**Response to Reviewer A**

Comment 1：

This is a work where the authors characterized, in both peripheral blood and bone marrow, the expression of 5 NB genes to find tumor relapse or residual disease.

Throughout the development of the work, the authors try to establish that the method chosen to perform these determinations (ddPCR) is superior to the detection of measurable residual disease (MRD) by flow cytometry. That's why I think the title doesn't reflect this.

On the other hand, the statistical analyzes that support the work show the robustness of its methodology.

Finally, there are some fixes that I list below, according to the line where they are found.

Reply 1: Thank you for your suggestion, we have modified the title.
Changes in the text: We have modified our text as advised "RT-PCR demonstrates superior sensitivity and specificity in detecting the five neuroblastoma genes compared to the flow cytometry method for measurable residual disease" (see Page 1, line 3-5)

Comment 2:

Line 42: define PFS

Reply 2: Thank you for your reminder, we have defined PFS.
Changes in the text: We have added some descriptive statements to define PFS. "Progression-free survival, the time from patient enrollment to the onset of disease progression" (see Page 2, line 46-47)

Comment 3:

Line 56: Correct it PCT

Reply 3: Thank you for pointing out the error, we have corrected it.
Changes in the text: We change "PCT" to "PCR" (see Page 2, line 64)

Comment 4:

Line 85: in vivo, cursive

Reply 4: Thank you for your reminder, we have modified it.
Changes in the text: We've italicized "*in vivo*" (see Page 3, line 95)


Comment 5:

Line 101: delete #

Reply 5: Thank you for your modification, we have deleted the "#".
Changes in the text: We have deleted the "#" (see Page 4, line 111)


Comment 6:

Lines 103 and 104: sentence is redundant

Reply 6: Thank you for your suggestion. I have deleted this sentence and the cited references.
Changes in the text: I have deleted this sentence. (see Page 4, line 113)


Comment 7:

Line 143: in vivo, cursive

Reply 7: Thank you for your reminder, we have modified it.
Changes in the text: We've italicized "*in vivo*" (see Page 5, line 149)


Comment 8:

Line 145: define OS

Reply 8: Thank you for your reminder, we have defined PFS.
Changes in the text: We have added some descriptive statements to define OS. "overall survival, time from enrollment to death from any cause" (see Page 5, line 154)


Comment 9:

Line 161: delete #

Reply 9: Thank you for your modification, we have deleted the "#".

Changes in the text: We have deleted the "#" (see Page 6, line 172)

Comment 10:

Line 162: delete #

Reply 10: Thank you for your modification, we have deleted the "#".
Changes in the text: We have deleted the "#" (see Page 6, line 173)

Comment 11:

Line 194: delete #

Reply 11: Thank you for your modification, we have deleted the "#".
Changes in the text: We have deleted the "#" (see Page 7, line 221)

Comment 12:

Lines 226 and 227: which values were assigned for each category? How do they define this?

Reply 12: We are very sorry that we did not explain clearly. We have now made a detailed explanation of each category.
Changes in the text: We modify the original sentence to At each disease evaluation time point during BM assessment, the responses assigned included complete remission (CR) (Regardless of BM infiltration at baseline, no BM infiltration was seen when BM was re-evaluated.), stable disease (SD) (BM infiltration, the extent of BM infiltration is >5% on re-evaluation but does not meet the criteria for CR, VGPR and PD), partial remission (PR) (There is no infiltration in the BM, and the extent of BM infiltration is >5% during re-evaluation, or there is infiltration in the BM, and the extent of BM infiltration is >2 times and the extent of infiltration is >20% during re-evaluation), and very good partial remission (VGPR) (If any of the following conditions occurs, it will be evaluated as VGPR. The range of BM infiltration is <5%, when re-evaluated, the BM infiltrates, but it is between 0 to 5%; There is no infiltration in the BM, when re-evaluated, there is infiltration in the BM, but the range of infiltration is between 0 to 5%; BM infiltration range >20%, BM infiltration was re-evaluated but between 0 to 5%). (see Page 8, line 251-264)

Comment 13:

Line 229: delete #

<u>Reply 13:</u> Thank you for your modification, we have deleted the "#".
<u>Changes in the text:</u> We have deleted the "#" (see Page 8, line 266)

Comment 14:

Line 241: delete #

<u>Reply 14:</u> Thank you for your modification, we have deleted the "#".
<u>Changes in the text:</u> We have deleted the "#" (see Page 9, line 279)

Comment 15:

Line 242: delete #

<u>Reply 15:</u> Thank you for your modification, we have deleted the "#".
<u>Changes in the text:</u> We have deleted the "#" (see Page 9, line 280)

Comment 16:

Line 253: delete #

<u>Reply 16:</u> Thank you for your modification, we have deleted the "#".
<u>Changes in the text:</u> We have deleted the "#" (see Page 9, line 291)

Comment 17:

Line 270: delete #

<u>Reply 17:</u> Thank you for your modification, we have deleted the "#".
<u>Changes in the text:</u> We have deleted the "#" (see Page 10, line 308)

Comment 18:

Line 285: delete #

<u>Reply 18:</u> Thank you for your modification, we have deleted the "#".
<u>Changes in the text:</u> We have deleted the "#" (see Page 10, line 323)

Comment 19:

Line 291: of them were

Reply 19: Thank you for your modification, we have added "them".
Changes in the text: The sentence is modified to "BM specimens, 30 (34.091%) of them were positive in the NB5 assay" (see Page 10, line 329)

Comment 20:

Line 317: delete #

Reply 20: Thank you for your modification, we have deleted the "#".
Changes in the text: We have deleted the "#" (see Page 11, line 355)

Comment 21:

Line 319: patients or cases?

Reply 21: Thank you for pointing out that our writing was not rigorous. The content has been added.
Changes in the text: The sentence is modified to "B For different specimens (BM, PB, and CSF), we performed ddPCR on 24 specimens (20 patients)." (see Page 11, line 357-358)

Comment 22:

Line 326: delete #

Reply 22: Thank you for your modification, we have deleted the "#".
Changes in the text: We have deleted the "#" (see Page 11, line 364)

Comment 23:

Line 339: delete #

Reply 23: Thank you for your modification, we have deleted the "#".
Changes in the text: We have deleted the "#" (see Page 11, line 377)

Comment 24:

Line 371: delete #

Reply 24: Thank you for your modification, we have deleted the "#".
Changes in the text: We have deleted the "#" (see Page 13, line 409)

Comment 25:

Line 483: delete #

Reply 25: Thank you for your modification, we have deleted the "#".
Changes in the text: We have deleted the "#" (see Page 16, line 521)

Comment 1：

First, the title should indicate the consistency between NB5 gene RT-PCR detection vs. MRD and the sensitivity and specificity outcomes.

Reply 1: Thanks for your suggestion, we have rewritten the title.
Changes in the text: We have modified our text as advised "RT-PCR demonstrates superior sensitivity and specificity in detecting the five neuroblastoma genes compared to the flow cytometry method for measurable residual disease" (see Page 1, line 3-5)

Comment 2：

Second, the abstract is inadequate. The background did not describe the knowledge gaps on the sensitivity and specificity of NB5 gene detection and its prognostic role. The methods should describe the calculation of sensitivity and specificity and how the clinical cohort was obtained and followed up. The results need to report the findings on the sensitivity and specificity and HR value for the prognostic role.

Reply 2: Thank you, we have added the summary content.
Changes in the text: We have modified our text as advised "Background: Exploring sensitive prognostic methods for patients with relapsed or refractory neuroblastoma (NB) is critical. The commonality among the five genes of neuroblastoma is their ability to be highly expressed in neuroblastoma. Previous research has found that the expression levels of these genes can be used as marker genes to guide micrometastasis. This study aimed to explore whether an improved NB5 detection method is more sensitive and specific than is flow cytometry of

measurable residual disease (MRD) in predicting NB metastasis or residual disease and prognosis, and whether this result can be used as an independent factor to influence PFS (Progression-free survival, the time from patient enrollment to the onset of disease progression).

Methods: Five neuroblastoma genes (NB5) detection involved reverse transcriptase polymerase chain reaction (RT-PCR) to detect the expression of CHGA, DCX, DDC, PHOX2B, and TH from the NB-associated genes. Bone marrow (BM), peripheral blood (PB), or cerebrospinal fluid (CSF) specimens were collected from 71 patients, and 113 detections were completed. The relationship between five genes expression changes with clinical characteristics and survival rate was analyzed. Evaluate the sensitivity and specificity of NB5 detection results in all patients. A total of 71 patients were collected from six research centers, with a median follow-up time of 14 months.

Results: PB specimens showed 100% concordance with the BM specimens in terms of positive results. Furthermore, the BM specimens exhibited an additional 45.455% (5/11) positive results compared to the 34.091% (30/88) of PB specimens. The BM specimens were positive for NB5 assay, which was significantly higher than the positive results of flow cytometric MRD (15/88, 17.045%). NB5 was mainly expressed in newly diagnosed patients (P=0.043) and positive patients with flow cytometric MRD (P<0.001) or BM morphology (P<0.001). Positive rates of droplet digital PCR (ddPCR) were consistent with those of quantitative PCR (qRT-PCR) in BM (13/18, 72.222%). However, in PB, the positive rate of ddPCR (2/5, 40.000%) was higher than that of qRT-PCR. A total of 38 specimens (BM, PB, CSF) were detected as positive under qRT-PCR. Among the positive results, the analysis revealed a significant difference between the CHGA and TH in pairwise comparisons (P=0.005). Progression-free survival (PFS) analysis showed that among MRD-negative patients, the survival time of the NB5-positive group was significantly lower than that of NB5-negative group (27.408±10.791 months vs. 35.961±3.084 months; P=0.034), and in the Cox regression model, risk stratification based on NB5 expression level was an independent prognostic factor for relapsed or refractory disease (95% CI 1.020 to 9.099, HR = 3.046, P=0.046). Combining the follow-up results, we found that the sensitivity and specificity of NB5 detection were both 100%." (see Page 2, line 38-76)

Comment 3：

Third, the introduction needs to review what has been known and unknown on the prognostic role of NB5 gene detection approach. The authors need to analyze the limitations of prior studies and clearly explain why the current study is needed.

Reply 3: Thank you for pointing out the issues. We have added content to the "Introduction".
Changes in the text: We have modified our text as advised "Several studies have identified multiple individual messenger RNA (mRNA) with stable expression in NB (19-21). Due to the limited sensitivity of a single marker, a set of multiple markers was identified for the qRT-PCR testing of patients with NB (22,25-26). A recent study identified five genes, chromogranin A (CHGA), doublecortin (DCX), dopadecarboxylase (DDC), paired-like homeobox 2b (PHOX2B), and tyrosine hydroxylase (TH), that were rarely or never expressed in BM or PB

but that were strongly expressed in NB in vivo or in cell lines (14,17). However, in previous studies, the calculation of multiple markers was primarily based on signature approach. This method of calculation can easily lead to false-negative outcomes because the expression levels of some genes do not increased in consideration of the stability of gene expression across various risk groups of NB, their correlation with PFS or OS (overall survival, time from enrollment to death from any cause), and their expression levels in other hematopoietic cells, the aforementioned five genes were chosen. They will be combine with appropriate calculation methods for further analysis. We detected the expression levels of five genes in different specimens [BM, PB, and cerebrospinal fluid (CSF)] and found the results were associated with clinical evaluation". (see Page 5, line 143-159)

Comment 4：

Fourth, the methodology of the main text needs to describe the clinical research design, explain the sample size estimation, inclusion criteria of the patient sample, and details of follow up. The authors need to specify the gold standard, otherwise there is no standard of comparison. In statistics, please describe the calculation of sensitivity and specificity and the P value for statistical significance.

Reply 4: Thank you for pointing out the issues. We have added content to the "Methods".
Changes in the text: We have modified our text as advised "From October 2018 to June 2023, a total of 71 cases aged 1 to 13 years old (median 5 years old) with NB were enrolled across 6 research centers (Hunan Provincial People's Hospital, Children's Hospital Affiliated to Medical College of Zhejiang University, Xinhua HospitaI Affiliated to Shanghai Jiao Tong University SchooI of Medicine, Anhui Provincial Children's Hospital, Children's Hospital of Chongqing Medical University, Children's Hospital of Fudan University). All cases were classified according to the 2009 International Neuroblastoma Risk Group Staging System (INRGSS), the Chinese expert consensus on diagnosis and treatment of pediatric neuroblastoma (2015 or 2021 edition), or the 2007 Children's Oncology Group (COG) Risk Staging System. They were categorized into low-risk, intermediate-risk, and high-risk groups. Among them, 3 patients were classified as low risk, 2 as intermediate risk, and the rest as high risk. All cases were diagnosed and treated according to the guidelines of the Expert Consensus on Diagnosis and Treatment of Pediatric Neuroblastoma of 2015 and the Expert Consensus on Diagnosis and Treatment of Pediatric Neuroblastoma 2021 (CCCG-NB-2021) protocol. Regular treatments were administered based on these guidelines. Three patients underwent prospective collection of BM, PB, or CSF specimens for NB5 assay. Additionally, all patients underwent concurrent BM morphology flow cytometric MRD, and qRT-PCR detection. We collected all patients with the above detect items and meeting the above criteria during the above time period for this study. A total of 182 standard disease assessments were performed and made available for analysis. The follow-up duration was 5 years, the final follow-up time was June 2023, and the median follow-up time was 14 months. During the treatment process, tumor markers were reassessed for each course of treatment, and imaging was reviewed for every two courses of treatment. Following the completion of treatment, tumor markers and imaging examinations will be

reviewed every three months in the first year, every four months in the second year, and every six months in the third and fourth years. Follow-up was conducted by telephone interviews and outpatient review. Loss to follow-up is defined as no follow-up for more than 6 months after the end of treatment, and the last follow-up time is as of July 9, 2023. If new lesions are detected during imaging reexamination during treatment and are accompanied by an increase in neuron-specific enolase (NSE) or vanillylmandelic acid (VMA), recurrence is diagnosed. A comprehensive disease evaluation was performed on BM aspirate/biopsy prior to treatment. During the course of treatment, tumor biomarkers and BM MRD (BM-MRD) levels were examined to assess tumor burden. Additionally, the expression levels of NB5 mRNA in BM and/or PB were also tested at the same time point. The gold standard for assessing whether there is BM infiltration is BM cell morphology. This study obtained approval from the research ethics committees of each participating institution and was conducted in accordance with the approved guidelines. Investigators obtained written informed consent from the legal guardians of all the patients involved. All the aforementioned patients' various detection parameters are compared with the qRT-PCR results to assess the correlation between qRT-PCR and other testing methods at the same time point. This analysis aims to evaluate the sensitivity, specificity, and prognostic guidance of detecting the five genes." (see Page 6, line 175-219)

"Statistical computations were performed using SPSS 26.0 software (IBM Corp., Armonk, NY, USA). Standard descriptive and analytic statistical methods were employed at appropriate points, including analysis of variance (ANOVA), $\chi 2$ test, and contingency table analysis (28). The significance in progression-free survival (PFS) was assessed using Kaplan-Meier survival analysis, and the 2-sided log-rank test was used to compare the survival curves. Univariate analysis of predicted values of NB5 $\Delta$Ct and other variables was completed via the Cox proportional hazards regression model. Evaluate the sensitivity and specificity of NB5 detection based on the follow-up results. P values <0.05 were considered statistically significant for all tests". (see Page 9, line 268-276)

Comment 5:

Finally, please consider to cite several related papers: 1. Pérez-García MJ, Segura MF. Maintaining excellent outcomes: the impact of age cutoff reclassification on reduced therapy for neuroblastoma patients. Transl Pediatr 2023;12(11):1926-1930. doi: 10.21037/tp-23-391. 2. Tang J, Lu H, Yang Z, Li L, Li L, Zhang J, Cheng J, Li Y, Li S, Zhou H, He J, Liu W. Associations between WTAP gene polymorphisms and neuroblastoma susceptibility in Chinese children. Transl Pediatr 2021;10(1):146-152. doi: 10.21037/tp-20-168

Reply 5: Thank you for the references you provided, we have added them to the text.
Changes in the text: We have added citations to these 2 references. (see Page 20, line 633-638)

**Reviewer C**

1. **Figure 2**

Please explain MRD in the legend.

**Reply: Thanks for the reminder, MRD is already explained in the legend. At the same time, we have also explained the MRD contained in other figure legends.**

**Changes in the text: See Page 21, line 692 and Page 28, line 762-763.**


2. **Figure 4**

Please explain PFS in the legend.

**Reply: Thanks for the reminder, PFS is already explained in the legend.**

**Changes in the text: See Page 22, line 708.**


3. **Figure S5**

Please explain AUC in the legend.

**Reply: Thanks for the reminder, AUC is already explained in the legend.**

**Changes in the text: See Page 29, line 789.**


4. **Table 3**

Please explain MRD in table footnote.

**Reply: Thanks for the reminder, MRD is already explained in the table footnote.**

**Changes in the text: See Page 25, line 732-733.**


5. **Table 4**

Please explain BM, PB, and MRD in table footnote.

**Reply: Thanks for the reminder. BM, PB and MRD are already explained in the table footnote.**

**Changes in the text: See Page 26, line 739.**


6. **Table S5**

Please explain HR and CI in table footnote.

**Reply: Thanks for the reminder. HR and CI are already explained in the table footnote.**

**Changes in the text: See Page 31, line 814-815.**


7. **Table S6**

Please explain CI in table footnote.

**Reply: Thanks for the reminder. CI is already explained in the table footnote.**

**Changes in the text: See Page 31, line 820-821.**


8. **References/Citations**

a) References 23, 24, and 37 were not cited in the main text, please add and cite them in order.

164    stable expression in NB (19-21). Due to the limited sensitivity of a single marker, a

165    set of multiple markers was identified for the qRT-PCR testing of patients with NB

166    (22,25-26). A recent study identified five genes, chromogranin A (*CHGA*),

511     survival (EFS) (38). Our results, consistent with Nino's findings, suggest that ddPCR

512     holds greater potential for the application of MRD monitoring in NB.

513      Using individual genes for evaluating the results can enhance the sensitivity of the

514     detection. However, to avoid false-positive results, we adopted a strategy inspired by

515     previous studies (17,36,38) and compared the performance of the five individual

**Reply: Thanks for the reminder. References 23, 24, and 37 have been added to the main text and the order has been modified according to the citation order.**
**Changes in the text: See Page 5, line 141, Page 14, line 451- 455.**

b) Please add the citation for Ninoet al. at the end of the sentence.

507     diagnostic evaluation could be a viable strategy worth exploring. Nino et al. used

508     ddPCR to detect MRD in PB stem cell grafts of patients with high-risk NB and found

509     that MRD had an impact on the prognosis. Through the analysis of seven genes, they

**Reply: Thank you for the reminder, a citation has been added to the end of the sentence.**
**Changes in the text: See Page 14, line 449.**