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Correlation of RGS4 and P16 expressions with pediatric nephroblastoma and its significance on prognosis

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Abstract. – OBJECTIVE: To investigate the correlation of regulator of G-protein signaling 4 (RGS4) and P16 expressions with pediatric nephroblastoma and its significance on prognosis.

PATIENTS AND METHODS: A total of 80 children with primary nephroblastoma who underwent surgical resection in our hospital from March 2009 to March 2012 were selected as objects of study. The expressions of RGS4 and P16 in cancer and cancer-adjacent tissues of patients were detected by reverse transcription-polymerase chain reaction (RT-PCR), Western blot and immunohistochemistry. All patients were followed up for 5 years. The relationship of RGS4 and P16 to nephroblastoma staging and patients' prognosis was analyzed.

RESULTS: The results of RT-PCR and Western blot displayed that the expressions of RGS4, P16 mRNA and protein in cancer tissue, were significantly lower than those in cancer-adjacent tissue ($p < 0.05$). Immunohistochemistry result revealed that the positive rates of RGS4 and P16 in cancer tissue were distinctly lower than those in cancer-adjacent tissue (76.54% *vs.* 34.32%, $p < 0.05$). RGS4 and P16 protein expressions were not associated with tumor, node metastasis (TNM) and pathological typing of nephroblastoma, but they were lowly expressed in patients with high metastasis ($p < 0.05$). The expressions of RGS4 and P16 were absent in pediatric nephroblastoma, and overall survival (OS) was significantly reduced ($p < 0.05$).

CONCLUSIONS: The absence of RGS4 and P16 in pediatric nephroblastoma tissue is correlated with poor prognosis of patients. RGS4 and P16 are of significance for the prognosis of pediatric nephroblastoma.

Key Words:
Pediatric nephroblastoma, RGS4, P16.

Introduction

Pediatric nephroblastoma is a complex mixed embryonal tumor. It is the most common malignant abdominal tumor in children, and its incidence ranks first in abdominal tumors[1,2]. This disease mainly occurs in children aged before 5 years old. The incidence of bilateral kidneys is similar, and about 5% of patients suffer from bilateral onset. The classic nephroblastoma contains three types of cells: blastema cell, epithelial cell, and stromal cell[3]. The tumor is excessively similar to the morphology and composition of Wilms' cell. At present, although 2-year survival rate of patients reaches 60% after the combined treatment with surgery, chemotherapy and radiotherapy for nephroblastoma, its adverse effects on pediatric patients are irreversible for life. The research on molecular mechanism of pediatric nephroblastoma has an important significance in treating tumor and ameliorating patients' prognosis.

P16 is also known as multiple tumor suppresser gene (MTS1) or cyclin-dependent kinase inhibitor (CDKN2); studies[4-6] reported that the absence of its expression can promote tumor proliferation thus leading to poor prognosis of patients. Regulator of G-protein signaling 4 (RGS4) can combine with many factors in the cell microenvironment (carbohydrate, lipid, peptide, protein, etc.) and activate a series of intracellular signaling pathways, eventually affecting multiple cell biological functions[7,8]. This work mainly aims to investigate RGS4 and P16 expressions in pediatric nephroblastoma and explore its possible influence on prognosis of patients, so as to provide a certain theoretical basis for the treatment and prognosis of nephroblastoma in children.

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Patients and Methods

Patients

A total of 80 children with primary nephroblastoma who underwent surgical resection in our hospital from March 2009 to March 2012 were selected. The nephroblastoma tissue and corresponding cancer-adjacent tissue (1.0 cm away from the margin of tumor) were collected from each patient, and the freshly collected tissue was placed into liquid nitrogen rapidly. Every fresh tumor tissue was divided into the following 3 parts: a) total RNA was extracted, and expressions of RGS4 and P16 in nephroblastoma and cancer-adjacent tissues were detected by reverse transcription-polymerase chain reaction (RT-PCR); b) total protein was extracted, and expressions of RGS4 and P16 in nephroblastoma and cancer-adjacent tissues were detected by Western blot; c) paraffin section was prepared to detect expressions of RGS4 and P16. No anticancer treatment was taken for all patients enrolled prior to the operation. This study was approved by the Ethics Committee of Haiyang People's Hospital. Signed written informed consents were obtained from all participants' parents or guardians.

RT-PCR Analysis

The pediatric nephroblastoma and cancer-adjacent tissues were taken from liquid nitrogen, followed by extraction of total mRNA using RNeasy kit (Qiagen, Hilden, Germany) according to operations of instructions. cDNA was synthesized by 1.0 μg total mRNA using reverse transcription kit (SuperScript® VILO cDNA synthesis Kit and Master Mix, Thermo Fisher Scientific, Waltham, MA, USA). The expressions of RGS4 and P16 were quantitatively detected by GeneCopoeia kit (Guangzhou, Guangdong, China) and Real-time PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA). The computational formula of relative mRNA expression level of each index: $2^{-\Delta Ct}$ [ΔCt = Ct (target gene) – Ct (glyceraldehyde-3-phosphate dehydrogenase, GAPDH)]. The corresponding primer sequences are shown in Table I.

Western Blot Analysis

Tumor tissue sample was homogenized by tissue homogenate machine, followed by adding 500 μL lysate Repa and appropriate amount of protease inhibitor, Cocktail, and splitting decomposition on ice for 1 h. The sample was centrifuged at 13000 g. The supernatant was taken to detect protein concentration, followed by electrophoresis separation of 40 μg total protein in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and being transferred onto nitrocellulose membrane; then, it was sealed in 10% bovine serum albumin (BSA) for 45 min and incubated with RGS4 (1:2000) and P16 (1:1000) primary antibodies overnight. Subsequently, the sample was incubated at room temperature for 1 h with β-actin as internal reference (1:1000). The target protein bands were displayed by enhanced chemiluminescence (ECL) system; the relative content of target protein was the ratio of target protein to corresponding gray value of internal reference band.

Hematoxylin-eosin (HE) Staining and Immunohistochemical Analysis

The paraffin wax with sample was cut into slices with 4 μm as thickness and toasted in an oven at 60°C for 75 min, followed by dewaxing with xylene and rehydration with gradient ethanol. The hematoxylin staining was conducted by HE staining reagent set (Google Biology, Wuhan, Hubei, China) for 1-3 min according to instructions; the differentiation was performed by 0.2% ethanol hydrochloride for few seconds, followed by eosin staining for 3 min, dewatering with gradient ethanol and mounting.

Immunohistochemistry: the slide containing sample was treated by antigen retrieval (98.5°C, 5 min) using citrate buffer (pH=6) after rehydration; after being cooled, the slide was incubated by 3% hydrogen peroxide at room temperature for 30 min, followed by incubation with commercial sealing liquid (serum-free protein, Dako, Glostrup, Denmark) for 60 min; rabbit-derived primary antibody RGS4 and P16 were purchased from Abcam (Cambridge, MA, USA); 200 μL RGS4 (1:500) and P16 (1:500) antibodies were added onto slide drip by drip, respectively, followed by incubation overnight; the conjugated polymer horseradish peroxidase (HRP) (Zhong-

Table I. RT-PCR primer sequences.

Gene		Primer sequence
RSG4	5'-3'	ACATCGGCTAGGTTTCCTGC
	3'-5'	GTTGTGGGAAGAATTGTGTTCAC
P16	5'-3'	GATCCAGGTGGGTAGAAGGTC
	3'-5'	CCCCTGCAAACTTCGTCCT
β-actin	5'-3'	GTGACGTTGACATCCGTAAAGA
	3'-5'	GCCGGACTCATCGTACTCC

shan Golden Bridge, Beijing, China) was used as secondary antibody. Diaminobenzidine (DAB) was adopted as a chromogenic substrate. The photography was taken by Leica inverted microscope (DMI6000B, Wetzlar, Germany), followed by analysis. Positive rate of protein (%) = positive cell number/total cell number × 100%.

5 fields in each slide were taken for statistical analysis.

Statistical Analysis

The experimental results were analyzed by GraphPad Prism software (Version 5.01, La Jolla, CA, USA) statistical software. The difference in index between the two groups was compared by *t*-test. The overall survival (OS) curve of patients in RGS4 and P16 high/low expression group was drawn by Kaplan-Meier method. The survival difference of patients between the two groups was compared by Log-rank test. $p < 0.05$ suggested that the difference was statistically significant.

Results

Detection of RGS4 and P16 mRNA Levels in Cancer and Cancer-Adjacent Tissues of Patients with Nephroblastoma Using RT-PCR

As shown in Figure 1, RGS4 and P16 mRNA expressions were significantly lower in cancer tissue than those in cancer-adjacent tissue in patients with nephroblastoma, and the differences were statistically significant ($p < 0.05$).

Detection of RGS4 and P16 Protein Levels in Cancer and Cancer-Adjacent Tissues of Patients with Nephroblastoma Using Western Blot

As shown in Figure 2, RGS4 and P16 protein expressions were significantly lower in cancer tissue than those in cancer-adjacent tissue in patients with nephroblastoma, and the differences were statistically significant ($p < 0.05$).

Detection of Morphologies of Cancer and Cancer-Adjacent Tissues of Patients with Nephroblastoma Using HE Staining

As shown in Figure 3, the partial cancer tissue was in mammillary and glomerular structural arrangement, but the cancer-adjacent tissue has normal glomerular structure.

Detection of Positive Rates of RGS4 and P16 Protein in Cancer and Cancer-Adjacent Tissues of Patients with Nephroblastoma Using Immunohistochemistry

RGS4 and P16 protein levels in cancer and cancer-adjacent tissues of patients with nephroblastoma were further verified by immunohisto-

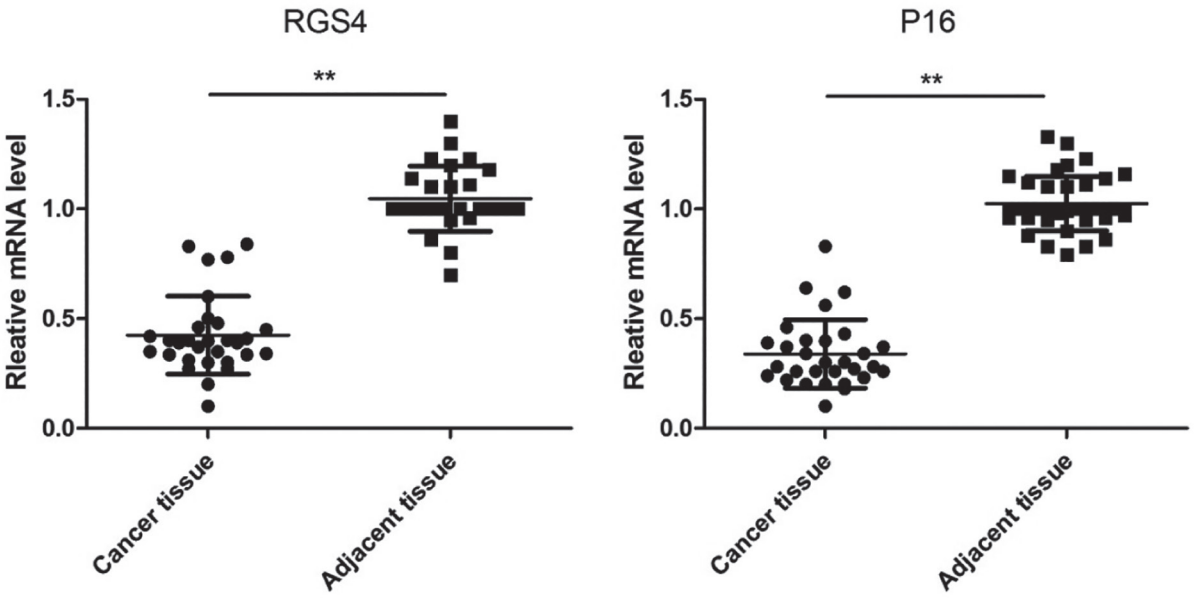

Figure 1. RGS4 and P16 mRNA levels in cancer and cancer-adjacent tissues in patients with nephroblastoma. $^{**}p < 0.01$, cancer tissue vs. cancer-adjacent tissue.

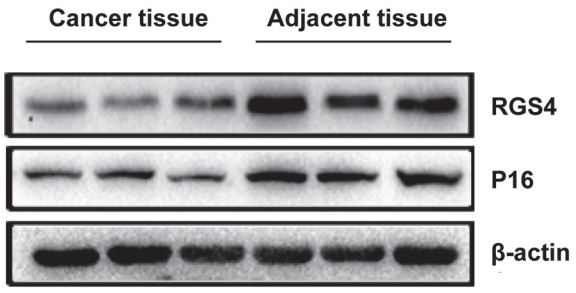

Figure 2. RGS4 and P16 protein levels in cancer and cancer-adjacent tissues in patients with nephroblastoma.

chemical method. RGS4 was mainly expressed in cytoplasm. RGS4 expression level in cancer-adjacent tissue was significantly higher than that in cancer tissue. P16 was mainly expressed in nucleus, which has the same expression tendency with RGS4, and its expression in cancer tissue was significantly lower than that in cancer-adjacent tissue (Figure 4).

RGS4 and P16 Expressions and Prognosis Staging

The gender, age, clinical staging, pathological type and lymphatic metastasis of nephroblastoma were selected as prognosis parameters. The relationship between RGS4 and P16 expressions and prognosis of pediatric nephroblastoma was analyzed. As shown in Table II, there were no statistically significant differences between RGS4 and P16 expressions and gender, age, clinical tumor node metastasis (TNM) staging and pathological type of nephroblastoma ($p > 0.05$). However, the positive rates of RGS4 and P16 in tissue of patients with metastasis were significantly lower than those without metastasis ($p < 0.05$), suggesting that the absence of RGS4 and P16 protein in pediatric nephroblastoma patients was significantly correlated with tumor lymphatic metastasis. The expressions of RGS4 and P16 are not associated with gender, age, TNM and pathological staging, but are correlated to lymphatic metastasis ($p < 0.05$).

Correlation of Low Expressions of RSG4 and P16 with Poor Prognosis of Pediatric Nephroblastoma

As shown in Figure 5, the overall survival (OS) in pediatric nephroblastoma patients with low expressions of RSG4 and P16 was distinctly lower than that in those with high expressions ($p < 0.05$). Therein, OS in patients with low expression of RGS4 mRNA was remarkably lower than that in high expression group (median survival: 40 months and 48 months, respectively, $p < 0.05$). Additionally, OS in patients with low expression of P16 mRNA was significantly lower than that in high expression group (median survival: 46 months and 55 months, $p < 0.05$), revealing that the absence of RGS4 and P16 was closely correlated with the death of pediatric nephroblastoma patients, and low expressions of RGS4 and P16 indicated the poor prognosis of patients.

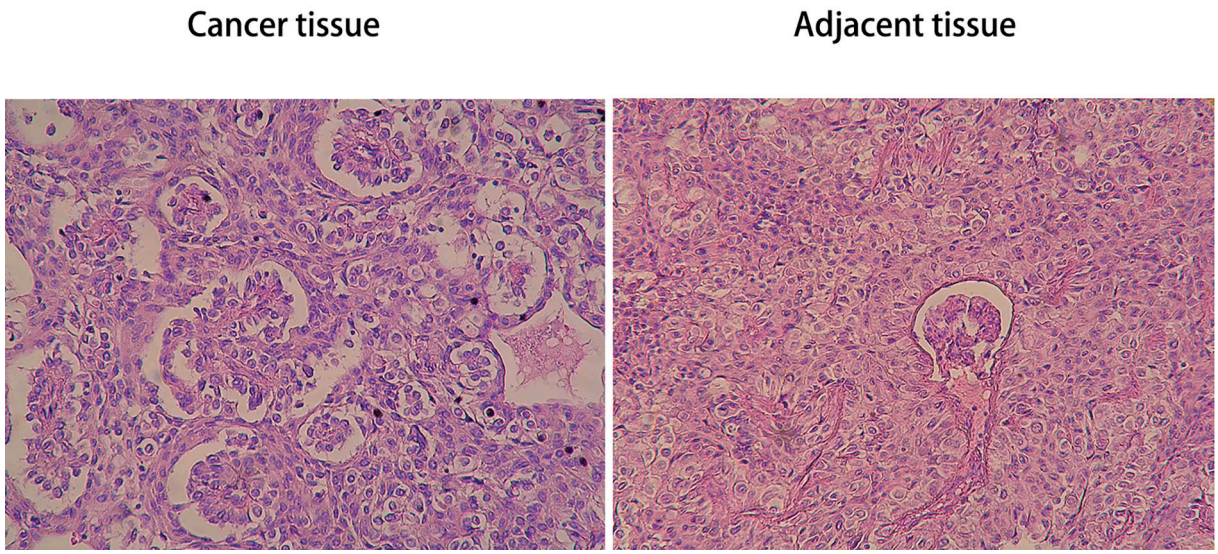

Figure 3. Morphologies of cancer and cancer-adjacent tissues of patients with nephroblastoma (×200).

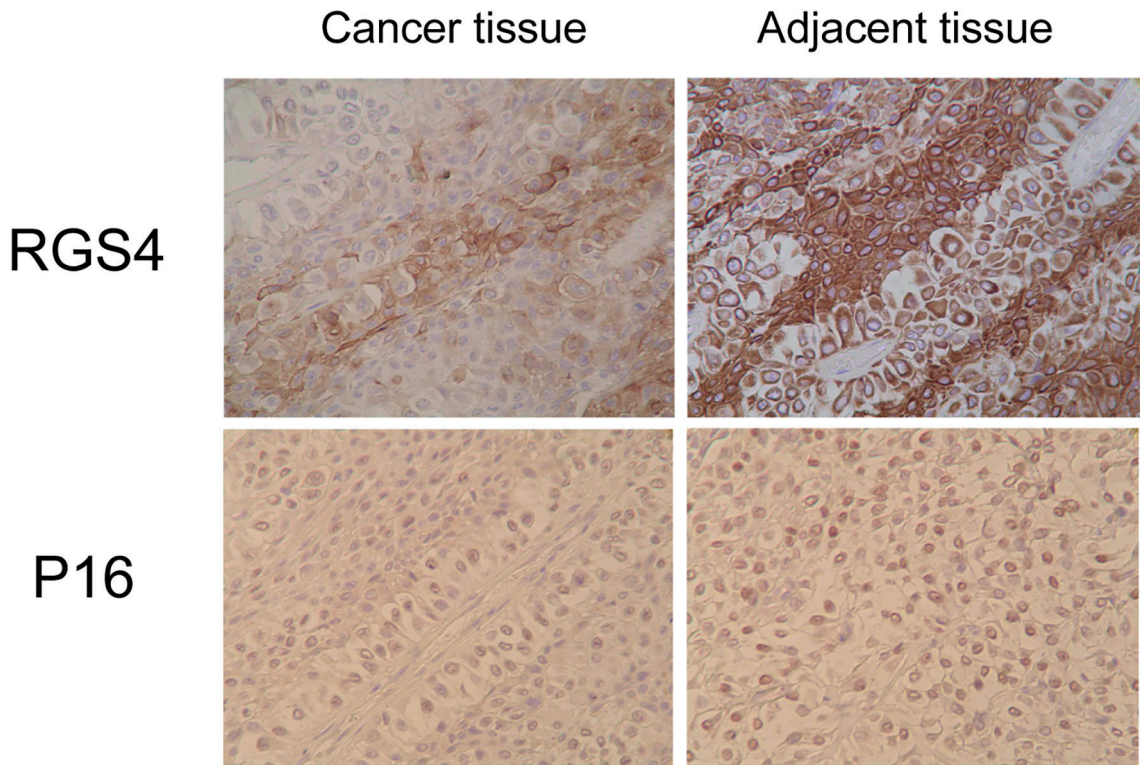

Figure 4. Expressions of RGS4 and P16 in cancer and cancer-adjacent tissues of patients with nephroblastoma (×200). The expressions of RGS4 and P16 in cancer tissue are lower than those in cancer-adjacent tissue ($p < 0.05$).

Discussion

At present, RGS protein in about 30 kinds of mammals has been ascertained, which is revealed by the evidence from preclinical and clinical study[9]. The RGS protein in different species has a conserved RGS domain and activity of GTPase-activating protein (GAP)[10]. Due to its GAP activity, RGS4 can significantly inhibit invasion and migration of breast cancer (MDA-MB-231)

Table II. Relationship between clinical pathological features and expressions of RGS4 and P16 in 160 patients with pediatric nephroblastoma ($\bar{x} \pm s$).

Classification	n	Positive rate of RGS4 (%)	*p*-value	Positive rate of P16 (%)	*p*-value
Gender			$p > 0.05$		$p > 0.05$
Male	38	21.34 ± 3.22		12.23 ± 2.68	
Female	42	19.73 ± 4.28		13.80 ± 3.64	
Age			$p > 0.05$		$p > 0.05$
< 3 years old	37	25.36 ± 4.82		15.74 ± 4.03	
> 3 years old	43	23.14 ± 3.79		13.36 ± 5.93	
TNM staging			$p > 0.05$		$p > 0.05$
I-II	41	22.83±3.65		14.55 ± 6.72	
III-IV	39	24.73 ± 5.93		15.68 ± 4.22	
Pathological typing			$p > 0.05$		$p > 0.05$
FH	42	20.40 ± 6.82		13.10 ± 6.14	
UH	38	22.54 ± 3.67		15.56 ± 6.33	
Lymphatic metastasis			$p < 0.05$		$p < 0.05$
No metastasis	47	29.84 ± 4.83		25.45 ± 5.80	
Metastasis	33	15.34 ± 5.82 **		8.32 ± 4.83 **	

Note: Lymphatic metastasis *vs*. no metastasis, *$p < 0.05$, **$p < 0.01$.

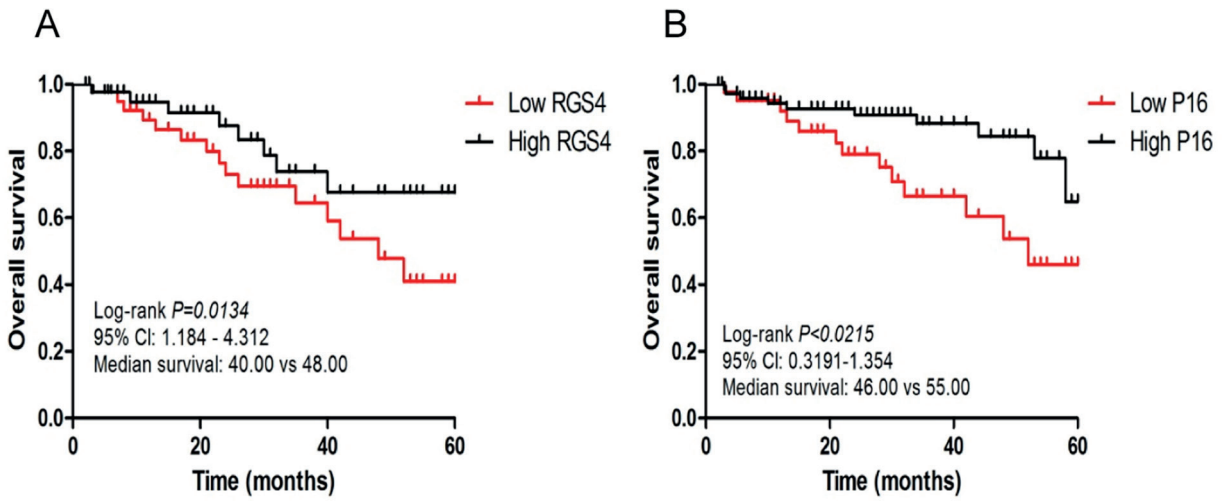

Figure 5. Correlation of low expressions of RSG4 and P16 with poor prognosis of pediatric nephroblastoma. ***A,*** Survival period of patients with low expression of RGS4 is shortened. Median survival: low RGS4 *vs.* high RGS4, 40 *vs.* 48 ($p < 0.05$); ***B,*** Survival period of patients with low expression of P16 is shortened. Median survival: low P16 *vs.* high P16, 46 *vs.* 55 ($p < 0.05$).

cells. The function of RGS protein has been reported in a variety of human diseases. The work indicates that G-protein-coupled receptor (GPCR) is involved in metastasis signal of cancer cell and down-regulation of RGS2, thus promoting the progress of prostatic carcinoma[11]. Additionally, it has been proved that RGS4 plays an important role in the activation of epithelial mesenchymal transformation (EMT) and promotion of invasion and metastasis of tumor[12]. Recently, Weiler et al[13] showed that the increased expression of GPCR such as RGS4 do not allow breast cancer cells to gain metastatic potential. On the contrary, the strict monitoring and regulation of GPCR signal loss can exert a more important role in the metastatic malignant tumor. By blocking the signal to start the G-coupled receptor, such as protease-activated receptor 1 (PAR1) and CXC chemokine receptor 4 (CXCR4), RGS4 plays a key role in inhibiting invasion and migration of tumor by disrupting the formation of Rac1-dependent lamellipodia[11,14].

P16 is a kind of important cyclin dependent kinase inhibitor apart from P27, P21 and P53[15] directly involving in cell cycle regulation and negative regulation of cell proliferation and division. Compared with P53, P16 is a type of more important new anticancer gene. The absence of P16 expression can lead to cell malignant proliferation, thus inducing the occurrence of malignant tumors. Currently, the absence of P16 gene has been confirmed in multiple tumors, which is frequently found in the invasive and metastatic tumors[16-19].

Conclusions

We observed that RGS4 and P16 mRNA and protein were significantly absent in cancer tissue of pediatric nephroblastoma. Additionally, immunohistochemical result displayed that the positive rates of RGS4 and P16 in cancer tissue were significantly lower than those in cancer-adjacent tissue, which was highly correlated with lymphatic metastasis, and the overall survival was significantly reduced. However, the specific molecular mechanism of regulation remains to be further investigated. In summary, this study indicated that the absence of RGS4 and P16 in pediatric nephroblastoma tissue was correlated with poor prognosis of patients, which provides a certain theoretical basis for the clinical screening of RGS4 and P16 as prognostic indicators.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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