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ORIGINAL ARTICLE

Overexpression of ubiquitin specific peptidase 14 predicts unfavorable prognosis in esophageal squamous cell carcinoma

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Abstract

Background: Ubiquitin specific peptidase 14 (USP14), a deubiquitinating enzyme, has been documented as a key element to regulate the proteolysis function of proteasomes and an attractive therapeutic target for several cancers. Herein, we elucidate the role of USP14 in predicting the prognosis of patients with esophageal squamous cell carcinoma (ESCC).

Methods: USP14 expression was detected in ESCC tissues and matched adjacent non-tumorous tissues by quantitative real-time reverse transcription-PCR and immunohistochemistry. Kaplan–Meier survival analysis was used to assess the correlation between USP14 expression and prognosis in ESCC patients. Univariate and multivariate analysis was conducted with a Cox proportional hazards model to determine whether USP14 is an independent prognostic factor.

Result: Overexpression of USP14 was observed in approximately 60% of tested ESCC samples compared to their paired non-tumor esophageal tissues at both RNA and protein levels, and was significantly associated with distant metastasis ($P = 0.001$). Kaplan–Meier analysis showed that USP14 overexpression was related to poorer overall patient survival. Univariate and multivariate analyses demonstrated that USP14 was an independent risk factor for overall survival.

Conclusion: The findings in this study suggest that USP14 could be used as a potential prognostic marker for ESCC patients.

Introduction

Esophageal cancer caused an estimated 400 200 deaths in 2012 worldwide, which makes it the sixth and the ninth leading cause of cancer death in men and women respectively.[1] Esophageal squamous cell carcinoma (ESCC), the most common histological type of esophageal cancer, is a threatening malignancy with aggressive biological behavior and a poor prognosis. In addition to unhealthy eating and lifestyle habits,[2] hereditary variability and molecular regulation have been found to be increasingly vital in the initiation and development of ESCC. Accordingly, clinical investigators are screening to locate potential biological molecules to predict ESCC prognosis.

Recently, we performed integrative RNA sequencing (RNA-Seq) to identify differentially expressed genes between three pairs of clinical samples of ESCC and their adjacent non-tumor tissues. Overexpression of ubiquitin specific peptidase 14 (*USP14*) was observed in all three ESCC tumor tissues compared to their matched non-tumor counterparts. USP14 belongs to the ubiquitin-specific protease (USP) family, which cleave the ubiquitin moiety from ubiquitin-fused precursors. USP14 was first associated with neurological disease, such as skeletal muscle atrophy, synaptic activity, and long-term memory formation.[3–5] Recent research has revealed that USP14 expression is related to liver and lymph node metastases in colorectal cancer,[6]

advanced stage of hepatocellular carcinoma,[7] and poor prognosis in epithelial ovarian cancer patients.[8] Overexpression of USP14 could increase tumor cell growth in lung adenocarcinoma.[9] Conversely, knockdown of USP14 inhibited cell proliferation of epithelial ovarian cancer, multiple myeloma, Waldenström macroglobulinemia, and acute myeloid leukemia.[8,10–12] USP14 is a tempting target for designing clinical drugs; however, its role in ESCC remains unknown.

Herein we detected USP14 expression in ESCC tissues and paired non-tumor specimens and explored its clinicopathological features and prognostic value in ESCC.

Methods

Patients and tissue samples

Forty six primary ESCC specimens and their non-tumor counterparts were obtained from Linzhou People's Hospital, Henan, China. Queen Mary Hospital, Hong Kong, China kindly donated a set of tissue microarray containing 132 ESCC samples. None of the patients in the sample had received any preoperative treatments. Permission was obtained from all patients prior to their inclusion in the study. The Medical Ethics Committee of Sun Yat-sen University Cancer Center, Zhenzhou University and Hong Kong University granted ethical approval for this study. Six ESCC cell lines (KYSE30, KYSE140, KYSE180, KYSE410, KYSE510, and KYSE520) were obtained from DSMZ (Braunschweig, Germany), the German Resource Centre for Biological Material.[13] Four Chinese ESCC cell lines (EC18, EC109, EC9706, and HKESC1) were kindly donated by Professor Tsao and Professor Srivastava (The University of Hong Kong).[14]

Quantitative real-time reverse transcription-PCR

Total RNA was extracted from clinical samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed using a PrimeScriptRT reagent Kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. Quantitative real-time reverse transcription (RT)-PCR was then performed using SYBR Green Super-Mix (Roche, Basel, Switzerland) and ABI7900HT Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as internal control. The primers used are as follows: *GAPDH* (5′-CCATGTTCGTCATGGG-TGTGAACCA-3′ and 5′-GCCAGTAGAGGCAGGGAT GATGTTG-3′) and *USP14* (5′-GAGT-TGGACCTTT-CCAGA-3′ and 5′-TGCTTGCACAG-ATGTGA-3′). The value of relative expression for each sample was averaged and compared using the Ct method. ΔΔCt(sample) = ΔCt (sample) − ΔCt(calibrator), ΔCt(sample) = Ct(sample) of *USP14* − Ct(sample) of *GAPDH*; ΔCt(calibrator) = Ct(calibrator) of *USP14* − Ct(calibrator) of GAPDH. The calibrator was defined as the pooled samples from 46 adjacent non-tumorous tissues.

Western blot analysis

Equal amounts of protein were separated by 12% dodecyl sulfate, sodium salt (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to microporous polyvinylidene difluoride (PVDF) Western blotting membranes (Roche, Basel, Switzerland). Membranes were incubated with antibody against USP14 (1:1000; Proteintech, Chicago, IL, USA) or β-actin (1:2000; Abgent, San Diego, CA, USA). Blots were developed using a Luminata Crescendo Western HRP Substrate (Millipore, Billerica, MA, USA). β-actin was used as a loading control.

Immunohistochemical staining

Immunohistochemical staining was conducted, as previously described.[15] Anti-USP14 antibody (1:100; Proteintech) was applied as a first antibody. Immunoreactivity was visualized using an Envision detection system (DAKO, Carpinteria, CA, USA), and the nuclei were counterstained with hematoxylin. An immunoreactivity score (IRS) system was applied, as previously described.[16] The percentage of USP14-positive cells was scored as: 0, <5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, 75–100%. The intensity of USP14-positive staining was scored as 0, negative; 1, weak; 2, moderate; and 3, strong. The total score was determined using the following formula: Staining index = intensity × positive percentage. According to receiver operating characteristic (ROC) curve analyses, the optimum cut-off value for USP14 was 6; therefore, a staining index ≤6 was considered low expression, and a staining index >6 was considered high expression.

Statistical analysis

All statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). An ROC curve was generated to determine the cut-off value for USP14 expression that yielded the highest combined sensitivity and specificity with respect to distinguishing overall 10-year survivors from non-survivors. A paired two-tailed *t*-test was used to compare the level of *USP14* messenger (m) RNA in primary ESCC samples and their paired adjacent non-tumorous tissues. The relationship between USP14 expression and clinicopathologic characteristics was measured by χ^2 or Fisher's exact tests. Overall survival was

calculated from the date of surgery to the date of death from ESCC or the last follow up. Kaplan–Meier analysis with log-rank test was employed to calculate the prognostic value. Univariate and multivariate survival analyses were performed using the Cox proportional hazard model with a forward stepwise procedure (the entry and removal probabilities were 0.05 and 0.10, respectively). Statistical significance was considered when $P < 0.05$.

Results

Ubiquitin specific peptidase 14 (USP14) expression is notably upregulated in esophageal squamous cell carcinoma (ESCC)

Our prior RNA-seq profiling data showed that *USP14* was upregulated in all three tested ESCC tumor tissues. Quantitative real-time RT-PCR was applied to measure the *USP14* expression level in 46 pairs of ESCC and paired adjacent non-tumor tissues. Overexpression of *USP14* was observed in 30/46 (65.2%) ESCC samples (defined as >twofold increase) compared to non-tumor counterparts. The average level of *USP14* expression was remarkably higher in ESCC tumor tissues than in their non-tumor counterparts (3.74 vs. 1.44; $P < 0.001$, paired Student's *t* test) (Fig 1a). To confirm our findings, we subsequently examined the USP14 protein level in 10 pairs of ESCC and adjacent non-tumor tissues. Western blotting showed that USP14 was upregulated in six out of 10 ESCC tissues (Fig 1b). The mRNA and protein expression of USP14 in 10 ESCC cell lines and one immortalized esophageal epithelial cell line (NE1) was then detected. High USP14 expression was detected in all ESCC cell lines but not in NE1 (Fig 1c).

Correlation of USP14 expression with clinicopathological variables

The ESCC tissue microarray containing 132 pairs of ESCC specimens was analyzed for USP14 expression by immunohistochemistry. Informative immunohistochemical results were obtained from 125 pairs of ESCCs. USP14 localized at the cytoplasm and nucleus of ESCC cells and exhibited different staining intensities (Fig 2a). Enhanced USP14 expression was detected in 68/125 (54.4%) informative ESCC tissues compared to their adjacent non-tumor tissues. To further evaluate the potential utility of USP14 expression as a diagnostic marker for ESCC, ROC curve

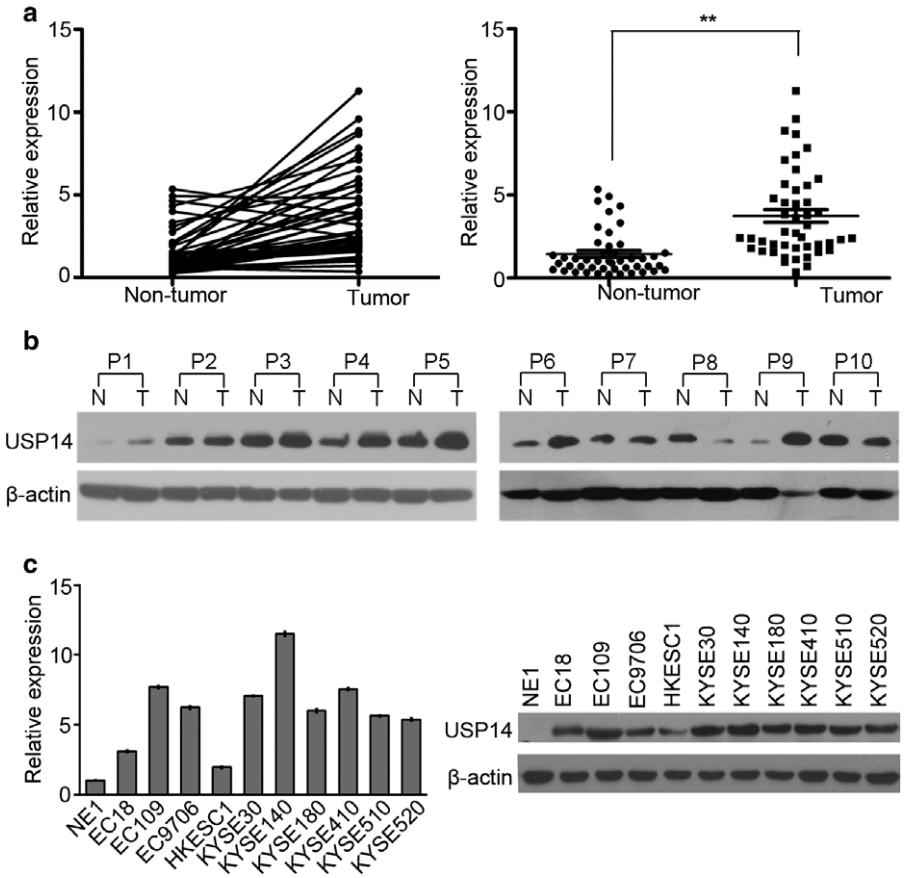

Figure 1 Upregulation of ubiquitin specific peptidase (USP)14 in esophageal squamous cell carcinoma (ESCC). (**a**) *USP14* messenger RNA expression (dot plots) in ESCC and matched adjacent non-tumor specimens from 46 cases. *β-actin* was used as an endogenous control. $P < 0.001$, paired *t*-test. (**b**) Western blotting exhibited that USP14 was upregulated in six out of 10 pairs of ESCC tissues. (**c**) USP14 messenger RNA (left) and protein expression (right) in 10 ESCC cell lines and one immortalized esophageal epithelial cell line (NE1). β-actin was used as an endogenous control.

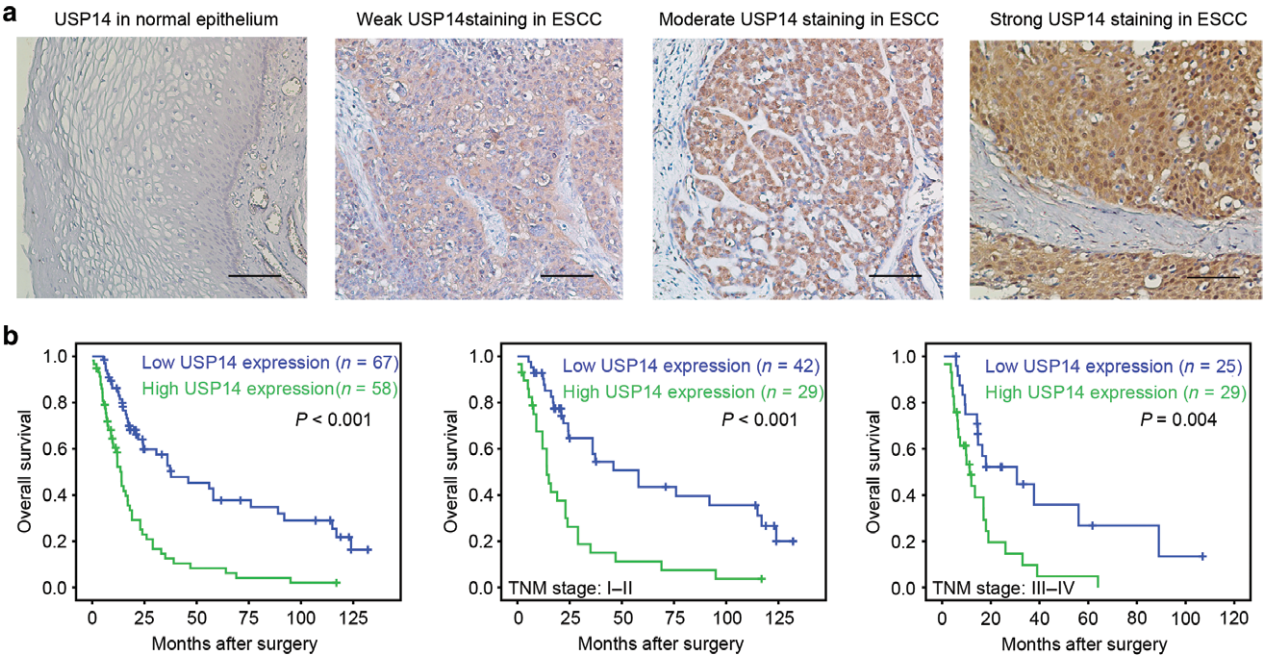

Figure 2 High expression of ubiquitin specific peptidase (USP)14 in esophageal squamous cell carcinoma (ESCC) tumor tissues was associated with poor prognosis in ESCC patients. (**a**) Representative images of USP14 immunohistochemistry staining in ESCC and normal esophageal tissues (original magnification, 200×; scale bar, 100 μm). (**b**) Kaplan–Meier analysis of overall survival for USP14 expression in: all 125 cases (left), ESCC patients with clinical tumor node metastasis (TNM) stage I–II (middle), and clinical stage III–IV (right).

analysis was performed and the optimal cut-off value of USP14 expression with the best discriminatory power was determined to be 6. Therefore, 125 informative ESCC cases were divided into two groups: high (index > 6, n = 58) and low (index ≤ 6, n = 67) expression. The association between USP14 expression and clinicopathological features was statistically analyzed and is summarized in Table 1. High USP14 expression was significantly associated with distant metastasis (P = 0.001). No correlation was observed between USP14 expression and other clinicopathological indexes.

Overexpression of USP14 is associated with poor prognosis

Kaplan–Meier analysis indicated that high USP14 expression was significantly associated with poorer overall survival (OS) (log-rank test, P < 0.001) (Fig 2b, left). The three, five, and eight-year OS rate in the high USP14 expression group was 13.4, 5.8, and 2.0%, respectively, compared to 57.5, 37.7, and 25.4%, respectively, in the low USP14 expression group (log-rank test, P < 0.001). In univariate analysis, the statistically significant predictors for a patient's OS were gender (P = 0.013), age (P = 0.004), tumor invasion (P = 0.004), lymph node metastasis (P = 0.015), and USP14 expression (P < 0.001) (Table 2). Multivariate Cox regression analysis showed that age ≥60 (P = 0.002) and USP14 overexpression (P < 0.001) are independent prognostic factors for poor survival in patients with ESCC (Table 2). Further, in a stratified survival analysis according to the clinical stage, USP14 expression could differentiate the prognosis of patients at clinical tumor node metastasis (TNM) stages I–II (P < 0.001) (Fig 2b, middle) and III–IV (P = 0.004) (Fig 2b, right).

Discussion

In a living mammalian cell, the ubiquitin–proteasome system (UPS) and deubiquitinases (DUBs) control the stability of around 90% of protein, including cell cycle regulating proteins such as cyclins, cyclin-dependent kinases, and their inhibitors.[17] DUBs have been implicated as important regulators of oncogenes and tumor suppressors as they promote destabilization by targeting them for degradation or regulating their activity (activation or inactivation).[18] The DUB superfamily consists of five subfamilies: USPs, otubain-domain containing proteins, Machado-Joseph Domain (Josephin domain)-containing proteins, and ubiquitin C-terminal hydrolases.[18] USP14 has gained increasing attention because of its high expression and important function in many types of tumors. We found that ESCC patients with high USP14 expression have poorer survival compared to those with low USP14 expression. Furthermore, multivariate Cox regression

Table 1 Association of USP14 expression with clinicopathological features in ESCC

Clinical features	Cases	USP14 protein expression		*P* value
		Low level (%)	High level (%)	
Age (years)				1.000
≤59	50	27 (54.0%)	23 (46.0%)	
>59	75	40 (53.3%)	35 (46.7%)	
Gender				0.076
Male	89	43 (48.3%)	46 (51.7%)	
Female	36	24 (66.7%)	12 (33.3%)	
Differentiation				0.149
Grade 1	18	6 (33.3%)	12 (66.7%)	
Grade 2	73	43 (58.9%)	30 (41.1%)	
Grade 3	34	19 (52.9%)	16 (47.1%)	
Tumor invasion				0.463
T1–2	48	28 (58.3%)	20 (41.7%)	
T3–4	77	39 (50.6%)	38 (49.4%)	
Lymph node metastasis				0.213
No	66	39 (59.1%)	27 (40.9%)	
Yes	59	28 (47.5%)	31 (52.5%)	
Distant metastasis				**0.001**
No	81	52 (62.4%)	29 (35.8%)	
Yes	44	15 (34.1%)	29 (65.9%)	
TNM stage				0.205
I–II	71	42 (59.2%)	29 (40.8%)	
III–IV	54	25 (46.3%)	29 (53.7%)	

Statistical significance ($P < 0.05$) is shown in bold. ESCC, esophageal squamous cell carcinoma; TNM, tumor node metastasis; USP, ubiquitin specific peptidase.

analysis showed that USP14 overexpression was an independent prognostic factor in ESCC patients.

Previous studies have shown that elevated USP14 expression is related to liver and lymph node metastases in colorectal cancer, advanced stage of hepatocellular carcinoma, and intrahepatic cholangiocarcinoma cell differentiation.[6,7,19] Consistent with these findings, we found that high USP14 expression was remarkably related to distant metastasis in ESCC. A recent study revealed that *USP14* knockdown inhibited the migration of breast cancer cells by increasing the expression of vimentin and reducing the expression of E-cadherin, suggesting that USP14 might enhance the metastatic ability of breast cancer cells by initiating the epithelial-mesenchymal transition process.[20] However, whether USP14 promotes ESCC metastasis through the same mechanism or other pathways remains unclear.

Ubiquitin specific peptidase 14 has been proven to inhibit the proteasome in vitro.[21] Ubiquitinated tau is a substrate protein for proteasomal degradation.[22] Expressing wild-type USP14 in *Usp14*$^{-/-}$ murine embryonic fibroblasts could increase tau expression levels, indicating that USP14 suppressed the ability of the proteasome to degrade tau.[23] The deubiquitinating activity of the 19S regulatory particle has recently been proven to be a promising target for cancer treatment. b-AP15, a small molecule inhibitor of two 19S regulatory-particle-associated deubiquitinases, USP14 and ubiquitin C-terminal hydrolase 5, could efficiently induce cell apoptosis or cell death in colorectal cancer cell line HCT116.[24] In addition, b-AP15 can suppress the growth of FaDu squamous cell carcinoma cells.[25] Our stratified survival analysis indicated that high USP14 expression could distinguish poor outcomes of patients with either early (TNM stage I–II) or advanced clinical stage (TNM stage III–IV), suggesting that USP14 may play a significant role throughout the development of ESCC. Therefore, inhibitors targeting USP14, such as b-AP15, may be specifically effective for ESCC patients with high USP14 expression.

In this study, elevated USP14 expression was detected at both mRNA and protein levels. It has been reported that USP14 expression could be regulated at mRNA level. In non-small cell lung cancer, miR-4782-3p was reported to bind the 3′ untranslated region region of USP14 mRNA and inhibited its expression.[26] The expression status of miR-4782-3p in ESCC and its influence on USP14 expression remain unknown. Xu *et al.* reported that serine/threonine kinase could phosphorylate USP14 at Ser432, thus activating its deubiquitinating activity by transforming its catalytic site from the inactive conformation to the active

Table 2 Cox proportional hazard regression analyses for overall survival

Clinical features	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	1.906 (1.234–2.946)	**0.004**	2.074 (1.319–3.262)	0.002
Gender	0.537 (0.330–0.875)	**0.013**	0.858 (0.506–1.455)	0.570
Differentiation	0.801 (0.552–1.163)	0.244	-	-
Tumor invasion	1.982 (1.245–3.155)	**0.004**	1.244 (0.782–2.310)	0.285
Lymph node metastasis	1.701 (1.110–2.605)	**0.015**	1.158 (0.708–1.893)	0.559
Distant metastasis	1.380 (0.913–2.085)	0.126	-	-
USP14 expression	2.953 (1.930–4.518)	< **0.001**	2.499 (1.607–3.886)	**< 0.001**

Statistical significance ($P < 0.05$) is shown in bold. CI, confidence interval; HR, hazard ratio; USP, ubiquitin specific peptidase.

form.[27] Whether the activity of USP14 is regulated by serine/threonine kinase in ESCC is still questionable.

In conclusion, USP14 may serve as a valuable marker for prognosis in ESCC.

Disclosure

No authors report any conflict of interest.

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