Identification of HBA2 and NR4A2 as Potential Prognostic Molecular Markers of MYCN Amplified Neuroblastoma

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Research Article

Keywords: Neuroblastoma, MYCN, WGCNA, prognostic molecular markers

Posted Date: September 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-877542/v1>

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Abstract

Background

Neuroblastoma is a common extracranial solid tumor in children, and the prognosis of children with MYCN amplified type is poor. The prognostic factors in children with MYCN amplified form are still unclear.

Methods

Download data sets GSE53371 and GSE49710 from GEO database. The GSE53371 data set is used to screen the differentially expressed genes (DEGs) of MYCN amplified and MYCN non-amplified NB patients, and then cross the key module genes in the WGCNA analysis results to obtain their key genes. Then these key genes were analyzed for GO and KEGG function enrichment to analyze their potential biological functions and signal pathways. Single-factor COX regression and multi-factor COX regression analysis are used to screen key genes related to prognosis from key genes to construct risk scores. Finally, qRT-PCR was used to verify the expression of HBA2 and NR4A2 in tissue samples collected from the clinic.

Results

20 core genes were obtained after intersecting the differential genes between MYCN amplified and non-amplified samples with WGCNA analysis results. Univariate COX regression analysis showed that a total of 4 genes were closely related to the overall survival of MYCN amplified NB patients ($p < 0.05$). Further multivariate COX regression analysis showed that HBA2 and NR4A2 have independent prognostic significance. Finally, the qRT-PCR test results of clinical samples showed that, compared with the MYCN unamplified group, the expression of HBA2 in the MYCN amplified group increased, and the expression of NR4A2 decreased.

Conclusion

HBA2 and NR4A2 are diagnostic prognostic markers and potential therapeutic targets for patients with MYCN amplified NB.

Background

Neuroblastoma (Neuroblastoma, NB) is the most common extracranial malignant solid tumor in children, with an incidence of about 1 in 7000. The incidence of children under 14 is the third, second only to leukemia and brain tumors[1, 2]. According to the Children's Oncology Group (COG) risk stratification system, neuroblastoma can be divided into a low-risk group, a medium-risk group, and a high-risk group;

the five-year overall survival rate of the medium-low-risk group is more than 90%, and The five-year overall survival rate of the high-risk group is less than 30–50%, even after various treatments, including surgical resection, multi-drug chemotherapy, radiotherapy, autologous hematopoietic stem cell transplantation, and immunotherapy[2–5]. In the latest COG risk stratification system, children with MYCN gene amplification have all been classified as high-risk groups. This is enough to show the importance of MYCN for the prognosis of children with neuroblastoma[5].

The MYCN gene is located on the 2p24 chromosome and encodes the transcription factor N-Myc, which is a member of the Myc proto-oncogene family[6]. Studies have confirmed the Inhibition of the ALDH18A1-MYCN positive feedback loop attenuates MYCN-amplified neuroblastoma growth[7]; Other studies have shown that N-Myc can fully regulate gene expression through histone acetylation and DNA methylation in NB[8]. Some scholars have used bioinformatics methods to preliminarily explore the target genes of MYCN, use MYCN-related genes to establish a prognostic model; identify potential miRNA-mRNA regulatory networks for high-risk neuroblastoma[9–11]. The biological process of MYCN expansion that causes the proliferation and metastasis of NB cells is extremely complicated. Therefore, it is particularly important to determine the differential genes between MYCN amplified and MYCN non-amplified neuroblastoma patients, so as to discover the potential prognostic molecular markers of MYCN amplified patients.

Previous studies mainly focused on the biological functions of differentially expressed genes (DEGs) between MYCN amplified and MYCN normal NB patients, and did not fully reflect the biological process of MYCN amplified neuroblastoma. Weighted Gene Co-Expression Network Analysis (WGCNA) analyzes the gene expression patterns of multiple samples, clusters genes with similar expression, and then divides them into different expression modules, which can divide the gene modules Correlation analysis with clinical phenotype[12]. Therefore, this study used a combination of DEGs and WGCNA to screen the key differential genes between MYCN amplified group and MYCN unamplified group; finally, survival analysis was used to screen for prognostic-related genes, and a risk score was constructed to explore MYCN amplified NB Potential survival marker genes in patients.

Methods

Data download

The GSE53371 and GSE49710 data sets are downloaded from the GEO database (Gene Expression Omnibus). The GSE53371 data set is based on the GPL887 platform and contains the gene expression profiling by array of 10 cases of MYCN amplified patients and 10 cases of MYCN non-amplified patients. The GSE49710 data set is based on the GPL16876 platform and contains 498 neuroblastoma patients, 401 MYCN unamplified samples, 92 MYCN amplified samples, and 4 patients have unknown MYCN amplification status. The GSE53371 data set is used for the screening of key genes; the GSE49710 data set is used for survival analysis.

Difference analysis and WGCNA analysis

Use the "limma" package of R (4.0.3) to screen the DEG (differentially expressed genes) of the MYCN amplified and MYCN unamplified samples in the GSE53371 data set ($|\log FC| > 1$, $P \leq 0.05$). WGCNA (Weighted Gene Co-Expression Network Analysis) was used to construct a scale-free gene expression network to identify gene modules that are obviously related to MYCN amplification. Subsequently, the gene modules amplified by MYCN and the co-expressed genes of DEG were screened out.

GO and KEGG pathway analysis

The R package "Bioconductor" was used to perform GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis on the intersection genes, and the "clusterProfiler" package was used to perform Fisher's exact test ($P < 0.05$).

PPI network construction and hub gene selection

Upload the gene list obtained by the intersection to String (V 11.0, <https://string-db.org/>) to construct the Protein-Protein Interaction Network (PPI). Cytoscape (V.3.8.2) is used for the visualization of the results. The "CytoHubba" plug-in is used to screen the core genes, and they are arranged in descending order of "Degree" scores. The hub genes of top20 are used for the next survival analysis.

Correlation between central gene and clinical stage

The INSS staging (International Neuroblastoma Staging System) of all patients with GSE49710 and the expression of central genes form a new matrix; in addition, the 2a and 2b stages are combined into the T2 stage, and the 4 and 4s stages are combined into the T4 stage, that is, all patients Divided into T1, T2, T3 and T4 phases. The T test was used to analyze the correlation between central gene expression and INSS staging ($P < 0.05$).

Identify hub genes with independent prognostic effects

The hub genes with prognostic significance in the univariate COX regression analysis were included in the multivariate COX survival analysis, and then the hub genes with independent prognostic effects ($P < 0.05$) were used to construct the risk score formula. The risk score is equal to the expression of the hub gene multiplied by the multivariate COX regression coefficient, and then the patients are divided into high-risk groups and low-risk groups based on the median value of the risk score. Finally, use Kaplan-Meier survival curves and ROC curve to evaluate the discrimination of the formula.

RT-qPCR to verify the expression of HBA2 and NR4A2

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Children's Hospital Affiliated to Chongqing Medical University, China. Written informed consent was obtained from individual or guardian participants. In this study, 15 cases of MYCN unamplified type and 8 cases of MYCN amplified tumor tissues were collected. Trizol reagent was used to extract total RNA from tissue samples, and the total RNA (1ug) was reverse transcribed with Servicebio qPCR RT kit. Use 2×SYBR Green qPCR Master Mix (Servicebio) to quantify HBA2 and NR4A2 mRNA on a CFX fluorescent quantitative PCR instrument (Bio-rad). The primer

sequences are as follows: HBA2, Forward GTAGCCGTTCTCCTGCC, Reverse TGCTGCCCACTCAGACTTTA. NR4A2, Forward CAGTGGAGGGTAACTCATCTTTTG, Reverse CCCGTGTCTCTCTGTGACCATAG. GAPDH was used as an internal control, and $2^{-\Delta\Delta CT}$ was used to calculate the relative gene expression.

Results

Differentially expressed genes and important gene modules of WGCNA

The research process is shown in Fig. 1. We screened 216 DEGs from the GSE53371 data set, including 75 up-regulated genes and 141 down-regulated genes. In addition, two gene modules were constructed based on WGCNA analysis, denoted by MEtuquoise and MEgrey. The MEtuquoise module is closely related to the clinical phenotype of NB ($r = 0.78$, $p \text{ value} \leq 0.05$), suggesting that the genes in the MEtuquoise module are closely related to the MYCN amplification of NB tissue. There are 162 genes in the MEtuquoise module. Subsequently, 76 important genes were obtained after the intersection of DEGs and the genes in the blue-green module (Fig. 2).

Enrichment analysis of GO and KEGG pathways

GO enrichment analysis results show that in terms of biological processes (BP), these 76 differential genes are mainly enriched in biological processes such as Skeletal muscle cell differentiation, gliogenesis, and Myelination; Cell component (CP) analysis shows that they are mainly distributed in Collagen-containing extracellular matrix and Myelin sheath. The molecular function (MF) display mainly focuses on DNA-binding transcription activator activity, and RNA polymerase II-specific (Fig. 3A,B). The results of KEGG enrichment analysis showed that the intersection genes were mainly enriched in PI3K-Akt signaling pathway and ECM-receptor interaction (Fig. 3C,D).

PPI network mapping and hub gene identification

The 76 genes obtained from the above intersection were uploaded to String to draw a PPI network diagram (Fig. 4), and then the results were downloaded and imported into Cytoscape (v.3.8.0) software, and the CytoHubba plug-in in the software was used to screen out the first 20 hub genes (Table 1).

Table 1
The top 20 hub genes

Up-Regulated	Down-Regulated		
HIST1H4I	EGR1	FOS	NR4A2
HIST1H4L	NR4A1	AHR	ANXA1
HIST1H4C	EGR2	PLAT	EMP
HIST1H4B	S100B	PLP1	CYR61
HBA2	PENK	PMP2	CEBPD

Univariate COX regression analysis further screens out prognostic-related hub genes

Substituting the top 20 hub genes into GSE49710 (including survival data) for univariate survival analysis showed that 4 genes (HBA2, FOS, NR4A2 and EMP1) were closely related to the overall survival of 92 patients with MYCN amplification in GSE49710 ($P \leq 0.05$). Among them, FOS, NR4A2 and EMP1 are protective genes, and HBA2 is a dangerous gene (Fig. 5A).

The hub gene is related to clinical staging

According to the INSS staging, the GSE49710 data collection was re-combined, and finally 121 patients with T1, 78 patients with T2, 63 patients with T3, and 236 patients with T4 were obtained. The results in Fig. 5B show that the higher the clinical stage of the patient, the higher the expression of HBA2 gene, and the expression of FOS, NR4A2 and EMP1 decreased ($P < 0.05$).

The risk score is constructed by the hub gene

In the multivariate COX regression analysis of the hub gene, HBA2 and NR4A2 were closely related to patient survival (P value < 0.05 , Table 2). In the K-M survival analysis, the survival time of patients in the high-risk group was significantly shorter than that in the low-risk group (P value < 0.05). The AUC value of the ROC curve of the risk score was 0.745 ($P < 0.05$, 95%CI: 0.687–0.798), the sensitivity was 0.916, and the specificity was 0.877 (Fig. 6A,B).

Table 2
Multivariate COX regression results of key genes

Gene symbol	Coef	HR(95%CI)	p value
HBA2	0.234	1.264(95%CI:1.081–1.477)	0.003
NR4A2	-0.222	0.800(95%CI:0.693–0.924)	0.002

RT-qPCR to verify the relationship between HBA2 and NR4A2 and the amplification status of MYCN

RT-qPCR results (Fig. 7) showed that HBA2 expression was higher in MYCN amplified patients than in non-amplified patients ($p < 0.05$), but NR4A2 was lower in MYCN amplified patients than in non-amplified patients ($p < 0.05$), this result is consistent with the results of bioinformatics analysis.

Discussion

This study combined the modular genes selected by DEGs and WGCNA to explore the key difference genes between MYCN amplification and MYCN normal NB. First of all, it was confirmed that there is a big difference in gene expression between MYCN amplified and MYCN unamplified samples. There are 76 intersection genes between DEGs and WGCNA blue-green modules. Secondly, GO analysis confirmed that the intersection genes in BP and CP are mainly distributed in glial cell regeneration and myelination. Molecular function (MF) shows that the main intersection genes are concentrated in DNA binding transcription activation and RNA polymerase II specificity. The results of KEGG show intersection. Genes are mainly enriched in the interaction between PI3K-Akt signaling pathway and extracellular matrix receptors. Based on these results, we infer that MYCN amplified NB can regulate DNA binding transcription activity and RNA polymerase specificity, affect myelination and glial cell regeneration, and participate in PI3K-Akt and extracellular matrix receptor interactions, etc. Signal pathways regulate tumor cell proliferation and differentiation[13, 14], resulting in MYCN-expanded NB cells that have stronger proliferation, differentiation and metastasis capabilities than normal NB cells [10], which ultimately leads to poor prognosis for MYCN-expanded NB patients[15].

In this study, 76 differentially expressed hub genes were uploaded to String to draw a diagram of the interaction network, and the top 20 core genes in the network were screened through the CytoHubba plug-in in Cytoscape. Univariate COX regression analysis showed that 4 genes in 20 cores (HBA2, FOS, NR4A2 and EMP1) were closely related to the overall survival of patients with MYCN amplified NB (P value < 0.05). In addition, these four key genes are closely related to the clinical stage of NB patients. The higher the clinical stage of the patient, the higher the expression of HBA2 gene, while the expression of FOS, NR4A2 and EMP1 decreases. Multivariate COX regression analysis confirmed that as the expression of HBA2 increased or the expression of NR4A2 decreased, the patient's survival time was shorter, indicating that HBA2 and NR4A2 not only expressed significant differences in MYCN amplified and MYCN normal NB patients, but also compared with MYCN amplified NB patients The survival time is closely related. In other words, HBA2 and NR4A2 can be used as potential prognostic markers for MYCN-amplified NB patients.

HBA2 is located on chromosome 16 with a base length of about 30 kb and mainly encodes human hemoglobin[16]. Retrospective studies have confirmed that NB patients are easily misdiagnosed as leukemia due to abnormal blood routine results such as increased hemoglobin[17], which is mainly due to the fact that NB tumor cells invade the bone marrow, their morphology is similar to primitive and naive lymphocytes, and glycogen staining Positive[18]. The results of this study indicate that the overexpression of HBA2 gene in NB cells may lead to active proliferation of bone marrow in patients with NB, and an increase in the proportion of erythroid. The higher the expression level, the more active the

metabolism of NB tumor cells. The expression of HBA2 gene in MYCN amplified NB patients was significantly higher than in MYCN normal patients, and the higher the clinical stage, the higher the expression level. Therefore, in the process of treating patients with MYCN amplified NB, attention should be paid to changes in the patient's blood picture to prevent misdiagnosis of the disease and delay treatment.

Studies have confirmed that the protein encoded by the NR4A2 gene can act as a transcription factor to limit the function of CAR T cells in solid tumors[19–21]. In other solid tumors, the role of NR4A2 is contradictory[22]. On the one hand, NR4A2 can be used for cancer treatment drug screening[23], for example, as a drug target for glioblastoma[24]. On the other hand, the high expression of NR4A2 in gastric cancer cells confers chemoresistance and attenuates 5-fluorouracil-induced apoptosis[25]. In this study, NR4A2 decreased with the increase of INSS stage, while the expression of NR4A2 decreased, the shorter the patient's survival time, indicating that the expression of NR4A2 in NB is different from other solid tumors. However, The form or pathway of NR4A2 in MYCN expansion of NB affects cell proliferation and differentiation. These mechanisms still need to be further studied.

This study constructed a prognostic risk score for patients with MYCN amplified NB based on HBA2 and NR4A2. According to the risk score, patients can be divided into high-risk and low-risk groups. The results of survival analysis show that the survival time of patients in the high-risk group is shorter than that in the low-risk group, and the area under the ROC curve AUC is 0.745 ($P < 0.05$, 95%CI: 0.687–0.798), the sensitivity is 0.916, and the specificity is 0.877. There have been previous studies on the overall prognosis model of neuroblastoma patients[9, 26], and the use of bioinformatics to identify key MYCN-related genes[10, 27], but temporarily There is no risk score prediction for the MYCN amplified NB population. This study is aimed at MYCN amplified NB, using only two genes HBA2 and NR4A2 to construct a risk score with an AUC value greater than 0.7. In general, HBA2 and NR4A2 are potential prognostic marker genes for MYCN amplified NB patients.

The results of this study have certain limitations, that is, even if the expression profile data in the GEO database can be used to quickly mine the potential prognostic marker genes of MYCN amplified NB patients, the bioinformatics algorithm is different, so the final result will be different. The difference. This study has initially successfully verified the expression of HBA2 and NR4A2 on clinical tissue samples. Therefore, the next step will focus on the functional experiments of HBA2 and NR4A2 genes in MYCN to expand NB cells to obtain more biological processes., To provide more evidence for treating NB and predicting the prognosis of children with NB.

Conclusion

HBA2 and NR4A2 can be used as potential prognostic molecular markers of MYCN amplified neuroblastoma. The risk score constructed with HBA2 and NR4A2 must be verified by a large sample and multi-center before being applied to clinical application. These findings provide new insights for the clinical treatment and potential target mining of MYCN amplified neuroblastoma.

Abbreviations

GEO

Gene Expression Omnibus

NB

Neuroblastoma

DEGs

Differential expression genes

DAVID

The Database for Annotation, Visualization and Integrated Discovery

KEGG

Kyoto Encyclopedia of Gens and Genomes

HR

hazard ratio

Declarations

Acknowledgements

We appreciate the generosity of the researches from GEO groups for sharing the huge amount of data.

Authors' Contributions

Peng designed and performed data analysis and wrote the manuscript. Lv reviewed the manuscript and supervised the work. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request and The GSE49710 and GSE53371 datasets were downloaded from <https://www.ncbi.nlm.nih.gov/geo/>.

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Children's Hospital Affiliated to Chongqing Medical University, China. Written informed consent was obtained from individual or guardian participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Maris JM, Hogarty MD, Bagatell R, Cohn SL: **Neuroblastoma**. *Lancet* 2007, **369**(9579):2106–2120.
2. Maris JM: **Recent advances in neuroblastoma**. *N Engl J Med* 2010, **362**(23):2202–2211.
3. Park JR, Bagatell R, London WB, Maris JM, Cohn SL, Mattay KK, Hogarty M, Committee COGN: **Children's Oncology Group's 2013 blueprint for research: neuroblastoma**. *Pediatr Blood Cancer* 2013, **60**(6):985–993.
4. Irwin MS, Park JR: **Neuroblastoma: paradigm for precision medicine**. *Pediatr Clin North Am* 2015, **62**(1):225–256.
5. Irwin MS, Naranjo A, Zhang FF, Cohn SL, London WB, Gastier-Foster JM, Ramirez NC, Pfau R, Reshmi S, Wagner E *et al*: **Revised Neuroblastoma Risk Classification System: A Report From the Children's Oncology Group**. *J Clin Oncol* 2021:JCO2100278.
6. Nakagawara A, Li Y, Izumi H, Muramori K, Inada H, Nishi M: **Neuroblastoma**. *Jpn J Clin Oncol* 2018, **48**(3):214–241.
7. Guo YF, Duan JJ, Wang J, Li L, Wang D, Liu XZ, Yang J, Zhang HR, Lv J, Yang YJ *et al*: **Inhibition of the ALDH18A1-MYCN positive feedback loop attenuates MYCN-amplified neuroblastoma growth**. *Sci Transl Med* 2020, **12**(531).
8. Beltran H: **The N-myc Oncogene: Maximizing its Targets, Regulation, and Therapeutic Potential**. *Mol Cancer Res* 2014, **12**(6):815–822.
9. Shao F, Wang Z, Wang S: **Identification of MYCN-Related Gene as a Potential Biomarker for Neuroblastoma Prognostic Model by Integrated Analysis and Quantitative Real-Time PCR**. *DNA Cell Biol* 2021, **40**(2):332–347.
10. Wang H, Wang X, Xu L, Zhang J, Cao H: **Prognostic significance of MYCN related genes in pediatric neuroblastoma: a study based on TARGET and GEO datasets**. *BMC Pediatr* 2020, **20**(1):314.
11. Shao FL, Liu QQ, Wang S: **Identify potential miRNA-mRNA regulatory networks contributing to high-risk neuroblastoma**. *Invest New Drugs* 2021, **39**(4):901–913.
12. Langfelder P, Horvath S: **WGCNA: an R package for weighted correlation network analysis**. *BMC Bioinformatics* 2008, **9**:559.
13. Li Z, Gao W, Fei Y, Gao P, Xie Q, Xie J, Xu Z: **NLGN3 promotes neuroblastoma cell proliferation and growth through activating PI3K/AKT pathway**. *Eur J Pharmacol* 2019, **857**:172423.
14. Santo EE, Stroeken P, Sluis PV, Koster J, Versteeg R, Westerhout EM: **FOXO3a is a major target of inactivation by PI3K/AKT signaling in aggressive neuroblastoma**. *Cancer Res* 2013, **73**(7):2189–2198.

15. Opel D, Poremba C, Simon T, Debatin KM, Fulda S: **Activation of Akt predicts poor outcome in neuroblastoma.** *Cancer Res* 2007, **67**(2):735–745.
16. Steinberg MH, Rodgers GP: **HbA2: biology, clinical relevance and a possible target for ameliorating sickle cell disease.** *Br J Haematol* 2015, **170**(6):781–787.
17. Panovska-Stavridis I, Ivanovski M, Hadzi-Pecova L, Ljatif A, Trajkov D, Spiroski M, Cevreska L: **A case report of aggressive adult neuroblastoma mimicking acute leukemia with fulminant course and fatal outcome.** *Prilozi* 2010, **31**(1):349–359.
18. Burchill SA, Beiske K, Shimada H, Ambros PF, Seeger R, Tytgat GA, Brock PR, Haber M, Park JR, Berthold F: **Recommendations for the standardization of bone marrow disease assessment and reporting in children with neuroblastoma on behalf of the International Neuroblastoma Response Criteria Bone Marrow Working Group.** *Cancer* 2017, **123**(7):1095–1105.
19. Yin K, Chhabra Y, Tropee R, Lim YC, Fane M, Dray E, Sturm RA, Smith AG: **NR4A2 Promotes DNA Double-strand Break Repair Upon Exposure to UVR.** *Mol Cancer Res* 2017, **15**(9):1184–1196.
20. Qi L, Guo N, Wei Q, Jin P, Wang W, Mao D: **The involvement of NR4A1 and NR4A2 in the regulation of the luteal function in rats.** *Acta Histochem* 2018, **120**(8):713–719.
21. Chen J, Lopez-Moyado IF, Seo H, Lio CJ, Hempleman LJ, Sekiya T, Yoshimura A, Scott-Browne JP, Rao A: **NR4A transcription factors limit CAR T cell function in solid tumours.** *Nature* 2019, **567**(7749):530–534.
22. Safe S, Karki K: **The Paradoxical Roles of Orphan Nuclear Receptor 4A (NR4A) in Cancer.** *Mol Cancer Res* 2021, **19**(2):180–191.
23. Cobb CM, Shultz RE, Brewer JH, Dunlap CL: **Chronic pulmonary histoplasmosis with an oral lesion.** *Oral Surg Oral Med Oral Pathol* 1989, **67**(1):73–76.
24. Karki K, Li X, Jin UH, Mohankumar K, Zarei M, Michelhaugh SK, Mittal S, Tjalkens R, Safe S: **Nuclear receptor 4A2 (NR4A2) is a druggable target for glioblastomas.** *J Neurooncol* 2020, **146**(1):25–39.
25. Han Y, Cai H, Ma L, Ding Y, Tan X, Chang W, Guan W, Liu Y, Shen Q, Yu Y *et al*: **Expression of orphan nuclear receptor NR4A2 in gastric cancer cells confers chemoresistance and predicts an unfavorable postoperative survival of gastric cancer patients with chemotherapy.** *Cancer* 2013, **119**(19):3436–3445.
26. Song J, Zhao Q, Xu Y, Zhu L: **A signature of 29 immune-related genes pairs to predict prognosis in patients with neuroblastoma.** *Int Immunopharmacol* 2020, **88**:106994.
27. Wang H, Wang X, Xu L, Zhang J, Cao H: **Age related gene DST represents an independent prognostic factor for MYCN non-amplified neuroblastoma.** *BMC Pediatr* 2021, **21**(1):272.

Figures

1

Figure 1

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Figure 2

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Figure 4

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Figure 6

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