of the research.

14.references

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