Clinical research protocol

Project name: The effect and mechanism of gene variation on neonatal hyperbilirubinemia

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Abstract

object name	The effect and mechanism of gene variation on neonatal hyperbilirubinemia
goal of study	(1) To analyze the carrying and pathogenicity of neonatal jaundice-related genes, and collect the sequencing data of jaundice-related genes (24 genes of 29 NHB-related genetic diseases) of 2000 newborns in the cooperative hospital to complete the construction of the gene database. (2) To analyze the incidence of NHB-related genes, pathogenic mutations, suspected pathogenic mutations, and the carrying rate of unknown mutations in 2000 cases, and to count the distribution of high-frequency mutation sites in the population, so as to provide a scientific basis for the selection of mutation sites for large-scale neonatal hyperbilirubinemia gene screening. (3) The influence of jaundice-related genes on neonatal jaundice was analyzed. The clinical data of newborns and the daily percutaneous bilirubin level were collected through the hospital hospitalization system and the " percutaneous jaundice instrument home monitoring + software doctor-patient interconnection " method to complete the construction of intelligent neonatal jaundice clinical database. (4) Analyze the integrated monitoring data, statistically analyze the gene and jaundice clinical data of 2000 newborns, understand whether there is a difference in the carrying rate of related pathogenic genes in different degrees of jaundice, and explore whether there is a difference in the degree of jaundice carrying multiple and single jaundice pathogenic genes. To understand the

	correlation between gene polymorphism and clinical manifestations (phenotypic polymorphism) of neonatal hyperbilirubinemia, so as to better evaluate the effect of genes on neonatal jaundice.
research design	prospective observational study
Research period	September 2022 to December 2024
Participating	Newborns
Inclusion criteria	(1) Informed consent of parents or guardians ; (2) Age : 1-28 days, gestational age ≥ 35 weeks ; (3) Birth weight ≥ 2.5 kg and < 4 kg.
Excluded criteria	(1) Neonatal data with unclear clinical basic information ; (2) Lack of traceability core information data ; (3) data that the test results cannot be analyzed and interpreted ; (4) Sample collection is not qualified and unwilling to cooperate with re-sampling. (5) Newborns with severe deformity and severe lethal inherited metabolic diseases
Grouping and sample size	A total of 2000 neonates were included, including 500 cases of non-significant hyperbilirubinemia (TSB / TCB < 205umol / L) ; (2) 500 cases of significant hyperbilirubinemia (205umol / L ≤ TSB / TCB < 342umol / L) ; (3) 500 cases of severe hyperbilirubinemia (TSB / TCB ≥ 342umol / L) ; 4 Extremely severe hyperbilirubinemia 500 cases (TSB / TCB ≥ 428umol / L).
data collection	(1) Basic information : gender, gestational age, date of birth, birth weight, height, head circumference, mode of conception, Apgar score and parents ' information ; (2) Perinatal information : parity, parity, mode of delivery, fetal

	membranes, amniotic fluid, placenta, umbilical cord, intrauterine distress, group B streptococcal infection, pregnancy complications such as gestational hypertension, diabetes, etc. (3) Jaundice information : blood types of parents and newborns, feeding methods, monitoring jaundice values, jaundice treatment methods and related test results ; (4) Test results : Collect genetic analysis results, notes, etc.
Statistical processing	IBM-SPSS 26.0 statistical software was used to analyze the test results. Logistic multivariate regression analysis was used to evaluate the correlation between genotype and clinical phenotype, and mediating variables were used to analyze the correlation between gene-gene interaction and genotype-phenotype.
abbreviation	neonatal hyperbilirubinemia, NHB; total serum bilirubin, TSB; transcutaneous bilirubin, TCB; Principal Investigator, PI.

1. Research background

Neonatal disease screening is one of the most effective public health initiatives, which has been carried out for more than 60 years. Since the single screening of phenylketonuria (PKU) in 1961, with the improvement of screening technology, it has developed into a screening of more than 50 diseases including endocrine system diseases (congenital hypothyroidism, congenital adrenal hyperplasia), hemoglobin disease, infectious diseases, cystic fibrosis, and congenital metabolic diseases. The incidence of birth defects is related to genetic and environmental factors. Among the patients with birth defects diagnosed by etiology, 94.4 % are caused by chromosomal abnormalities and genetic variations. Because most patients have a genetic basis, early screening and diagnosis have important scientific value and social significance for the prevention and control of these diseases.

At present, neonatal disease screening methods are diverse, including : neonatal metabolic disease screening (hypothyroidism, phenylketonuria, adrenocortical hyperplasia, G-6-PD deficiency and metabolic disease tandem mass spectrometry screening), hearing screening, fundus screening, cardiac ultrasound, etc. However, although the above-mentioned routine screening techniques play an important role in the prevention and control of birth defects, there are still obvious deficiencies : 1) There are many interference factors, such as susceptibility to sample quality, blood collection time, gestational age, birth weight and other factors, and false positive or false negative are more likely to occur, which brings a greater psychological burden to the children 's families. At the same time, biochemical tests, tandem mass spectrometry, urinary organic acids and other methods are needed to assist diagnosis after recall ; 2) For children with positive screening, further gene sequencing is needed to confirm the diagnosis and typing, and further develop an accurate treatment plan. During this period, the diagnosis may be delayed for 1-2 months. For children with severe illness, the damage to the nervous system is often irreversible, and even the treatment is not timely and life-threatening during the detection period ; 3) Traditional methods can only make a preliminary diagnosis, and can not be clearly classified, and more birth defects of

different types, treatment options vary. At present, the mainstream technology of neonatal screening has the problems of limited methods, long time for diagnosis, easy delay in disease treatment, and direct impact on the prognosis of children. Therefore, efficient and accurate screening techniques need to be developed and applied.

The development of genomics technology has pushed the diagnosis and prevention of hereditary birth defects to a new height. With the rapid development of gene sequencing technology and the deepening of medical genetics research, more and more genetic screening and related clinical studies of genetic diseases caused by genetic variation have been widely carried out at home and abroad, highlighting the broad prospects of neonatal disease screening and diagnosis based on gene detection in early diagnosis and early prevention of birth defects.

Neonatal hyperbilirubinemia, also known as neonatal jaundice, is the most common clinical manifestation of newborns. It has many causes and is difficult to diagnose. Genetic factors are one of the common causes of metabolic-related jaundice. According to statistics, about 60 % to 80 % of newborns can have varying degrees of jaundice, and severe cases can cause a series of body damage, including nerve, hearing, cardiovascular and kidney function, and even cause death. Within 7-27 days after birth, the incidence of death in children due to neonatal jaundice is about 107.1 / 100,000, ranking ninth in the global causes of late neonatal death. The common causes of neonatal jaundice include neonatal ABO hemolysis, RH hemolysis, neonatal sepsis, and congenital hypothyroidism. Genetic factors play an important role in the metabolism of bilirubin. Hyperbilirubinemia is mainly caused by bilirubin metabolism or circulatory disorders. Clinically, it can be manifested as high unconjugated hyperbilirubinemia and hyperconjugated hyperbilirubinemia. At present, the gold standard for the detection of neonatal jaundice is to use venous blood to detect bilirubin as the main laboratory examination method. In addition, it also includes micro-blood bilirubin meter detection, percutaneous bilirubin measurement and visual inspection. However, such detection does not have high diagnostic sensitivity and specificity and cannot determine whether it is jaundice caused by genetic factors. Genetic metabolism-related jaundice has great

individual differences in age of onset and clinical manifestations. Even if the same pathogenic gene mutation is carried out, the clinical phenotype can be different. Therefore, the application of genetic testing in this type of disease can improve the early diagnosis of the disease. Genetic testing of children without jaundice or hospitalized jaundice can obtain the proportion of genetic factors and their genotypes in children with jaundice, so as to continuously improve the efficiency of disease diagnosis.

However, previous studies have focused on the role of one or two specific genes in patients with hyperbilirubinemia. For example, a number of studies have shown that the UGT1A1 gene hotspot c.211G > A polymorphism is a risk factor for NHB, and is closely related to the severity of jaundice, and can aggravate the symptoms of breast milk jaundice, glucose-6-phosphate dehydrogenase deficiency, hereditary spherocytosis, and hemolytic jaundice caused by congenital erythropoietic anemia. Yang et al.showed that the c.211G > A locus was a risk factor for severe hyperbilirubinemia in patients with glucose-6-phosphate dehydrogenase deficiency. Watchko et al.showed that a single G6PD gene mutation or UGT1A1 gene mutation only caused mild jaundice, but the coexistence of the two mutations may cause severe NHB or even nuclear jaundice. Up to now, there is no literature report on the study of gene polymorphism and clinical manifestation polymorphism of jaundice in large data population.

The transcutaneous jaundice instrument (YSJ-20) has obtained the ' People 's Republic of China Class II Medical Device Registration Certificate '.It can accurately, quickly, safely and non-invasively detect the current bilirubin level of the newborn through the skin appearance. It is portable, easy to operate, and has a low threshold for use. It is very suitable for family members to monitor the level of neonatal jaundice at home.

In summary, this study intends to collect heel peripheral blood samples from 2000 newborns [including 500 non-significant hyperbilirubinemia newborns, 500 significant hyperbilirubinemia newborns, 500 severe hyperbilirubinemia newborns and 500 extremely severe hyperbilirubinemia newborns]. The gene capture sequencing

technology containing 24 genes of neonatal common hereditary jaundice genes was used to detect and collect the clinical data of these 2000 newborns. At the same time, the percutaneous jaundice meter (YSJ-20) was used to monitor the daily jaundice at home. According to the analysis results of gene detection and clinical data of jaundice in 2000 children, the carrying rate of pathogenic genes of common hereditary jaundice in neonates was studied, and the carrying rate of pathogenic genes in different degrees of jaundice was understood. Whether there is a difference in the degree of jaundice with multiple and single jaundice pathogenic genes, and the correlation between gene polymorphism and clinical manifestations (phenotypic polymorphism) of neonatal hyperbilirubinemia were analyzed.

2. Research objective

(1) To analyze the carrying and pathogenicity of neonatal jaundice-related genes, and to collect the sequencing data of jaundice-related genes (24 genes of 29 NHB-related genetic diseases) of 2000 newborns in the cooperative hospital, and to complete the construction of the gene database.

(2) To analyze the incidence of NHB-related genes, pathogenic mutations, suspected pathogenic mutations, and the carrying rate of unknown mutations in 2000 cases, and to count the distribution of high-frequency variation sites in the population, so as to provide a scientific basis for the selection of mutation sites for large-scale neonatal hyperbilirubinemia gene screening.

(3) The influence of jaundice-related genes on neonatal jaundice was analyzed. The clinical data of newborns and the daily percutaneous bilirubin level were collected through the hospital hospitalization system and the " percutaneous jaundice instrument home monitoring + software doctor-patient interconnection " method, and the construction of intelligent neonatal jaundice clinical database was completed.

(4) Analyze the integrated monitoring data, statistically analyze the gene and jaundice data of 2000 newborns, understand whether there is a difference in the carrying rate of related pathogenic genes in different degrees of jaundice, and explore whether there is a difference in the degree of jaundice carrying multiple and single jaundice pathogenic genes. To understand the correlation between gene polymorphism and clinical manifestations (phenotypic polymorphism) of neonatal hyperbilirubinemia, thereby increasing the accuracy of genetic diagnosis.

3. Research Design

This study is a prospective observational study. Three drops of heel peripheral blood samples were collected from 2000 newborns, and 24 genes of 29 NHB-related genetic diseases were analyzed. All dry blood spot samples that met the inclusion criteria could be included in the genetic analysis. At the same time, the clinical data of these 2000 newborns were collected, and the daily jaundice monitoring at home was performed using the percutaneous jaundice meter (YSJ-20). The data were synchronized to the mobile phone jaundice follow-up doctor assistant small program for storage statistics. The family members can view the jaundice risk level and obtain free doctor consultation in the small program.

The purpose of this study is to study the carrying rate of pathogenic genes of common hereditary jaundice diseases in newborns, to understand whether there is a difference in the carrying rate of pathogenic genes in different degrees of jaundice, to explore whether there is a difference in the degree of jaundice carrying multiple and single jaundice pathogenic genes, and to analyze the correlation between gene polymorphism and clinical manifestations (phenotypic polymorphism) of neonatal hyperbilirubinemia, so as to evaluate the feasibility of jaundice gene screening program in the detection of jaundice-related genetic metabolic diseases.

Based on the ' Classification Criteria and Guidelines for Genetic Variation (2017) ', the variation pathogenicity of genetic testing is divided into five categories : known pathogenic, possible pathogenic, unclear significance, possible benign and benign. In this study, known pathogenic and possible pathogenic are classified as positive results. The pathogenicity of the mutation site is mainly based on the pathogenic mutation database and the latest literature reports.

The classification criteria of jaundice were based on the American Academy of Pediatrics (AAP) 2022 edition of neonatal hyperbilirubinemia management guidelines for gestational age ≥ 35 weeks and the latest version of 2023 guidelines to interpret the management of neonatal hyperbilirubinemia-the diagnosis and intervention criteria of neonatal hyperbilirubinemia in the American Academy of Pediatrics and multi-national clinical management guidelines. The 2000 neonates were divided into 500 cases of non-

significant hyperbilirubinemia (TSB / TCB < 205umol / L), 500 cases of significant hyperbilirubinemia (205umol / L ≤ TSB / TCB < 342umol/L), 500 cases of severe hyperbilirubinemia (TSB/TCB ≥ 342umol/L) and 500 cases of extremely severe hyperbilirubinemia (TSB/TCB ≥ 428umol /L).

4. Inclusion criteria

(1) Informed consent of parents or guardians ;

(2) Age : 1-28 days, gestational age ≥ 35 weeks ;

(3) Birth weight ≥ 2.5 kg and < 4 kg.

5. Excluded criteria

(1) Neonatal data with unclear clinical basic information ;

(2) Lack of traceability core information data ;

(3) data that the test results cannot be analyzed and interpreted ;

(4) Sample collection is not qualified and unwilling to cooperate with re-sampling.

(5) Newborns with severe deformity and severe lethal inherited metabolic diseases

6. termination criteria

(1) The family members of the newborn included in the study requested termination at any time ;

(2) The relevant management departments require termination.

7. outcome index

Through the comprehensive analysis of genetic variation information and jaundice clinical data, the carrying rate of pathogenic genes of common hereditary jaundice diseases in newborns was counted, and the difference of carrying rate of pathogenic genes in different degrees of jaundice was understood. To explore whether there is a difference in the degree of jaundice carrying multiple and single jaundice pathogenic genes, and to analyze the correlation between gene polymorphism and clinical manifestations (phenotypic polymorphism) of neonatal hyperbilirubinemia.

8. Grouping and sample size

This study is a prospective observational study. According to the positive mutation rate of NHB-related genes in neonatal hyperbilirubinemia, P is about 30 %, the test level α

= 0.05 and the allowable error δ = 2 %, n (sample size) = (Uα / δ) 2 * p * (1-P) ≈ 2000 cases. A total of 2000 neonates were included, including 500 cases of non-significant hyperbilirubinemia (TSB / TCB < 205umol / L) ; (2) 500 cases of significant hyperbilirubinemia (205umol / L ≤ TSB / TCB < 342umol / L) ; (3) 500 cases of severe hyperbilirubinemia (TSB / TCB ≥ 342umol / L) ; (4) Extremely severe hyperbilirubinemia 500 cases (TSB / TCB ≥ 428umol / L).

9. Recruiting

This study will recruit subjects in the outpatient and inpatient departments of pediatrics, neonatology, and obstetrics in the South China Neonatal Genetic Screening Alliance (including cooperation units of 49 hospitals).

10. Clinical evaluation index

(1) The carrying rate, positive rate, negative rate and high-frequency mutation sites of common NHB-related gene mutation sites in neonates ;

(2) The difference in the carrying rate of NHB-related pathogenic genes in neonates with different degrees of jaundice ;

(3) The difference of jaundice degree between carrying multiple and single jaundice pathogenic genes ;

(4) Gene-gene interaction and genotype-phenotype correlation.

11. Methods

11.1 gene sequencing

A total of 24 genes were included in 29 NHB-related genetic diseases, including bilirubin metabolic disorders, congenital bile acid synthesis or transport disorders, red blood cell metabolic defects, lipid metabolic abnormalities, and metal metabolic abnormalities. The detected genes included ABCB11, ABCB4, ABCC2, ABCD3, ACOX2, AKR1D1, AMACR, ATP7B, ATP8B1, CYP7B1, G6PD, GBA, HBB, HSD3B7, JAG1, NOTCH2, NPC1, NPC2, NR1H4, SLC10A1, SLC25A13, SMPD1, TJP2 and UGT1A1. Gene and genetic disease related information refer to OMIM and Orphanet database.

Target gene sequence capture and library construction. Three drops of heel peripheral

blood were collected by filter paper, and genomic DNA was extracted by magnetic bead method. The target gene sequence was captured by multiplex PCR amplification method, covering all exon regions and adjacent intron regions (± 50bp) of the target gene. A total of 900 amplicons were designed for 24 genes and completed in two amplification systems. The target sequence library was obtained after amplification-purification-re-amplification-re-purification. The library was quantified using a Qubit ® 3.0 fluorescence quantitative instrument (Thermo Fisher Scientific), and the library length was determined using an Aglient 2100 biological analyzer (Agilent) (228-378 bp).

High-throughput sequencing and bioinformatics analysis. The library was diluted, added sequencing primers, and sequenced on the Illumina NextSeq 550 platform (PE150). The effective sequencing data were aligned to the human reference genome (Human _ B37) by BWA (Burrows-Wheeler Aligner) software. GATK (Genome Analysis ToolKit) was used to analyze the variation information, and ANNOVAR software was used to annotate the variation, including the annotation information of dbSNP database, Thousand Human Genome Project and other existing databases. The annotation content covers population frequency, mutation type, function prediction (SIFT, Polyphen2, Mutation Taster, etc.), HGMD, Clinvar and other pathogenic classification information. Based on the ' Classification Criteria and Guidelines for Genetic Variation (2017) ', the pathogenic interpretation of genetic variation was divided into five categories : ' pathogenic ', ' possible pathogenic ', ' unclear significance ', ' possible benign ' and ' benign '. Among them, ' pathogenic ', ' possible pathogenic ' and ' unclear significance ' were included in the statistics of test results.

Validation of positive loci. For children with autosomal recessive double heterozygosity and children with autosomal dominant single heterozygosity, Sanger sequencing was used to verify the loci of their parents.

11.2 Information and data collection

Researchers should truthfully fill in the test results of the subjects and other relevant information, and ensure that the information is true and reliable. The specific

information and data to be collected are as follows :

(1) Sign informed consent ;

(2) Jaundice gene detection : Take out the special filter paper blood collection card, disinfect with alcohol and dry the heel blood collection site, collect heel peripheral blood, drop three drops on the filter paper.

(3) Detection of jaundice value by percutaneous jaundice meter : The family members of the subjects rented the percutaneous jaundice meter for free under the guidance of the doctor, and used the ' single measurement mode ' to measure the forehead, chest and inner thigh of the subjects every day for a total of 2 weeks.

(4) Collect demographic characteristics : screening number, name, gender, gestational age, date of birth, birth weight, body length, head circumference, mode of conception, Apgar score and parental information ;

(5) Perinatal information : parity, parity, mode of delivery, fetal membranes, amniotic fluid, placenta, umbilical cord, intrauterine distress, group B streptococcal infection, pregnancy complications such as gestational hypertension, diabetes, etc.

(6) Jaundice information : parents and newborns blood type, hemolysis three tests, feeding methods, percutaneous jaundice value, jaundice treatment and related test results ;

(7) Gene detection results : Collect gene analysis results, notes, etc.

12. Statistical processing

IBM-SPSS 26.0 statistical software and R language (Version 4.1.0) were used for statistical analysis of the test results. The carrying rate of common NHB-related gene mutation sites in neonates was counted, and the differences in the carrying rate of NHB-related pathogenic genes in neonates with different degrees of jaundice were compared. To explore the difference in the degree of jaundice carrying multiple and single jaundice-related genes. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and the t test was used for comparison between groups. The count data were expressed as values or rates (%), and the chi-square test was used for comparison between groups. Unconditional logistic multivariate regression analysis

was used to evaluate the correlation between genotype and clinical phenotype, and mediating variables were used to analyze the correlation between gene-gene interaction and genotype-phenotype.

13. data monitoring

(1) Case Report FormThe case report form (CRF) was used in this study. The researcher ensures the authenticity, accuracy and integrity of the data in the original case of each enrolled (signed informed consent) newborn. The researcher or authorized researcher confirms and fills in the original medical record and submits it to the researcher / clinical coordinator. The data points in the original medical record are entered into the case report form system by the researcher / clinical coordinator.

(2) Data audit and locking After all data-related queries have been resolved, the main researchers, data managers, and statistical analysts will confirm the statistical analysis of the population, lock the database, and sign the audit report at the same time. The locked data must not be changed.

(3) Data privacy regulations All members of the research team, including lead researchers and their authorized researchers, must comply with all local and regional regulatory requirements and applicable privacy regulations (such as the ' Health Insurance Carriage and Liability Act ').

Each newborn participating in this study will be assigned a unique number and an institutional identifier.

All research data will be stored in a confidential electronic system and stored for at least 3 years after the completion of the study.

The coding table for recording neonatal information and research will be kept by the researcher (PI) in a locked file cabinet or in a password-protected database. Only researchers and their authorized researchers have the right to access the coding table.

The coding table that records the name of the hospital and the institution identification number will be saved by the research team in a locked file cabinet, which is only limited to access by limited research members.

The identity of each subject of observation should be kept confidential in research

reports and related publications.

14. adverse reactions

In this study, only 3 drops of neonatal heel peripheral blood were collected for genetic analysis and statistical analysis of percutaneous jaundice values. No human trials were involved. Family members were willing to know the genetic test results and the genetic test results were positive. Family members may have a certain psychological burden. We will provide free professional genetic counseling, as detailed as possible to inform the hazards of this genetic defect, treatment and other related information to help. Psychiatrists can be arranged for free psychological counseling if necessary.

15. Ethical approval

This clinical study follows the Helsinki Declaration (2013 edition), the ethical principles of human medical research and the relevant clinical research norms and regulations in China.

This clinical study was approved by the Research Ethics Committee / Institutional Review Boards (REC / IRBs).

This clinical study is an observational clinical study initiated by researchers. The clinical research protocol (including informed consent and case report forms, etc.) and other information provided to the family members or guardians of the newborn were reviewed and approved by the ethics committee of the clinical research team leader unit and the participating units.

16. informed consent

Before the start of the clinical study, the researchers completely and comprehensively introduced the purpose, process, method, and the interests and risks of the newborn to the parents or legal representatives who met the inclusion criteria. A written informed consent form was given to each parent or guardian of the newborn before enrollment, so that he / she had sufficient time to consider whether to consent to the collection of neonatal information to participate in this clinical study. After obtaining the informed consent of each newborn 's parent or legal representative and signing the informed consent form, researchers can begin to collect relevant data.

17. privacy protection

All members of the research team, including the primary researchers and their authorized researchers, must comply with all local and regional regulatory requirements and applicable privacy regulations.

Each newborn participating in this study will be assigned a unique number and an institutional identifier.

All research data will be stored in a confidential electronic system and stored for at least 3 years after the completion of the study.

The coding table for recording neonatal information and research will be kept by the researcher (PI) in a locked file cabinet or in a password-protected database. Only researchers and their authorized researchers have the right to access the coding table.

The coding table that records the name of the hospital and the institution identification number will be saved by the research team in a locked file cabinet, which is only limited to access by limited research members. The identity of each subject of observation should be kept confidential in research reports and related publications.

18. Research progress

September 01, 2023 to March 31, 2024: The samples were grouped and tested : (1) contact parents for informed consent ; (2) Collect 3 drops of heel peripheral blood samples, extract genomic DNA by magnetic bead genomic DNA extraction kit, and detect the samples by high-throughput sequencing technology ; (3) Collect neonatal clinical data and daily percutaneous bilirubin levels through the hospital inpatient system and the ' percutaneous jaundice instrument home monitoring + software doctor-patient interconnection ' method.

April 01, 2024 to June 30, 2024: Data analysis : (1) Statistics of neonatal common jaundice-related gene screening results : the positive rate of common jaundice gene screening (common positive disease cases, high-frequency pathogenic sites), the carrying rate of pathogenic mutations, the carrying rate of pathogenic genes with different degrees of jaundice, and the degree of jaundice carrying multiple and single jaundice pathogenic genes. (2) Combined with clinical data and data uploaded by

transcutaneous jaundice meter : Correlation analysis and comparison of jaundice-related gene detection results and jaundice clinical data to evaluate the feasibility of jaundice gene detection for neonatal hyperbilirubinemia screening.

July 01, 2024 - December 31, 2024: Write the conclusion report, complete the paper, submission and publication work.

19. Reference

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