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RESEARCH

Collagen Triple Helix Repeat Containing-1 (CTHRC1) Expression in Invasive Ductal Carcinoma of the Breast: The Impact on Prognosis and Correlation to Clinicopathologic Features

Joo Heon Kim · Tae-Hwa Baek · Hyun Sun Yim · Kyo Hyun Kim · Seong-Hoo Jeong · Ho Bum Kang · Sang-seok Oh · Hee Gu Lee · Jae Wha Kim · Kwang Dong Kim

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Abstract CTHRC1 has been known as a regulator of collagen expression and cell migration. The aim of this research was to clarify the clinicopathologic significance of *CTHRC1* expression in human breast cancer. 22 cases of breast cancer tissues, randomly selected from clinically diagnosed patients, showed a significant increase of *CTHRC1* mRNA expression compared to the normal tissue from the same patients using RT-PCR and real-time PCR. Additionally we investigated breast cancers from 189 patients by immunohistochemistry (IHC). A high level of *CTHRC1* expression was observed in 111 (58.7 %) out of 189 breast cancer patients and the expression was significantly correlated with histologic grade (P=0.026), nodal status (P<0.001), and TNM pathologic stage (P=0.002). High *CTHRC1* expression was associated with a shorter recurrence free survival (P=0.008). Taken together, the results showed that *CTHRC1* over-expression was significantly associated with clinicopathological factors of poor prognosis in invasive ductal carcinoma. CTHRC1 could be used as a supplementary prognostic biomarker and a potential therapeutic target in breast cancer.

Keywords *CTHRC1* · Immunohistochemistry · Metastasis · Breast cancer

J. H. Kim · T.-H. Baek · H. S. Yim
Department of Pathology, Eulji University School of Medicine, Daejeon 301-070, Republic of Korea

K. H. Kim
Preventive Medicine, Eulji University School of Medicine, Daejeon 301-070, Republic of Korea

S.-H. Jeong
Department of Surgery, Medical School, Chonbuk National University, Chonju 560-182, Republic of Korea

H. B. Kang · H. G. Lee · J. W. Kim
Medical Genomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Republic of Korea

S.-s. Oh · K. D. Kim (✉)
Division of Applied Life Science (BK21), PMBBRC, Gyeongsang National University, 501 Jinju-daero, Jinju 660-701, Republic of Korea
e-mail: kdkim88@gnu.ac.kr

Introduction

Breast cancer is the leading cause of cancer mortality in women worldwide [1]. Despite advances in early clinical detection and the understanding of the molecular bases of breast cancer biology, some patients with early-stage breast cancer have recurrent disease [2]. It is a clinically heterogeneous and complex disease, and now widely acknowledged that accumulation of genetic anomalies contributed to the acquisition of an increasingly aggressive, invasive, and drug-resistant tumor phenotype [3–5]. Advances in molecular biology have allowed us to identify important prognostic cell markers and potentially useful targets for the treatment of breast cancer.

Collagen triple helix repeat containing-1 (*CTHRC1*) is a secreted protein that inhibits collagen expression and increases cell migration [6]. This gene was originally identified

as a novel gene regulated by TGF-beta family members with expression restricted to the adventitia and neointima of injured arteries, during the screen for differentially expressed gene profiles of normal and balloon injured rat arteries using suppressive subtractive hybridization [7]. The enhanced expression of *CTHRC1* was found in fibroblasts of the remodeling adventitia and smooth muscle cells of the neointima on arterial injury, and functionally impact collagen type I and III deposition, neointimal formation, and dedifferentiation of smooth muscle cells [8–10]. In transcription or protein survey, *CTHRC1* was increased in malignant melanoma and cancers of the gastrointestinal tract, lung, breast, thyroid, ovary, cervix, liver and the pancreas, suggesting its functional role in tumor progression by increasing cancer cell migration [11–13]. Recently, germline mutation in *CTHRC1* gene was identified in patients with Barrett esophagus and esophageal adenocarcinoma [14]. However, there is little information available about the expression and its clinical significance of *CTHRC1* in a series of human breast carcinoma. Recent reports showed *CTHRC1* expression in stromal cells of breast cancer by in situ hybridization [15, 16]. A study of combined evaluation of *CTHRC1* and periostin showed these genes as a potential marker for bone metastasis [17].

In this study, to investigate the clinicpathologic roles of the CTHRC1 in human breast cancer, we examined its expression pattern at the level of transcript and protein, and analyzed the possible correlations between the expression of *CTHRC1* and the various clinicopathologic factors in 189 invasive ductal carcinomas of the breast.

Materials and Methods

Patients and Samples

The biospecimens for this study were provided by the Biobank of Chonbuk National University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare and Family Affairs. A total of 189 patients were diagnosed and received their first line treatment at Chonbuk National University between January 1997 and January 2005. All samples derived from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols. All 189 breast carcinoma tissues and paired normal breast tissues taken from a site distant from the tumor lesion were fixed in 10 % neutral buffered formalin solution for 24 h and embedded in paraffin wax. The histologic grade was assessed using a modified Bloom-Richardson classification, and nuclear grade was evaluated according to modified Black's nuclear grade [18, 19]. Breast cancer phenotypes were classified according to the immunohistochemistry results for ER, PR, Her2, and Ki-67 combined with the FISH results for Her2 as follows: luminal A or B, Her2 overexpressed, and triple negative breast cancer (basal like) type [20]. Medical records were reviewed for clinical information including follow-up status and histologic parameters were determined from the hematoxylin and eosin (H&E)-stained slides. The cutoff date for vital status analysis was December 2010.

RT-PCR Analysis

Total RNA was isolated by a standard protocol [21], and cDNA was synthesized with oligo-dT primers and reverse transcriptase (Fermentas). RT-PCR was performed using the DNA Engine Dyad® Peltier Thermal cycler (Bio-Rad Laboratories). Real-time PCR was performed using SSoFast™ EvaGreen Supermix® and CFX96™ Real-time detection system (Bio-Rad Laboratories). *CTHRC1* gene-specific primers used for PCR were 5′-TGGACACCCA ACTACAAGCA-3′ (sense) and 5′-GAACAAGT GCCAACCCAGAT-3′ (antisense). The primers used for *CTHRC1* real-time RT-PCR were 5′-GGAAT GTCTGAGGGAAAGC-3′ (sense), 5′-AGCA CTATTTGAACGCATCT-3′ (antisense) and for *GAPDH* were 5′-AGTCAGCCGCATCTTCTT-3′ (sense), 5′-GCC CAATACGACCAAATC C-3′ (antisense). The relative levels of gene expression were normalized to *GAPDH* expression.

Immunohistochemistry (IHC)

Serial sections of 4-μm thickness were cut and mounted on charged glass slides (Superfrost Plus; Fisher Scientific, Rochester, NY, USA). IHC conditions for CTHRC1 were optimized and evaluated by two independent pathologists. In brief, tissue sections were microwaved twice for 10 min in citrate buffer (pH 6.0) for antigen retrieval. Then the sections were treated with 3 % hydrogen peroxide in methanol to quench the endogenous peroxidase activity followed by incubation with 1 % BSA. Mouse monoclonal antibodies against CTHRC1 (Abnova, Taipei, Taiwan) was used at dilutions of 1:100. The tissue sections were incubated with antibody overnight at 4°C in a wet chamber. The sections were stained using a standard EnVision-HRP kit (Dako, Glostrup, Denmark) and developed with diaminobenzidine as a substrate. An irrelevant mouse IgG of the same isotype or antibody dilution solution was served as a negative control.

Assesment of Immunostaining

We evaluated the intensity of immunohistochemical staining relative to the staining intensity of adjacent ductal epithelium cells within the same section as well as occasionally in

relation to the paired normal tissue as positive and negative controls. Each slide was evaluated for CTHRC1 immunoreactivity by using a semi-quantitative scoring system for both the intensity of the staining and the percentage of positive neoplastic cells under microscope. Immunohistochemistry conditions for CTHRC1 were optimized and evaluated by two independent pathologists. The intensity of staining was coded as: 0, lower than the adjacent normal-appearing ductal epithelium; 1, similar to the adjacent ductal epithelium; 2, stronger than the adjacent ductal epithelium. The percentage of cells displaying a stronger staining intensity than the adjacent ductal epithelium was scored as 1 (0 to 24 % tumor cells stained); 2 (25 to 49 % tumor cells stained); 3 (50 to 74 % tumor cells stained); 4 (75 to 100 % tumor cells stained). For the purpose of statistical analysis, the median of this series (25 % of malignant cells showing a stronger intensity than adjacent ductal epithelium) was used as a cutoff value to distinguish tumors with a low (< 25 %) or high (≥ 25 %) level of CTHRC1 expression. We separately analyzed the immunoreactivity for the stroma around the invasion front and tumor center, respectively.

Statistical Analysis

Statistical analysis was performed using the SPSS software package (version 14.0; SPSS Inc.). The correlation between staining index scores and other categorical factors was analyzed using the Pearson's chi-square test of independence. Recurrence-free survival was defined as the time from the date of surgery to the first date of recurrence of cancer, or death from any cause. Overall survival was defined as the time from the date of surgery to the date of last follow-up or death from any cause. The median follow-up period for all patients was 80.2 months (interquartile range: 61.9–92.6). Survival curve and median survival curve were estimated by the Kaplan-Meier method. Log-rank test was used to evaluate the statistical significance of differences in survival distribution. Multivariate analysis was done using the Cox proportional hazard regression analysis. Cox proportional hazard model, that included variables from the chi-square test for factors affecting survival and age variables, was identified. All other data were analyzed using unpaired Student's *t*-test. Results were considered statistically significant if $P<0.05$.

Results

Collagen Triple Helix Repeat Containing-1 (*CTHRC1*) Over-Expression in Human Breast Cancer Tissues

To compare the *CTHRC1* expression levels in the breast cancer tissues, we examined the mRNA level of *CTHRC1* by performing RT-PCR or real-time RT-PCR analysis on pairs of frozen tissue containing normal and tumor tissue samples from the same donor. *GAPDH* was used as a reference gene to correct for the variations for mRNA in individual samples. As shown in Fig. 1a and b, 22 cases of breast cancer tissues, randomly selected from clinically diagnosed patients, showed a significant increase of *CTHRC1* mRNA expression compared to the normal tissue from the same patients. CTHRC1 protein expression was evaluated by immunohistochemical analysis. CTHRC1 was expressed at higher levels in the breast tumorous tissues, but was not or detected at lower levels in corresponding non-tumorous breast tissues (Fig. 2a). Normal ductal epithelium showed negative expression of CTHRC1, but non-tumorous proliferating ductal epithelium did weakly positive expression. CTHRC1 immunoreactivity was found primarily on the cytosol, although membrane coexpression of CTHRC1 was occasionally noted in cells. CTHRC1 was also expressed in the stromal cells. It was highly expressed not only in the invasive front than in inner part of the tumor, but also in tumor cells of the lymphovascular spaces (Fig. 2b and c). CTHRC1-positive staining looked very heterogeneous even within the tumor, as the transformed cells might have heterogeneous characteristics.

Association of CTHRC1 Expression Levels with Clinicopathologic Characteristics

Clinical and pathologic characteristics of the 189 breast cancer patients who underwent a surgical resection are summarized in Table 1. The median age at the time of resection was 49 years; the median age of patients with a low level and a high level of CTHRC1 expression was 49.8 years and 48.6 years, respectively. A high level of CTHRC1 expression was observed in 111 (58.7 %) patients out of 189. When we tested for an association between the CTHRC1 expression levels and the clinicopathological parameters, we found that lymph node metastasis ($P<0.001$), histology grade ($P=0.026$), and pTNM (tumor, node, metastasis) stage ($P=0.002$) were significantly associated with CTHRC1 expression status. But a level of CTHRC1 expression for the stroma around the invasion front and tumor center did not show statistically significant correlation. *P* values obtained from the Pearson's chi-square test of independence are shown in Table 1.

A High CTHRC1 Expression Correlates with Worse Recurrence-Free Survival, but not Overall Survival

We first carried out univariate analyses to examine whether the expression status of CTHRC1 correlates with recurrence-free survival and overall survival. A total of 38 patients (20.1 %=38/189) presented with recurrence during

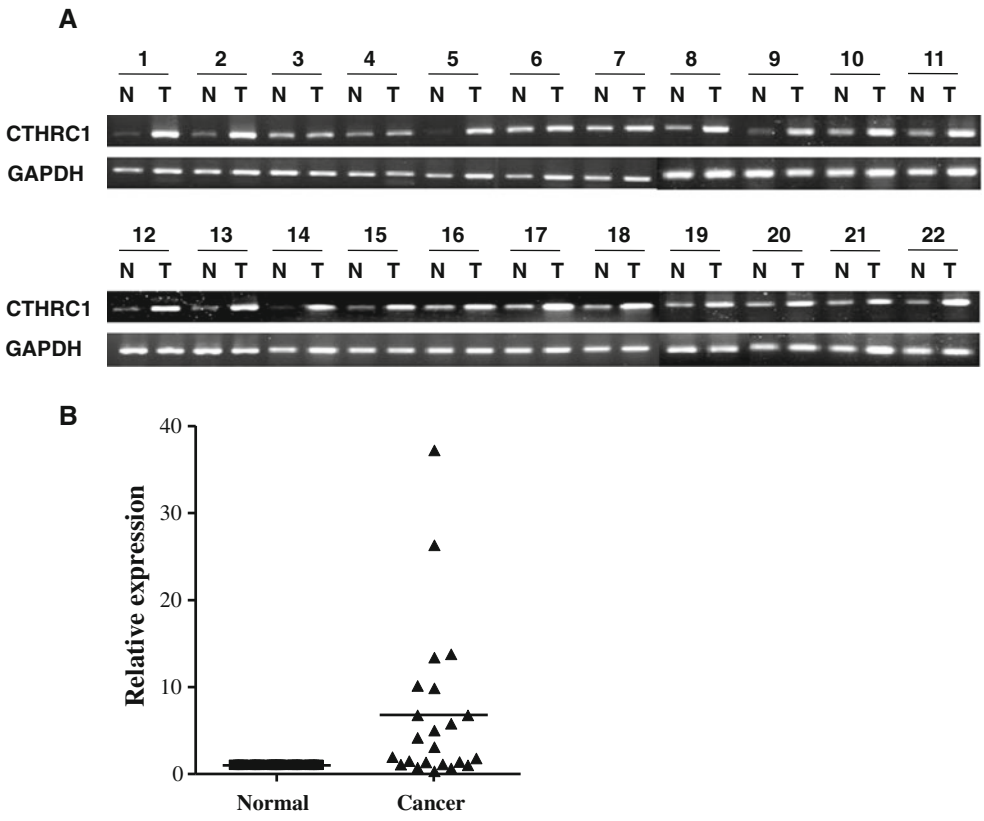

Fig. 1 Expression of *CTHRC1* in human breast tissues. **a** In RT-PCR analysis, comparative expression levels of *CTHRC1* are determined with 22 normal breast tissue (N) and breast carcinoma tissue (T) samples. Expression of the *CTHRC1* was higher in the breast carcinoma tissue than the corresponding normal tissue. **b** Real-time RT-PCR analysis was used to determine the level of relative expression of *CTHRC1* by using the ratio of *CTHRC1* to *GAPDH*. The value of tumor-originated *CTHRC1* was relative to the *CTHRC1/GAPDH* of matched normal controls

the follow-up period. At the end of the follow-up, 166 (87.8 %) patients were alive, and 23 had died. The analysis showed that a high level of CTHRC1 expression in tumor cells was associated with worse recurrence-free survival (P=0.008), shown in Fig. 3a. The mean recurrence-free survival for patients with a high level of CTHRC1 expression was 94.6 months [95 % confidence interval (95 % CI), 87.2–102.0], whereas the time was increased to 100.1 months (95 % CI, 94.3–106.0) for those with a low level of CTHRC1. But, a level of CTHRC1 expression was not statistically correlated with overall survival (Fig. 3b).

We carried out multivariate Cox proportional hazards analyses to assess the predictive value of CTHRC1 expression status for recurrence-free survival and overall survival by adjusting other potentially prognostic factors including age, histologic grade, pTNM tumor stage, and hormonal status. The results corroborated a worse recurrence-free survival outcome in patients with a high level of CTHRC1 expression (P=0.061), however, the value did not reach statistical significance at the level of 0.05. The relative risk (RR) of recurrence was about twice times greater in patients with high CTHRC1 (RR, 2.287; 95 % CI, 0.962–5.438) than

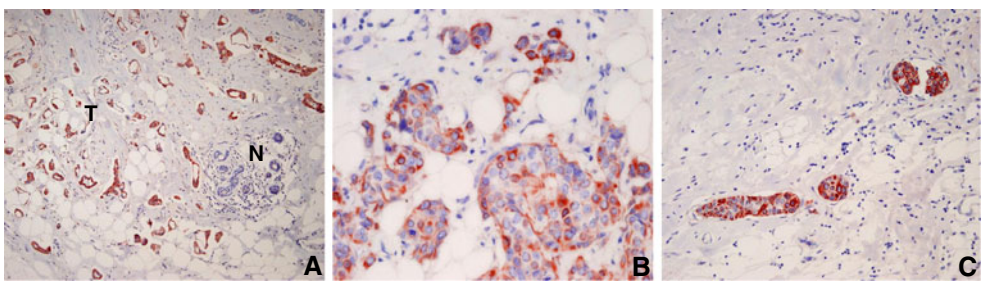

Fig. 2 Immunohistochemical expression of CTHRC1 in human breast cancer tissues. **a.** CTHRC1 was highly expressed in the breast tumor cells, but not or weakly expressed in the normal breast tissue. Original magnification: ×100. N and T represent normal and tumor tissue, respectively. **b** CTHRC1 was strongly expressed primarily in the cytosol of infiltrating tumor cells and highly at the invasive front than in inner part of the tumor. Original magnification: ×400. **c** CTHRC1 was highly expressed on tumor cells in the lymphovascular spaces; it was localized in both the cytosol and/or cell membrane of tumor cells. Original magnification: ×200

those with low CTHRC1. In a multivariate Cox regression analysis, the independent prognostic factors significantly associated with recurrence-free survival were histologic grade (P=0.028) and pTNM tumor stage (P <0.001). A level of CTHRC1 expression was not predictive of overall survival.

Table 1 Clinicopathologic variables and the expression status of CTHRC1

Characteristics	Total	CTHRC1 expression level				P
		Negative/ Low		High		
		n	%	*n*	%	
Age (years)						
<50	115	44	56.4	71	64.0	0.295
≥50	74	34	43.6	40	36.0	
Size						
≤2 cm in diameter	60	25	41.7	35	58.3	0.083
2–5 cm in diameter	116	52	44.8	64	55.2	
>5 cm in diameter	13	1	7.7	12	92.3	
Nodal status						<0.001
N0	95	61	78.2	34	30.6	
N1	59	10	12.8	49	44.1	
N2	20	2	2.6	18	16.2	
N3	15	5	6.4	10	9.0	
Histologic grade						0.026
I	37	27	21.8	20	18.0	
II	101	33	42.3	68	61.3	
III	51	28	35.9	23	20.7	
TNM stage						0.002
I	37	22	28.2	15	13.5	
II	109	47	60.3	62	55.9	
III	42	8	10.3	34	30.6	
IV	1	1	1.3	0	0	
Estrogen receptor						0.113
Negative	84	40	51.3	44	39.6	
Positive	105	38	48.7	67	60.4	
Progesterone receptor						0.075
Negative	92	44	56.4	48	43.2	
Positive	97	34	43.6	63	56.8	
Her2/neu						0.537
Negative	104	45	57.7	59	53.2	
Positive	85	33	42.3	52	46.8	
IHC-based subtype						0.620
Luminal	102	39	50.0	63	56.8	
Her2	34	16	20.5	18	16.2	
Triple negative	53	23	29.5	30	27.0	

Discussion

CTHRC1 was identified as a novel gene expressed in the adventitia and neointima on arterial injury [7], and was known as a regulator of vascular remodeling, osteoblastic bone formation, and wound repair [6, 8–10, 22, 23]. Furthermore, *CTHRC1* is up-regulated in several cancers, including breast, esophagus, gastric, and colorectal cancers [11, 12, 15–17], although the exact function of *CTHRC1* in cancer has not been elucidated. *CTHRC1* are implicated in a variety of normal and pathologic processes such as tissue injuries and cancers, supporting that tissue repair with remodeling and tumorigenesis are tightly linked.

In the present study, we have shown that mRNA of *CTHRC1* is increased in the breast cancer compare to the corresponding normal breast tissue, and over-expression of its protein level significantly correlated with nodal metastasis and TNM pathologic stage in patients with breast cancer. The immunohistochemical staining for CTHRC1 showed not only expression in the stroma but also in tumor cells with localization to the cytoplasm and/or cell membrane. Its expression in stroma and tumor cells is higher at the invasive front than in inner part of the tumor. CTHRC1 has been to be highly active and potent in degrading ECM protein and have known as a motility related gene influenced by MALAT-1 (Metastasis-associated lung adenocarcinoma transcript I) in lung cancer cells [24]. This gene is known to participate in non-canonical Wnt pathway that controls cell motility and taxis [10]. Cell surface anchored CTHRC1 can also stabilize the Wnt-Frizzled receptor interaction and selectively activates the PCP pathway of Wnt signaling [25]. Recently, interest in Wnt/PCP signaling pathway as well as tumor microenvironment in tumorigenesis has grown in the field of cancer progression. Wnt/PCP signaling pathway controls tissue polarity and cell movement, and was mainly involved in tumor dissemination and metastasis. In tumor microenviroment, the extracellular matrix (ECM), which is composed of basement membrane and interstitial connective tissue, provides a physical framework for the process of tumor cell proliferation, migration, and metastasis. An increase of a secreted glycoprotein CTHRC1 both in tumor cells and stromal cells may be facilitate tumor progression and lymph node metastasis by influencing changes in the structural composition of the stromal ECM in tumor microenviroment. Invasion and metastasis are biologic hallmarks of malignant tumors. These are the major cause of cancer-related morbidity and mortality. Therefore, CTHRC1 may be one of the tumor prognostic markers in human breast cancers.

Recent immunohistochemical study of CTHRC1 showed that the gene was aberrantly expressed in various human

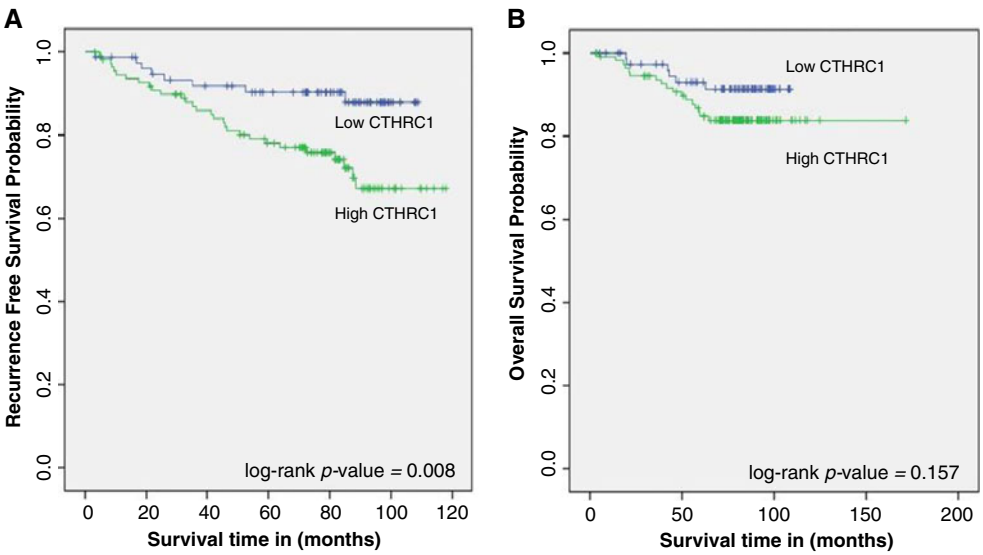

Fig. 3 Kaplan-Meier survival analysis by CTHRC1 expression status. **a** Cumulative recurrence-free survival differences between patients with high and low CTHRC1 expression. **b** Cumulative overall survival differences between patients with high and low CTHRC1 expression. The *P* value of the difference was obtained using the log-rank test of the difference

solid tumors [11–13, 17, 26]. Kharaishvili et al. reported that stromal expression of CTHRC1 was enhanced in triple negative breast cancers and also in patients with bone metastasis [17]. Its epithelial expression was negatively correlated with E-cadherin and membraneous β-catenin expression in the tumor cells. Our result showed that epithelial over-expression of CTHRC1 was correlated with high histologic grade and nodal metastasis in the breast cancers. It has been well established that the tumor cells at invasive front and metastasis are closely related to the epithelial-mesenchymal transition (EMT) and associated with Wnt/β-catenin signaling pathway [27–29]. Also, E-cadherin expression level is often inversely correlated with the tumor grade and stage [30, 31]. Therefore, we can postulate that CTHRC1 may be participated in EMT process by Wnt/β-catenin-E-cadherin regulation in breast cancer. Wang et al. have report that CTHRC1 is a positive marker for dermatofibrosarcoma protuberans, which is a locally aggressive spindle cell neoplasm that frequently recurs and can metastasize [26]. Taken together, these results suggest that CTHRC1 may be involved to tumorigenesis and contribute to a more aggressive biologic behavior of tumor cells.

In the present study, we demonstrated that there are significant correlation between *CTHRC1* over-expression in tumor cells and adverse recurrence-free survival rates in patients of breast cancers. Our results suggest that the over-expression of *CTHRC1* may indicate a poor clinical outcome. Taken together, *CTHRC1* over-expression in breast cancer may play important roles in tumor progression and metastasis, and would predict adverse clinical outcome.

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Conflict of interest The authors declare that they have no competing financial interests.

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