Project Name： Identification of a new gene spliceosome in neuroblastoma and its translational application in clinically accurate diagnosis and therapy

Project Number： LCYJ20242005

Project Deadline： 2024.2.1-2026.12.31

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Date： 2024.01.05

1、Project research plan

1.1Research background, purpose, argument basis, domestic and foreign research status, etc

Neuroblastoma (NB) is an early embryonic malignant tumor in children, originating from neural crest cells.NB shows high heterogeneity in biological, morphological, genetic, and clinical features.The Deep Seuqencing technology of genomics has deepened researchers' understanding of the molecular mechanisms underlying the pathogenesis of NB. NB has relatively fewer somatic single nucleotide variations (SNVs) and small indels, with a mutation load of approximately 0.5–1/MB[1-2].The mutation signature of NB is mainly C>A and C>T[2], with a low frequency of recurrent mutations. Therefore, exploring the genomic attributes of NB based on monogenic variation and small segment indels have certain limitations.Current researchs indicate that genomic structural variation is a key driving factor for NB[3–4]. Genomic structural variation typically refers to changes in the sequence and positional relationships of large segments of the genome. Its main types include long segment sequence indels (usually over 50 bp), tandemrepeat, chromosomal inversion, sequence translocation within or between chromosomes, copy number variation (CNV), and more complex forms of chimeric variation.In NB, genomic structural variation features represented by MYCN amplification and chromosome 11q deletion have been included in the risk grouping of clinical children with NB. With the progress of NB genome sequencing, more and more structural variation features of the genome are being explored.Therefore, understanding the new progress in structural variations of the NB genome can help deepen our understanding of the role of these variations in the occurrence, development, invasion, and metastasis of NB.And researchers combine them with clinical features ,which will guide personalized precision diagnosis and therapy of patients with NB.This article summarizes the genomic structural variation characteristics of NB, aiming to explore its highly invasive and heterogeneous genomic basis, and provide a theoretical basis for exploring the mechanisms of NB occurrence, development of NB, and precise diagnosis and therapy. As mentioned above, more in–depth research has been conducted on the changes of NB in the genome, but there is still a limited understanding of the isomeric changes in genome transcription.Research has found that a single gene often produces multiple isoforms, which have certain functional and tissue expression differences.However, traditional high pathway techniques have technical difficulties in studying gene transcriptional isomers, which makes it difficult for us to fully understand the differential expression of gene transcriptional isomers in NB and their downstream biological

functions. The emergence of the Third Generation sequencing technology has made it possible to systematically study the transcriptional isomers of NB genes.

1.1.1The variation of NB chromosome copy number

The variation of chromosome copy number refers to a change in the number of copies of the entire chromosome or chromosome segment.The variation of chromosome copy number is the main form of genomic structural variation in NB, which is closely related to clinical staging and risk grouping.Common chromosomal copy number variations in NB, such as MYCN amplification, ALK amplification, 11q deletion, 1p deletion, and 17q acquisition, indicate a high risk of recurrence and poor prognosis[5]。

（ⅰ） MYCN amplification

MYCN is located in the 2p24 region of the chromosome and is a member of the MYC oncogene family. As a transcriptional regulator, MYCN plays a regulatory role in the differentiation, proliferation, survival, metabolism, metastasis, and angiogenesis of NB.Abnormal amplification of the MYCN gene is an important driving factor for high-risk NB and the most important prognostic factor for NB. Research has shown that around 20% of NB is accompanied by MYCN amplification, and in high-risk NB, this proportion can reach 40% to 50%[6].MYCN amplification is closely related to the high invasiveness of NB. Researchs have indicated that MYCN amplification in NB is an early initiating event that leads to the development of high-risk NB[6-7].The N-myc protein encoded by the MYCN gene is the main transcriptional regulatory factor for cell growth, metabolism, and differentiation.The amplification of MYCN leads to its overexpression, which is an important way for MYCN to affect the occurrence and development of NB. Chromosomal translocation leading to amplification of distal enhancer elements or hijacking of highly active enhancers is an important mechanism of MYCN overexpression[8].润色后的句子：

"Other factors that contribute to MYCN overexpression, such as dysregulation of MYCN phosphorylation or MYCN mutation (e.g. P44L), leading to increased stability of the MYCN protein, are also important mechanisms involved in the onset and progression of NB."

（ⅱ） ALK amplification

ALK is a receptor of tyrosine kinase that is typically only expressed in developing embryos and neonatal brains.ALK is the most common mutated gene in NB, with 6% to 12% of NB carrying ALK somatic mutations.Researchs have found that ALK amplification is also a prognostic feature of NB. Research has shown that 1% to 2% of children with NB in high-risk group are associated with ALK

amplification, thereby increasing ALK activity[9]。 ALK amplification almost does not occur simultaneously with ALK hotspot mutations, but ALK amplification tends to occur simultaneously with MYCN amplification.In the study by the International Society of Pediatric Oncology Europe Neuroblastoma Group(SIOPEN), 97% of tumors with ALK ampilication were accompanied by MYCN amplification [10], which may be related to this two genes which are located close to each other on the chromosome(ALK located at 2p23).The prognosis of NB with ALK amplification is significantly poor.The 5-year overall survival rate of patients with ALK amplification is about only 26%.NB with ALK amplification exhibits a synergistic biological effect of ALK pathway activation and MYCN amplification-related pathway activation, resulting in poorer survival rates.Preclinical trial data shows that, unlike NB children with ALK mutations, children with ALK amplification lack response to ALK inhibitors such as Crizotinib[11].The above data suggests that children with ALK amplification may be an independent and poor prognostic subgroup who need targeted treatment plans in clinical diagnosis and treatment.

（iii）11q missing

Long arm deletion of chromosome 11 (11q deletion) is one of the most common genomic structural variation events in NB, with 20% to 45% of NB carrying this mutation.In clinical practice, 11q deficiency is associated with higher disease staging and lower survival probability. In 2009, the International Neuroblastoma Risk Group (INRG) included 11q deficiency in the clinical risk grading system for pediatric patients with NB.11q deficiency and MYCN amplification are mutually exclusive events, but their tendency occurs together with 17q amplification and 3p deficiency [4].Due to the long arm region of chromosome 11, which contains numerous small regions and genes, further exploration is needed to determine the specific functional regions and genes of chromosome 11.Different research groups have identified candidate genes located in the 11q region, such as CADM1, ATM, H2AFX, etc. Although the biological functions of these genes are relatively clear, their roles in the occurrence and development of NB need to be verified.In order to further determine the functional regions where 11q affects NB, the researchers further narrow down the research scope and determine that the 11q23 region may be a potentially important region. Currently, detection probes with missing 11q are also designed in this region in clinical testing and basic research.Due to the fact that 11q is the entire long arm region of chromosome 11, such a long segment also results in inconsistent variations in its different microregions， which reflects the upregulation of CCND1(located at 11q13.3) detected in NB samples and cell lines.CCND1 forms a complex with

CDK4/CDK6 to regulate cell cycle G1/S transition.In NB, inhibiting CCND1 can significantly reduce cell proliferation and promote neuronal differentiation [12].The above research shows that the 11q region in NB is functionally important and relatively complex. How to conduct basic and clinical research, and determine specific functional regions or important functional genes with the analysis of genomic data is currently an urgent issue that needs to be explored and researched.

（iv） 1p (1p36) missing

1p36 deficiency is commonly present in human cancers, particularly in neurological tumors. About 30% of children with NB carry 1p heterozygous deficiency [13-14].Although 1p36 heterozygous loss is associated with the prognosis of pediatric patients, multivariate analysis shows that 1p36 heterozygous loss cannot be used as an independent prognostic factor [14]; there is a tendency for 1p36 heterozygous deletion to occur simultaneously with MYCN amplification, with approximately 70% of MYCN amplified tumors carrying 1p36 heterozygous deletion; children with NB who carry both variants simultaneously are in an extremely high-risk group, prone to bone marrow metastasis and poor clinical prognosis.Although studies have shown that the deletion of tumor suppressor genes such as CHD5 and ARID1A located in the 1p36 region is an intrinsic mechanism that affects tumor occurrence and development in this region [15], further exploration of the mechanism of 1p36's action in NB and its concurrent occurrence with MYCN is still needed.

1.1.2 Chromosomal structure variation

In addition to chromosomal copy number abnormalities, there are also types of chromosomal structural variations such as deletions, duplications, inversions, and translocations, as well as more complex genomic structural variations that occur simultaneously with several types of variations, such as chromosomal fragmentation.Chromosomal structural variation is an important driving factor for tumor occurrence and development. There are common chromosomal structural variations, their frequency, type, and localization vary greatly in NB, especially in high-risk NB.A study using whole genome sequencing to analyze 19 high-risk NB cases found that each sample carried an average of 40 chromosomal breakpoints [1].TERT gene rearrangement is a common chromosomal structural variation in NB. Among 75 cases of high-stage NB, 15 cases detected TERT gene rearrangement [16]. This study found that for high-risk NB, the tandem repeats of tumor without MYCN amplification increased significantly .The complexity of structural variation is more pronounced in high-risk tumors without MYCN amplification [3].Analyzing the genes affected by chromosomal structural

variations, it was found that structural variations are more likely to disrupt the neural development related genes of NB.Studies have shown that some genes involved in neuronal differentiation or playing a role in neural transmission and synapses, such as SHANK2, DLG2, AUTS2, CNTNAP2, NRXN1, and CTNND2, are enriched in genes disrupted by genomic structural variation breakpoints [3].The mechanism by which chromosomal structural variations affect the occurrence and development of NB is complex and currently lacks a profound understanding.The enhancer hijacking or localized enhancer amplification mediated by chromosomal structural variation is an important mechanism affecting NB, which affects telomere maintenance through abnormal regulation of MYCN oncogenes or activation of telomerase reverse transcriptase (TERT) genes.At the same time, genomic structural variations in tumors generally do not appear in a single type, but rather in complex forms where multiple types occur simultaneously, further increasing complexity and making the biological behavior and clinical manifestations of NB more complex.Although compound structural variations increase the difficulty of identifying genomic structural variations, exploring new compound structural variations will deepen our understanding of the complexity of NB and provide new perspectives for exploring its mechanisms of occurrence and development.

(i) Chromothripsis

Chromothripsis is a complex chromosomal structural variation that refers to the division of chromosomal regions into smaller fragments, rearrangement, and connection in a single catastrophic event, resulting in the assembly of a new genome.Chromothripsis is closely related to tumor growth and drug resistance. NB also exhibits such complex chromosomal structural variations. Research has shown that chromosomal rupture is detected in up to 18% of high stage NB and is associated with poor clinical prognosis [17].In the TARGET NB dataset, approximately 19% of high-risk NBs experience chromosomal rupture events [3].In NB, chromosomal rupture events largely overlap with MYCN amplification at 2p and TERT amplification at 5p, indicating that chromosome purification selection is an important cause of these changes after chromosomal variation events occur [3,16].At the same time, partial chromosome rupture and co localization of the TERT gene can increase the expression level of the TERT gene, leading to an increase in the telomere length of NB, indicating that it can affect tumor progression by affecting the proliferation ability of tumor cells.Research has found that chromosome rupture is closely related to loss of 1p heterozygosity, possibly due to its inhibition of NB cell differentiation through the loss of some tumor suppressor gene alleles located on chromosome 1p36.

(ii) Extra chromosomal circular DNA

During the process of chromosome fragmentation, some chromosomal fragments are reordered and assembled, while others exist in a circular shape outside the chromosome, known as extra chromosomal circular DNA (ecDNA).EcDNA can be generated through various cell cycle events, but the exact mechanism of its formation is still unclear.In the most widely used ecDNA formation model, the stagnation of the replication fork leads to the collapse of the replication fork, which then causes replication bubbles to detach from chromosomes and connect with each other, resulting in the formation of ecDNA.In ecDNA, in addition to complete genes, it also contains non coding sequences such as promoters and enhancers. EcDNA is associated with the amplification of some oncogenes and is an important cause of tumor resistance, which is associated with poor prognosis.Kohl et al. first discovered the MYCN gene in the ecDNA of NB, which is also the first time an oncogene has been discovered in ecDNA[18].Afterwards, a complete map of extracellular DNA cyclization in NB was gradually established, which also confirmed the amplification of MYCN gene on the ecDNA of NB. Subsequently, 93 cases of NB were detected and found to contain an average of 0.82 ecDNAs per tumor [19].In NB, ecDNA can be roughly divided into two types: 1)the type with amplification of proximal enhancers ，which is controlled by the norepinephrine core regulatory circuit (CRC); 2) the type without proximal enhancers and with CRC driven enhancers in distant chromosomal regions, which hijacks distal enhancers to compensate for the deficiency of local gene regulatory elements. This type occupies a major portion of ecDNA containing MYCN and causing its amplification[20]At the same time, ecDNA can also promote the expression of TERT, thereby participating in telomere maintenance. Overall, ecDNA typically carries oncogenes such as MYCN, JUN, MDM2, SOX11 for amplification [19–20]. Through uneven separation during mitosis, ecDNA can accumulate in cancer cells, giving them a competitive advantage in responding to the tumor microenvironment and selecting cytotoxic therapeutic drugs, thereby promoting tumor occurrence and development.

1.1.3 Genomic instability

Tumor genomic instability includes a series of genomic changes, from small indels to alterations throughout the entire chromosome. Genomic instability, as an important mechanism of tumor development, has always been of great concern.It is generally believed that genomic instability is a poor prognostic factor for pediatric patients, but it is an exception in NB.NB has two different genomic instability patterns: 1) chromosomal aneuploidy, which is associated with better prognosis; 2)

Segmental chromosomal changes,which are a poor prognostic factor [21].

For NB, tumor cells in children with stage 1, 2, or 4S have the characteristic with a high degree of aneuploidy, indicating that aneuploidy status is associated with a good prognosis in the child.On the contrary, cells of NB in stage 4 exhibit severe structural chromosomal damage, including chromosomal deletions, chromosomal increases, and chromosomal rearrangements.Tumors with these characteristics are highly invasive [22].The analysis of 493 NB samples using comparative genomic hybridization chips showed that children carrying only chromosomal aneuploidy had a 4-year progression free survival rate of over 90%, while children with MYCN amplification or segmental chromosomal changes had a 4-year progression free survival rate of 37% to 45% [23].Similar results also appeared in the analysis of chromosomal changes in 190 cases of NB, where the total number of chromosomal breakpoints was significantly associated with poor prognosis [24]. The mechanism of these leads to the above state is currently unclear.It is generally believed that chromosomal aneuploidy is caused by a malfunction of the mitotic apparatus. If the spindle fails, it can lead to uneven distribution of chromosome numbers in daughter-cells.Cells with these characteristics are more sensitive to internal and external pressure and are more susceptible to further genomic damage (such as DNA damage, chromosome breakage, or chromosome exfoliation), leading to segmental changes in chromosomes.Therefore, genes such as TP53 involved in cell cycle regulation and DNA repair play important roles in NB progression and risk stratification [25].Based on this, it is speculated that drugs that inhibit the regulation of cell cycle, mitosis related genes, and target DNA damage repair may benefit children.

1.1.4 The maintenance mechanism of NB telomeres mediated by genomic structural variations

Telomeres, located at the end of chromosomes ,in most cases shorten with each cell replication, ultimately leading to the inability of cells to replicate.Tumor cells maintain telomere length by activating the telomere maintenance mechanism, thereby achieving sustained proliferation ability. Tumors with activated telomere maintenance mechanisms have poor prognosis.In NB, genomic changes that affect telomere maintenance mechanisms are often found in the high-risk group, while low-risk groups are less associated with telomere elongation activity [16, 26-27].Meanwhile, it was found that telomere maintenance is closely related to the differentiation of NB tumor cells [28].The levels of telomere related factors are significantly different in adrenergic (ADRN) and mesenchymal (MES)

type cells, and inhibition of telomerase reversibly triggers ADRN conversion to MES cell type[15], indicating that telomere maintenance plays an important role in NB.NB mainly maintains telomere length through two mechanisms, including upregulation of telomerase activity caused by overexpression of TERT and the alternative length of telomeres (ALT) pathway.Genomic structural variation is an important molecular mechanism for maintaining telomeres.MYCN amplification, ATRX inactivation, and TERT promoter rearrangement are the main telomere maintenance mechanisms in NB, which perform telomere maintenance functions in different ways.The TERT gene encodes telomerase catalytic units, and its overexpression is an important telomere maintenance mechanism in NB. TERT overexpression mainly involves two mechanisms, namely segmental chromosomal rearrangement near TERT and MYCN amplification.20% to 25% of high-risk NB are associated with chromosomal rearrangements in the proximal 5p15.33 region of the TERT gene. This chromosomal rearrangement induces upregulation of TERT transcription by juxtaposing the TERT coding sequence with enhancer elements, leading to chromatin remodeling and DNA methylation in the affected region [16, 26-27].TERT is a transcription target of N-Myc, and MYCN amplification is also an important mechanism for promoting TERT overexpression.ALT achieves telomere extension through homologous recombination, which is another mechanism for telomere maintenance.In the high-risk group NB, 20% to 25% of tumors are accompanied by ALT activation, while in the low to medium risk group, this proportion is only 5% to 12% [27,29].The RNA helicase encoded by the ATRX gene is a member of the chromatin remodeling protein SWI/SNF family, and its functional deficiency is an important mechanism for the occurrence of ALT.The ATRX genome changes in NB mainly include single nucleotide variations, large fragment deletions, and rearrangements that produce new fusion proteins, all of which are associated with ALT [4,30-31].ATRX mutations can be detected in 55% to 60% of ALT positive NB[25]. From the clinical presentation, similar to MYCN amplified children, ALT activated children also have poor prognosis.Research has found that TERT rearrangement, ATRX variation, and MYCN amplification, which are three different forms of variation, may represent three subgroups of high stage NB that do not overlap with each other [26].Although the mechanism of telomere maintenance in NB is currently relatively clear, further exploration and research are still needed. In some cases where TERT rearrangement and MYCN amplification have not occurred, some tumors may also exhibit high expression of telomerase.The driving factors for the occurrence of ALT in NB are not yet clear, even in the absence of ATRX mutation.Research has found [32] that in NB cell lines, telomere sequences are included in chromosomal imbalanced

translocations, suggesting that this structural variation may contribute to defects in telomere maintenance pathways. At present, some telomere maintenance mechanisms have also been found in other tumors, but whether these mechanisms can serve as supplements and improvements to NB telomere maintenance mechanisms remains to be further explored and verified.

1.1.5 Conclusion

The study of genomic variation characteristics, especially genomic structural variation characteristics, provides an important theoretical basis for exploring the underlying mechanisms and precise diagnosis and treatment of NB.However, there is still limited understanding of the abnormal expression of gene transcriptional isomers and the function of tumor specific isomers without altering the genome sequence.The use of third-generation sequencing techniques such as Third Generation and Biono optical profiling can increase the resolution and accuracy of identifying genomic structural variations.At the same time, basic and clinical trials are needed to explore the relationship between newly discovered genomic structural variations and NB biology and clinical applications, in order to promote the transformation of basic research into clinical applications.

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2、The main content of the study (including experimental design, study grouping, intervention measures, primary and secondary research indicators)

2.1 Research purpose and significance

Neuroblastoma (NB) is a common malignant tumor in children. The current main treatment methods are surgical resection combined with postoperative chemotherapy and immunotherapy.However, clinical studies have found that 40% –50% of children who undergo postoperative chemotherapy have poor efficacy and progress in their condition.This study aims to screen the gene types of children with neuroblastoma through Deep Sequencing Technology and Third Generation Sequencing Technology, and treat them according to different treatment modes. It is expected to find new splicing bodies and new treatment targets, providing a basis for precise treatment of pediatric neuroblastoma in the future.

2.2 Research content and design

2.2.1 research contents

ⅰ）Collect 20 clinical neuroblastoma tumor specimens;

ⅱ）Deep analysis of sequencing data and tumor specific transcripts;

2.2.2experimental design

ⅰ) 7 types of neuroblastoma cell lines：SK-N-SH、SH-SY5Y、SH-EP、IMR-32、NB-19、SK-N-BE、SK-K-AS; Detection of gene transcriptional isomer expression usingThird Generation Sequencing Technology, divided into NMYC amplification group：IMR-32, NB-19, SK-N-BE and NMYC non amplification group：SK-N-SH, SH-SY5Y, SH-EP， SK-N-AS，and compare the two；SH-SY5Y, SH-EP are differentiated from SK-N-SH， and comparison of three cell lines reveals differentiation related specific transcripts。

ⅱ）Deep sequencing of tissues from 20 clinical neuroblastoma specimens；

2.2.3 Research object

The subjects included in this study were children with neuroblastoma disease. The study will be

conducted in the medical institutions of Nantong University Affiliated Hospital, lasting for 2 years. It is expected that 20 cases will participate in this study.

2.2.4 Research objectives

ⅰ) Identifying tumor differentiation related specific transcripts through Third Generation Sequencing Technology of cell lines;

ⅱ) Identify possible differential expression through Deep Sequencing Technology of clinical tissue samples.

3、Specific research methods (including inclusion criteria, sample size calculation, study grouping, intervention and control, specific research process, primary and secondary research indicators, and statistical analysis)

3.1.Inclusion and exclusion criteria

3.1.1 Inclusion criteria

① Children with neuroblastoma range in age from 0 to 14, regardless of gender;

② Preoperative imaging examinations indicate patient with neuroblastoma;

③ Before postoperative chemotherapy, the patient's physical strength is good. At the same time, the results of white blood cells, neutrophils, hemoglobin, platelets, and other test indicators are all within normal range, which is in line with the treatment conditions of the relevant chemotherapy and immunotherapy;

④ Preoperative pathological examination of the patient reveals neuroblastoma;

⑤ Patients have no other history of malignant tumors;

⑥ Patients voluntarily participate and sign informed consent and can comply with the study visit plan and other protocol requirements.

3.1.2 Exclusion Criteria:

① Patients who are found to have distant metastasis or ascites on preoperative examination are excluded;

② Individuals with severe liver and kidney dysfunction, as well as those with autoimmune diseases are excluded;

③ Patients with severe cardiovascular disease who can not tolerate general anesthesia are excluded;

④ Patients who have other malignancies or blood disorders are excluded.

3.1.3Exit and Termination Criteria

① If serious adverse events occur, the experiment should be terminated in a timely manner;

② External information (such as other high-quality research or evidence) proves that the intervention plan is ineffective or effective, and there is no need to continue the current clinical trial;

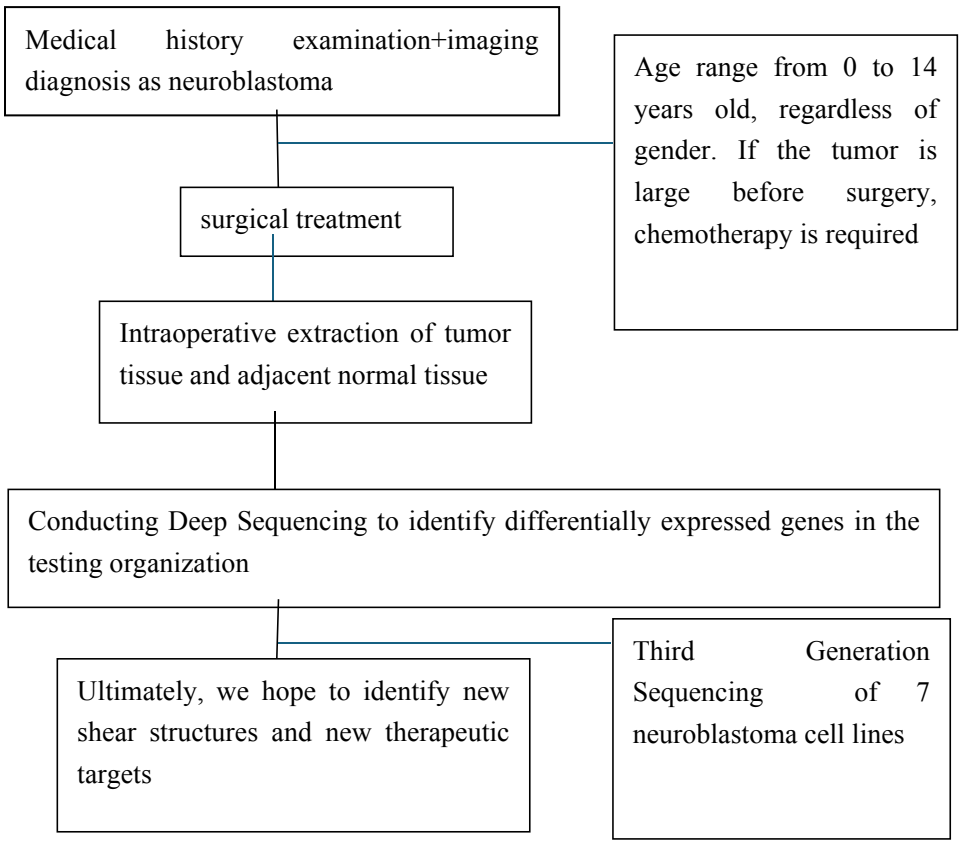
③ According to the planned mid-term analysis, the expected therapeutic differences were achieved, and it has been observed that the intervention plan of the experimental group is significantly better than that of the control group.

3.2.Sample size calculation and research grouping

Recruitment is mainly conducted in children with neuroblastoma within the past 2 years, excluding cases that are not suitable for enrollment. This study expects recruitment of 20 children with neuroblastoma.

3.3. Research specific processes

3.3.1technology roadmap



3.3.2 Specific sequencing

7 types of neuroblastoma cell lines：SK-N-SH、SH-SY5Y、SH-EP、IMR-32、NB-19、SK-N-BE、SK-K-AS; Detection of gene transcriptional isomer expression using third-generation

sequencing, divided into NMYC amplification group：IMR-32, NB-19, SK-N-BE and NMYC non amplification group：SK-N-SH, SH-SY5Y, SH-EP， SK-N-AS，and compare the two；SH-SY5Y, SH-EP are differentiated from SK-N-SH， and comparison of three cell lines reveals differentiation related specific transcripts。

i) Sequencing experiment process

The process of full-length transcriptome sequencing experiment includes three steps:

a.Sample extraction and testing;

b.Library construction;

c.On machine sequencing.

Kangsheng Zhenyuan strictly controls every production step of sample testing, database construction, and sequencing to ensure the output of high-quality data.

ii)Sample extraction and testing

After extracting total RNA from tissue samples, we use the following methods for detection. Only after the detection results meet the requirements can we proceed with library construction:

a.Nanodrop detection of RNA purity (OD 260/280), concentration, and whether the nucleic acid absorption peak is normal; A single database construction requires a total RNA content of 1 μg, a concentration of ⩾ 200ng/μL, and an OD260/280 between 1.8 and 2.2.

b.Agilent 2100 accurately detects the integrity of RNA, with detection indicators including: RIN value, 28S/18S, baseline elevation, and 5S peak;

c.Electrophoretic detection of RNA sample bands for dispersion, presence of impurities, or contamination of genomic DNA.

iii)Library construction

After passing the sample testing, the library is constructed, and the main process is as follows:

a.Synthesize full-length cDNA of mRNA using Clonetech SMARTerTM PCR cDNA Synthesis Kit. The 3 'end of eukaryotic mRNA has a polyA tail structure. Primers with Oligo dT are used to pair with poly-A as A-T base pairs for reverse synthesis of cDNA, and primers are added at the end of the full-length cDNA synthesized by reverse synthesis. Amplify the full-length cDNA obtained through PCR, purify the product with magnetic beads, and quantify using Qubit 3.0.

b.Purify and amplify the full-length cDNA using magnetic beads, and remove some small fragments of cDNA below 1kb.

c.Perform end repair on full-length cDNA and connect to SMRT dumbbell-shaped connectors.

d.Digest the unconnected fragments using exonuclease and purify them again using magnetic beads to obtain a sequencing library.

e.After the construction of the library is completed, the quality of the library is tested, and only

after the test results meet the requirements can machine sequencing be carried out.

The detection method is:

a.Use Qubit 3.0 for accurate quantification;

b.Use Agilent 2100 to detect the library size, and only after the library size meets expectations can machine sequencing be performed.

iv)On-machine sequencing

After passing the library inspection, use PacBio sequencer to perform full-length transcriptome sequencing according to the target offline data volume.

3.4 Main and secondary research indicators and statistical analysis

Main and secondary indicators: comparison between genes :IMR-32, NB-19, SK-N-BE and NMYC non-amplified groups:SK-N-SH, SH-SY5Y, SH-EP, and SK-N-AS; SH-SY5Y and SH-EP are derived from SK-N-SH differentiation, and differentiation related specific transcripts can be found by comparing the three cell lines.

Statistical analysis: ① Sample size estimation and grouping method, collecting clinical data of laparoscopic/open neuroblastoma surgery performed at Nantong University Affiliated Hospital from 2024 to 2026.② Grouping method: Random grouping ③ Research data collection and statistical analysis. Researchers should ensure the accuracy, completeness, and timeliness of the data in the Case Report Form (CRF) and all required reports provided to them.Statistical analysis used survival analysis and repeated measurement methods to analyze the data of the experimental group and control group. t-test was used for quantitative data, chi square test was used for count data, Kaplan Meier method and Cox regression analysis were used for survival analysis, and Stata 26.0 was used for statistical software. $P<0.05$ was considered statistically significant.

4、Ethic principles

Meet the requirements of the Ethics Committee of Nantong University Affiliated Hospital.

5、Plan and Progress

2024.04.01-2024.08.31 Long segment sequencing of 7 neuroblastomas, collecting neuroblastoma and adjacent control groups;

2024.09.01-2025.11.31 Analyze sequencing data and discover over 100 new splicing fragments; Defective cell lines with successful 2-3 gene new splicing.

2025.12.01-2026.03.31 Organize and analyze data, and write a paper.