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Chronic diarrhea related to neuroblastoma: the important role of vasoactive intestinal peptide in tumor pathology and survival

Meng-ying Cao[1,2†], Kun Zhang[1†], Jing Guo[1], Fang Dong[3] and Ling-fen Xu[1*]

Abstract

Background To analyze the correlation between vasoactive intestinal peptide (VIP) protein expression in neuroblastoma (NB) tumors and NB clinical features and prognosis.

Methods Clinical data were collected from 91 patients with NB aged < 18 years who underwent tumor resection at the Shengjing Hospital of China Medical University between January 2015 and December 2021. VIP expression levels in tumor tissues were evaluated by immunohistochemistry, and the correlation between VIP expression intensity and NB clinical characteristics and prognosis was analyzed.

Results VIP expression was detected in 25/91 patients with NB (27.5%). VIP expression intensity was significantly increased in children with diarrhea and hypokalemia ($P < 0.001$, and $P < 0.001$, respectively), and was significantly associated with histopathological classification, prognosis, bone marrow metastasis, and tumor stage ($P = 0.003$, $P = 0.036$, $P = 0.018$, and $P = 0.027$, respectively). VIP expression intensity was positively correlated with synaptophysin expression ($r_s = 0.342$, $P = 0.001$), and negatively correlated with expression of chromogranin A and proliferating cell nuclear antigen (Ki67) ($r_s = -0.265$, $P = 0.011$; $r_s = -0.317$, $P = 0.002$, respectively). There were no significant differences in VIP expression levels according to sex, age, tumor site, or levels of neuron specific enolase, 24-h urine vanillylmandelic acid, and lactate dehydrogenase.

Conclusions VIP is one of the main causes of refractory diarrhea in patients with NB, and may be a potential biomarker of good prognosis.

Trial registration Retrospectively registered.

Keywords Chronic diarrhea, Neuroblastoma, Prognosis, Vasoactive intestinal peptide

Introduction

Neuroblastoma (NB) is an embryonal tumor arising from primitive neural crest cells that accounts for 7–10% of all pediatric malignancies, and for up to 15% of all childhood tumor-related mortalities [1, 2]. The clinical manifestations of NB are highly variable depending on whether the tumor is localized or metastatic, and may include fever, anemia, abdominal mass or pain, exophthalmos, and ataxia. Moreover, a minority of patients with NB present with refractory watery diarrhea, which makes it difficult to diagnose NB in a timely manner.

†Meng-ying Cao and Kun Zhang have contributed equally as first co-authors.

*Correspondence:
Ling-fen Xu
xulingfen7408@163.com
[1] Department of Pediatrics, Shengjing Hospital of China Medical University, 36 Sanhao Street, Shenyang, Liaoning 110004, P.R. China
[2] Department of PICU, Children's Hospital, Zhejiang University School of Medicine, National Clinical Research Center for Child Health, National Children's Regional Medical Center, Hangzhou, Zhejiang 310052, P.R. China
[3] Department of Pathology, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110004, P.R. China

In 1975, Swift et al. first detected abnormally high serum levels of vasoactive intestinal peptide (VIP) in childhood gangliomas with refractory diarrhea [3]. The new concept of VIP-secreting tumor (VIPoma) was proposed, characterized by watery diarrhea, hypokalemia, and alkalosis (WDHA syndrome) [4–6]. These tumors predominantly occur in the pancreatic tail in adults, but occur more frequently in extra-pancreatic areas as neurogenic tumors in children, such as gangliomas and ganglioneuroblastomas (GNB) [7, 8].

VIP is a 28 amino acid neuropeptide that was first isolated in 1970 as a member of the secretin/glucagon peptide superfamily. It is mainly secreted by neurons and endocrine and immune cells, and is widely distributed in the body [9]. In the digestive tract, VIP binds to VPAC1 on intestinal epithelial cells, causing secretory diarrhea and ion disturbance [10, 11]. Recent observations have demonstrated that VIP plays an important role in the proliferation, migration, and invasion of gastric [12, 13], lung [14], prostate [15, 16] and renal cell cancers [17].

In the present study, we investigated the expression rate of VIP in NB, in order to explore the correlation between VIP expression and NB clinicopathological features, and the interaction between VIP expression and NB prognosis.

Materials and methods

Participants and tissue specimens

This study included 91 patients treated between January 2015 and December 2021 at the Shengjing Hospital of China Medical University. The inclusion criteria were age less than 18 years, tumor resection, and a postoperative pathological diagnosis of NB. Clinical data, including sex, age, tumor site, diarrhea, hypokalemia, histopathological patterns, bone marrow (BM) metastasis, and tumor stage were extracted from medical records. Infectious diarrhea was ruled out in patients with diarrhea. All personal information was anonymized, and none of the patients received chemotherapy or radiotherapy before diagnosis. This study was approved by the Ethics Committee of Shengjing Hospital of China Medical University (2021PS521K).

The 91 tissue specimens were obtained from surgically resected tissues of NB primary tumors preserved at the Department of Pathology, Shengjing Hospital of China Medical University. Histopathological patterns were based on the International Neuroblastoma Pathology Classification (INPC) [18]. Tumor stage was defined according to the International Neuroblastoma Risk Group (INRG) Staging System [19].

Study design

Clinical data of patients were collected; pathological staging, and staging and grading of resected tumors were performed; the VIP expression rate and VIP intensity in NB tumor tissues were determined by immunohistochemical staining; the expression of synaptophysin (Syn), chromogranin A (CgA), and proliferating cell nuclear antigen (Ki67) were also detected by immunohistochemical staining; the correlation between VIP expression intensity in tumors and clinical performance and prognosis was analyzed. Patients with refractory diarrhea were followed up postoperatively to record improvements after surgery.

Immunohistochemistry and scoring

All tissue specimens were fixed in buffered formalin, and tissue sections were hydrated after dewaxing. After antigen repair in citrate buffer, endogenous peroxidase was blocked using 3% hydrogen peroxide in phosphate buffered saline (PBS). Slides were then incubated overnight at 4 ℃ with primary antibodies against VIP (1:1000, CUSA-bio), Syn (ready-to-use, ZSGB-Bio), CgA (ready-to-use, ZSGB-Bio), and Ki67 (ready-to-use, ZSGB-Bio). The slides were washed with PBS, and were subsequently treated with secondary antibodies at 37 ℃ for 30 min, followed by the addition of the 3, 3-diaminobenzidine for color development under the microscope. Hematoxylin and hydrochloric acid were used for counterstaining and differentiation, respectively. Immunohistochemical staining intensities were scored using image analysis software, and were graded as negative (−), weak (+), moderate (++), or strong (+++). Scoring was done in a double-blind manner. Staining range: < 10% is 0 points, 11%−25% is 1 point, 26%−50% is 2 points, > 50% is 3 points; staining intensity:no coloration is 0 points, light yellow is 1 point, tan is 2 points, brown is 3 points. Staining range x staining intensity to determine the intensity of expression: 0 points (-); 1, 2, 3 points (+); 4, 6 points (++); 9 points (++++) [20].

Statistical analysis

All statistical analyses were performed with SPSS version 26.0. Variables of clinicopathological features (gender, age, presence of diarrhea, presence of hypokalemia, histopathological classification, histopathological prognosis, presence of bone marrow infiltration, and INRG stage) were used Mann–Whitney U test. Subgroups stratified by VIP expression intensities (tumor site) were used the Kruskal–Wallis tests. Variables of laboratory tests were compared using analysis of variance. Correlations between VIP and Syn, CgA and Ki67 were compared

using Spearman's coefficient. $P < 0.05$ was considered statistically significant.

Results

Clinicopathological features

The clinicopathological features of the 91 included patients are summarized in Table 1. The median patient age was 27 months (1–139 months), with an approximate male-to-female ratio of 1.1:1. Tumor location showed a predominance of the retroperitoneum (38.5%) and adrenal glands (27.5%). Six patients (6.6%) presented with refractory watery diarrhea, and seven patients (7.7%) had clinical manifestations of hypokalemia. The histopathological patterns showed a predominance of NB (65.9%) and favorable histology (FH) (68.1%). Absence of BM metastasis or stage L1/L2 were observed in 75.8% and 66.6% of patients, respectively.

Clinicopathological features of patients with diarrhea in NB

Refractory watery diarrhea was observed in six patients (6.6%), all of whom were immediately relieved after NB surgical resection. The specific characteristics of these patients are summarized in Table 2. Their median age was 15 months (12–20 months), and the male-to-female ratio was 2:1. All six cases visited hospitals for varying periods (1–12 months) for chronic diarrhea, and laboratory tests revealed an average serum potassium level of 2.5 mmol/L (1.5–3.6 mmol/L). The tumor stage in all cases was L1/L2. There was a predominance of abdominal low-risk group including the retroperitoneum ($n=3$) and the adrenal glands ($n=2$), well-differentiated NB or GNB ($n=2$), low tumor stage (L1, $n=5$ and L2, $n=1$), and no MYCN expression ($n=5$).

VIP expression in NB tumors

The expression of VIP in tumor tissues of the 91 included patients was evaluated. Twenty-five cases were found VIP expression (27.5%), of which three (3.3%) were strong, three (3.3%) were moderate, and 19 (20.9%) were weak. The six patients with clinical manifestations of refractory diarrhea showed 100% VIP expression, which were mainly found in the cytoplasm.

Correlation of VIP expression with clinical characteristics

These results suggested differences in VIP expression in NB tumors. Hence we further investigated the

Table 1 Summary of clinicopathological features

Variables		Total	Rate	Variables		Total	Rate
Sex	Male	47	51.6%	Hypokalemia	Yes	7	7.7%
	Female	44	48.4%		No	84	92.3%
Age(months)	≤ 18	36	39.6%	Histopathological classification	NB	60	65.9%
	> 18	55	60.4%		GNB	31	34.1%
Tumor site	Retroperitoneum	35	38.5%	Histopathological prognosis	FH	62	68.1%
	Adrenal	25	27.5%		UH	29	31.9%
	Mediastinum	24	26.4%	BM metastasis	Yes	23	24.2%
	Others	7	7.6%		No	68	75.8%
Diarrhea	Yes	6	6.6%	Stage	L1	39	43.3%
					L2	21	23.3%
	No	85	93.4%		M	15	16.7%
					MS	15	16.7%

Abbreviations: NB Neuroblastoma, *GNB* Ganglioneuroblastoma, *FH* Favorable Histology, *UH* Unfavorable Histology

Table 2 Summary of clinicopathological features in 6 cases with refractory watery diarrhea

Case	Sex	Age (month)	Period (month)	K^+ (mmol/L)	Histopathological diagnosis	MYCN expression
1	Male	12	1	3.6	Retroperitoneum NB, poorly differentiated, low MKI, FH	Unknown
2	Female	18	1.5	3.0	Mediastinum NB, well differentiated, low MKI, FH	No
3	Male	20	2	2.8	Adrenal NB, well differentiated, low MKI, FH	No
4	Female	13	3	2.2	Adrenal NB, well differentiated, low MKI, FH	No
5	Male	15	6	2.1	Retroperitoneum GNB, mixed, low MKI, FH	No
6	Male	20	12	1.5	Retroperitoneum NB, well differentiated, low MKI, FH	No

Abbreviations: NB Neuroblastoma, *MKI* mitosis-karyorrhexis index, *FH* Favorable histology

relationship between VIP expression and clinical parameters and symptoms, such as sex, age, tumor site, diarrhea, and hypokalemia. Furthermore, we analyzed the correlation between VIP expression and levels of the laboratory parameters neuron specific enolase (NSE), 24-h urine vanillylmandelic acid (VMA), and lactate dehydrogenase (LDH). The differences in VIP expression intensity between diarrhea and hypokalemia were statistically significant ($P<0.001$, and $P<0.001$, respectively); however, the differences in VIP expression intensity between different sexes, ages, and tumor sites were not ($P=0.410$, $P=0.884$, and $P=0.372$, respectively) (Table 3). The differences in NSE, 24-h urinary VMA, and LDH levels between patients with different VIP expression intensities were also not significant ($P=0.134$, $P=0.578$, and $P=0.686$, respectively) (Table 3).

Correlation between VIP expression and prognosis

All patients with NB in this study underwent surgical resection, and for the six patients with refractory diarrhea the symptoms associated with diarrhea disappeared immediately after tumor removal. We, therefore, investigated the relationship between VIP expression intensity and patient prognosis. The differences in VIP expression intensity between different pathological type, Shimada stage, presence or absence of BM infiltration, and INRG score were significant ($P=0.003$, $P=0.036$, $P=0.018$, and $P=0.044$, respectively) (Table 4). We also investigated the correlation between VIP expression intensity and the expression of prognosis-related molecules such as Syn, CgA, and Ki67. Microscopy showed that Syn and CgA were localized in the cytoplasm, while Ki67 was localized in the nucleus. Among the 91 patient samples: 75 showed expression for Syn (82.4%), of which 10 (11%) were strong, 29 (31.9%) moderate, and 36 (39.6%) weak; 72 showed expression for CgA (79.1%), of which 6 (6.6%) were strong, 27 (29.7%) moderate, and 39 (42.9%) weak; 59 showed expression for Ki67 (64.8%), of which 10 (11%) were strong, 20 (22%) moderate, and 29 (31.8%) weak (Fig. 1). VIP expression intensity positively correlated with the expression of Syn (Table 5, $r_s=0.342$, $P=0.001$), and negatively correlated with the expression of CgA (Table 5, $r_s=-0.265$, $P=0.011$) and Ki67 (Table 5, $r_s=-0.317$, $P=0.002$). Finally, we compared the mortality rates of patients, seven of whom were excluded due to missed visits, and we found that patients expressing VIP had a lower mortality rate compared to those not expressing VIP (13.3%, 4.2%).

Discussion

NB is the most common and aggressive extracranial solid tumor in childhood [21]. It is also the tumor most likely to cause pediatric paraneoplastic syndrome (PNS), mainly involving neurological instead of gastrointestinal disorders [22]. However, recent studies have updated the typical watery-diarrhea syndrome caused by VIP secretion in NB; this is now considered one of the rare digestive tract-related PNS, accounting for less than 3% of all patients with NB [23–25]. Diarrhea may appear as the first and only symptom or may occur during

Table 3 Relationship between VIP expression intensity and clinical information

Variables		VIP expression intensity				Expression rate/ F	P
		−	+	++	+++		
Sex	Male	36	8	1	2	23.4%	0.410
	Female	30	11	2	1	31.8%	
Age	≤ 18 M	26	7	1	2	27.8%	0.884
	> 18 M	40	12	2	1	27.3%	
Tumor site	Retroperitoneum	26	6	1	2	25.7%	0.372
	Adrenal	21	2	1	1	16.0%	
	Mediastinum	14	9	1	0	47.6%	
	Others	5	2	0	0	28.6%	
Diarrhea	Yes	0	1	2	3	100%	< 0.001
	No	66	18	1	0	22.4%	
Hypokalemia	Yes	2	0	2	3	71.4%	< 0.001
	No	64	19	1	0	23.8%	
NSE		111.28 ± 114.39	58.23 ± 34.31	35.45 ± 9.39	57.87 ± 21.24	1.922	0.134
VMA		8.69 ± 16.41	4.38 ± 6.84	3.87 ± 2.27	1.70 ± 0.70	0.661	0.578
LDH		540.67 ± 554.05	472.26 ± 551.99	267.67 ± 21.94	272.00 ± 36.04	0.496	0.686

Abbreviations: VIP Vasoactive intestinal peptide, *M* months, *NSE* neuron-specific enolase, *VMA* Vanillylmandelic acid, *LDH* Lactate dehydrogenase

$P<0.05$ is statistically significant

Table 4 Differences in clinicopathological variables among subgroups of patients stratified by VIP expression intensities

Variables		Total	VIP expression intensity				*P*
			−	+	++	+++	
Classification	NB	60	50	6	2	2	0.003[a]
	GNB	31	16	13	1	1	
Prognosis	FH	62	41	15	3	3	0.036[a]
	UH	29	25	4	0	0	
BM metastasis	Yes	23	21	2	0	0	0.018[a]
	No	68	45	17	3	3	
Tumor stage	L1	39	23	12	2	2	0.044[b]
	L2	21	15	5	0	1	
	M	15	13	1	1	0	
	MS	15	14	1	0	0	

Abbreviations: VIP Vasoactive intestinal peptide, *NB* Neuroblastoma, *GNB* ganglioneuroblastoma, *FH* Favorable Histology, *UH* Unfavorable Histology

[a] $P < 0.05$ means that the intensity of VIP expression differed between groups NB vs GNB (Classification); FH vs UH (Prognosis); Yes vs No (BM metastasis)

[b] $P < 0.05$ represents a difference in VIP expression intensity between tumor stages

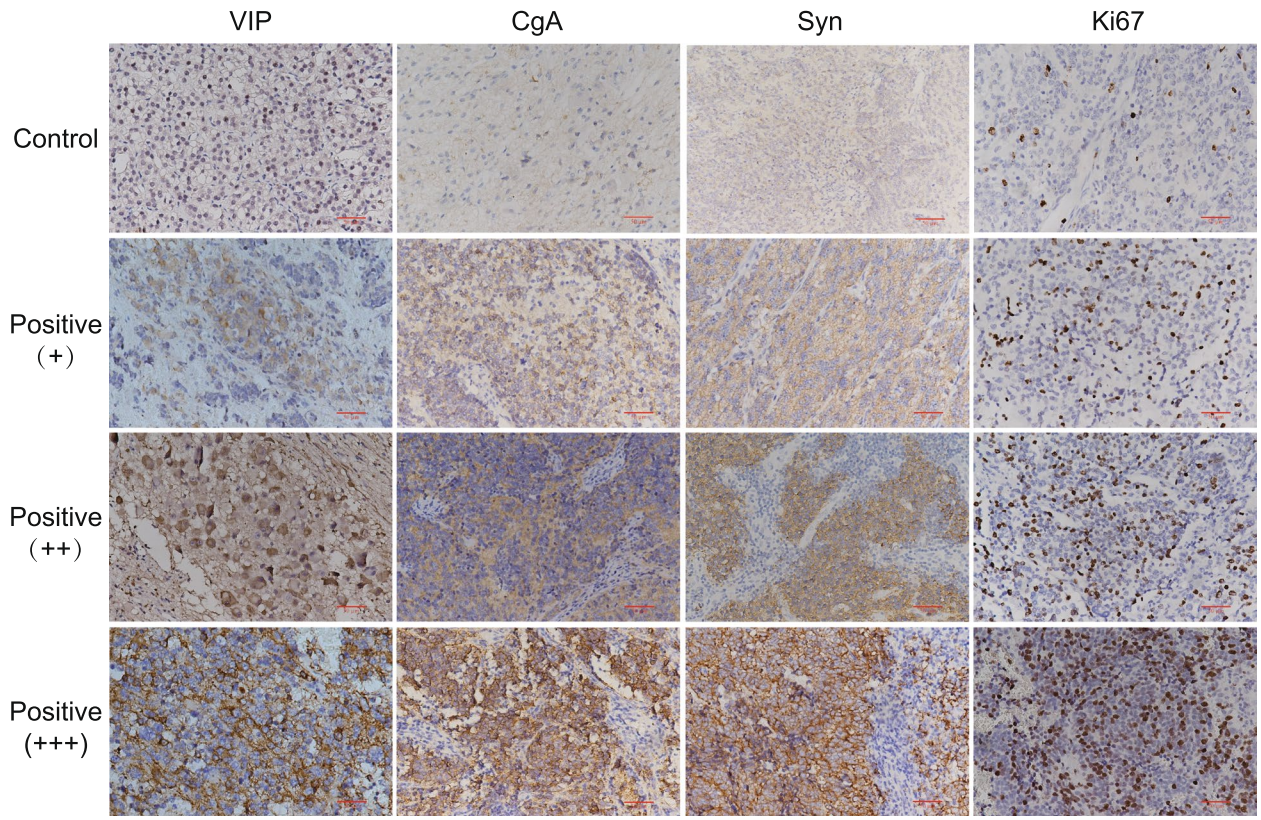

Fig. 1 Histopathology of VIP, Syn, CgA, and Ki67. Histopathology of tumor tissues showing different degrees of expression (+−+++) (200×)

chemotherapy, which is probably related to secondary VIP expression after induction of NB differentiation [26, 27].

In view of the secretion interval and short half-life of VIP, which is rapidly degraded in the serum, we used immunohistochemical staining to detect VIP expression in NB tissues [28]. Our study showed that VIP was expressed in NB at a rate of 27.5%, and the expression intensity of VIP was significantly increased in patients with diarrhea and hypokalemia. Numerous studies have

Table 5 The correlation between VIP and Syn, CgA, and Ki67 in expression intensities

VIP expression intensity	Negative	Positive	r_s	*P*
Syn	66	25	0.342	0.001
CgA	60	31	−0.265	0.011
Ki67	66	31	−0.317	0.002

Abbreviations: *Syn* synaptophysin, *CgA* chromogranin A, *Ki67* proliferating cell nuclear antigen

$P < 0.05$ is statistically significant

shown that VIP can reduce NB invasion in high-risk groups by decreasing MYCN expression. MYCN expression was not observed in patients with NB with refractory diarrhea (Table 2), suggesting a good prognosis for these patients. The absence of diarrhea in children with VIP expression may be related to insufficient VIP expression [28]. Instead, diarrhea in children with negative VIP expression may be related to infectious, allergic, or antibiotic-associated diseases during hospitalization. Careful medical evaluation should be performed based on the typical characteristics of such diarrhea.

Our study suggests that VIP expression intensity was increased in patients diagnosed with GNB and FH. Meanwhile, VIP expression intensity also increased in patients without BM metastasis and with low tumor stage, suggesting an indolent course of VIP-expressing NB, which is in agreement with earlier reports. High levels of TrkA receptor are expressed in low-stage NBs, which indicates a good prognosis. TrkA is a transmembrane receptor tyrosine kinase for nerve growth factor (NGF). In an embryonic mouse model, VIP was shown to be a regulator of NGF and to stimulate an increase in the molecular weight isoform of NGF, suggesting that VIP may be a potential marker of good prognosis in NB [29, 30]. NB is a tumor originating from the sympathetic nervous system and therefore has certain neuroendocrine characteristics. Syn, CgA, and Ki67 are immunohistochemical biomarkers commonly used for neuroendocrine tumors and are adjunctive in the pathological diagnosis of NB after operation [31, 32]. Previous studies have reported that Syn expression is positively correlated with NB prognosis [33], CgA expression is negatively correlated with NB prognosis [34], while Ki67 is a marker of poor NB prognosis [35]. As expected, our findings showed that the expression intensity of VIP positively correlated with Syn expression, and negatively correlated with the expression intensity of CgA and Ki67, suggesting that VIP may be a potential biomarker of good prognosis in NB.

However, this study has some limitations. First, this study was a single-center retrospective study with a relatively insufficient sample size. Therefore, future studies should be expanded to conduct multicenter, large-sample, prospective studies to further assess whether the results are applicable to a larger number of NB patients and, if possible, will combine long-term follow-up with simultaneous detection of serum VIP expression levels to increase the credibility of VIP seat as a good prognostic indicator. In addition, in vitro experiments were added to combine joint testing with other molecular markers to improve the accuracy of diagnosis and prognostic assessment of VIP.

In summary, in addition to published literature, this study also supports the fact that VIP levels could help identify poorly differentiated NB tumors at an earlier timepoint leading to better disease management and increased overall survival. As such, VIP could be a potential biomarker for early prognosis of NB.

Conclusions

This study systematically investigate the correlation between VIP expression in NB tumors and NB clinical features and prognosis, and suggests VIP as a potential biomarker of good prognosis in NB.

Abbreviations

BM	Bone marrow
CgA	Chromogranin A
GNB	Ganglioneuroblastoma
INPC	International Neuroblastoma Pathology Classification
INRG	International Neuroblastoma Risk Group
Ki67	Proliferating cell nuclear antigen
LDH	Lactate dehydrogenase
NB	Neuroblastoma
NGF	Nerve growth factor
NSE	Neuron specific enolase
PBS	Phosphate buffer saline
PNS	Paraneoplastic syndrome
Syn	Synaptophysin
VIP	Vasoactive intestinal peptide
VIPoma	Vasoactive intestinal polypeptide-secreting tumor
VMA	Vanillylmandelic acid
WDHA syndrome	Watery diarrhea, hypokalemia, and alkalosis syndrome

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Authors' contributions

All authors contributed to the conception and design of this study. Meng-ying Cao collected the data and wrote the paper; Kun Zhang performed the analysis and conceptualized the structure of the paper; Jing Guo and Fang Dong performed the analysis and revised the paper. Ling-fen Xu was the communication author for overall guidance. All the authors have read and approved the final version of the manuscript.

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Data availability
All raw data including the imaging data can be provided from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This was a retrospective study involving human subjects. The consent to participate was waived by the Ethics Committee of the Shengjing Hospital of China Medical University (approval no. 2021PS521K Shenyang China). This study was conducted in accordance with the principles of the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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