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Original Article

CTHRC1 as a novel biomarker in the diagnosis of cervical squamous cell carcinoma

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Abstract: To verify whether the collagen triple helix repeat containing 1 (CTHRC1) can be used as a potential biomarker in diagnosis of cervical cancer, by evaluating the expression level of CTHRC1 in cervical squamous cell carcinoma patients. In this study, CTHRC1 expression in cervical squamous cell carcinoma, CIN (cervical intraepithelial neoplasia) and healthy cervical squamous epithelium were measured by immunohistochemistry. The serum levels of CTHRC1 and SCC-Ag within the all three groups were performed using ELISA. In addition, the ROC curve of CTHRC1 and SCC-Ag as well as combined CTHRC1 and SCC-Ag was demonstrated and analyzed. CTHRC1 was significantly overexpressed in cervical squamous cell carcinoma compared with CIN and healthy control group. And CTHRC1 concentration in serum of cervical squamous cell carcinoma group was also remarkably higher than that in other two groups. The ROC curve showed AUC of CTHRC1 and SCC-Ag was 0.665±0.034 and 0.878±0.027, the sensitivity of them were 57.1% and 77.6%, and the specificity of them were 85.4% and 86%, respectively. Furthermore, AUC of combined CTHRC1 and SCC-Ag was 0.879±0.027, sensitivity was 87.2% and specificity were 84%. Our study indicated that CTHRC1 was highly upregulated not only in the tissue but also in the serum of cervical squamous cell carcinoma patients, which pointed out it can be used as a novel prognostic and metastatic biomarker of cervical squamous cell carcinoma. And combined SCC-Ag and CTHRC1 serological detection may have potential value in the early diagnosis of cervical squamous cell carcinoma and CIN.

Keywords: Collagen triple helix repeat containing 1 (CTHRC1), squamous cell carcinoma antigen (SCC-Ag), cervical squamous cell carcinoma, ELISA

Introduction

Cervical cancer is the most common gynecologic cancer and the leading cause of death in gynecologic malignancies worldwide. According to the latest report from the International Agency for Research on Cancer (IARC), new cases of the disease may reach up to 12820 and the death is about 4210 in USA 2017 [1]. Until now, the pathogenesis of cervical cancer is still unknown. A large number of studies have shown that the main cause of cervical squamous cell carcinoma and middle cervical intraepithelial neoplasia (CINII/III) is relapsing and persistent infection of high-risk HPV, however one single HPV infection is not enough to turn the host epithelial cells into cancer cells [2, 3]. Additionally, there is lack of remarkable biomarker similar to AFP or CA 125 in the cervical cancer, which can be using for the clinical diagnosis.

Recently, the relationship between tumor microenvironment and occurrence as well as development of cervical cancer has attracted more attention. Among of the microenvironment components, extracellular secreted proteins have various crucial roles on activating the downstream signaling pathway and promoting tumor proliferation, invasion, matrix reconstruction, dissemination and so on [4]. And emerging studies have shown that they are also notable molecular biomarkers on many cancer diagnoses. Collagen triple helix repeat containing 1 (CTHRC1) is an extracellular secreted protein that involved in wound repair, bone remod-

eling, liver fibrosis, and adipose tissue formation and other progressions. High CTHRC1 expression has been found in many advanced cancers and is considered to be an indicator of poor prognosis. For example, CTHRC1 can be detected in primary invasive and metastatic melanoma, but not in benign or non-invasive samples, and inhibition of CTHRC1 reduced migration of melanoma cell [5]. In addition, CTHRC1 expression in pancreatic carcinoma was significantly higher than in normal or precancerous tissue, and it could regulate pancreatic cancer cell adhesion activity and thus play a role in tumor progression and metastasis [6].

However, the investigation of CTHRC1 in cervical cancer especially in assessing as a tumor biomarker is still in the initial stage. Our previous results showed that CTHRC1 and HPV E6/E7 synergistically promote proliferation and invasion of cervical cancer cell [7]. To further determine whether CTHRC1 could be used as a diagnosis biomarker of cervical cancer, base on expanded samples, we firstly detected CTHRC1 expression level in cervical squamous cell carcinoma, CIN and normal tissue by immunohistochemistry, and analyzed the relationship between CTHRC1 expression and clinicopathological features in cervical squamous cell carcinoma. Then we detected and analyzed CTHRC1 and SCC-Ag concentrations in serum of cervical squamous cell carcinoma patients to evaluate whether CTHRC1 can be used as a tumor biomarker in the diagnosis and prognosis of cervical squamous cell carcinoma.

Materials and methods

Patients

A total of 334 cervical squamous cell carcinoma tissue samples were obtained between 2005 and 2016 from Department of Obstetrics and Gynecology, Fengxian Hospital, Changzhou 2nd People's Hospital, the Department of Obstetrics and Gynecology, Changzhou Maternal and Child Care Hospital, Jinhua Central Hospital. The cases of cervical squamous cell carcinoma were collected in this study only if clinical data were available and follow up was obtained. All patients underwent a modified radical or complete hysterectomy, bilateral salpingo oophorectomy and pelvic lymphadenectomy with or without para-aortic lymph node sampling. None of the selected patients had received chemotherapy, radiotherapy, hormone therapy or other related anti-tumor therapies prior to surgical excision. 100 cases of healthy cervical epithelium and 96 cervical intraepithelial neoplasia (CIN) were selected as controls at same time for immunohistochemical analysis. In addition, serum samples were collected from 102 healthy people, 108 CIN and 175 cervical squamous cell carcinoma patients for ELISA analysis. All samples were obtained with informed consent and all procedures were performed in accordance with the Human Investigation Ethical Committee of the four hospitals.

Tissue microarrays

Core of carcinoma (diameter 2 mm) from individual cervical squamous cell carcinoma biopsy specimens (donor blocks) were transferred into recipient paraffin blocks (tissue assay blocks). The control specimens were also arranged into each of the assay blocks.

Immunohistochemistry

Four-micron-thick sections were cut from tissue microarrays and incubated with 0.3% H_2O_2/PBS for 30 minutes, then blocked with 10% BSA (Sangon, Shanghai, China) for 30 minutes. The sections were detected with primary polyclonal antibody for CTHRC1 (Huamei Biological Engineering Co., Ltd. CUSABIO Wuhan, China), overnight at 4°C in a moist chamber, followed by an incubation with the secondary antibody (Thermo Scientific, US) labeled with HRP (rabbit) for 1 hour at room temperature, then the microarrays sections were treated with diaminobenzidine and counterstained with hematoxylin. Finally, all the microarrays sections were observed and photographed with a microscope (Carl Zeiss). Scoring was conducted according to the ratio and intensity of positive-staining cells: 0-10% scored 0, 11-30% scored 1, 31-60% scored 2, 61-100% scored 3. Then scored 0-1 was designated as low expression and scored 2-3 as high expression. All the CTHRC1 expression levels were quantified double-blindly by two independent pathologists.

ELISA

The concentration of serum CTHRC1 was determined using a sandwich technique of a commercial ELISA kit (Cusabio Biotech Co. Ltd) and

Table 1. Age factor in each group

Content		Cervical squamous carcinoma	CIN	Normal cervical squamous epithelium
Cases	N	334	96	100
Age (year)	Mean (Std)	47.449	38.82	42.57
	Min, Max	21, 79	22, 66	20, 70

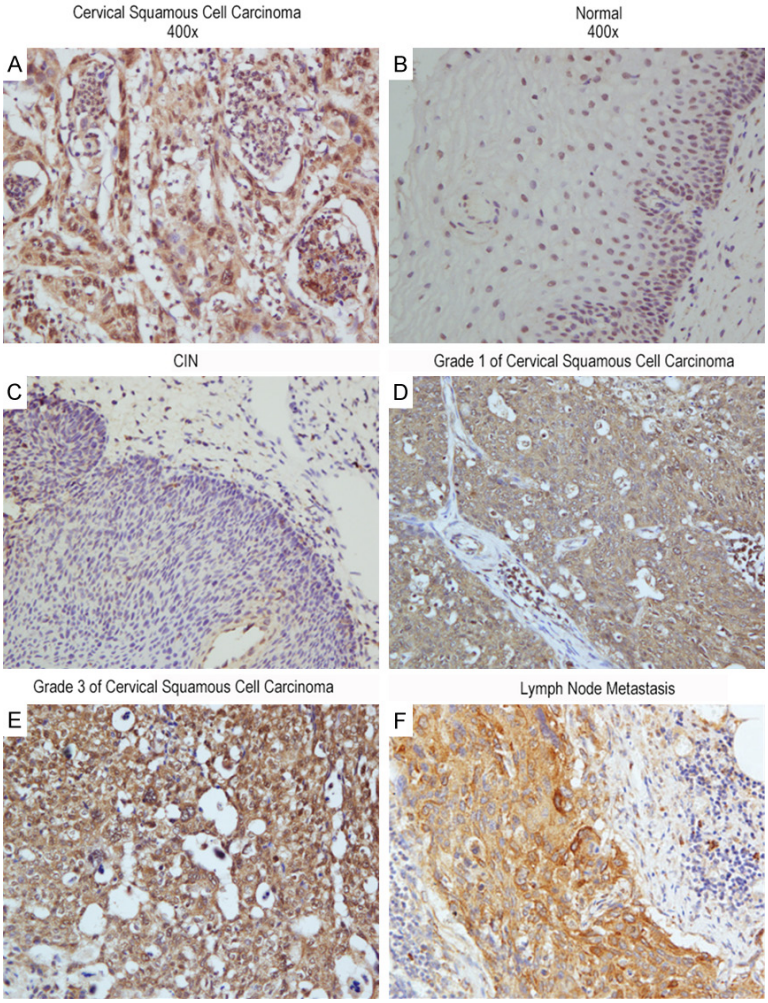

Figure 1. Immunohistochemical expression of CTHRC1 in cervical squamous cell carcinoma, healthy group and CIN. A. High expression of CTHRC1 in cervical squamous cell carcinoma (brown colour). B, C. Low expression of CTHRC1 in healthy group and CIN. D, E. High expression of CTHRC1 in Grade 1 and Grade 3 of cervical squamous cell carcinoma. F. High expression of CTHRC1 in metastatic lymph node (original magnification ×400).

Table 2. Comparison of CTHRC1 expression between cervical cancer, CIN and controls

Contents	Low	High	Total	X^2	*P*-value
Cancer	159	175	334	26.53303	<.0001
CIN	77	19	96	8.372	0.004*
Normal	94	6	100		0.0671*

Note: *0.004 was compared with cervical squamous cell carcinoma and CIN, 0.0671 was CIN and normal squamous epithelium.

the dosage of serum soluble SCC-Ag was also measured with an ELISA kit (Cusabio Biotech Co. Ltd) according to manufacturer's instructions.

Statistical analyses

Data were shown as the means ± SEM (standard error of the mean). The chi-square was used to analyze the correlations between CTHRC1 expression and clinicopathologic features in patients with CIN or cervical squamous cell carcinoma. The student's t-test was used for comparison between different groups. The accuracy of CTHRC1, SCC-AG and CTHRC1 combined SCC-Ag was evaluated by using the ROC (receiver operating characteristics) curve analysis. Statistical analyses were done using SPSS 17.0 for windows (IBM). Values of $P<0.05$ were considered statistically significant.

Results

CTHRC1 expression in cervical squamous cell carcinoma, CIN and normal cervical tissue

Three groups of patients and volunteers are involved in this study. First group is 334 cases with cervical squamous carcinoma, average age is 47.449 (range 21-79 years old), Next is 96 cases with CIN, average age of 38.82 (range 22-66 years old). The control group is 100 cases with healthy cervical squamous epithelium, average age is 42.57 (range 20-70 years old). There was no clear difference in age between all three groups, as shown in **Table 1**.

The expression of CTHRC1 was detected in all three groups by immunohistochemistry. High CTHRC1 expression rate was 52.3% (175/334) in cervical squamous cell carcinoma group

Table 3. Relationship between CTHRC1 and clinicopathological parameters of cervical squamous cell carcinoma

Contents		Low expression	High expression	Total	X^2	*P*-value
Stage Clinical stages	Stage 1	149	127	276	25.943	<0.01
	Stage 2+3	10	48	58		
Pathology grade	Grade 1	38	15	53	17.109	<0.01
	Grade 2	101	116	217		
	Grade 3	44	20	64		
Lymph node metastasis	Yes	20	83	103	47.439	<0.01
	No	139	92	231		
Lymph vessel invasion	Yes	39	85	124	20.631	<0.01
	No	120	90	210		
Stromal	>1/2	106	151	257	18.076	<0.01
	≤1/2	53	24	77		
Tumor Size	≤4 cm	100	81	181	9.256	0.002
	>4 cm	59	94	153		
Age (years)	≤45	61	79	140	1.572	0.210
	>45	98	96	194		

(**Figure 1A**), 6.0% (6/100) in healthy group (**Figure 1B**), which showed significant difference (P<0.0001). Moderate CTHRC1 expression rate was 19.8% (19/96) in CIN (**Figure 1C**), significantly lower than cervical squamous cell carcinoma group (P=0.004). But no significantly different between CIN and normal groups (P=0.0671). See **Table 2**.

Relationship between CTHRC1 level and clinicopathological parameters in cervical squamous cell carcinoma

High CTHRC1 expression rate was 46% (127/276) in stage 1 of cervical squamous carcinoma, 82.7% (n=48/58) in stages 2 and 3, which showed significant difference (P<0.01). CTHRC1 expression was also correlated with the grade of tumor pathology, high expression rate was 28% (15/53) in Grade 1, 54% (116/217) in Grade 2 and 31% (20/64) in Grade 3 (**Figure 1D**, **1E**). The difference was significant (P<0.01). Significant difference in CTHRC1 expression was also observed on the lymph node metastasis, which is high CTHRC1 expression rate was 68.5% (83/103) with lymph node metastasis (**Figure 1F**) and 39.8% (92/231) without lymph node metastasis (P<0.01). In addition, CTHRC1 expression was also related to lymph vessel invasion, stromal infiltration depth and tumor diameter (**Table 3**).

The concentrations of CTHRC1 and SCC-Ag in serum of cervical cancer, CIN, and healthy subjects

The CTHRC1 concentration in cervical cancer patients was also one of the main targets of our study. As showing in **Table 4**, the sera were collected from 102 healthy persons (control group), 108 CIN and 175 cervical cancer patients. ELISA analysis indicated that there was significant difference between cervical cancer patients and healthy human as well as CIN (P<0.001 and P<0.01 respectively), but no significant difference between CIN and the control group (P>0.05).

We further detected serum SCC-Ag levels as it is a common tumor biomarker in cervical squamous cell carcinoma. The results show that the concentration of serum SCC-Ag of cervical cancer group was significantly increased compared with CIN and normal persons (both P<0.01), but no clear difference between CIN and control group (P>0.05).

ROC curve of CTHRC1 and SCC-Ag in cervical squamous cell carcinoma

To address whether serum CTHRC1 could be recognized as a biomarker in the diagnosis of cervical cancer, ROC curve was drawn according to serum CTHRC1 and SCC-Ag concentrations with cervical squamous cell carcinoma patients.

The results showed that AUC was 0.665±0.034 by using CTHRC1 in diagnosis of squamous cell carcinoma of the cervix, the diagnosis critical value was 9.6 ng/ml using Youden's index method with the sensitivity of 57.1% and specificity of 85.4%. The AUC was 0.878±0.027 by using SCC-Ag in diagnosis of squamous cell carcinoma of the cervix, the diagnosis critical

Table 4. Comparison of CTHRC1 and SCC-Ag levels in serum of 3 groups

Group	Cases	CTHRC1 (ng/ml)	*P*-value	SCC-Ag (ng/ml)	*P*-value
Normal	102	8.031±3.864	0.055*	0.9922±.9922	0.2996*
CIN	108	8.303±3.827	0.0008	1.1779±0.9633	0.0003
Cancer	175	11.791±9.416	0.00073	6.5528±9.4017	<0.0001

Note: *0.055 and *0.2996 are normal group compared with CIN.

value was 1.485 ng/ml with the sensitivity of 77.6% and specificity of 86%. However, when combined CTHRC1 and SCC-Ag, the AUC was 0.879±0.027, and the diagnosis critical value decreased to 0.502 ng/ml with the sensitivity up to 87.2% and specificity of 84%, as shown in **Table 5**; **Figure 2**.

Discussion

Tumor biomarkers are chemical substances that reflect the occurrence of tumors and studying them is one of the hottest topics in the field of cancer research [8, 9]. They can be recognized by immunohistochemical or serological strategies and the former is the most common approach using for prognostic prediction. They either significantly overexpress in tumor tissue or remarkably indicate one specific property of the cancer. Therefore we can investigate the pathology of tumor, as well as differentiation and dysfunction of cancer cells, which can be a key contribution of diagnosis, classification, prognosis judgment and treatment guidance of carcinoma. Various immunohistochemical markers of cervical cancer had been reported by previous studies [10-12] which might be associated with relapse and prognosis of cancer, however these markers still have a large limitation in clinical applications. Therefore, to a great extent, clinicians still rely on CT, MRI or PET-CT and other imaging modalities for diagnosis and determination the relapse and metastasis of tumor diseases. It is urgent that how to precisely evaluate the relapse and metastasis of cervical cancer and how to estimate the prognosis of the disease pre- and post-operation objectively.

The extracellular secreted protein CTHRC1 is highly expressed in many organs during embryonic period [13] and its expression is very low in normal adult tissues. However, in the pathological situations, the level of CTHRC1 is significant upregulated. For instance, overexpression of CTHRC1 is been detected in non-small cell lung cancer, colorectal cancer and gastric cancer [14-16]. It has also been reported that CTHRC1 could promote the invasion and metastasis of a variety of tumors, Truly, CTHRC1 was found to be associated with the risk of tumor cell metastasis in osteosarcoma [17]. In addition, overexpression of CTHRC1 in hepatocellular carcinoma (HCC) is often associated with advanced or late phase of cancer and multivariate analysis has already shown that CTHRC1 is an independent prognostic factor for HCC [18]. CTHRC1 was also reported to contribute to the migration and invasion of tumor cells in ovarian cancer by activating the EGFR signal [19].

It has been reported that cervical lymph node metastasis, tumor diameter, lymphatic invasion are independent risk factors for cervical cancer [20]. Among prognostic factors of cervical cancer, the most important one is the clinical stage of FIGO [21]. Cervical cancer lymph node metastasis predicts poor prognosis as well as relapsing and is also one of the most important prognostic factors [22]. However, they are all histological prognostic factors, which have innate limitations on assessing the prognosis of cervical cancer. CTHRC1 level was significantly higher in cervical squamous cell carcinoma, suggesting that CTHRC1 may be involved in development of CIN and in transformation of health cervical squamous epithelium to cervical squamous cell carcinoma. The high expression of CTHRC1 was positively correlated with clinical stage, lymph node metastasis, vascular invasion, cervical matrix infiltration depth >1/2 and tumor diameter >4 cm. That is, CTHRC1 expression is consistent with the poor prognostic factors of cervical squamous cell carcinoma, and also with previous studies which mentioned CTHRC1 is upregulated in many other malignant tumors [23]. The results above suggest that CTHRC1 may be involved in the occurrence, development and metastasis of cervical squamous cell carcinoma, which in protein level is further evidence of our previous studies [7]. Thus, we speculate that CTHRC1 can be used as an immunohistochemical marker to assess the prognosis and relapsing of cervical squamous cell carcinoma.

Table 5. The ROC related parameters of CTHRC1 and SCC-Ag separately in cervical cancer patients

	Critical value	AUC	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Youden index
CTHRC1	9.608	0.665±0.034	57.1%	85.4%	72.14	48.89	0.425
SCC-Ag	1.485	0.878±0.027	77.6%	86.0%	77.80	86.00	0.636
CTHRC1+SCC-Ag	0.502	0.879±0.027	87.2%	84.0%	87.29	84.00	0.712

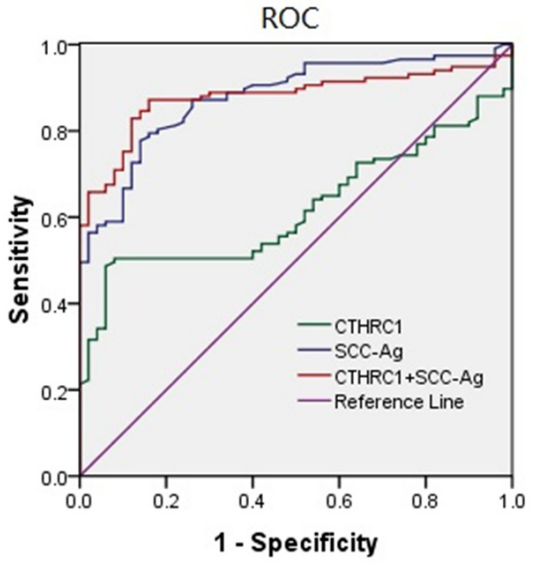

Figure 2. ROC curves for CTRHC1, SCC-Ag, and CTHRC1 combined SCC-Ag.

In the diagnosis of early stage of tumor, serum immunological detection has great advantages including small trauma, large flux, easy operation and economy. Thus it is suitable for screening the patients, such as detecting of alpha-fetoprotein (AFP) and CA125, which has already used in clinics [24, 25]. To get precisely clinical evaluation of relapsing and prognosis, especially at the time points of diagnosis, post-operation as well as during the follow up, specific serum biomarkers are needed to be identified within different stages of cervical cancer. To this end, we detected the concentration of CTHRC1 in serum via ELISA as extracellular secreted proteins can be detected in serum. The results showed that CTHRC1 concentration in cervical squamous cell carcinoma patients serum were significantly higher than in serums of the healthy and CIN controls (**Table 4**), indicating that CTHRC1 could be treated as a serum biomarker for monitoring the transformation of precancerous lesions to invasive cancer and for assessing the prognosis of cervical squamous cell carcinoma.

In order to further authenticate CTHRC1 as serum biomarker of cervical cancer, serological examination of SCC-Ag of the subjects and comparison of sensitivity and specificity of CTHRC1 and SCC-Ag in serum via ROC were performed. The results showed that the AUC of CTHRC1 itself was 0.665±0.034, the specificity was 85.4% and the sensitivity was 57.15%. However, the combined AUC of CTHRC1 and SCC-Ag was 0.879±0.027, the sensitivity was 87.2% and the specificity was 84%. SCC-Ag is one of the most commonly used diagnostic markers for cervical cancer [26]. It has been reported that serum SCC-Ag levels are highly correlated with the development of cervical cancer and with further transformation of cervical invasive carcinoma [27, 28]. This study indicated that CTHRC1 was a potential candidate of serum biomarker on early diagnosis and prognosis of cervical squamous cell carcinoma. Furthermore, combination of CTHRC1 with SCC-Ag can improve sensitivity and specificity of the diagnosis of cervical precancerous lesions as well as cervical squamous cell carcinoma.

In summary, immunohistochemical CTHRC1 was associated with clinical stage, lymph node metastasis, vascular invasion, tumor grade, cervical matrix infiltration depth and tumor size of cervical squamous cell carcinoma, which can be used as one of the best biomarker for cervical cancer prognosis. Additionally, serum concentration of CTHRC1 was significantly increased in cervical squamous cell carcinoma patients. And sensitivity and specificity of diagnosis on early disease stage was positively improved when combined CTHRC1 and SCC-Ag. Taken together, our study for the first time pointed out CTHRC1 can be used as immunohistochemical and serum biomarker of cervical squamous cell carcinoma, which provides the basis for individualized treatment and evaluation of cervical squamous cell carcinoma prognosis as well as relapsing.

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Disclosure of conflict of interest

None.

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