RESEARCH ARTICLE

Expression and Clinical Significance of REPS2 in Human Esophageal Squamous Cell Carcinoma

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Abstract

Objective: REPS2 plays important roles in inhibiting cell proliferation, migration and in inducing apoptosis of cancer cells, now being identified as a useful biomarker for favorable prognosis in prostate and breast cancers. The purpose of this study was to assess REPS2 expression and to explore its role in esophageal squamous cell carcinoma (ESCC). Methods: Protein expression of REPS2 in ESCCs and adjacent non-cancerous tissues from 120 patients was analyzed by immunohistochemistry and correlated with clinicopathological parameters and patient outcome. Additionally, thirty paired ESCC tissues and four ESCC cell lines and one normal human esophageal epithelial cell line were evaluated for REPS2 mRNA and protein expression levels by quantitative RT-PCR and Western blotting. Results: REPS2 mRNA and protein expression levels were down-regulated in ESCC tissues and cell lines. Low protein levels were significantly associated with primary tumour, TNM stage, lymph node metastasis and recurrence (all, $P < 0.05$). Survival analysis demonstrated that decreased REPS2 expression was significantly associated with shorter overall survival and disease-free survival (both, $P < 0.001$), especially in early stage ESCC patients. When REPS2 expression and lymph node metastasis status were combined, patients with low REPS2 expression/lymph node (+) had both poorer overall and disease-free survival than others (both, $P < 0.001$). Cox multivariate regression analysis further revealed REPS2 to be an independent prognostic factor for ESCC patients. Conclusions: Our findings demonstrate that downregulation of REPS2 may contribute to malignant progression of ESCC and represent a novel prognostic marker and a potential therapeutic target for ESCC patients.

Keywords: REPS2 - esophageal squamous cell carcinoma - clinicopathology - metastasis - prognosis

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Introduction

Esophageal cancer occurs worldwide with a variable geographic distribution and ranks 6th in order of incidence and 5th as the leading cause of cancer mortality, affecting men more than women (Jemal et al., 2011). Northern China where the incidence rate can be as high as 800 cases per 100000 people owing to its location in the ''esophageal cancer belt'' area, and the majority of esophageal cancer diagnoses are esophageal squamous cell carcinoma (ESCC) (Chai et al., 2012). The incidence and the mortality rates of esophageal cancer have been steadily decreasing during the past several decades in China, whereas esophageal cancer is continuing its march as the fastest growing malignancy in the western world. Though the widespread application of systemic chemoradiotherapy and radical esophagectomy, ESCC patients still have a high mortality rate and poor prognosis due to the high prevalence of invasion and metastasis. Therefore, it is important to identify and characterize clinically applicable tumour-specific molecular biomarkers involved in early stage of ESCC that may contribute to ESCC carcinogenesis for improving the survival of this dreaded malignancy.

REPS2 (also known as POB1) was initially identified in a yeast two-hybrid screening as a partner of RalBP1, a molecule in the Ras/Ral signaling pathway (Ikeda et al., 1998). This gene is located on the human X chromosome at Xp22 and it has an EH domain in its N-terminal region and two proline-rich motifs and a coiled-coil structure in its C-terminal region. The two proline-rich regions of REPS2 have been shown to interact with the growth factor receptor adaptor protein Grb2 (Ikeda et al., 1998) and the paxillin-associated protein PAG2 (Oshiro et al., 2002), while the EH domain of REPS2 is found to bind directly to Epsin and Eps15 (Chen et al., 1998; Morinaka et al., 1999; Kariya et al., 2000).

REPS2 is involved in the regulation of receptor-mediated endocytosis for EGF and insulin, moreover, deletion mutants of REPS2 inhibit the internalization of EGF and insulin (Nakashima et al., 1999). Recently, it has been shown that overexpression of REPS2 and its binding with RalBP1 induce apoptosis and loss of

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REPS2 expression results in dysregulation of growth factor signaling in prostate cancer cells (Oosterhoff et al., 2003; Oosterhoff et al., 2005). Likewise, augmentation of cellular levels of REPS2 and Hsf-1 result in dramatic apoptosis in non-small cell lung cancer cell line H358 through RalBP1 inhibition (Singhal et al., 2008). What is more, the binding of REPS2 to PAG2 inhibits cell migration (Oshiro et al., 2002). At present, REPS2 isoform 2 downregulation has been demonstrated in the progression of prostate cancer and the overexpression of REPS2 might serve as a useful biomarker for favorable prognosis in prostate and breast cancers (Oosterhoff et al., 2003; Oosterhoff et al., 2005; Doolan et al., 2009). On the contrary, REPS2 overexpression protects against dopaminergic cell death induced by paraquat (Rodriguez-Rocha et al., 2012) and REPS2 is upregulated in clinical specimens of aggressive oral squamous cell carcinoma (Loudig et al., 2011). So far there were no published reports evaluating the role of REPS2 protein expression in ESCC, particularly with respect to clinical outcome. Thus, in order to gain better insight into the clinical relevance of REPS2 protein in ESCC, the present study was carried out to investigate REPS2 protein expression in a series of archival ESCC tissue specimens and cell lines, and further to assess whether REPS2 expression was correlated with clinicopathological parameters and prognosis in ESCC patients.

Materials and Methods

Patients and tissue specimens

For immunohistochemical assays, 120 pairs of paraffin-embedded ESCC samples and adjacent non-cancerous tissues were obtained from patients who underwent curative resection from January 2004 to June 2007 at Xiangya Hospital. All patients had no history of previous malignancies, no history of chemotherapy or radiotherapy. Recurrence and metastasis were diagnosed by imaging evaluation, clinical examination, operation and postoperative pathological examination. The main clinical and pathological variables of the patients were recorded in detail in Table 1. Ninety patients were men and thirty were women, with an average age of 57.38 years (range: 38-73 years, SD = 11.12). According to the 2009 TNM classification of malignant tumours by the International Union Against Cancer (UICC) and American Joint Committee on Cancer (AJCC), there were 28 cases in stage I (IA 13 cases, IB 15 cases), 49 cases in stage II (IIA 20 cases, IIB 29 cases), 43 cases in stage III (IIIA 30 cases, IIIB 6 cases, IIIC 7 cases). Considering pathological grading, 63 were staged as well differentiated (G1), 30 as moderately differentiated (G2), 27 as poorly differentiated and undifferentiated (G3 + G4). Forty-four patients with lymph node metastasis were validated by conventional postoperative pathological examinations. The follow-up time was 5 years for 120 patients, ranging from 5 months to 60 months. Three patients lost to follow-up because of telephone number changes or home moving.

Moreover, 30 cases of ESCC samples, paired adjacent non-cancerous tissues (from 2 to 3 cm away from the tumour margin) and normal tissues (greater than 7 cm away from the tumour margin) were randomly collected from each patient during operation from January 2010 to June 2011 at Xiangya Hospital in Central South University, all of which were validated by two pathologists. All specimens were immediately snap-frozen in liquid nitrogen and stored at -80 °C until RNA and total protein extraction. Before surgery informed consents were acquired from all patients, whose specimens were handled and made anonymous according to the ethical and legal standards. The study was approved by the Research Ethics Committee of Central South University, Changsha, China.

Cell culture

EC-1 and EC9706 cell lines were obtained from the Central Experiment Laboratory of Xiangya Medical School in Central South University. TE-1 and Eca109 cell lines were purchased from the Chinese Academy of Sciences Cells Library. A normal human esophageal epithelial cell line (HEEpic) was bought from the American type culture collection. All cell lines were maintained as monolayer cultures in Roswell Park Memorial Institute 1640 containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 IU/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

Quantitative RT-PCR (qRT-PCR)

Total RNA from tissue specimens and cell lines was isolated by TRIzol reagent (Invitrogen, USA). Total RNA (2 μg) was reverse transcribed by cDNA Reverse Transcription Kits (Invitrogen) according to the manufacturer's instructions. Primers were designed and synthesized by Sangon Biological Engineering Technology and Services Co. Ltd (Shanghai, China). The primers for REPS2 and β-actin were designed as follows: REPS2 primer (126 bp), forward 5'-CTGAAGACCAGCAGACACCA-3', reverse 5'-TTTAGGATCTGGCCCTGTTG-3'; β-actin primer (205 bp), forward 5'-TGACGTGGACATCCGCAAAG-3', reverse 5'-CTGGAAGGTGGACAGCGAGG-3'. QRT-PCR was performed with SYBR Green PCR Master Mix according to the manufacturer's instructions by using the Bio-Rad CFX96 sequence detection system and accompanying analytical software. The reaction was first denatured at 95 °C for 10 min, then 40 cycles at 95 °C for 10 s, 60 °C for 20 s and followed by 72 °C for 10 s.

Western blotting

Harvest total protein lysates from tissue specimens and cell lines were extracted using a Total Protein Extraction Kit (Beyotime, China). Briefly, total protein (40 μg) was resolved on 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis in running buffer, and transferred to polyvinylidene difluoride membranes (Millipore, USA). After blocking with 5 % milk for 2 h, the membrane were incubated with primary antibodies against REPS2 (Abcam, Britain), and GAPDH antibody (Beyotime) at room temperature for 2 h. Then, the membranes were washed with Phosphate Buffered Saline (PBS)-Twen-20 and incubated with goat anti-rabbit secondary antibodies (1:1000) (Beyotime) for 1 h at room temperature. Bands were visualized by employing the BeyoECL Plus Detection System (Beyotime). REPS2 protein expression

Table 1. Correlation Between REPS2 Expression and Clinicopathologic Features of ESCC Patients (n=120)

Parameters	Case	REPS2 expression (n)		χ^2	*P*-value
		Low	High		
Tissues					
Cancer	120	77	43	8.148	**0.004**
Adjacent	120	55	65		
Age					
≤60	81	54	27	0.677	0.41
>60	39	23	16		
Gender					
Male	90	59	31	0.302	0.583
Female	30	18	12		
Drinking					
Yes	75	49	26	0.118	0.731
No	45	28	17		
Tumour site					
Upper	35	20	15	4.063	0.131
Middle	40	23	17		
Lower	45	34	11		
Grade(G)					
G1	63	39	24	0.601	0.74
G2	30	19	11		
G3+G4	27	19	8		
Primary tumour(T)					
T1	30	13	17	8.61	**0.035**
T2	31	20	11		
T3	42	32	10		
T4	17	12	5		
TNM stage					
I	28	10	18	12.948	**0.002**
II	49	35	14		
III	43	32	11		
Lymph node metastasis(N)					
N0	76	42	34	7.146	**0.008**
N+	44	35	9		
Recurrence*					
Yes	85	49	36	5.628	**0.018**
No	32	26	6		

Bold values represent *P* values are considered to be statistically significant at < 0.05; *Three patients lost to follow-up because of telephone number changes or home moving

levels were quantified by Bio–Rad Image Lab Software and represented as the densitometric ratio of the targeted protein to GAPDH.

Immunohistochemistry

All specimens were fixed with 4 % formaldehyde, dewaxed, embedded, and cut into 4 μm serial sections. Briefly, antigen retrieval was carried out in 10 mmol/L citrate buffer (pH 6.0) for 15 min at 100 °C in a microwave oven. Endogenous peroxidase activity was blocked with 3 % hydrogen peroxide for 10 min at room temperature. The sections were then incubated overnight at 4 °C with anti-REPS2 antibody (Abcam, Britain). After washing with PBS, sections were incubated with secondary antibodies for 30 min at 37 °C. Then, the sections were washed three times with PBS and treated with 3,3'-diaminobenzedine for approximately 5 min. Finally, the sections were counterstained with hematoxylin, dehydrated, mounted, and examined by light microscopy. Negative controls were probed with PBS under the same experimental conditions. Immunohistochemical staining was assessed

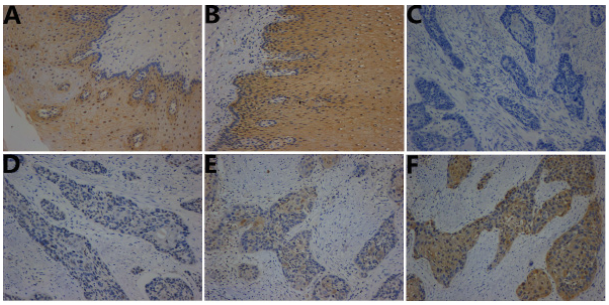

Figure 1. Representative Immunohistochemical Staining for REPS2 in ESCC Samples, Adjacent Non-cancerous Tissues and Normal Esophageal Tissues (original magnification ×100). Representative strong staining of REPS2 in normal esophageal tissues (A) and adjacent non-cancerous tissues (B). Representative negative staining (C), weak staining (D), moderate staining (E) and strong staining (F) of REPS2 in ESCC tissues

by two independent experienced pathologists who were blinded to all clinicopathological features. Five high power fields in each specimen were selected randomly, in which cytoplasmic staining was considered to be positive staining for REPS2. A staining index (values 0-9), obtained as a product of staining intensity (0-3: 0 point = no intensity; 1 point = weak intensity; 2 points = moderate intensity; 3 points = strong intensity) multiplied by proportion of immunopositive cells of interest (≤10% = 1, 10%-50% = 2, ≥50% = 3). Tumours were categorized into three groups according to the final staining index: negative or weak staining (scored 0-3), moderate staining (scored 4-6) and strong staining (7-9). ESCC patients were dichotomized into low expression group (negative, weak, or moderate staining: 0-6) and high expression group (strong staining: 7-9) in order to better analyze the prognosis between groups.

Statistical Analysis

All continuous variables were expressed as mean ± SD from at least three separate experiments. REPS2 mRNA and protein expression levels in ESCC tissues and cell lines were examined by Wilcoxon signed-rank test. The association between REPS2 protein expression and clinicopathological features was analyzed using χ^2 test. Survival curves were obtained using Kaplan-Meier curves and log-rank tests. Multivariate prognostic factors were examined by Cox's proportional hazards model. A value of $P < 0.05$ was considered to be statistically significant. All statistical calculations were performed with SPSS 18.0 software.

Results

Decreased REPS2 mRNA and protein expression in ESCC tissue and cell levels

To investigate the protein expression profile of REPS2 in ESCC, immunohistochemistry was initially performed in 120 paraffin-embedded, archival ESCC primary tumour samples and paired adjacent non-cancerous samples. Positive REPS2 immunostaining was predominantly observed in the cytoplasm of carcinoma and non-cancerous epithelial cells (Figure 1). Among all the tumour

Table 2. Univariate Survival Analysis of Overall and Disease-free Survival in 120 Patients with ESCC

Features	Overall survival		Disease-free survival	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Age (≤60/>60)	0.736 (0.463-1.170)	0.195	0.693 (0.448-1.070)	0.098
Gender (Female/Male)	1.040 (0.612-1.768)	0.883	1.080 (0.680-1.715)	0.746
Drinking (Yes/No)	1.448 (0.907-2.311)	0.121	1.308 (0.861-1.987)	0.208
Tumour site (Upper+Lower/Middle)	0.734 (0.463-1.166)	0.19	0.733 (0.481-1.117)	0.148
Grade(G) (G2+G3+G4/G1)	1.106 (0.702-1.744)	0.663	1.130 (0.744-1.716)	0.567
Primary tumour (T) (T3 +T4/ T1 +T2)	1.633(1.014-2.632)	**0.044**	1.435 (0.938-2.196)	0.096
TNM stage (III/ I +II)	7.493 (4.399-12.763)	**<0.001**	5.094 (3.150-8.237)	**<0.001**
Lymph node metastasis(N) (-/+)	0.363 (0.223-0.590)	**<0.001**	0.434 (0.275-0.685)	**<0.001**
REPS2 expression (Low/High)	0.480 (0.284-0.812)	**0.006**	0.486 (0.304-0.777)	**0.003**

Bold values represent *P* values are considered to be statistically significant at <0.05

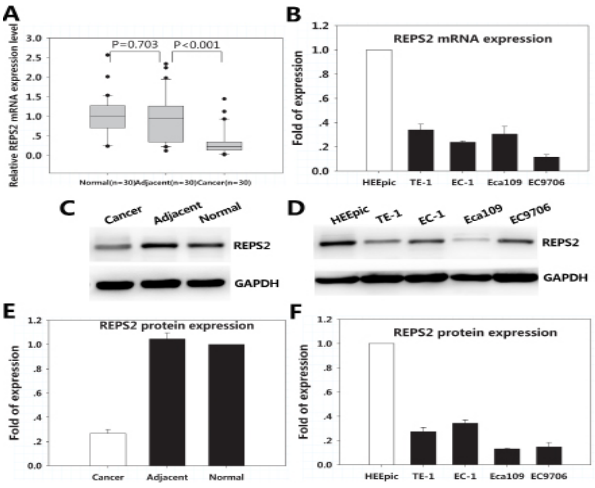

Figure 2. Comparison of REPS2 mRNA and Protein Levels in ESCC Tissues and Cell Lines by qRT-PCR and Western Blotting, Respectively. A, Relative REPS2 mRNA expression levels in different tissues: Cancer, 30 ESCC samples; Adjacent, paired adjacent non-cancerous tissues; and Normal, normal esophageal tissues. B, Relative REPS2 mRNA expression levels in ESCC cell lines and HEEpic cell line. C, Representative western blotting analysis of REPS2 protein expression in all specimens. D, Representative western blotting analysis of REPS2 protein expression in all cell lines. E, Relative REPS2 protein expression levels in different esophageal tissues. F, Relative REPS2 protein expression levels in different cell lines

samples analyzed, 43 (35.83%) cases demonstrated high REPS2 protein expression (Scored 7–9; Figure 1F), 20 (16.67%) cases with moderate REPS2 protein expression (Scored 4–6; Figure 1E) and 57 (47.50%) cases with negative or weak expression (Scored 0–3; Figure 1D). However, REPS2 expression was stronger in the normal and adjacent non-cancerous epithelial cells (Figure 1A and B).

More importantly, REPS2 mRNA and protein expressions were detected in the 30 cases of primary ESCC samples, matched adjacent non-cancerous tissues and normal tissues. The results in Figure 2A showed that REPS2 mRNA level in the ESCC tissues was lower than that in the adjacent non-cancerous tissues (0.35 ± 0.35 vs. 0.95 ± 0.60, $P < 0.05$). However, no significant differences were found between adjacent non-cancerous tissues and normal tissues ($P > 0.05$). Western blotting analysis showed that the REPS2 protein expression in ESCC tissues was significantly lower than that in adjacent non-cancerous tissues and normal tissues (Figure 2C, E; $P < 0.05$). Furthermore, REPS2 mRNA and protein expressions in the cultured four human ESCC cell lines and one normal HEEpic cell line were also investigated by qRT-PCR and western blotting. As presented in Figure 2B and D, REPS2 mRNA and protein expressions were detected in all the selected ESCC cell lines, but revealed the strongest REPS2 mRNA and protein expressions in the HEEpic cell line.

Correlation between decreased REPS2 protein expression and clinicopathological parameters

The association between REPS2 protein expression and clinicopathological characteristics of ESCC was explored by the χ^2 test. As summarized in Table 1, the low expression of REPS2 protein was significantly associated with primary tumour ($P < 0.035$), TNM stage ($P < 0.002$), lymph node metastasis ($P < 0.008$) and recurrence ($P < 0.018$), respectively. However, no significant relationship existed between REPS2 protein expression and variables such as age ($P = 0.410$), gender ($P = 0.583$), drinking ($P = 0.731$), tumour site ($P = 0.131$) or tumour grade ($P = 0.740$). In addition, there was a statistically significant difference between tumour tissues and adjacent non-cancerous tissues ($P = 0.004$).

Correlation between REPS2 protein expression and patients' survival

At the end of clinical follow-up, survival information was available in 117 of 120 cases, three patients lost to follow-up because of telephone number changes or home moving. As determined by the Kaplan–Meier method, the protein expression of REPS2 in ESCC was significantly correlated with overall survival ($P = 0.001$; Figure 3A) and disease-free survival ($P = 0.001$; Figure 3B). The log-rank test further verificated that the survival time was significantly different between groups with low and high expression of REPS2, indicating that low level of REPS2 was closely correlated with a shorter survival time. As shown in Table 2, univariate analysis showed that overall survival and disease-free survival were correlated with primary tumour, TNM stage, lymph node metastasis and REPS2 expression. Furthermore, Cox multivariate regression analysis indicated that REPS2 expression, lymph node metastasis and TNM stage were considered as independent prognostic factors for overall survival and disease-free survival in Table 3.

The prognostic value of REPS2 protein expression

Table 3. Multivariate Survival Analysis of overall and Disease-free Survival in 120 Patients with ESCC

Features	Overall survival		Disease-free survival	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Primary tumour (T) (T3 +T4/ T1 +T2)	1.591(1.007-2.515)	**0.047**	1.373(0.911-2.069)	0.129
TNM stage (III/ I +II)	6.990(4.215-11.593)	**<0.001**	4.913(3.095-7.800)	**<0.001**
Lymph node metastasis(N) (-/+)	0.354(0.219-0.572)	**<0.001**	0.420(0.268-0.657)	**<0.001**
REPS2 expression (Low/High)	0.542(0.328-0.895)	**0.017**	0.548(0.353-0.849)	**0.007**

Bold values represent *P* values are considered to be statistically significant at <0.05

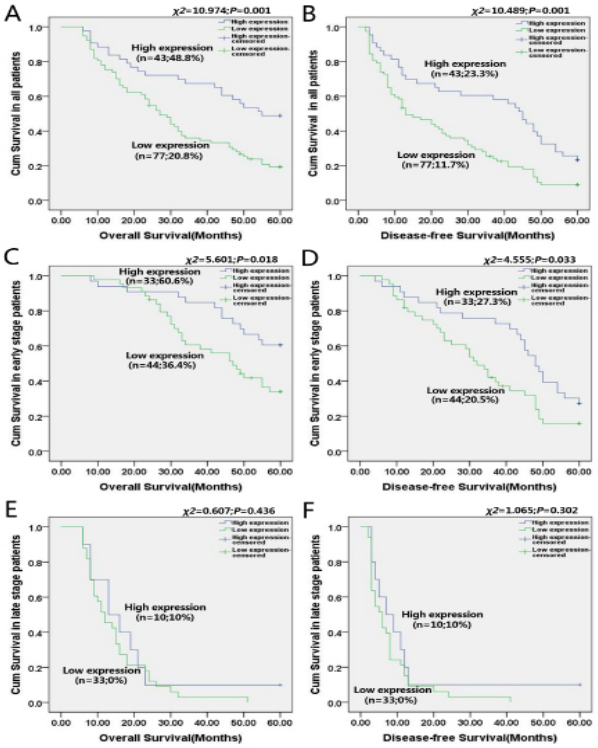

Figure 3. Kaplan–Meier Survival Analysis of overall survival (A) and disease-free survival (B) in all patients according to REPS2 protein expression; survival analysis of overall survival patients in early stage (I + II) (C) and in late stage (III) patients (E) according to REPS2 protein expression; survival analysis of disease-free survival in early stage (D) and in late stage patients (F) according to REPS2 protein expression. The log-rank test was used to calculate *P*-value

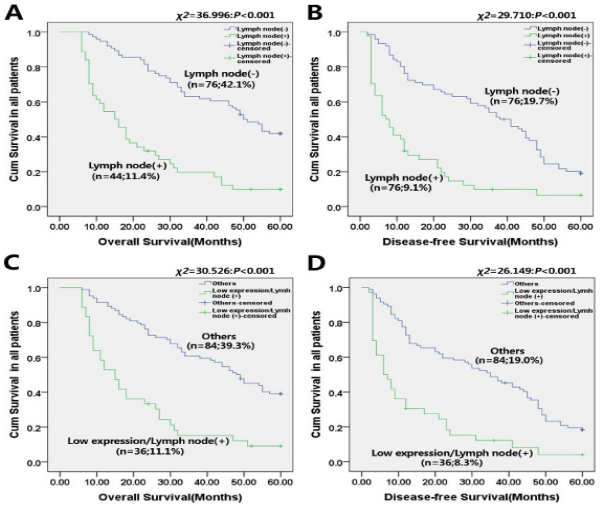

Figure 4. Kaplan–Meier Survival Analysis of overall survival (A) and disease-free survival (B) in all patients according to the lymph node metastasis status; survival analysis of overall survival (C) and disease-free survival (D) in all patients according to REPS2 expression/lymph node status. The log-rank test was applied to calculate *P*-value

in selective patient subgroups, stratified according to the tumour clinical stage was also analysed. In the late stage group (III), no statistically significant difference was found in overall survival ($P = 0.436$; Figure 3E) and disease-free survival ($P = 0.302$; Figure 3F). While in the early stage group (I + II), patients with a low level of REPS2 protein expression had an obviously shorter overall survival and disease-free survival time when compared with patients with a high level of REPS2 protein expression ($P = 0.018$; $P = 0.033$; Figure 3C and D). Thus, REPS2 protein expression pattern might be a valuable prognostic marker for early stage patients with ESCC.

It has been reported that lymph node metastasis was an important prognostic factor in patients with ESCC (Chen et al., 2013). Therfore, we investigated the correlation between lymph node metastasis and patients prognosis in ESCC. The results revealed that lymph node metastasis was significantly associated with short overall and disease-free survival ($P < 0.001$; $P < 0.001$, respectively; Figure 4A and B) and was an independent prognostic factor for overall and disease-free survival by multivariate analysis (both, $P < 0.001$; Table 3). Consequently, a subset analysis was carried out by combining REPS2 expression with lymph node metastasis status. Our results demonstrated that patients with the phenotype of low REPS2 expression/lymph node (+) had poorer overall and disease-free survival than that of others ($P < 0.001$; $P < 0.001$, respectively; Figure 4C and D).

Discussion

REPS2 is an essential protein that has been involved in the regulation of the cell shape and growth. Overexpression of REPS2 inhibits cell growth, causing the host cells to become round and swollen (Toya et al., 1999). In this study, we investigated the protein expression of REPS2 in a series of 120 clinical paraffin-embedded specimens with intact follow-up data. Immunohistochemical results revealed that REPS2 protein was obviously lower in ESCC tissues compared with adjacent non-cancerous tissues and normal esophageal tissues. We also demonstrated that the expression levels of REPS2 mRNA and protein in the ESCC tissues were significantly lower than in the adjacent non-cancerous tissues and normal tissues by qRT-PCR and western blotting analysis. Furthermore, REPS2 mRNA and protein expression levels in the HEEpic cell line were significantly higher than that in the ESCC cell lines. These findings were consistent with several other cancer reports (Oosterhoff et al., 2003; Penninkhof et

al., 2004; Oosterhoff et al., 2005; Badway et al., 2011), meanwhile suggestting that REPS2 played an important role in the progression of ESCC as a tumour suppressor. However, the underlying mechanisms for the decreased expression of REPS2 in tumour are still incompletely understood but likely involved in growth factor receptor-mediated endocytosis and signalling through its influence on the Ral signalling pathway. The activated Ral forms a complex with REPS2 through RalBP1 (a GTPase-activating protein), whereas REPS2 could function as an inhibitor of the transport activity of RalBP1. Recently, it has been reported that increased REPS2 expression negatively affects EGF receptor internalisation and subsequent signaling, while loss of REPS2 expression in prostate cancer cells results in dysregulation of growth factor signaling and consequently in loss control of cell proliferation (Oosterhoff et al., 2003). Meanwhile, REPS2 isoform 2 downregulation has been observed during progression of prostate cancer from androgen to EGF dependency (Oosterhoff et al., 2003; Oosterhoff et al., 2005). It is also found that downregulation of REPS2 is accompanied by upregulation of NF-kB activity during progression of prostate cancer from androgen-dependent to androgen-independent growth, and that NF-kB may promote cell proliferation through interacting with the EH domain of REPS2 (Penninkhof et al., 2004). In addition, REPS2 may function to keep RalBP1 cytosolic where it displays a GAP activity towards Rac1 and Cdc42 to signal cell survival so that overexpression of REPS2 may lead to a strong inhibition of Rac1 and Cdc42 signalling, which may consequently result in the attendant induction of apoptosis (Jullien-Flores et al., 1995; Joneson et al., 1999; Rincon et al., 2009). Above molecular mechanisms may provide the theoretical basis of REPS2 for participating in the the progression of ESCC, but that need further research to testify in future clinical practice.

From immunohistochemical analysis, we correlated REPS2 protein expression with respect to various clinicopathological factors in 120 ESCC patients. Our results showed that low expression of REPS2 was significantly associated with primary tumour, TNM stage, lymph node metastasis and recurrence respectively. Patients with low expression of REPS2 were significantly associated with shorter overall and disease-free survival by using the Kaplan–Meier analysis. In particular, low expression of REPS2 was significantly associated with a poor prognosis in early stage patients with ESCC. Previous research works have demonstrated that decreased REPS2 expression is observed in earlier work during prostate cancer progression, and which could add to the androgen-independent state of advanced prostate cancer (Chang et al., 1997; Oosterhoff et al., 2003). Actually, low expression of REPS2 should be more obviously associated with a poor prognosis in advanced stage patients, whereas our results did not provide strong support. After careful consideration, we thinked that it might be caused by low sample size. Anyhow, REPS2 protein expression pattern might be a valuable prognostic marker for patients with ESCC. More importantly, from univariate and multivariate analysis we obtained sufficient evidence to educe that REPS2 expression level was an independent prognostic indicator for ESCC patients. Previous studies have shown that downregulation of REPS2 is correlated with poor therapeutic outcome in human prostate cancer and breast cancer, and furthermore, overexpression of REPS2 might serve as a useful biomarker for favorable prognosis in prostate and breast cancers (Oosterhoff et al., 2003; Oosterhoff et al., 2005; Doolan et al., 2009).

It is well known that lymph node metastasis could be used as a prognostic factor for ESCC patients, which was consistent with our present study. Our preceding research has revealed that low REPS2 protein expression was well correlated with lymph node metastasis. Thus, a subset-combined survival analysis was carried out by both REPS2 protein expression and the lymph node status. The results revealed that patients with the phenotype of low REPS2 expression/lymph node metastasis (+) had both shorter overall and disease-free survival time than patients with other phenotypes. Therefore, the evaluation of REPS2 protein together with the lymph node status may further provide new information for patients' prognosis, and provide better planning of appropriate treatment strategies and better management after surgery. REPS2 has been thinked as a molecular scaffold recruiting proteins involved in vesicular trafficking and linking them to actin cytoskeleton, owing to its links to actin cytoskeleton remodeling and to receptor endocytosis (Tomassi et al., 2008). We have already mentioned the overexpression of REPS2 may lead to a strong inhibition of Rac1 and Cdc42 signalling that is required for the actin cytoskeleton organization and cell morphogenesis. Rac1 and Cdc42, two members of the Rho family of small GTPases, are all involved in cell transformation, survival, proliferation, invasion and metastasis of human cancer cells (Rincon et al., 2009; Bashir et al., 2010; Feng et al., 2012). Overexpression of Rac1 and Cdc42 have been reported in several types of human cancer including esophageal cancer (Bashir et al., 2010; Feng et al., 2012). Moreover, the latest published report indicated that REPS2 inhibited cell migration by interaction with the paxillin-associated protein PAG2 through its proline-rich motif (Oshiro et al., 2002). It is widely accepted that cell migration is critical for tumour formation and metastasis. Thus, we speculate that downregulation of REPS2 promotes cell migration by its binding to PAG2 or enhncement of Rac1 and Cdc42 signalling so that contribute to the high probability of lymph node metastasis. Although close association between REPS2 expression and ESCC metastasis has been established in our study, the possible mechanisms are still unclear that need further investigations.

In conclusion, our current investigation demonstrated that REPS2 was downregulated in human ESCC and that decreased REPS2 expression was significantly associated with the progression and poor prognosis in ESCC patients, indicating that REPS2 might serve as a valuable prognosis biomarker for ESCC patients. However, further research will be required to determine the molecular mechanism of REPS2 involved in ESCC progression and prognosis, which may lead to further development of new approaches targeting REPS2 for effective cancer management.

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References

Badway JA, Baleja JD (2011). Reps2: a cellular signaling and molecular trafficking nexus. *Int J Biochem Cell Biol*, **43**, 1660-3.

Bashir M, Kirmani D, Bhat HF, et al (2010). P66shc and its downstream Eps8 and Rac1 proteins are upregulated in esophageal cancers. *Cell Commun Signal*, **8**, 13.

Chai J, Jamal MM (2012). Esophageal malignancy: A growing concern. *World J Gastroenterol*, **18**, 6521-6.

Chang GT, Blok LJ, Steenbeek M, et al (1997). Differentially expressed genes in androgen-dependent and -independent prostate carcinomas. *Cancer Res*, **57**, 4075-81.

Chen H, Fre S, Slepnev VI, et al (1998). Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature*, **394**, 793-7.

Chen SB, Weng HR, Wang G, et al (2013). Prognostic factors and outcome for patients with esophageal squamous cell carcinoma underwent surgical resection alone: evaluation of the seventh edition of the american joint committee on cancer staging system for esophageal squamous cell carcinoma. *J Thorac Oncol*, **8**, 495-501.

Doolan P, Clynes M, Kennedy S, et al (2009). TMEM25, REPS2 and Meis 1: favourable prognostic and predictive biomarkers for breast cancer. *Tumour Biol*, **30**, 200-9.

Feng JG, Liu Q, Qin X, et al (2012). Clinicopathological pattern and Annexin A2 and Cdc42 status in patients presenting with differentiation and lymphnode metastasis of esophageal squamous cell carcinomas. *Mol Biol Rep*, **39**, 1267-74.

Ikeda M, Ishida O, Hinoi T, et al (1998). Identification and characterization of a novel protein interacting with Ral-binding protein 1, a putative effector protein of Ral. *J Biol Chem*, **273**, 814-21.

Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA Cancer J Clin*, **61**, 69-90.

Joneson T, Bar-Sagi D (1999). Suppression of Ras-induced apoptosis by the Rac GTPase. *Mol Cell Biol*, **19**, 5892-901.

Jullien-Flores V, Dorseuil O, Romero F, et al (1995). Bridging Ral GTPase to Rho pathways. RLIP76, a Ral effector with CDC42/Rac GTPase-activating protein activity. *J Biol Chem*, **270**, 22473-77.

Kariya K, Koyama S, Nakashima S, et al (2000). Regulation of complex formation of POB1/epsin/adaptor protein complex 2 by mitotic phosphorylation. *J Biol Chem*, **275**, 18399-406.

Loudig O, Brandwein-Gensler M, Kim RS, et al (2011). Illumina whole-genome complementary DNA-mediated annealing, selection, extension and ligation platform: assessing its performance in formalin-fixed, paraffin-embedded samples and identifying invasion pattern-related genes in oral squamous cell carcinoma. *Hum Pathol*, **42**, 1911-22.

Morinaka K, Koyama S, Nakashima S, et al (1999). Epsin binds to the EH domain of POB1 and regulates receptor-mediated endocytosis. *Oncogene*, **18**, 5915-22.

Nakashima S, Morinaka K, Koyama S, et al (1999). Small G protein Ral and its downstream molecules regulate endocytosis of EGF and insulin receptors. *EMBO J*, **18**, 3629-42.

Oosterhoff JK, Kuhne LC, Grootegoed JA, Blok LJ (2005). EGF signalling in prostate cancer cell lines is inhibited by a high expression level of the endocytosis protein REPS2. *Int J Cancer*, **113**, 561-7.

Oosterhoff JK, Penninkhof F, Brinkmann AO, et al (2003). REPS2/POB1 is downregulated during human prostate cancer progression and inhibits growth factor signalling in prostate cancer cells. *Oncogene*, **22**, 2920-25.

Oshiro T, Koyama S, Sugiyama S, et al (2002). Interaction of POB1, a downstream molecule of small G protein Ral, with PAG2, a paxillin-binding protein, is involved in cell migration. *J Biol Chem*, **277**, 38618-26.

Penninkhof F, Grootegoed JA, Blok LJ (2004). Identification of REPS2 as a putative modulator of NF-kappaB activity in prostate cancer cells. *Oncogene*, **23**, 5607-15.

Rincon SA, Ye Y, Villar-Tajadura MA, et al (2009). Pob1 participates in the Cdc42 regulation of fission yeast actin cytoskeleton. *Mol Biol Cell*, **20**, 4390-9.

Rodriguez-Rocha H, Garcia Garcia A, Zavala-Flores L, et al (2012). Glutaredoxin 1 protects dopaminergic cells by increased protein glutathionylation in experimental Parkinson's disease. *Antioxid Redox Signal*, **17**, 1676-93.

Singhal SS, Yadav S, Drake K, et al (2008). Hsf-1 and POB1 induce drug sensitivity and apoptosis by inhibiting Ralbp1. *J Biol Chem*, **283**, 19714-29.

Tomassi L, Costantini A, Corallino S, et al (2008). The central proline rich region of POB1/REPS2 plays a regulatory role in epidermal growth factor receptor endocytosis by binding to 14-3-3 and SH3 domain-containing proteins. *BMC Biochem*, **9**, 21.

Toya M, Iino Y, Yamamoto M (1999). Fission yeast Pob1p, which is homologous to budding yeast Boi proteins and exhibits subcellular localization close to actin patches, is essential for cell elongation and separation. *Mol Biol Cell*, **10**, 2745-57.