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

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...
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 RabBP1 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 Joint Committee on 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, IIC 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 (HEEp) 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% CO₂.

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'-CTGAGAACACGAGACACCA-3', reverse 5'-TITAGGTCTGCCCCCTTGTG-3'; β-actin primer (205 bp), forward 5'-TGCAGGATCCACCCGAAAG-3', reverse 5'-CTGAGAGTGGTCGACGGAGG-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...
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Table 1. Correlation Between REPS2 Expression and Clinicopathologic Features of ESCC Patients (n=120)

| Parameters          | Case | REPS2 expression (n) | χ² | 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

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′-diaminobenzidine 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 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 χ² 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) | <0.001 | 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

Correlation between decreased REPS2 protein expression and clinicopathological parameters

The association between REPS2 protein expression and clinicopathological characteristics of ESCC was explored by the χ² 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
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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 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 esophageal tissues. Further, we 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
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Analysis we obtained sufficient evidence to deduce 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 thought as a molecular scaffold recruiting proteins involved in vesicular trafficking and linking them 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 enhancement 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.
Acknowledgements

This work was supported by grants from the National Natural Scientific Foundation of China (No. 30670990, 30871189) and the Province Natural Scientific Foundation of Hunan (No. 2010FJ3134). We thank Liyuan Feng (Department of pathology, Xiangya Hospital, Central South University) and Xueping Feng (Central Laboratory of Medical Research, Xiangya Hospital, Central South University) for their evaluation of these clinical samples. The author(s) declare that they have no competing interests.

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