Soluble purified recombinant C2ORF40 protein inhibits tumor cell growth *in vivo* by decreasing telomerase activity in esophageal squamous cell carcinoma

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Abstract. The chromosome 2 open reading frame 40 (*C2ORF40*) gene is a candidate tumor suppressor gene for a variety of tumors. Previous results by the present authors revealed that the C2ORF40 protein is a secreted protein. However, the exact biological function of secreted C2ORF40 protein in carcinogenesis has not been thoroughly investigated. In the present study, the signal peptide sequence of the *C2ORF40* cDNA was initially removed to produce secreted recombinant human C2ORF40 protein (rhC2ORF40). Soluble rhC2ORF40 was successfully expressed and purified, which was evaluated for the first time, to the best of our knowledge, for tumor-suppressing function *in vivo* in esophageal cancer. The present results revealed that soluble purified rhC2ORF40 was concentrated with a purity of >95%. Furthermore, rhC2ORF40 inhibited esophageal cancer cell growth *in vivo* in a dose-dependent manner compared with a control group ($P<0.05$). In addition, the present study demonstrated for the first time that rhC2ORF40 decreased telomerase activity using telomeric repeat amplification protocol-enzyme-linked immunosorbent assay ($P<0.05$), without affecting the expression levels of telomerase-component RNA ($P>0.05$), as shown with polymerase chain reaction. Overall, the present results demonstrated that soluble rhC2ORF40 inhibited tumor cell growth *in vivo* by decreasing telomerase activity in esophageal squamous cell carcinoma. Therefore, soluble rhC2ORF40 with a high purity and biological activity may be a potential biological therapy drug for esophageal cancer.

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Introduction

Esophageal carcinoma is foremost for cancer incidence and mortality rates in males and females in developing countries (1). In addition, ~50% of esophageal cancer cases in the world occur in China, and esophageal squamous cell carcinoma (ESCC) accounts for ~90% of all esophageal cancers diagnosed in China each year (1). To date, the molecular pathogenesis of ESCC remains unclear. At present, the focus for research is transitioning between the cloning of novel tumor-associated genes and characterizing the biological function of the protein product (2). As a result, a major research effort has been directed at expressing and identifying the function of novel specific esophageal cancer-associated proteins and elucidating the relevant molecular mechanisms in the carcinogenesis in ESCC.

Human chromosome 2 open reading frame 40 (*C2ORF40*) gene, also referred to as *ECRG4*, is expressed in various normal tissues, including the esophageal epithelium, heart, brain, placenta, lung, liver, kidney and pancreas (3,4). The *C2ORF40* gene is important in processes associated with physiological functional regulation, including inflammation, injury, senescence, the neuroendocrines environment, differentiation and apoptosis (5-13). Notably, previous studies have indicated that *C2ORF40* is a candidate tumor suppressor gene associated with prognosis in a variety of tumors (4,14-22). In addition, *C2ORF40* has been demonstrated to be chemosensitive to 5-fluorouracil and cisplatin (23,24). Previous research by the present authors demonstrated that *C2ORF40* is a candidate tumor suppressor gene and an independent prognostic factor in ESCC, and *C2ORF40* gene overexpression inhibits tumor cell proliferation and invasion in ESCC (25-29). Notably, additional bioinformatics analysis indicated that pro-C2ORF40 protein was a secreted protein with a signal peptide. In addition, previous studies indicated that secreted C2ORF40 protein exists in *C2ORF40* gene-transfected

esophageal cancer cell medium (30). However, the exact biological function of secreted C2ORF40 protein in carcinogenesis has not been thoroughly investigated.

The present study initially expressed and purified soluble recombinant human C2ORF40 protein (rhC2ORF40), validated the tumor-suppressing biological activities of rhC2ORF40 protein *in vivo,* and explored the possible molecular mechanism of rhC2ORF40 in ESCC, to the best of our knowledge, for the first time.

Materials and methods

Production and purification of soluble rhC2ORF40. The pGEM-T-C2ORF40 vector and pET30a (+) plasmid used in the present study were constructed at the State Key Laboratory of Molecular Oncology and Department of Etiology and Carcinogenesis of the Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). The shortened rhC2ORF40 cDNA (with the 1-28 amino acid signal peptide sequence deleted) was excised from the preserved pGEM-T-C2ORF40 vector and subcloned into the pET30a (+) plasmid using a previously described method (30). The resulting product was an inducible pET30a-C2ORF40 expression vector encoding His-tagged soluble rhC2ORF40 (without a signal peptide).

The cDNA was amplified by polymerase chain reaction (PCR) using the GoldScript one-step RT-PCR kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primers were as follows: Forward, 5'-TCGGATCCATAA GTGGAAATAACTC-3' and reverse 5'-TCAAGCTTTTAG TAGTCATCGTAGTT-3' (Invitrogen™; Thermo Fisher Scientific, Inc.). The thermal cycling conditions were: 95˚C for 5 min; 35 cycles of 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 45 sec; followed by extension at 72˚C for 7 min. The PCR product was digested by *Bam*HI and *Hin*dIII. Subsequently, the recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) cells (Takara Biotechnology Co., Ltd., Dalian, China), according to a previous study (30), to produce N-terminal His-tagged soluble rhC2ORF40.

rhC2ORF40 expression in *E. coli* BL21 cells was induced with 0.3 mM isopropyl-D-thiogalactopyranoside and detected by western blotting, according to a previous study (31). Briefly, total protein was extracted from *E. coli* BL21 cells using the Complete Bacterial Protein Extraction Reagent (cat. no. 89821; Pierce Biotechnology, Inc., Rockford, IL, USA), and the resulting protein lysate was separated by 15% SDS-PAGE, followed by transfer onto polyvinylidene fluoride membranes. The mebranes were blocked with 5% bovine serum albumin (Pierce Biotechnology, Inc.) for 1 h at room temperature, followed by incubation with rabbit anti-ECRG4 polyclonal antibody (cat. no. sc-135139; 1:150 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated chicken anti-rabbit secondary antibody (cat. no. sc-516087; 1:2,000 dilution; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The membranes were visualized by enhanced chemiluminiescence to confirm the presence of rhC2ORF40.

rhC2ORF40 was purified and renatured by affinity chromatography with nickel-nitrilotriacetic acid resin (Merck Millipore, Darmstadt, Germany), according to the manufacturer's protocol. Purified rhC2ORF40 was dialyzed in phosphate-buffered saline (PBS), 0.1 M sodium phosphate and 0.15 M sodium chloride (pH 7.4) to remove the denaturant. Soluble rhC2ORF40 was used for additional experiments.

Tumor growth in vivo. A total of 24 six-week-old female BALB/c nude mice weighing 16-18 g were obtained from Beijing Vital River Laboratory Animal Technology, Co., Ltd. (Beijing, China). The mice were housed at four mice per cage and were maintained at 25-27˚C and 45-50% humidity, under a 12-h light/dark cycle. The mice were fed *ad libitum* with autoclaved food. Esophageal cancer EC9706 cells ($5x10^6$; Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) that had been cultured in RPMI-1640 medium containing 10% fetal bovine serum (both Invitrogen; Thermo Fisher Scientific, Inc.) in 5% CO_2 at 37˚C for 48 h, were incubated in Trypsin-EDTA (Invitrogen; Thermo Fisher Scientific, Inc.), washed with PBS, centrifuged at 1,500 x g for 5 min, resuspended in PBS, and injected subcutaneously into the armpit region of the nude mice. When the mean tumor volume reached 100 mm^3, the nude mice were randomly divided into two groups (eight mice per group). The rhC2ORF40 treatment group received various concentrations of rhC2ORF40 (0.1, 1.0 and 10.0 mg/kg) injected subcutaneously around tumors every other day, and the control group mice were injected with 200 μl PBS. Tumor volumes were recorded twice per week thereafter for 14 days. At the end of the 14 days, the mice were sacrificed by cervical dislocation. Tumor sizes were estimated from the length (a) and width (b) of the tumors, as measured using calipers, according to the following formula: Tumor volume = ab^2 / 2. Nude mice experiments were approved by Zhengzhou University Ethics Committee (Zhengzhou, China; approval no. U1304817).

Telomerase activity assay. The telomerase activities of EC9706 cells treated with 10 μg/ml rhC2ORF40 or PBS for 48 h were examined by telomeric repeat amplification protocol (TRAP)-ELISA kits (JRDUN Biotechnology (Shanghai), Co., Ltd., Shanghai, China), according to the manufacturer's protocol. The mean values for statistical analysis was the average of three independent experiments.

Telomerase-component RNA reverse transcription PCR (RT-PCR). The human telomerase-component RNA (hTR) of EC9706 cells, which were treated with 10 μg/ml rhC2ORF40 or PBS for 48 h, was examined using RT-PCR. Briefly, total RNA was extracted from EC9706 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the extracted RNA was treated with DNase (Invitrogen; Thermo Fisher Scientific, Inc.) to remove contaminating genomic DNA. The purity and concentration of the extracted RNA was assessed using an ultraviolet spectrophotometer. RT-PCR was performed using the GoldScript one-step RT-PCR kit, with the following primers: hTR, forward 5'-CTAACCCTATCT GAGTTGGGCGTA-3' and reverse 5'-CAACGGACAGAC AGCAGCTGACAT-3'; and GAPDH forward, 5'-AGAAGG CTGGGGCTCATTTG-3' and reverse, 5'-AGGGGCCATCCA CAGTCTTC-3' (Invitrogen™; Thermo Fisher Scientific, Inc.). The PCR conditions were 35 cycles at 95˚C for 45 sec, 55˚C for

45 sec and 72˚C for 65 sec, followed by an extension at 72˚C for 10 min. The PCR products were separated by 1.5% agarose gel electrophoresis and referenced to a DNA molecular weight marker (Takara Biotechnology Co., Ltd.). The GDS-8000 Gel Image Analysis system (UVP, LLC, Upland, CA, USA) was used to quantify the band intensities.

Statistical analysis. All statistical analysis was performed with SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). Statistical significance was determined using Student's t test and analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Soluble purified rhC2ORF40 was obtained for in vivo functional experiments. The present authors previously demonstrated that C2ORF40 protein was secreted from a sub-cellular location in esophageal cells (30). The Universal Protein Resource (http://web.expasy.org) predicted that C2ORF40 protein was a secreted protein with a signal peptide. In addition, it was revealed that secreted C2ORF40 protein existed in esophageal cancer EC9706 cell medium transfected with a *C2ORF40* expression plasmid compared with an empty plasmid control group, as shown by western blotting (30). Therefore, C2ORF40 protein was a secreted protein, which is secreted into the extracellular environment where it exhibits biological functions.

In the present study, the signal peptide sequence of the *C2ORF40* cDNA was removed to produce soluble secreted rhC2ORF40. The constructed expression plasmid pET30a-C2ORF40 was identified by PCR restrictive enzyme digestion and DNA sequencing. Subsequently, recombinant *E. coli* BL21 strains, which expressed soluble rhC2ORF40, were obtained. Soluble rhC2ORF40 was specifically recognized by anti-His and anti-C2ORF40 antibodies (data not shown). Soluble rhC2ORF40 was purified with a purity of >95%. Therefore, soluble rhC2ORF40 with a high purity was successfully obtained for *in vivo* functional experiments by the present study.

rhC2ORF40 suppresses tumor growth in vivo in ESCC. The effect of rhC2ORF40 on tumor growth *in vivo* was additionally evaluated in ESCC. Esophageal cancer EC9706 cells were subcutaneously injected into athymic nude mice. One week following tumor cell injection, the experimental group of mice received various doses of rhC2ORF40 (0.1, 1.0 and 10.0 mg/kg) and the control group was treated with 200 μl PBS. The mice were sacrificed 2 weeks following rhC2ORF40 protein treatment and tumor volumes were measured. The results revealed that purified rhC2ORF40 inhibited the growth of EC9706 tumor xenografts in nude mice in a dose-dependent manner. Compared with the PBS control group, rhC2ORF40 treatment (1 and 10 mg/kg) significantly inhibited the development of xenograft growth *in vivo* (P=0.014 and P=0.001 for 1 and 10 mg/kg rhC2ORF40, respectively); however, 0.1 mg/kg rhC2ORF40 treatment did not demonstrate a suppressive effect compared with the control mice (P=0.86; Fig. 1). Therefore, the results suggest that soluble rhC2ORF40 inhibits tumor cell growth *in vivo* in ESCC in a dose-dependent manner.

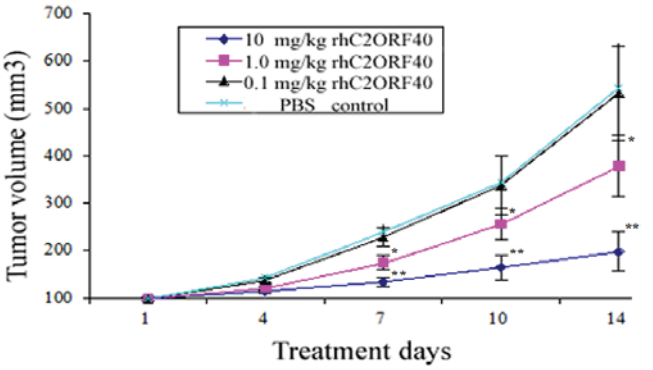

Figure 1. Effect of rhC2ORF40 on tumor growth *in vivo*. Subcutaneous tumor growth curves of rhC2ORF40 treatment and control (PBS) groups *in vivo*. Compared with the PBS control group, the rhC2ORF40 treatment (1 and 10 mg/kg) groups significantly inhibited xenograft tumor growth. The 0.1 mg/kg rhC2ORF40 treatment group did not demonstrate a suppressive effect on tumor growth compared with the control. Error bars represent standard deviation from mean value. *P<0.05, **P<0.01 vs. PBS control cells. rhC2ORF40, recombinant human chromosome 2 open reading frame 40 protein; PBS, phosphate buffered saline.

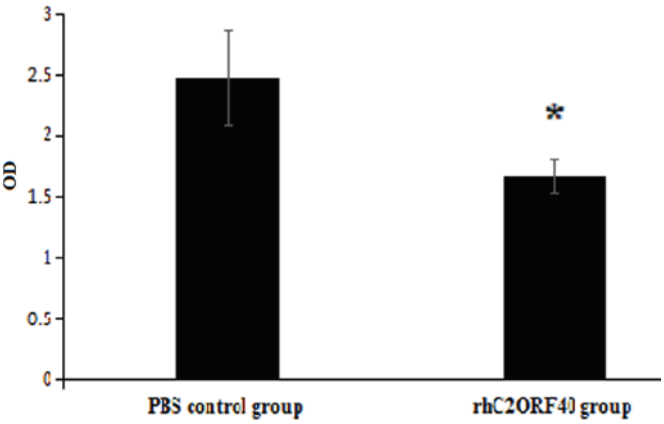

Figure 2. A telomeric repeat amplification protocol (TRAP)-ELISA was performed to investigate the effect of rhC2ORF40 on the telomerase activity of EC9706 cells. EC9706 cells were treated with 10 μg/ml rhC2ORF40 or PBS for 48 h, after which the telomerase was analyzed using a TRAP-ELISA kit. Data are presented as the mean ± standard deviation. *P<0.05 vs. the PBS control group. rhC2ORF40, recombinant human chromosome 2 open reading frame 40 protein; PBS, phosphate-buffered saline; OD, optical density.

rhC2ORF40 decreases telomerase activity. The telomerase activity of EC9706 cells was also examined, in order to investigate the molecular mechanism of the tumor-suppressive function exhibited by rhC2ORF40. A TRAP-ELISA revealed that the telomerase activities in the control cell group and rhC2ORF40 treatment (10 μg/ml) cell group were 2.475±0.395 and 1.672±0.138, respectively (P=0.017; Fig. 2). This result indicated that rhC2ORF40 decreased the telomerase activity in EC9706 cells.

Effect of rhC2ORF40 on telomerase-component RNA transcription. The GC-rich region of telomerase-component RNA is essential for ribonucleoprotein enzyme biological function (1). To investigate the detailed molecular mechanisms of the decrease in telomerase activity induced by rhC2ORF40, the present study evaluated the transcriptional alteration of telomerase-component RNA in EC9706 cells. There was no

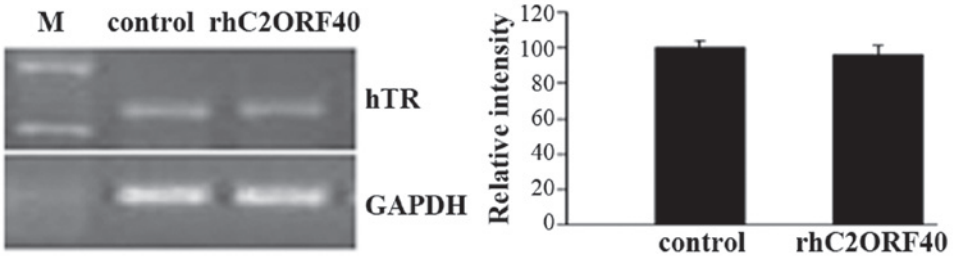

Figure 3. Effect of rhC2ORF40 on hTR transcription using reverse transcription-polymerase chain reaction. hTR level in the rhC2ORF40 treatment group (10 μg/ml) was similar compared with the control group (P>0.05). Data are presented as the mean ± standard deviation. M, molecular weight ladder; rhC2ORF40, recombinant human chromosome 2 open reading frame 40 protein; hTR, human telomerase-component RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

apparent alteration in telomerase-component RNA levels in the rhC2ORF40 treatment group (10 μg/ml) compared with the control group (P=0.98; Fig. 3). This result demonstrated that rhC2ORF40 does not affect telomerase-component RNA transcription; however, it may post-transcriptionally modulate telomerase activity in ESCC.

Discussion

ESCC is a highly invasive and clinically challenging cancer in China. Despite advances in clinical comprehensive treatment, ESCC prognosis remains poor, due to its diffuse and invasive nature (32). Novel biological therapy drugs with high anti-tumor efficacy are being constantly sought to improve the survival of ESCC patients. A previous study by the present authors revealed that C2ORF40 was secreted into cell medium, and rhC2ORF40 inhibits EC9706 cell proliferation *in vitro* (30). In the present study, soluble secreted rhC2ORF40 was expressed and purified, and subsequent experiments revealed for the first time, to the best of our knowledge, that rhC2ORF40 inhibits ESCC tumor cell growth *in vivo* in a dose-dependent manner. Although all the mice treated with rhC2ORF40 possessed tumors at the end of the experimental phase, mice treated with 1 and 10 mg/kg rhC2ORF40 had tumors that grew more slowly compared with those in the control group (P<0.05). In addition, compared with the control group, the rhC2ORF40 treatment group (1 and 10 mg/kg) had significantly inhibited xenograft tumor growth (P<0.05); the tumor weights of the control group mice were increased compared with those of the rhC2ORF40 treatment group (P<0.05). Therefore, soluble rhC2ORF40 with high purity and biological activity was obtained by the present study. Furthermore, the *in vivo* tumor-suppressing functional experiments by the present study demonstrated that soluble rhC2ORF40 may be a candidate biological therapy for ESCC.

Transformed cells acquire a series of malignant traits, during ESCC development and progression. Among them, escape from cellular death by constitutive telomerase activation, or alternative telomere maintenance, represents a trait that tumor cells acquire for indefinite growth during carcinogenesis (32). The present study revealed that rhC2ORF40 decreased telomerase activity in ESCC cells (P<0.05). However, there was no apparent alteration in telomerase RNA level (P>0.05). Therefore, the present study hypothesizes that C2ORF40 post-transcriptionally modulates telomerase, either by direct protein interaction or by indirect protein signal transduction.

Cell cycle alteration is also a key phase in carcinogenesis. Once cell cycle regulation is broken, tumorigenesis may result. In addition, recent studies have revealed that a decrease in telomerase activity correlates closely with cell cycle arrest (33,34). The present authors previously demonstrated that rhC2ORF40 causes a cell cycle G1/S phase block in esophageal cancer cells *in vitro* (30). In the present study, rhC2ORF40 was shown to decrease the telomerase activity in EC9706 cells, which may underlie the mechanism of rhC2ORF40-mediated inhibition of ESCC tumor cell growth *in vivo*.

Numerous oncogenes and tumor suppressor genes are directly involved in the regulation of the cell cycle. Among them p21 and p16 genes, critical cyclin-dependent kinase inhibitors, are hypothesized to be functionally relevant to the regulation of cell cycle G1 phase. Previous results have revealed that *C2ORF40* transfection induces the upregulation of *p21* expression in ESCC (25-27). However, there is no significant upregulation of *p16* expression. Therefore, the increased expression of *p21*, not p16, is likely to be responsible for the cell cycle G1 phase block induced by C2ORF40 in ESCC. An additional study also demonstrated that C2ORF40 causes cell cycle G1 phase block via interaction with Transmembrane Protease, Serine 11A in ESCC (27). Previous studies have indicated that C2ORF40 may be processed and released from the cell surface to function biologically (35,36). However, a detailed molecular mechanism for the tumor suppressing function of C2ORF40 protein remains to be clarified in ESCC.

Overall, the present study revealed that soluble rhC2ORF40 inhibits tumor cell growth *in vivo* by decreasing telomerase activity in ESCC. Soluble rhC2ORF40 was purified by the present study with a high purity and had a biological activity that may be a potential biological therapy for esophageal cancer in the future.

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