Original Article

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Correlation between reticulum ribosome-binding protein 1 (RRBP1) overexpression and prognosis in cervical squamous cell carcinoma

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SUMMARY Our purpose was to evaluate the correlation between endoplasmic reticulum ribosomal binding protein 1 (RRBP1) expression in cervical squamous cell carcinoma (CSCC) and poor patient prognosis. RRBP1 is a nascent transporter that is situated on the rough endoplasmic reticulum (ER). It adjusts to the secretion of proteins in cells and alleviates ER stress, thus stimulating cell proliferation. An immunohistochemical (IHC) study was conducted to detect the expression level of RRBP1 on 96 CSCC tissue samples. Western blot and Quantitative real-time polymerase chain reaction (qRT-PCR) were performed to compare the expression levels of RRBP1 in cervical squamous cell carcinoma with healthy cervical tissues. An overexpression of RRBP1 was observed in CSCC tissues, and the expression level was associated with FIGO stage (Stage I *vs.* II: 52.6% *vs.* 74.1%, $p = 0.030$), and lymph node metastasis (No *vs.* Yes: 61.5% *vs.* 92.3%, $p = 0.031$) but not patient age and tissue differentiation. Univariate survival analysis indicated that prognosis was associated with the expression level of RRBP1 and tissue differentiation and lymph node metastasis. Analysis of the multi-factor survival Cox model proved that RRBP1 was an independent prognostic factor. In conclusion, compared with healthy cervical tissues, RRBP1 was overexpressed in CSCC tissues, illustrating that RRBP1 may be a new biomarker for the diagnosis of CSCC. The study on RRBP1 may contribute to exploring the pathogenesis of CSCC and may also guide targeted therapy for CSCC in the future.

Keywords cervical squamous cell carcinoma (CSCC), endoplasmic reticulum ribosome binding protein 1 (RRBP1), diagnosis, prognosis

1. Introduction

Cervical cancer (CC) is the fourth most common cancer in women and the fourth leading cause of cancer-related death (*1*). With the popularity of cervical cancer screening and the application of the human papilloma virus (HPV) vaccine in recent years, its incidence and mortality are declining, but the 5-year overall survival (OS) rate for advanced CC patients remains at only 52% and still plagues women (*2*). In recent years, individualized treatment based on gene targets has become a trend, and it has been proven that MALAT1 is involved in the development of CC (*3*). Although the discovery of these genes has certain significance for the early diagnosis and personalized treatment of CC, some of these diagnostic techniques or treatments cannot be widely applied to the clinic due to low accuracy or high expense. Cervical squamous cell carcinoma (CSCC) is the most common pathologic type of CC (*4*). Therefore, the identification of high-precision CSCC molecular markers is imperative, which will guide the early diagnosis and treatment of CSCC.

Endoplasmic reticulum ribosomal-binding protein 1 (RRBP 1) is a transport protein with a molecular weight of 180 KD that is located on the rough endoplasmic reticulum (ER) membrane (*5,6*). Its most notable feature of primary structure is a highly conserved sequence containing 10 amino acids that is repeated 54 times for a series near the NH2 end of the protein (*7*). According to previous studies, we found that low RRBP1 expression is related to deposition of the extracellular matrix in myogenic progenitor cells (*8*). RRBP1 plays an important role in intestinal maturation and can be expressed during osteoblast differentiation and at neuromuscular junctions (*9,10*). In recent years, RRBP1 has been determined to be overexpressed in lung cancer and involved in the mRNA stability control of unfolded protein response (UPR) components, thus diminishing ER stress and assisting tumor cell survival (*11,12*). In addition, we determined that RRBP1 was

related to multiple cancers, such as, liver cancer, prostate cancer, colorectal cancer, lung cancer, breast cancer, esophageal cancer, endometrial cancer and ovarian cancer according to previous studies (*11,13-19*). All of the above findings suggest that RRBP1 may be related to the proliferation of tumor cells, indicating that it may also act as a new biomarker and become a new target for therapy of malignant tumors.

However, few studies have explored expression levels of RRBP1 in CSCC, and the relationship with clinicopathological features. Our study aimed to investigate whether RRBP1 was expressed in CSCC using Immunohistochemical (IHC), Western blot and Quantitative real-time polymerase chain reaction (qRT-PCR) methods.

2. Materials and Methods

2.1. Patients and clinical samples

A total of 96 CSCC tissue samples were collected from 96 patients who had undergone cervical cancer stage surgery at Department of Gynecology Harbin Medical University Cancer Hospital between January 10, 2010 and November 20, 2012. None of these patients had any therapy before surgery, including immunotherapy, chemotherapy, or radiotherapy. The paraffin-embedded sections of the 96 clinical samples were made for IHC analysis after fixation, dehydration, transparency, wax transparency, embedding, sectioning, patching, staining, transparency, and paraffin embedding. The clinical pathological features of the patients enrolled were obtained from the medical record system of the hospital. The patients were followed from the day of surgery until November 30, 2018 (the follow-up period was 11-105 months, average 81 months). We also collected 36 fresh surgical specimens for Western blot and qRT-PCR analysis at Harbin Medical University Cancer Hospital from November 2018 to May 2019, including 10 normal cervical tissues and 26 CSCC tissues. The study was approved by the Harbin Municipal Ethics Committee and all enrolled participants have signed informed consent after being fully informed.

2.2. IHC

First, the reagents were prepared according to the manufacturer's instructions. Then the 96 paraffin-embedded sections were treated with antigen retrieval and serum blocking. The primary anti-RRBP1 antibody (1:1,000, Abcam, Ab95983, UK) was added to all samples and then incubated at 4°C overnight. After rinsing with phosphate buffered saline (PBS), the biotin-labelled secondary antibody (goat anti-rabbit lgG-HRP, Wanleibio, WLA023, China) was added to samples and incubated at 37°C for 30 minutes. After staining all samples with Diaminobenzidine (DAB) chromogen, the samples were incubated at room temperature for 1 hour. Finally, the samples were counterstained with hematoxylin after rinsing with PBS. The negative control is a diluent.

2.3. IHC result judgment

IHC results were evaluated by two pathologists and RRBP1 staining was analyzed by semi-quantitative methods. The intensity was scored as follows: colourlessness (0), light yellow (1), brownish yellow (2), and brown (3). The percentage of positive cells was scored as follows: 0 indicates < 5%, 1 indicates 5-25%, 2 indicates 26-50%, 3 indicates 51-75%, and 4 indicates > 75%. The final score was evaluated by multiplying the above two scores together, and a score ≥ 4 was considered overexpression and a score < 4 was considered low expression.

2.4. Western blot analysis

First, the reagents and polyacrylamide gels were prepared according to manufacturer's instructions. For protein extraction, the lysate (containing 1% PMSF) was aliquoted on the basis of the demands of the experiment and added to each sample. The lysate was centrifuged at 12,000 rpm for 5 minutes at 4°C. Then, total protein was quantified using a BCA protein concentration determination kit (Wanleibio, WLA004, China). The complex protein mixture was separated using Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (Wanleibio, WLA013, China) and transferred to a Polyvinylidene difluoride (PVDF) membrane (Millipore, IPVH00010, USA) using normal methods. The primary RRBP1 antibody (1:1,000) was added to 36 samples which were then incubated at 4°C overnight. Then, the secondary antibody, goat anti-rabbit lgG-HRP (1:5,000), was added and incubated for 45 minutes at 37°C. Finally, the chemiluminescent reagent was added to the membrane and slowly shaken. The membrane was placed on X-ray film in a dark room before final development, and the exposure time was adjusted according to the strength of the signal. Gel-Pro-Analyzer software was used to analyze the optical density value of the target band. A β-actin antibody (Wanleibio, WL01845, China) was used as an internal reference antibody.

2.5. Real-time PCR analysis

First, mRNA was extracted from 36 samples according to instructions, and the concentration of RNA was measured using a NanoDrop 2000 UV spectrophotometer (NanoDrop 2000, Thermo, USA). cDNA was then synthesized in a PCR instrument (Real-Time PCR, Exicycler 96, BIONEER, Korea) using Super M-MLV reverse transcriptase (BioTeke, PR6502, Beijing), and the products underwent quantitative fluorescence analysis

with the $2^{-\Delta\Delta Ct}$ method. The RRBP1-F primer sequence is 5'-TCCATCCAGAGTCTCACTTC-3', and the RRBP1-R primer sequence is 5'-GCCCTCGTTGAACACCAT-3'. The GAPDH-F primer sequence of is 5'-GGCACCCAG CACAATGAA-3', and the GAPDH-R primer sequence of is 5'-TAGAAGCATTTGCGGTGG-3'.

2.6. Statistical analysis

The IHC results were analyzed using the chi-square test. The Western blot result was assessed by the gray value of the electrophoretic band, which was analyzed by GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, California, United States) and plotted as a peak curve where the peak area represented protein concentration. Finally, the results were plotted as a histogram. The PCR results were analyzed by the $2^{-\Delta\Delta Ct}$ method, and the products of PCR were subjected to quantitative fluorescence analysis. Finally, the results were drawn into a histogram by GraphPad Prism. OS and DFS of all samples were estimated by Kaplan-Meier method, and were tested by the log-rank test. Finally, multivariate analysis was conducted with Cox regression models (proportional risk models). A P value < 0.05 was deemed statistically significant. All the above data analyses were performed using Windows SPSS software V25.0 (IBM SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Patient pathological characteristics

To analyze the immunity of RRBP1 in all tissue samples, we examined 96 untreated CSCC tissue samples by IHC. As shown in Table 1, 45 were obtained from patients > 49 years old, and 51 were ≤ 49 years old. Of the 96 patients, 38 patients were in stage I (according to FIGO cervical cancer staging in 2009), and 58 patients were in stage II.

Table 1. Association analyses between the expression levels of RRBP1 and the clinicopathological characteristics of Cervical squamous cell carcinoma (CSCC)

Variables	Patients (*n*)	RRBP1 expression		P^a
		Low	High	
Age (years)				0.667
> 49	45	14	31	
≤ 49	51	19	32	
FIGO stage				0.030
I	38	18	20	
II	58	15	43	
Histological grade				0.071
G1	23	12	11	
G2	65	20	45	
G3	8	1	7	
lymph node metastasis				0.031
No	83	32	51	
Yes	13	1	12	

G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; [a]Chi-square test.

3.2. Expression of RRBP1 was extremely obvious in CSCC

We analyzed 26 fresh CSCC specimens by Western blot and qRT-PCR and compared them with 10 normal cervical specimens, and the results showed that the expression of RRBP1 increased obviously at both the protein (Figure 1B, $p < 0.001$) and mRNA level (Figure 2, $p < 0.001$) in CSCC tissue.

To analyze the association between the expression of RRBP 1 and the pathological features of CSCC patients, we performed an IHC analysis on 96 samples. The IHC outcomes showed that RRBP1 was situated in the cytoplasm of CSCC (Figure 3). As shown in Table 1, there were 33 patients with low RRBP1 expression and 63 patients with high RRBP1 expression. Moreover, in CSCC tissue, the high expression of RRBP1 was related to FIGO stage (Stage I *vs.* II: 52.6% *vs.* 74.1%, $p = 0.030$), and lymph node metastasis (No *vs.* Yes: 61.5% *vs.* 92.3%, $p = 0.031$), but not patient age ($p = 0.667$) and tissue differentiation ($p = 0.071$) (Table 1).

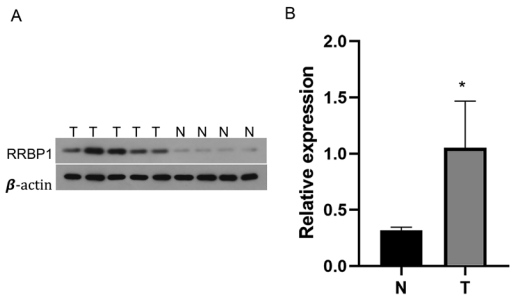

Figure 1. (A), Representative protein samples obtained from frozen normal cervical tissues (N) and cervical squamous cell carcinoma tissues (T) were analyzed by Western blot. The levels of β-actin were used as an internal control; **(B)**, Histogram of pooled data from N (n = 10) and cervical squamous cell carcinoma cells (CSCCs) ($n = 26$**).** RRBP1 expression was elevated in CSCCs compared with that in N. The data are presented as the mean ± SD ($^*p < 0.001$).

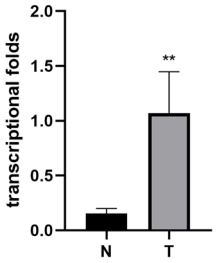

Figure 2. Histogram of RRBP1 mRNA expression in normal cervical tissues and cervical squamous cell carcinoma tissues (N, normal cervical tissues; T, cervical squamous cell carcinoma tissues). The levels of β-actin were used as an internal control, and the RRBP1 mRNA expression was calculated by $2^{-\Delta\Delta Ct}$ method. RRBP1 mRNA expression was elevated in CSCCs compared with normal cervical tissues. The data are presented as the mean ± SD ($^{**}p < 0.001$).

3.3. Association between overexpression of RRBP1 in CSCC and patient unfavorable prognosis.

We analyzed the OS and DFS of 96 patients using the Kaplan-Meier method. The results showed that high RRBP1 expression significantly shortened OS (Figure 4A, $p = 0.018$) and DFS (Figure 4B, $p = 0.008$). Univariate survival analysis indicated that RRBP1 high expression and tissue differentiation and lymph node metastasis were related to unfavorable prognosis of CSCC (Table 2); Table 2 manifests OS ($p = 0.001$) and DFS ($p < 0.001$) of patients with RRBP1 overexpression, and OS ($p = 0.045$) and DFS ($p = 0.016$) of patients with lymph node metastasis, and OS ($p = 0.010$) and DFS ($p = 0.003$) of patients with tissue differentiation.

Furthermore, multivariate survival evaluation was conducted with Cox regression models. RRBP1 is an independent prognostic factor. The estimation of OS (95% Cl = 1.305 -73.315, $p = 0.026$) and DFS (95% Cl = 1.712- 95.598, $p = 0.013$) is shown in Table 3.

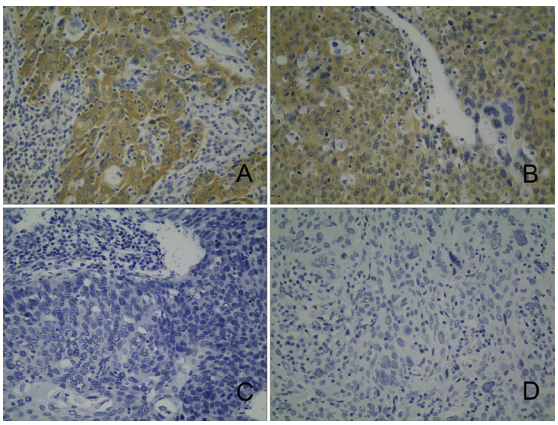

Figure 3. Immunohistochemical staining of RRBP1 in CSCC specimens. A and B, High expression of RRBP1 in CSCCs; C and D, Low expression of RRBP1 in CSCCs.

4. Discussion

In this study, we analyzed the association between the expression level of RRBP1 in CSCC and patient prognosis. It seems to be the first published assessment

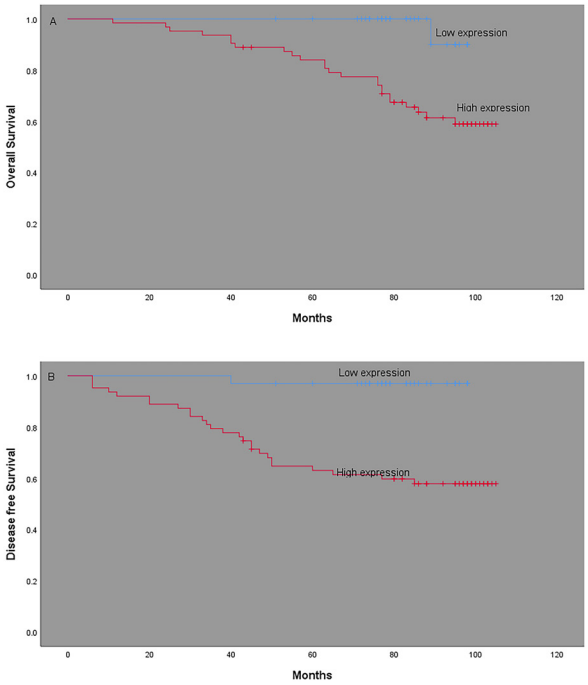

Figure 4. Kaplan-Meier analysis of overall survival and disease-free survival related to expression of RRBP1. Patients with high expression of RRBP1 had a poorer prognosis than those with low expression of RRBP1. **(A),** overall survival curves of CSCC according to their RRBP1 expression status, ($p = 0.018$); **(B)**, disease-free survival curves of CSCC patients according to their RRBP1 expression status, ($p = 0.008$).

Table 2. Univariate survival analysis of OS and DFS in 96 patients with Cervical squamous cell carcinoma (CSCC)

Variables	*n*	OS		P^a	DFS		P^a
		Mean ± SE (month)	95% CI		Mean ± SE (month)	95% CI	
Age(years)							
> 49	45	96 ± 3	91 - 102	0.200	91 ± 4	82 - 99	0.164
≤ 49	51	88 ± 4	81 - 95		80 ± 5	70 - 89	
FIGO stage							
I	38	93 ± 3	86 - 100	0.767	85 ± 5	76 - 95	0.667
II	58	91 ± 3	85 - 97		84 ± 4	76 - 93	
Histological grade							
G1	23	95 ± 3	90 - 100	0.010	89 ± 6	77 - 100	0.003
G2	65	94 ± 3	88 - 99		87 ± 4	79 - 95	
G3	8	65 ± 10	44 - 85		54 ± 11	32 - 76	
lymph node metastasis							
No	83	94 ± 2	89 - 99	0.045	88 ± 3	82 - 95	0.016
Yes	13	82 ± 5	71 - 92		65 ± 9	47 - 83	
RRBP1							
Low expression	38	97 ± 1	95 - 99	0.001	96 ± 2	93 - 100	< 0.001
High expression	58	87 ± 3	81 - 94		77 ± 5	68 - 86	

G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; OS, overall survival; DFS, disease-free survival; [a]Log-rank test.

Table 3. Multivariate survival analysis of OS and DFS in 96 patients with Cervical squamous cell carcinoma (CSCC)

Variables	OS			OS		
	Exp(B)	95% CI	P^a	Exp(B)	95% CI	P^a
lymph node metastasis	0.617	0.729 - 4.714	0.195	0.672	0.822 - 4.664	0.129
Histological grade	0.693	0.913 - 4.380	0.083	0.709	0.983 - 4.203	0.056
RRBP1	2.281	1.305 - 73.315	0.026	2.549	1.712 - 95.598	0.013

OS overall survival; DFS disease-free survival; CI confidence interval; [a]Cox regression test.

of RRBP1 expression levels in CSCC tissue.

RRBP1 was originally found in Saccharomyces cerevisiae, which is also located on the ER membrane, with a primary structure containing an immensely repetitive tandem sequence (*7*). Previous studies have shown that the function of the internal ribosome entry site (IRES) is to maintain or enhance the expression of regulatory proteins (*20,21*). Gao *et al.* demonstrated that in liver cancer cells, the 5'-untranslated region (UTR) of the RRBP1 protein contains an IRES and the overexpression of RRBP1 was mainly to enhance protein synthesis (*13*). Fulda *et al.* have shown that enhanced ER activity is necessary for the rapid proliferation of tumor cells, and ER stress will promote the UPR, initiating the mitogen-activated protein kinase (MAPK) family signaling pathway (*12*). Tsai *et al.* demonstrated in lung cancer tissue that RRBP1 may participate in the adjustment of the normality of GRP78 (a UPR component) mRNA, thereby reducing ER stress and helping tumor cells survive (*11,22,23*). Diefenbach *et al.* demonstrated that RRBP1 interacted with cell microtubules by binding to kinase family member 5B (KIF5B) (*24*). Lee *et al.* discovered that the RRBP1-ALK fusion genes were a novel and recurrent carcinogenic mechanism in invasive epithelioid inflammatory myofibroblastic sarcoma (*25*). In recent years, overexpression of RRBP1 has been found in many kinds of cancers and is closely related to poor prognosis (*11, 13-19*). Through our study, it is indicated that RRBP1 may be involved in the occurrence of CSCC and has important clinical significance for exploring the carcinogenic mechanism of CSCC in the future. We confirmed that RRBP1 is an independent prognostic factor, indicating that RRBP1 may be a potential biological marker for CSCC.

We used the same methods as previous studies. The pathogenesis of RRBP1 in CSCC is not clearly understood yet and still needs further investigation. All of our samples were squamous cell carcinomas; therefore, many other kinds of pathological types are needed to fully evaluate the association between the expression level of RRBP1 in CC and patient prognosis.

In conclusion, RRBP1 may become a new biomarker for CSCC and has important clinical significance for exploring the carcinogenic mechanism of CSCC in the future. RRBP1 may play an important role in early diagnosis, individualized therapy of CSCC patients.

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