


Exportin-T: A Novel Prognostic Predictor and Potential Therapeutic Target for Neuroblastoma

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Abstract

Exportins as the key mediators of nucleocytoplasmic transport have been identified as the controllers of the passage of numerous types of crucial cancer-related proteins. Targeting exportins in cancer cells might represent an emerging strategy in cancer intervention with the potential to affect clinical outcomes. Here, we focused on the prognostic and therapeutic values of Exportin-T (XPOT) in neuroblastoma. The correlation between the expression and prognostic values of XPOT in patients with neuroblastoma was investigated based on both published transcriptome data and our clinical data. Then, decision curve analysis (DCA) was implemented to identify a XPOT risk prediction model. In addition, RNA interference was performed to silence the expression of XPOT to further investigate the specific roles of XPOT in the progression of neuroblastoma in vitro. Overexpression of XPOT mRNA was associated with poor clinical characteristics, such as age at diagnosis more than 18 months, amplification of MYCN, and advanced International Neuroblastoma Staging System (INSS) stage, and XPOT expression was identified as an independent poor prognosis factor for neuroblastoma using Cox proportional hazards model ($P < .001$). DCA suggested that neuroblastoma patients could benefit from XPOT risk prediction model-guided interventions (status of MYCN + INSS stage + XPOT). Experimentally, knockdown of XPOT by small interfering RNA inhibited the proliferation and migration in neuroblastoma cells. XPOT is identified as a novel prognostic predictor and potential therapeutic target for neuroblastoma patients. Further investigation should focus on the profound molecular mechanism underlying the tumor inhibition activity of XPOT inhibitors.

Keywords

Exportin-T, neuroblastoma, prognostic marker, therapeutic target, prognosis

Abbreviations

DCA, Decision curve analysis; DMEM, Dulbecco's modified eagle medium; FBS, Fetal bovine serum; INRG, International Neuroblastoma Risk Group; INSS, International Neuroblastoma Staging System; PMSF, Phenylmethanesulfonyl fluoride; RT-qPCR, Quantitative real-time PCR; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SINE, Inhibitors of nuclear export; XPOT, Exportin-T; $1\alpha,25[\text{OH}]_2\text{D}_3$, $1\alpha,25$ -dihydroxyvitamin D₃

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Introduction

Neuroblastomas are the most common extracranial solid malignancy in children originating from the adrenal glands or sympathetic ganglia.^{1,2} About 1.1 per 100 000 children are diagnosed with neuroblastoma annually, accounting for ~6% of all childhood cancers.³ Neuroblastoma is a highly heterogeneous disease with a broad spectrum of clinical behavior.⁴ Tumors may spontaneously regress or mature, or they can progress and metastasize despite multimodality treatment.⁴ Therefore, pretreatment risk stratification is essential for determining the risk group and therapeutic strategy of patients.

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Over the past decade, International Neuroblastoma Risk Group (INRG) classification system has provided invaluable information for determining the prognosis and therapy stratification for patients diagnosed with neuroblastoma.⁵ The INRG classification system is a strict schema, considering the criteria International Neuroblastoma Staging System (INSS) stage, age at diagnosis, histologic category, tumor differentiation, MYCN status, chromosomal aberrations, and tumor cell ploidy.⁵ However, this system has not yet been adopted by all regions,⁶ and its clinical application is limited internationally because of the difficulty of acquisition of some factors, such as tumor cell ploidy and chromosomal aberrations. Meanwhile, patients with low- and intermediate-risk neuroblastoma have achieved favorable prognosis, while the prognosis of treatment remains unfavorable for the high-risk ones, with the 5-year survival rate under 50%.⁷ This stagnation in therapeutic advances for high-risk neuroblastoma motivates efforts in excavating the underlying molecular biology of neuroblastoma and exploring novel therapeutic targets for further survival improvement.

Exportins are a group of karyopherins that regulate the transferring of proteins from nucleus to cytoplasm, which have been identified as the controllers of the passage of numerous types of crucial cancer-related proteins.⁸ Emerging evidence has considered exportin inhibitors as therapeutic targets against cancer and they have shown preclinical anticancer activity.⁹ Exportin-T (XPOT) is a member of the karyopherin- β family and acts as a specific mediator of the export of transfer RNA (tRNA).^{10,11} It shuttles from the nucleus to the cytoplasm bidirectionally through nuclear pore complexes by interacting with GTP-bound Ran (Ran-GTP).¹² Previous studies have reported the critical roles of XPOT in the development and progression of various tumors, such as breast cancer,¹³ hepatocellular carcinoma,¹⁴ and promyelocytic leukemia.¹⁵ In addition, Exportin 1 (XPO1, another exportin family member, has been reported to play important roles in neuroblastoma. Inhibition of XPO1 suppresses neuroblastoma cell proliferation via reduction of the phosphorylation level of Forkhead box O3 (FOXO3a).¹⁶ The neuroblastoma animal models proved that the second-generation XPO1 inhibitor selinexor (KPT-330) showed anti-tumor effect in vivo.¹⁷ Despite these advances, XPOT remains 1 mysterious player in tumorigenesis, and only a few studies investigated the biological function and prognosis value of XPOT in the pathogenesis of neuroblastoma.

Here, we investigated the expression and prognostic value of XPOT in patients with neuroblastoma using 2 published datasets, and presented an XPOT risk prediction model by decision curve analysis (DCA). Then, its expression and the prognostic value of XPOT were verified clinically based on 64 neuroblastoma patients. In addition, the specific role of XPOT in the progression of neuroblastoma was analyzed using RNA inference by *in vitro*. Together, this study attempted to explore a promising predictor for predicting prognosis of neuroblastoma and reveal the significance of XPOT in

neuroblastoma oncogenesis. Targeting XPOT in neuroblastoma cells might represent a novel strategy in treatment intervention of neuroblastoma.

Materials and Methods

Transcriptome Data

The mRNA expression of XPOT and clinical annotation were evaluated using R2: Genomics Analysis and Visualization Platform (<http://r2.amc.nl>) on the datasets from the Sequencing Quality Control Consortium (SEQC, GEO accessing number GSE49710¹⁸) and the European Neuroblastoma Research Consortium (NRC, GEO accessing number GSE85047¹⁹)

Cell Lines and Cell Culture

The human neuroblastoma cell lines SK-N-BE(2), SH-SY5Y, and SK-N-SH were obtained from Guangzhou Jennio Biotech Co., Ltd and were cultured in Dulbecco's modified Eagle medium (DMEM; HyClone) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37 °C in a humidified incubator with 5% CO₂.

Specimens and Tissue Microarray

Tissue samples were obtained from 64 neuroblastoma patients, who received radical resection at the Department of Pediatric Surgery, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine from 2012 to 2015. The diagnosis was confirmed by pathological biopsy. The study protocol was approved by the Ethics Committee of Xinhua Hospital (Approval number: XHEC-D-2018-034). Written informed consent was obtained from all donors and their relatives. The age at diagnosis ranged from 2 to 156 m, and the male:female ratio was 9:7. The 5-year follow-up overall survival was 26.7 \pm 16.1 m. Tissue microarrays were constructed by Servicebio Technology Co., Ltd and scanned by Panoramic MIDI²⁰ (3D HISTECH).

Immunohistochemistry Staining

Immunohistochemistry staining was performed according to the procedure described by Meseure et al.²¹ XPOT antibodies for immunohistochemistry staining was obtained from Lifespan Biosciences (LS-C160669, rabbit, 1:1000). A semi-quantitative histologic score (H-score) was applied for the evaluation of the immunohistochemical staining results. The H-score considers both the degrees of staining intensity and the %age of positive neoplastic cells. The intensity was scored on a scale of 0 to 3 (0, negative; 1, weak; 2, medium; 3, strong). The formula for the H-score is as follows: $H\text{-score} = \sum(P_i \times I) = (\% \text{age of cells with weak intensity} \times 1) + (\% \text{age of cells with moderate intensity} \times 2) + (\% \text{age of cells with strong intensity} \times 3)$,²² where

P_i = %age of positive tumor cells and I = staining intensity. A total of 2 pathologists assessed the scoring independently without prior knowledge of the clinical outcomes.

Small Interfering RNA Transfection

Small interfering RNAs (siRNAs) targeting XPOT were designed and synthesized by Huagene Biotech Co. Ltd. The sequences of XPOT-siRNAs were as follows: XPOT-siRNA1, 5'- GCUAGUGCUUUGCAGGAUATT-3'; XPOT-siRNA2, 5'- GCACAUCCAUGUGUACUATT-3'; and XPOT-siRNA3, 5'- GCUAGUGUGCUAUUGUUATT-3'. Negative control-siRNA (NC-siRNA) or XPOT-siRNA were transfected to SK-N-BE(2), SH-SY5Y, and SK-N-SH cells using the Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen).

Real-Time-Quantitative Polymerase Chain Reaction

Total RNA of SK-N-BE(2), SH-SY5Y, and SK-N-SH cells was extracted using Trizol (Takara) following the manufacturer's instructions. Then, 1 µg of total RNA was extracted to synthesize cDNA as the polymerase chain reaction (PCR) template using PrimeScript Reverse Transcriptase (Takara). The primers used for amplification were as follows: XPOT 5'-AGGGAGACGCTCATATCATGG-3' (forward), 5'-TTGGGCGGCTTTATTTTCGTAT-3' (reverse); glyceraldehyde 3-phosphate dehydrogenase 5'-CAACAGCCTCAAGATCATCAGC-3' (forward), 5'-TTCTAGACGGCAGGTCAGGTC-3' (reverse). Real-time (RT)-qPCR was conducted using the StepOnePlus™ RT-PCR system (Applied Biosystems) and XPOT expression levels were detected using the SYBR-Green kit (Takara). XPOT expression levels were calculated using the $2^{-\Delta\Delta C_q}$ methods.²³

Western Blot

Total protein was extracted from SK-N-BE(2), SH-SY5Y and SK-N-SH cell lines using radio immunoprecipitation assay (RIPA) buffer (Cell Signaling Technology) with 1 × phenylmethanesulfonyl fluoride (PMSF; Beyotime). Proteins (15 µg/lane) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under constant voltage and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) under constant current for 90 min. Then, all blots were blocked with fresh 5% skimmed milk for 2 h at room temperature. Next, PVDF membranes were incubated with polyclonal antibody against XPOT (LS-C160669, rabbit, 1:1000) purchased from Lifespan Biosciences and polyclonal antibody against β-actin (#4970, rabbit, 1:1000) acquired from Cell Signaling Technology overnight at 4 °C. Finally, all blots were incubated with a horseradish peroxidase-conjugated secondary antibody (A0208, rabbit, 1:1000) for 2 h at room temperature, and analyzed using BIO-RAD ChemiDoc XRS+(Bio-Rad).

Cell Proliferation Assays

Cell proliferation ability was tested by a Cell Counting Kit-8 reagent (Dojindo). In brief, cells were seeded in 96-well plates at a density of 1×10^3 cells per well and cultured with DMEM containing 10% FBS at day 0. Different rows are marked as day1 to day5 to be measured at different time points. For next every 24 h (total 5 days), after ensuring the cells were not contaminated by observation via a microscope, cells were cultured in DMEM with 10% Cell Counting Kit-8 (CCK-8) reagent for 2 h at 37 °C. Then, cell proliferation ability was assessed by the absorbance value at 450 nm using an Automated Microplate Reader (Bio-Rad).

Colony Formation Assay

Colony formation assay was used to assess single cell proliferation ability. Firstly, cells were cultured at a density of 1×10^4 cells per well in 6-well plates for 1 week. Next, cells were fixed by 4% paraformaldehyde (Sigma) for 15 min at room temperature. Then, cells were stained by 0.5% crystal violet (Beyotime) for 15 min at room temperature. Finally, after washing with water, colonies were observed under the optical microscope (Leica), and the number of colonies was counted.

Cell Migration Assays

Cell migration ability was measured in 24-well Transwell chambers (Corning). Cells were plated at a density of 2×10^4 per well onto the upper chamber with 200 µL serum-free DMEM. The lower chamber was filled with 500 µL DMEM supplemented with 10% FBS. After incubation for 48 h at 37 °C in a humidified incubator with 5% CO₂, cells attached to the low surface of the membrane were fixed by 4% paraformaldehyde (Sigma) for 15 min at room temperature and stained with 0.5% crystal violet (Beyotime) for another 15 min. Finally, the migrated cells were visualized under an optical microscope (Leica), and counted from 5 randomly selected fields.

Statistical Analysis

Statistical analysis was conducted by SAS v8.0 (SAS Institute Inc.), Prism 6 software (GraphPad Software, Inc.) and R v3.5.0 (R Core Team, 2018). Pearson's χ test or Cochran-Mantel-Haenszel (CMH) test was used to analyze the association between XPOT expression and clinical characteristics in neuroblastoma. Univariate and multivariate Cox regression models were performed for prognostic analysis. Log-rank (Mantel–Cox) test was used to determine the significant difference. DCA was performed by R package.²⁴ X-tile software was used to determine the best cut-off value of XPOT expression in stratification analysis.²⁵ All experiments were conducted independently 3 times. The results were considered statistically significant when $P < .05$.

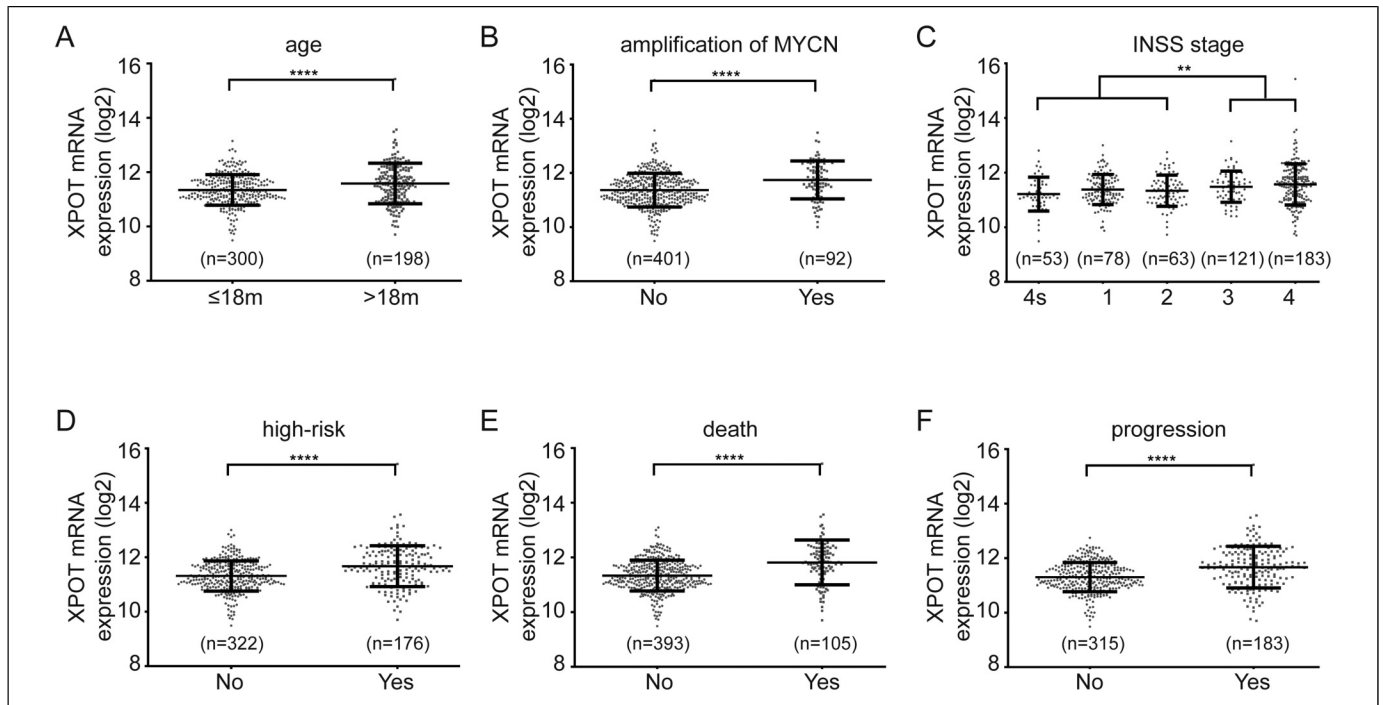


Figure 1. Patients with high-risk clinical characteristic have higher XPOT expression in neuroblastoma tissues than patients with low-risk clinical characteristics. Higher XPOT mRNA is expressed in patients with age at diagnosis > 18 months (A), amplification of MYCN (B), advanced INSS stage (C), high-risk group (D), death from disease (E), and tumor progression (F).

* $P < .05$.

Abbreviations: INSS, International Neuroblastoma Staging System; XPOT, Exportin-T.

Results

Patients with high-risk clinical characteristic have higher XPOT expression in neuroblastoma tissues than patients with low-risk clinical characteristics. To investigate the correlation between mRNA expression level of XPOT and clinical characteristics, 2 independent datasets, SEQC and NRC, were analyzed, respectively. According to the SEQC dataset, higher XPOT mRNA expression level was associated with several high-risk parameters, such as age at diagnosis >18 months, amplification of MYCN, high-risk group, and advanced INSS stage (Figure 1A to D, $P < .05$). Moreover, patients with death or tumor progression showed higher XPOT mRNA expression level (Figure 1E and F, $P < .05$). Further analysis of the NRC dataset identified consistent results (Figure S1).

To further clarify the relevance between XPOT mRNA expression and these clinical characteristics, a stratification analysis was performed. The best cut-off value of XPOT mRNA expression level was determined by X-tile software (best P value). According to the SEQC dataset, higher XPOT mRNA expression was associated with age diagnosed >18 months, amplification of MYCN, advanced INSS stage, high-risk group, tumor progression, and death from disease (Table 1, $P < .05$). NRC analysis results also supported the above results (Table S1).

Higher XPOT Level is Correlated to Poor Prognosis

Univariate and multivariate analysis suggested XPOT expression as an independent poor prognosis factor for neuroblastoma according to the SEQC dataset (Table 2, $P < .001$). NRC analysis results also supported the above results (Table S2). These results indicated that high XPOT expression level might be associated with poor clinical outcomes, and their correlation should be confirmed by further analysis.

Overall survival analysis was performed to evaluate the prognostic impact of XPOT mRNA expression in neuroblastoma patients. According to the SEQC dataset, patients with high XPOT mRNA expression presented shorter overall survival time relative to those with low XPOT expression (Figure 2A, $P < .05$). For the subgroup with high XPOT expression, patients with high risk presented shorter overall survival time (Figure 2B, $P < .05$). In the stratification analysis for clinical parameters, higher XPOT level was related to poor prognosis in patients with age diagnosed <18 months, with age diagnosed >18 months, without amplification of MYCN, and with advanced INSS stage (Figure 2C to F, $P < .05$). NRC analysis showed consistent results (Figure S2). These results demonstrated that neuroblastoma patients with higher XPOT levels presented poor prognosis.

Neuroblastoma Patients Could Benefit From XPOT Risk Prediction Model-Guided Interventions

DCA analysis was used to detect whether patients could benefit from a risk prediction model that included XPOT. According to the SEQC dataset, DCA analysis identified that model 5 (status of MYCN + stage + XPOT) had a higher net benefit compared with other models in the threshold probability of 0–0.5 (Figure 3A). Given that age at diagnosis <18 months is a favorable prognostic factor, we performed a stratification analysis. For patients with diagnosed age <18 months, model 5 also had a higher net benefit (Figure 3B). Besides, net reduction

curves, which show the potential to avoid unnecessary intervention, implied that model 5 had the best reduction rates compared with other models (Figure 3C). For patients with diagnosed age <18 months, model 5 also had excellent reduction rates (Figure 3D). In the NRC dataset, models 2, 4, and 5 showed good net benefit and reduction rates in the threshold probability of 0–0.5, with similar net benefit among 3 models (Figure S3A and S3B) for all patients. Although, for patients with diagnosed age <18 months, model 5 (threshold probability 0–0.25) had a higher net benefit and good reduction rates (Figure S3C and S3D). These results suggested that neuroblastoma patients, especially those with diagnosed age <18 months, could benefit from XPOT risk prediction model-guided interventions.

Table 1. Association Between XPOT mRNA Expression and Clinical Characteristics in Neuroblastoma Patients (SEQC).

Factors	Cases No.	XPOT expression		P value
		low, No. (%)	high, No. (%)	
Gender				.544
female	211	188 (89.1)	23 (10.9)	
male	287	261 (90.9)	26 (9.1)	
Age (month)				<.001
≤18	300	287 (95.7)	13 (4.3)	
>18	198	162 (81.8)	36 (18.2)	
MYCN amplification				<.001
No	401	388 (96.8)	13 (3.2)	
Yes	92	57 (62.0)	35 (38.0)	
Tumor stage (INSS)				<.001
4s + 1 + 2	252	247 (98.0)	5 (2.0)	
3 + 4	246	202 (82.1)	44 (17.9)	
High risk				<.001
No	322	317 (98.5)	5 (1.5)	
Yes	176	132 (75.0)	44 (25.0)	
Progression				<.001
No	315	307 (97.5)	8 (2.5)	
Yes	183	142 (77.6)	41 (22.4)	
Death				<.001
No	393	377 (95.9)	16 (4.1)	
Yes	105	72 (68.6)	33 (31.4)	

Pearson's χ^2 test was used for statistical analysis.

Abbreviations: INSS, International Neuroblastoma Staging System; XPOT, Exportin-T; SEQC, Sequencing Quality Control Consortium.

Higher XPOT Protein Expression is Associated with Poor Prognosis

Staining intensity was divided into weak, medium, and strong under an optical microscope (Figure 4A). XPOT protein was significantly overexpressed in patients with diagnosed age >18 months, advanced INSS stage, advanced risk group, preoperative chemotherapy, death due to disease (Figure 4B to F, $P < .05$), suggesting that higher XPOT protein expression was correlated with poor clinical characteristics. To further investigate association between XPOT protein expression and clinical characteristics, the best cut-off value of XPOT protein expression level was determined by X-tile software (best P value), and 64 neuroblastoma patients were divided into high XPOT protein expression group and low XPOT protein expression group. Overall survival analysis showed that patients with high XPOT protein expression presented lower overall survival (Figure 4G, $P < .05$). In the stratification analysis for those with age diagnosed >18 months, without amplification of MYCN and without preoperative chemotherapy, high XPOT expression presented shorter survival time (Figure 4H to J, $P < .05$).

Tissue microarray showed that higher XPOT protein expression was associated with diagnosed age >18 months ($P = .013$), advanced INSS stage ($P = .008$), preoperative chemotherapy ($P = .006$), and death from disease ($P < .001$) (Table S3). As showed in Table S4, univariate analysis suggested XPOT as a risk factor with a hazard ratio (HR) (95% CI) of 4.008 (1.892–8.491). However, multivariate analysis did not support

Table 2. Cox Proportional Hazards Model for Prognostic Factor Analysis in Neuroblastoma Patients (SEQC).

Variables	Favorable/Unfavorable	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value
Gender	female/male	0.799 (0.545–1.173)	.253		
Age (month)	≤18/>18	8.111 (4.978–13.214)	<.001	1.895(1.542–0.832)	.169
MYCN amplification	No/Yes	7.793 (5.262–11.541)	<.001	1.502(0.934–2.415)	.093
Tumor stage (INSS)	4s + 1 + 2/3 + 4	14.515 (7.323–28.768)	<.001	2.693(1.124–6.455)	.026
High risk	No/Yes	21.422 (11.931–38.462)	<.001	5.744(2.371–13.915)	<.001
XPOT expression	low/high	8.613 (5.644–13.142)	<.001	2.645(1.637–4.274)	<.001

Cox regression was used for statistical analysis.

Abbreviations: 95%CI, 95% confidence interval; HR, hazard ratio; INSS, International Neuroblastoma Staging System; XPOT, Exportin-T; SEQC, Sequencing Quality Control Consortium.

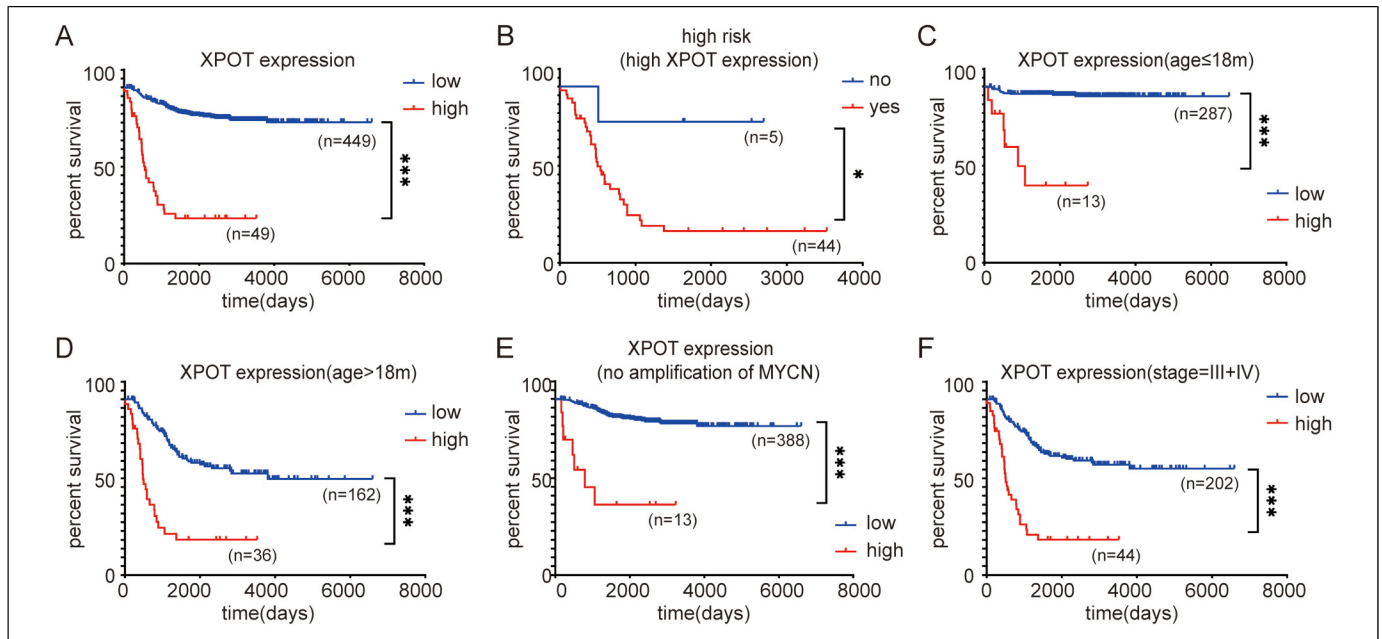


Figure 2. Higher XPOT level is correlated to poor prognosis. (A) Patients with high XPOT mRNA expression present shorter overall survival time relative to those with low XPOT expression. (B) For the subgroup with high XPOT expression, patients with high risk have a poor prognosis. (C) For patients with age diagnosed <18 months, higher XPOT expression shows lower overall survival. (D) For patients with age diagnosed >18 months, higher XPOT expression shows lower overall survival. (E) For patients without amplification of MYCN, higher XPOT expression achieves lower overall survival. (F) For patients with advanced INSS stage, higher XPOT expression achieves lower overall survival. INSS, International Neuroblastoma Staging System.

* $P < .05$.

Abbreviations: INSS, International Neuroblastoma Staging System; XPOT, Exportin-T.

XPOT as an independent prognostic factor. This might be due to too few samples.

knockdown of XPOT inhibited neuroblastoma cell proliferation and migration.

Knockdown of XPOT Inhibits Neuroblastoma Cell Proliferation and Migration

To evaluate the biological function of XPOT in the development of neuroblastomas, SK-N-BE(2), SH-SY5Y, and SK-N-SH were transfected with 3 siRNAs targeting XPOT. XPOT mRNA and protein expression were significantly down-regulated after transfected with siRNAs in SK-N-BE(2), SH-SY5Y, and SK-N-SH cells (Figure 5A to B, $P < .05$). The CCK-8 assay indicated that knockdown of XPOT significantly decreased cell proliferation ability in SK-N-BE(2), SH-SY5Y, and SK-N-SH cells after transfection (Figure 5C, $P < .05$). The colony formation assay identified that the number and the size of colonies formed by neuroblastoma cells transfected with XPOT-siRNA were significantly reduced compared to those transfected with control siRNA (Figure 5D, $P < .05$). A Transwell assay was conducted to investigate the effect of XPOT knockdown on cell migration ability. As shown in Figure 5E, the migratory ability from the upper chamber to the lower chamber was significantly suppressed in SK-N-BE(2), SH-SY5Y, and SK-N-SH cells transfected with XPOT-siRNA ($P < .05$). These results suggested that

Discussion

In eukaryotic cells, RNAs needed to be transported to the cytoplasm to function.²⁶ The regulation of genes in biological processes depends in part on the controlled exchange of those RNAs between the nucleus and the cytoplasm.²⁷ Therefore, understanding the molecular pathways of nuclear export receptor in tumor development could contribute to the development of novel clinical intervention.²⁸ In the present study, XPOT, as one of the key nuclear export receptors, was investigated to explore its biological functions and prognostic value in neuroblastomas. Our study indicated that XPOT overexpression was associated with poor clinical characteristics, such as age at diagnosis >18 months, amplification of MYCN, and advanced INSS stage, and poor prognosis in both bioinformatics and clinical experiment analyses. Moreover, we built a risk prediction model based on the status of MYCN, clinical stage (INSS), and XPOT mRNA expression using DCA analysis. In addition, RNA interference studied *in vitro* indicated that knockdown of XPOT inhibited the proliferation and migration of neuroblastoma cells.

Inhibition of nuclear export results in subsequent change of gene expression by disordering RNAs bidirectionally

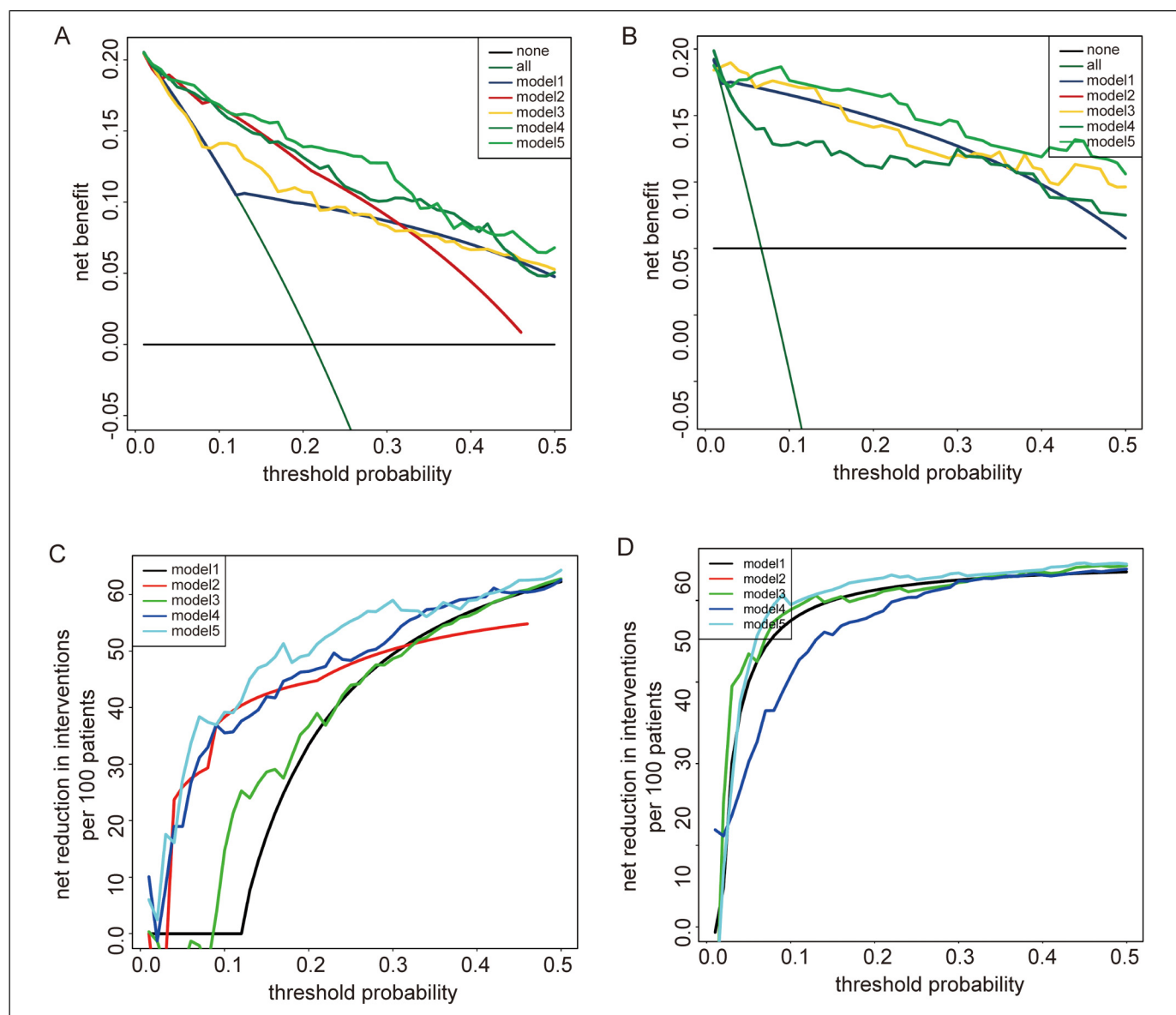


Figure 3. Decision curve analysis and net reduction curves show that neuroblastoma patients can benefit from XPOT risk prediction model-guided interventions. For all patients, model 5 achieves the highest net benefit (A) and the greatest ability to avoid unnecessary intervention (C) in the threshold probability of 0 to 0.5. For patients with diagnosed age <18 months, model 5 has a higher net benefit (B) and greater ability to avoid unnecessary intervention (D) than the other models in the threshold probability of 0 to 0.5. Abbreviations: INSS, International Neuroblastoma Staging System; none = no patients received interventions; all = all patients received interventions, model 1 = status of MYCN, model 2 = INSS stage, model 3 = status of MYCN + XPOT, model 4 = INSS stage + XPOT, model 5 = status of MYCN + INSS stage + XPOT; XPOT, Exportin-T.

transportation. It has become a promising therapeutic strategy for several tumors.^{29,30} Selinexor, also called KPT-330, is a selective inhibitor of nuclear export (SINE) against nuclear export protein exportin 1, and has been proven to exhibit broad antitumor activity.³¹ In myelofibrosis CD34+ cells, inhibition of nuclear export causes nuclear accumulation of p53 and enhanced ruxolitinib-mediated proliferation suppression and apoptosis.³² Inhibition of XPO1 has also been indicated to decrease myeloid cell leukemia sequence 1 (Mcl-1) levels and enhances cell death induced by Venetoclax (ABT-199), a B-

cell lymphoma 2 (Bcl-2)-selective inhibitor, in acute myeloid leukemia.³³ XPO1 inhibitor impedes Mcl-1 and the B-cell lymphoma-extra large (Bcl-xL) complex, causes increased mitochondrial membrane permeability, and therefore triggers cell apoptosis.³⁴ The combination of XPO1 and fms like tyrosine kinase 3 (FLT3) exerts synergistic pro-apoptotic effects through elevated nuclear levels of extracellular regulated protein kinases (ERK), AKT, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and FOXO3a in FLT3-mutated acute myeloid leukemias.³⁵ These studies

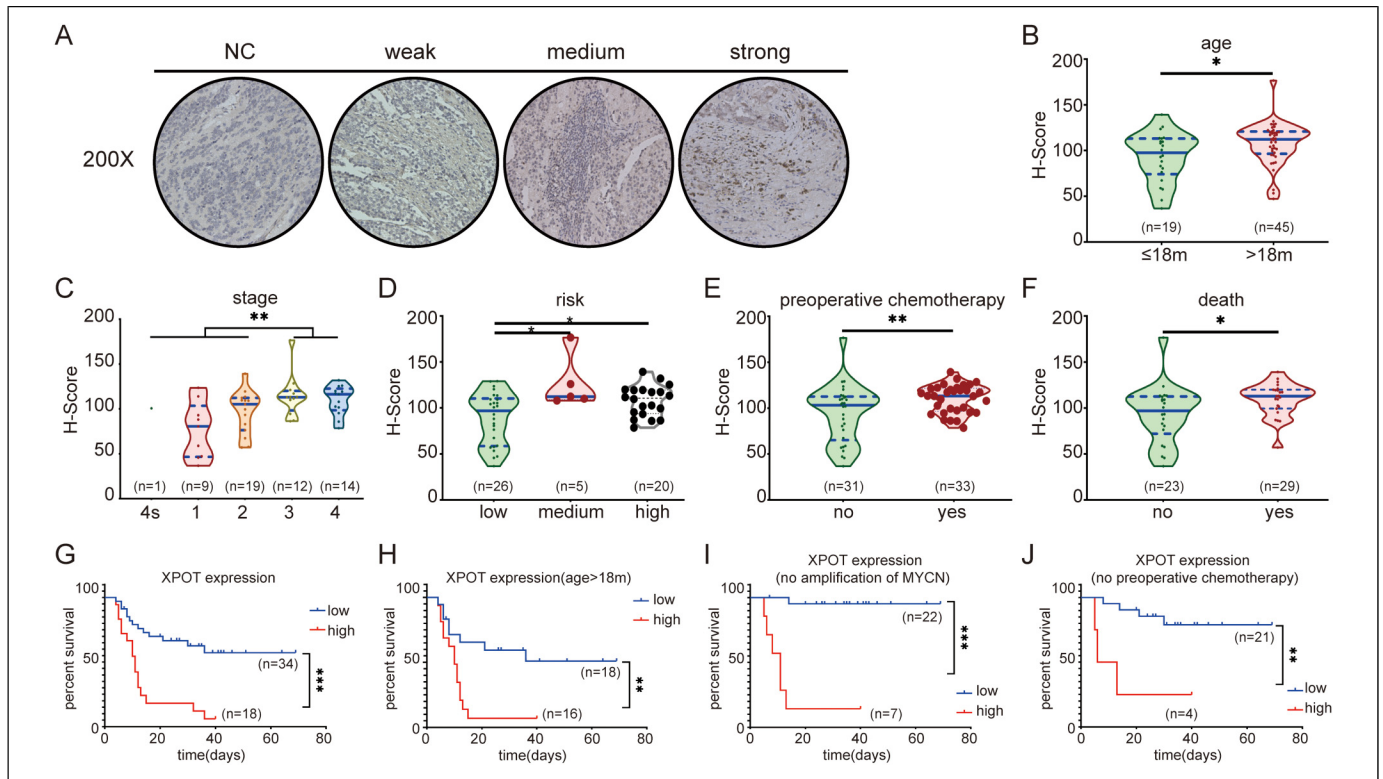


Figure 4. Higher XPOT protein expression is associated with poor prognosis. (A) Representative photographs of different XPOT staining intensity of neuroblastoma tissue immunohistochemistry. Higher XPOT protein is expressed in patients with age at diagnosis >18 months (B), advanced INSS stage (C), advanced risk group (D), preoperative chemotherapy (E), and death from disease (F). Overall survival analysis shows that patients with high XPOT protein expression present lower overall survival (G). For patients with age diagnosed >18 months (H), without amplification of MYCN (I), and without preoperative chemotherapy (J), high XPOT expression present shorter survival time. Abbreviation: INSS, International Neuroblastoma Staging System; XPOT, Exportin-T. * $P < .05$.

demonstrate the enormous clinical potential of SINE combination therapy. However, few studies focus on the molecular action of XPOT in tumorigenesis currently, and the underlying mechanism of XPOT in the progression of tumor remains unknown. Given that XPOT is also a key member of nuclear export receptors, it might play an important role in cancer by transporting key mediators of oncogenesis across the nuclear membrane in cancer cells.

In our study, we built a risk prediction model based on status of MYCN, clinical stage (INSS), and XPOT mRNA expression using DCA analysis. Compared with receiver operating characteristic curve analysis, DCA analysis focuses on clinical net benefit. According to DCA analysis, neuroblastoma patients could benefit from the XPOT risk prediction model-guided interventions. Compared with other models, the accession of XPOT significantly increased the clinical net benefit of neuroblastoma patients. At the translation level, XPOT protein overexpression was also significantly associated with poor prognosis through tissue microarray analysis. These results implied that XPOT might be a novel predictor of neuroblastoma prognosis. Considering the convenience and feasibility of XPOT testing, this could be more practical in clinic.

XPOT depletion by siRNA could significantly suppressed neuroblastoma cell proliferation and migration, highlighting XPOT as a therapeutic target in neuroblastoma. However, siRNA as a drug remains far to clinical application due to the off-target effects as well as the difficulties of siRNA delivery.³⁶ Unfortunately, specific inhibitors directly against XPOT are not available yet. Recently, $1\alpha,25\text{-dihydroxyvitamin D}_3$ ($1\alpha,25[\text{OH}]_2\text{D}_3$) has been reported to suppress the expression of XPOT in human promyelocytic leukemia HL-60 cells and consequently inhibited the proliferation of HL-60 cells.¹⁵ This implied that $1\alpha,25(\text{OH})_2\text{D}_3$ can be administrated as an XPOT inhibitor. However, the therapeutic window of $1\alpha,25(\text{OH})_2\text{D}_3$ is very narrow in clinical practice because of dose-limiting hypercalcemia. Fortunately, several low-calcemic analogs with vitamin D3-mediated anticancer have been reported.^{15,37} $24\text{R},25(\text{OH})_2\text{D}_3$, another metabolite of $25(\text{OH})\text{D}_3$, potentiates a great ability of inducing cell apoptosis and suppressing metastasis in breast cancer,³⁷ although it is still unclear if the anti-tumor activity of $24\text{R},25(\text{OH})_2\text{D}_3$ is achieved by suppressing XPOT. For neuroblastoma, a novel $1\alpha,25(\text{OH})_2\text{D}_3$ analog, QW1624F2 to 2 induced cell-cycle arrest in the G1 phase and induced cell differentiation by increasing neurite length.³⁸

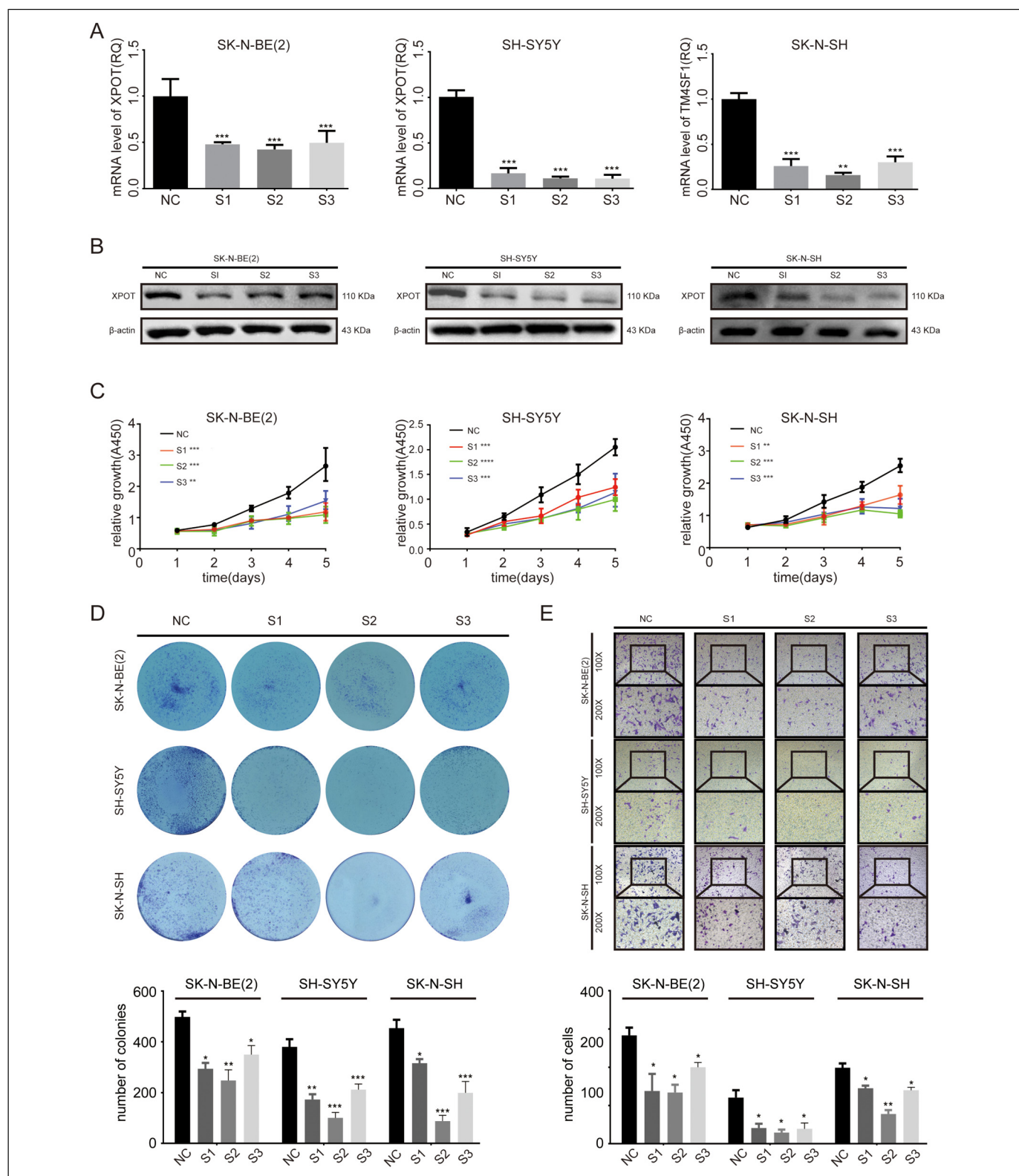


Figure 5. Knockdown of XPOT inhibits neuroblastoma cell proliferation and migration. (A) RT-qPCR identifies the efficiency of siRNA against XPOT in SK-N-BE(2), SH-SY5Y, and SK-N-SH cells. (B) Western blot identifies the knockdown efficiency of siRNA against XPOT. (C) A CCK-8 assay indicates the relative growth of SK-N-BE(2), SH-SY5Y, and SK-N-SH cells after knockdown by siRNA against XPOT. (D) Colony images and histogram for SK-N-BE(2), SH-SY5Y, and SK-N-SH cell lines after knockdown of XPOT. (E) Cell migration images and histogram for SK-N-BE(2), SH-SY5Y, and SK-N-SH cell lines after knockdown of XPOT. All experiments were performed 3 times independently. Error bars represent the mean \pm SD. * $P < .05$.

Abbreviations: XPOT, Exportin-T; RT-qPCR, quantitative real-time- polymerase chain reaction.

Considering its role in human promyelocytic leukemia, we highly believe that these analogs may provide an alternative approach to inhibit neuroblastoma by downregulating XPOT. Further research can focus on the interaction between these analogs and XPOT.

Conclusions

In this study, we systematically mined 2 transcriptome datasets of neuroblastoma and identified XPOT as a novel prognostic predictor of neuroblastoma. Tissue microarray analysis including 64 neuroblastoma patients confirmed the results. Moreover, DCA analysis suggested neuroblastoma patients benefited from XPOT risk prediction model-guided interventions. Knockdown of XPOT presented anti-tumor effect in neuroblastoma in vitro, highlighting the potential therapeutic target of XPOT in neuroblastoma. However, profound molecular mechanism underlying the tumor inhibition activity of XPOT needs more work for further investigation. Another logical extension of this work would be to investigate the molecular roles of XPOT in the anti-tumor activity of the $1\alpha,25(\text{OH})_2\text{D}_3$ analogs.

Ethics Approval

The study protocol was approved by the Ethics Committee of Xinhua Hospital (XHEC-D-2018-034). Written informed consent was obtained from all donors and their relatives.

Availability of Data and Materials

The datasets analyzed during the current study are available in the Gene Expression Omnibus repository, (GSE85047, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85047>; GSE49710, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49710>).

All data generated and analyzed during this study are included in this published article and its supplementary information files.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

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