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Preliminary observations regarding the expression of collagen triple helix repeat-containing 1 is an independent prognostic factor for Wilms' tumor

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ABSTRACT

Purpose: Overexpression of collagen triple helix-repeat containing 1 (CTHRC1) has been reported in many malignancies, where it plays an important role in tumorigenesis and progression. This study aimed to examine the clinical significance of CTHRC1 expression in patients with Wilms' tumor (WT).
Methods: The expression of CTHRC1, and its correlations with various clinicopathological parameters, was analyzed using immunohistochemistry in 42 WT tissues and 42 adjacent non-cancerous tissues. Samples from 8 patients with WT were examined using Western blotting and quantitative real-time polymerase chain reaction (qRT-PCR). Kaplan–Meier analysis and Cox proportional hazards regression models were used to investigate the correlations between CTHRC1 expression and the prognosis of patients with WT.
Results: Immunohistochemistry, Western blotting, and qRT-PCR revealed that the expression of CTHRC1 was significantly higher in WT tumors, compared to the expression in the adjacent non-cancerous tissues. Furthermore, high tumor expression of CTHRC1 was associated with tumor size, clinical stage, histopathological type, and vascular invasion/metastasis. Moreover, the proportions of expressing cells in the WT specimens was higher than the proportions in the matched adjacent non-cancerous tissues. Kaplan–Meier analysis revealed that patients with high CTHRC1 expression exhibited a shorter survival, compared to patients with low CTHRC1 expression. Univariate and multivariate analyses also revealed that CTHRC1 expression was an independent prognostic factor for overall survival.
Conclusions: Our preliminary results suggest that CTHRC1 is an independent prognostic factor, which may play an important role in tumorigenesis and progression, and may be a potential biomarker for WT.

Wilms' tumor (WT) is one of the most common pediatric tumors, with a prevalence of 1:8000–10,000 among North American children [1,2]. Although combination therapy has greatly improved the short-term prognosis for most patients, approximately 10% of patients with WT experience poor survival because of metastasis or recurrence [3,4]. Furthermore, conventional prognostic factors (e.g., histopathological type and stage) are currently thought to predict the clinical outcomes of WT, although its complex pathogenesis can make it difficult to predict the biological behavior of WT using histopathological findings alone.

Recent medical developments have led to the discovery of various biomarkers that are associated with tumor occurrence and progression [3,5,6]. For example, the collagen triple helix-repeat containing 1 protein (CTHRC1) is a secretory glycoprotein that was first identified in injured arteries, although more recent research has revealed that it is involved in both physiological and pathological processes [7]. In addition, CTHRC1 has recently been demonstrated to be expressed in various human cancers, where it plays a role in tumor cell migration and invasion [8–11]. However, the expression of CTHRC1 in WT and its association with the clinicopathological characteristics of WT remain unclear. Thus, the present study aimed to examine the expression of CTHRC1 in WT and matched adjacent non-cancerous tissues, and to examine the correlation between CTHRC1 expression and patient prognosis.

1. Materials and methods

1.1. Patients and tissue specimens

Our institutional ethics review board approved the retrospective use of all patients' clinical data and tissue samples. In addition to the patients' clinicopathological data (age, sex, tumor size, stage, vascular invasion status, and histopathological type), we collected 42 WT tissues

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and 42 adjacent non-cancerous tissues from patients who had undergone unilateral radical nephrectomy without other preoperative treatment between January 2006 and December 2012. All patients received sequential treatment at the Urology Department of the Children's Hospital of Hebei Province and the Urology Department of the Children's Hospital of Tianjin, based on the Children's Oncology Group (COG) guidelines. In addition, eight fresh WT tissues and matched adjacent non-cancerous tissues were collected and stored at −80 °C for Western blotting and quantitative real-time polymerase chain reaction (qRT-PCR) analysis. All samples were analyzed by two experienced pathologists, who confirmed that the tumor samples were correctly identified as WT. The patients' follow-up data were collected via telephone or mail and the deadline for follow-up was December 2014.

1.2. Immunohistochemistry

Expression of CTHRC1 was assessed using immunohistochemical staining. First, all specimens were embedded in paraffin and sectioned at a thickness of 4 μm. After baking at 65 °C for 1 h, the sections were dewaxed in xylene, hydrated using a graded series of alcohols (100%, 95%, and 85%), and rinsed with deionized water. Next, the CTHRC1 antigen was retrieved using a citric acid buffer (pH 6.0) via microwave antigen retrieval for 25 min. To block any endogenous peroxidase activity, the sections were treated using a 0.3% hydrogen peroxide solution for 15 min. The sections were then incubated overnight at 4 °C in a humid chamber with the primary monoclonal antibody to CTHRC1 (ab85739, diluted 1:100; Abcam, USA). On the next day, the sections were incubated with a biotinylated goat anti-rabbit secondary antibody for 60 min. After a final washing step (with phosphate-buffered saline; PBS), the sections were incubated in a substrate solution (3,3'-diaminobenzidine), counterstained in hematoxylin, dehydrated using alcohols and xylene, and mounted. For the negative controls, the primary antibody was replaced with PBS. The positive controls were slides from patients with breast cancer and hyperplasia of the lobular mammary gland, which exhibited positive CTHRC1 expression.

The immunostaining results were scored by two investigators who independently evaluate a random selection of at least 5 fields (200×) for each section, and the mean score for the randomly selected areas was calculated as the immunostaining score for that section. Cases with significant disagreement were simultaneously rechecked by the two original pathologists and a senior pathologist, who evaluated additional areas on the section until a consensus was reached. The total immunohistochemistry scores for CTHRC1 expression were semi-quantitatively calculated based on the relative area and intensity of the brown 3,3'-diaminobenzidine signal. The area was scored as 0 for 0–5% of the cells being positive, 1 for 5–50% of the cells being positive, and 2 for >50% of the cells being positive. The intensity of the immunostaining was scored as 0 for no staining, 1 for yellow-brown staining, and 2 for brown staining. The final score was defined as the sum of the intensity and area scores, with negative staining defined as a final score of 0–2, and a positive score of 3–4 [12].

1.3. Western blot analysis

To further verify the expression of CTHRC1, eight fresh WT tissues and eight matched non-cancerous tissues were lysed using pre-chilled radioimmunoprecipitation assay buffer that was supplemented with 1 mM phenylmethanesulfonyl fluoride. The mixture was centrifuged at 20,817 ×*g* for 5 min, the supernatant was retrieved, and the protein levels were quantified using the bicinchoninic acid method. Equal amounts of protein extract (80 μg) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride filters (Invitrogen, Carlsbad, CA, USA). Immunoblotting was performed overnight at 4 °C with the primary anti-CTHRC1 antibody (diluted 1:1000; Abcam, USA) and anti-β-actin (diluted 1:2000; Santa Cruz, CA, USA) as an internal control. On the following day, the samples were incubated with the secondary antibody for 1 h, and the expression was visualized using an enhanced chemiluminescence system (Amersham, Uppsala, Sweden). The bands' intensities were quantified using Quantity One software (version 4.6).

Table 1
CTHRC1 expression in WT and matched adjacent non-cancerous tissues.

Parameter	n	CTHRC1expression		*P*
		Negative	Positive	
WT	42	20	22	0.007*
Adjacent non-cancerous tissues	42	32	10	

* Statistically significant.

1.4. Quantitative real-time polymerase chain reaction

The expression of *CTHRC1* was also evaluated using qRT-PCR. Total RNA was isolated from the eight fresh WT specimens and eight matched adjacent non-cancerous tissues using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Next, 1 μg of total mRNA from each sample was used for reverse transcription with the Omniscript RT kit (Qiagen, Germany). According to the manufacturer's instructions, qRT-PCR for *CTHRC1* was performed using the Roche FastStart Universal SYBR Green Master kit (Roche Diagnostic Corporation), and the following primers: 5'-TCATCGCACTTCTTCTGTGGA-3' (forward) and 5'-GCCAACCCAGATAGCAACATC-3' (reverse). The internal control was *GAPDH*, and we used the following primers: 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse). The relative expression of *CTHRC1* was normalized and the data were calculated using the $2^{-\Delta\Delta Ct}$ method.

1.5. Statistical analysis

All statistical analyses were performed using SPSS software (version 20.0; SPSS Inc., Chicago, IL). The associations between the patients' clinicopathological parameters and CTHRC1 expression were examined using the chi-square test or Fisher's exact test. The analyses of the relatively expressions or percentages of expressing cells were performed using the t test. Survival curves were plotted using the Kaplan–Meier method and differences between the survival curves were tested using the log-rank test. Cox's proportional hazards model was adopted for the univariate and multivariate analyses of the prognostic factors. A two-tailed *P*-value of <0.05 was considered statistically significant.

Table 2
The relationship between CTHRC1 expression and the clinicopathological characteristics.

Parameter		N	CTHRC1 expression		*P*
			Negative	Positive	
Age(years)	<3	28	14	14	0.662
	⩾3	14	6	8	
Gender	Female	16	7	9	0.694
	Male	26	13	13	
Tumor size(cm)	<10	27	16	11	0.043*
	⩾10	15	4	11	
Clinical stage	I–II	31	18	13	0.023*
	III–IV	11	2	9	
Histopathological type	Favorable	29	17	12	0.033*
	Unfavorable	13	3	10	
Invasion/metastasis	(−)	30	19	11	0.001*
	(+)	12	1	11	

* Statistically significant.