

Original Article

The clinical significance of isocitrate dehydrogenase 2 in esophageal squamous cell carcinoma

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Abstract: Isocitrate dehydrogenase 2 (IDH2) is the rate-limiting enzyme in the tricarboxylic acid (TCA) cycle in cellular metabolism. Growing evidence indicates that IDH2 plays a crucial role in the development of cancer. We aimed to investigate the expression level of IDH2 and its prognostic value in esophageal squamous cell cancer (ESCC). We evaluate the IDH2 expression and prognostic value in ESCC by immunohistochemical (IHC) staining, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting. The cell counting kit-8 (CCK8), clonogenic and invasion assays were performed to verify the IDH2 function in vitro. The protein expression level of IDH2 was significantly upregulated in ESCC tissues (IHC, Western blotting, all $P < 0.001$) despite no significant difference at mRNA expression level ($P > 0.05$). Kaplan-Meier analysis showed that IDH2 overexpression in ESCC patients was significantly related to worse overall survival (OS) and progression-free survival (PFS), $P = 0.003$ and 0.002 , respectively. The univariate and multivariate analyses revealed that IDH2 overexpression served as an independent prognostic factor for OS and PFS (all $P < 0.005$) in ESCC. The OD_{450} value, colony formation and invasive cell number were decreased in the shIDH2 groups (all $P < 0.0001$). The upregulation of IDH2 in ESCC cells showed opposite effects (all $P < 0.05$). Additionally, IDH2 knockdown phenotype can be rescued by shRNA-resistant IDH2 (all $P < 0.05$). These results demonstrated that IDH2 was upregulated in ESCC and could be used as a valuable prognostic marker for ESCC patients.

Keywords: ESCC, IDH2, expression, prognosis

Introduction

Esophageal cancer is the eighth most common cancer type and the sixth leading cause of cancer-related mortality globally [1]. According to the study on global cancer statistics of the National Cancer Institute, an estimated 16,910 new esophageal cancer cases and 15,690 death cases will occur in 2016 nationwide [2]. Despite the fact that the incidence of esophageal adenocarcinoma (EAC) is growing faster than other malignancies in the Western countries, squamous cell carcinoma (ESCC) is still the most common histological type of esophageal cancer worldwide [3]. New advances for the diagnosis and treatment of esophageal cancer have not changed the reality that the clinical outcome of ESCC patients remains poor, the 5-year overall survival (OS) rate ranges from 15% to 25% [4]. The most common bio-

logical changes during the development of ESCC include activation of oncogenes (such as EGFR and c-MYC), inactivation of tumor suppressor genes (such as p53, TOC and DLC1), and modification of cell cycle control by several mechanisms (such as amplification of Cyclin D1 and homozygous deletion or promoter methylation of MTS1) [5]. However, unlike alpha fetal protein (AFP) in hepatocellular carcinoma and carcinoembryonic antigen (CEA) in colon cancer, no good biomarker for ESCC has been widely applied in clinical practice. Accordingly, identification of novel biomarkers for ESCC should be emphasized.

Besides being involved in maintaining proliferative signaling, evading growth suppressors, immune escape, replicative immortality, tumor-promoting inflammation, invasion and metastasis, angiogenesis, genome instability and muta-

tion and resisting cell death, the reprogramming of energy metabolism has been regarded as an emerging hallmark of cancer [6]. Isocitrate dehydrogenase (IDH) is a reversible enzyme in the tricarboxylic acid (TCA) cycle that can both catalyze the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG) and the reductive carboxylation of the reciprocal process, which depend on NADP^+ and NADPH/CO_2 respectively [7]. Meanwhile, IDH plays a crucial role in processing reactive oxygen species (ROS) induced by hypoxic tumor microenvironment to support cell growth through the production of nonmitochondrial NADPH [8]. IDH2, which is located in the mitochondria and utilizes NADP^+ as a cofactor, is one of the three members of the IDH family [9]. Detoxification mechanisms could be impaired in cancer cells due to the inactivation of IDH2, and further result in DNA damage [10].

To date, most of the studies about IDH in cancer have focused on its mutation status. Mutant IDH gains a novel enzymatic activity of converting α -KG to the metabolite 2-hydroxyglutarate (2-HG) by a reduction reaction [8]. A tumor-derived IDH mutant inhibits histone and DNA demethylation, which hinder cell differentiation [11, 12]. Mutations in IDH1 or 2 (mutant IDH1/2) are detected in several malignancies, including acute myeloid leukemia (AML), glioma, cholangiocarcinoma as well as chondrosarcoma and chondromas [13-16]. Moreover, mutant IDH1/2 is a poor prognostic factor in cytogenetically normal (CN)-AML and a positive prognostic marker in low-grade gliomas [17, 18].

However, expression of wild-type IDH2 and its clinical relevance in cancers have been less investigated. IDH2 is downregulated in hepatocellular carcinoma (HCC) and gastric cancer (GC) and patients with low IDH2 had lower 5-year survival [19, 20]. In contrast, IDH2 is expressed at high level in endometrial, prostate and testicular cancers [21, 22]. In addition, IDH2 expression is downregulated in *in situ* carcinoma and upregulated in infiltrating carcinoma in colon cancer patients [23]. The expression status and prognostic value of IDH2 has not been examined in patients with ESCC. In this study, the expression of IDH2 was evaluated in cancerous tissue and compared with its expression in paracancerous tissue by quantitative real-time PCR (qRT-PCR), Western blot

analysis and immunohistochemistry (IHC). The prognostic potential of IDH2 was also analyzed. Furthermore, we conducted *in vitro* experiments by interfering IDH2 expression to verify its effect on proliferation and invasion of ESCC cells.

Methods

Patients and specimens

A total of 28 ESCC tissue samples were collected from surgical tissue blocks in the Qilu Hospital of Shandong University from April 2015 to August 2015. Adjacent noncancerous tissue samples were obtained and used as control groups. All the tissues were immediately stored in a freezer at -80°C until used for experiments. A total of 119 formalin-fixed, paraffin-embedded (FFPE) tissue samples from patients who received subtotal esophagectomy and esophagogastric anastomosis plus regional lymph node dissection in 2009 were used for survival analysis. None of the patients enrolled in our cohort had received neoadjuvant therapy (chemotherapy and/or radiotherapy). All the specimens were pathologically confirmed. The AJCC Cancer Staging Manual, 7th edition was used for assessing tumor stages. Our study was approved by the Ethics Boards of Qilu Hospital of Shandong University and informed written consent was obtained from all patients.

IHC

After the ESCC tissue was fixed with 10% formalin and embedded in paraffin, they were cut into 4 μm sections, dried at 80°C for 15 min, dewaxed in xylene, rinsed in ethanol at various concentrations and rehydrated in double-distilled water. Citrate-EDTA buffer (2 mM EDTA, 10 mM citric acid, 0.05% Tween 20, pH 6.2) was used for antigen retrieval. Sections were incubated with hydrogen peroxide for blocking the peroxidase enzyme. Then the anti-IDH2 antibody (1:60; Proteintech, Chicago, IL, USA) was applied to the sections at 4°C overnight. Negative controls were incubated with PBS instead of the primary antibody. The sections were reacted with horseradish peroxidase (HRP)-labeled streptavidin by adding it together with the biotinylated secondary antibody. Then the sections were stained with DAB and counterstained with hematoxylin. We selected five fields ($\times 400$ magnification) at random for each sample and invited two pathologists to evalu-

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ate and score them independently. The intensity of the dye color and the number of positive cells were both used for assessing the scores. The dye color was classified as: 0 (no staining), 1 (weak), 2 (moderate) and 3 (intense). The number of positive cells was graded as 0 (<5%), 1 (5-25%) and 2 (25-50%), 3 (51-75%) and 4 (>75%). The final score was the multiplication value of these two scores. 0-1 scores (-), 2-4 scores (+), 5-8 scores (++) and 9-12 scores (+++). Samples with (+++) were regarded as overexpression.

qRT-PCR

The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for extracting total RNA from fresh tissues following the manufacturer's instructions. IDH2 expression at the mRNA level was assessed by SYBR Green Real Time PCR Master Mix (TOYOBO, Osaka, Japan) using the Bio-Rad Single Color Real-Time PCR system (Bio-Rad, Hercules, California, USA). The primers (Sangon Biotech, Shanghai, China) were designed and synthesized as follows: IDH2: 5'-CAAAAACATCCCACGCCTAGTC-3' (forward primer), 5'-CCCGGTCTGCCACAAAGT-3' (reverse primer); GAPDH: 5'-GAAGTCCGAGTCAACGG-AT-3' (forward primer), 5'-TGAAACACCGTCTG-GCCC-3' (reverse primer). The $2^{-\Delta\Delta CT}$ method was used for calculating the relative expression of IDH2. We performed all assays in triplicate and present the data as the mean \pm SD.

Western blot

Tissue blocks were grinded into homogenate. The proteins were extracted from 100 mg of each tissue sample using 1 ml RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin) and 10 μ l PMSF (Beyotime, Shanghai, China). Supernatant was divided and stored at -20°C for later use. Samples containing an identical amount of protein were electrophoresed on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Defatted dry milk (5%) was used to diminish nonspecific binding on the membranes. After incubation with the primary IDH2 antibody and β -actin antibody (1:100, Proteintech, Chicago, IL, USA), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. The bands were detected by the chemilumi-

nescence detection system (EMD Millipore, Billerica, MA, USA).

Cell culture

The human ESCC cell lines Eca109 (CCTCC) and Eca9706 (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 media (Gibco, Life Technologies Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Life Technologies Inc.). Cells were incubated in a 5% CO_2 atmosphere at 37°C .

Transfection

Plasmid containing a short hairpin RNA (shRNA) targeting IDH2 (Cat. No. HSH009353-LVRU6GP; GeneCopoeia, Inc., Rockville, MD, USA) and a nonsense shRNA (Cat. No. CSHCTRO01-LVR-U6GP; GeneCopoeia, Inc.) were used for RNA interference experiments. Cells were seeded in 24-well plates for 24 h. Lipofectamine solution was prepared by mixing 2 μ l of Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) and 50 μ l of Opti-MEM1 medium (Invitrogen) for 5 min. The plasmid solution was prepared by adding 1 μ g of recombinant plasmid into 50 μ l Opti-MEM1 medium, followed by incubation for 5 min. Then, the plasmid and the lipofectamine solutions were combined to obtain the transfection solution. A 100 μ l volume of the transfection solution was mixed with pen-strep-free RPMI 1640 and 0.5 ml of this mixture was added per well. After 24 h, the transfection medium was replaced with pen-strep-free normal medium. Puromycin (1.5 mg/ml) was used for selecting stably transfected cells.

Lentivirus transduction

We purchased lentiviruses (GeneCopoeia, Inc.) expressing open reading frame (ORF) of wild-type IDH2 (IDH2W) and a shRNA-resistant form of IDH2 (IDH2R) that harbors eight silent mutations within the sequence targeted by sh-IDH2-1. The targeting sequence by sh-IDH2-1 in the IDH2 was mutated from GACCGACTTCGA-CAAGAATAA to GACCTACCTCCACCAGGATCA by site-directed mutagenesis. Cells were plated in 24-well plate 24 hours prior to viral infection. 2 μ l of lentivirus was diluted in 0.5 ml complete medium with polybrene at a final concentration of 6 μ g/ml. The plates were placed for 2 hours at $4-8^{\circ}\text{C}$ and transferred to a 37°C incubator with 5% CO_2 for overnight incubation. The cells

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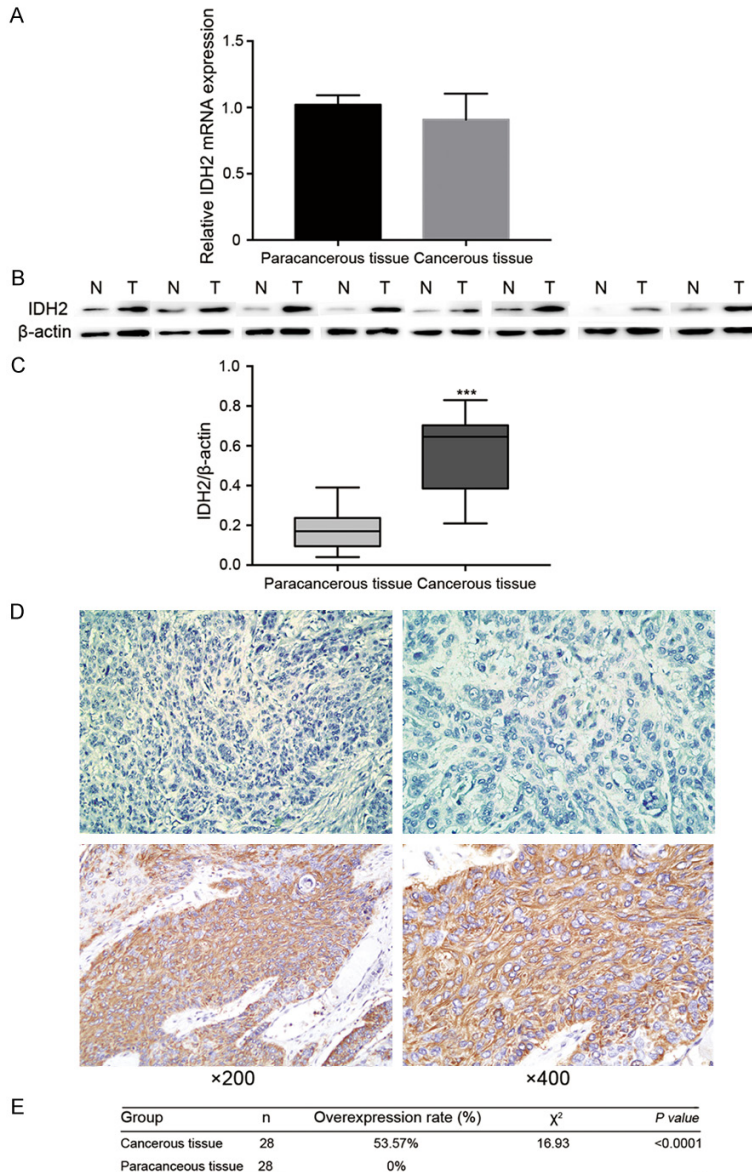


Figure 1. IDH2 expression in ESCC cancerous and paracancerous tissues from 28 patients. A: IDH2 expression at the mRNA level. B: Western blot bands of 8 pairs of cancerous and paracancerous tissues from ESCC patients. C: IDH2/ β -actin values of 8 pairs of ESCC tissues. D: Representative immunostaining of IDH2 in ESCC tissues: the upper ESCC tissue did not overexpressed IDH2, while the lower ESCC tissue overexpressed IDH2. E: Comparison of the results of the IHC analysis of cancerous and paracancerous tissues.

were trypsinized and re-seeded onto 6-well plates and incubated for 48 hours. Puromycin selection (1.5 mg/ml) was applied to select stably transduced cells.

Cell viability assays

A cell counting kit-8 assay (CCK8; Dojindo, Japan) was used to evaluate the proliferation of

Eca109 and Eca9706 cells. Cells were seeded into 96-well plates at a density of 2×10^3 per well. After culturing for 0, 24, 48, 72, 96 h, 120, and 144 h at 37°C , the medium was replaced with 100 μl fresh medium and 10 μl CCK-8 solution and incubated for an additional 1 h. The absorbance at 450 nm was measured using the Thermo Scientific Varioskan Flash spectrophotometer (Thermo Scientific, Finland). The mean value of 4 wells was used as the result of each sample. The data are expressed as the mean values \pm SD of three independent experiments.

Clonal efficiency assay

Transfected cells were trypsinized into single cell suspension and diluted in proportion. A total of 200 cells per dish were cultured for 2 weeks for knockdown experiments. 1000 cells per dish were used for overexpression and rescue experiments. Cells transfected with nonsense shRNA were used as controls. Colonies were fixed with ethanol and stained with crystal violet. Clone formation were defined as those containing at least 50 cells.

Transwell invasion assay

Matrigel (BD Bioscience, San Jose, CA, USA) was used for coating the membrane of the transwell chambers (Corning Costar, Cambridge, MA, USA).

Eca109 and Eca9706 cells were added into the upper chamber at a density of $2.5 \times 10^5/\text{ml}$ in serum-free medium, while the lower chamber contained normal medium with 10% FBS. The cells remaining on the upper chamber after 24 h were wiped off with a cotton swab. The cells attached to the lower membrane were fixed with methanol and stained with crystal violet.

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Table 1. The correlations of clinicopathologic variables of ESCC with IDH2 expression in FFPE cancerous tissues

Clinicopathological features	IDH2 overexpression		P ^a value
	No (n = 54)	Yes (n = 65)	
Age			0.852
<65	30	35	
≥65	24	30	
Gender			0.602
Female	25	29	
Male	27	38	
Smoking			0.933
No	27	33	
Yes	27	32	
Drinking			0.235
No	24	36	
Yes	30	29	
Differentiation			0.214
Well	26	22	
Moderate	13	24	
Poor	15	19	
T stage			0.011*
T1	10	5	
T2	16	26	
T3	24	18	
T4	4	16	
N stage			0.381
N0	25	24	
N1	11	18	
N2	8	15	
N3	10	8	
TNM stage			0.287
I	19	15	
II	11	19	
III	24	31	

P^a: Chi-square test. Abbreviation: FFPE, formalin-fixed paraffin-embedded. *P<0.05.

The number of invasive cells in 5 different fields under the inverted microscope was regarded as the mean value. Each samples were tested in triplicate and each assay was repeated 3 times.

Statistical analysis

The difference between the cancerous and paracancerous tissue groups was assessed by a paired Student's t-test. The difference between each pair combination among sh-NC, sh-IDH2-1, and sh-IDH2-2 was evaluated using

ANOVA with the post-hoc Tukey's test. The correlation of IDH2 and clinicopathological factors was determined with the Bilateral χ^2 test. Kaplan-Meier method and the log-rank test were used to obtain survival curves and compare the difference between subgroups. Univariate and multivariate Cox survival analyses were used for evaluating the hazard ratios (HRs). Receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) were used to determine the concordance level between the observed value and the actual value. Statistical analyses were performed with the SPSS software (Statistical Package for Social Sciences, Chicago, IL, USA). P<0.05 was considered statistically significant.

Results

The expression level of IDH2

The expression status of IDH2 in cancerous and paracancerous tissues was evaluated by qRT-PCR, Western blot and IHC analyses. The qRT-PCR analysis results indicated that the expression of IDH2 had no difference between two groups (**Figure 1A**, 1.02 ± 0.07 vs. 0.91 ± 0.20 , $P>0.05$). The Western blot analysis, which was performed on 8 pairs of cancerous and paracancerous tissues (**Figure 1B**), revealed that IDH2 was upregulated in cancerous tissues at the protein level (**Figure 1C**, IDH2/ β -actin: 0.57 ± 0.21 vs. 0.18 ± 0.11 , $P<0.001$). To test IDH2 expression further, FFPE tissue samples were prepared and the expression level of IDH2 protein was identified by IHC analysis. IDH2 was mainly distributed in the cytoplasm of ESCC cells (**Figure 1D**). The scores of the cancerous tissue samples were: 15 (+++), 8 (++) , 4 (+) and 1 (-), while those of the paracancerous tissues were: 24 (-) and 4 (+). Overall, the results revealed that IDH2 was overexpressed in 15 cancerous and 0 paracancerous tissue samples, and their difference was statistically significant (**Figure 1E**, $P<0.0001$). These results suggest that IDH2 expression was higher in cancerous tissues than in paracancerous tissues.

The prognostic value of IDH2

During the follow-up period, 43 patients (36.13%) were alive and (63.87%) died. The median survival time was 40 months (11-83 months). The IDH2 expression was assessed in FFPE tissue samples from 119 patients. Ac-

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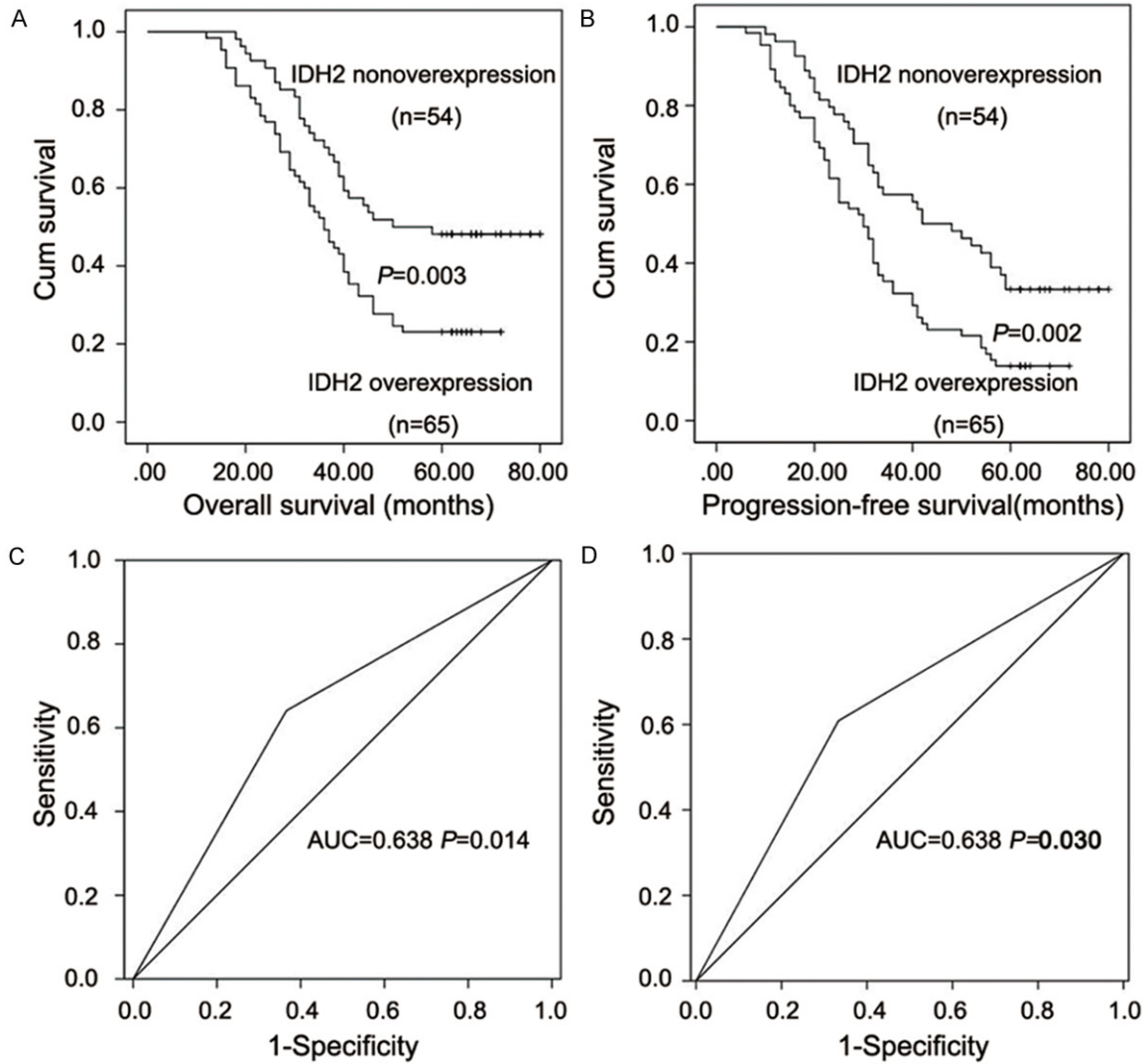


Figure 2. Kaplan-Meier curves and ROC curves analyses of IDH2 in ESCC tissues. A: Kaplan-Meier curve for OS. B: Kaplan-Meier curve for PFS. C: Receiver operating curve for OS. D: Receiver operating curve for PFS.

cording to the evaluation criteria, 65 (+++), 32 (++) , 17 (+) and 5 (-) samples were observed and the overall overexpression rate was 54.62%. The correlations of IDH2 expression with the clinical features of the patients, as determined by the bilateral χ^2 test, are shown in **Table 1**. T stage ($P=0.011$) was significantly related to the IDH2 expression. Age, gender, smoking, drinking, differentiation, N stage and TNM stage did not show any significant correlation with IDH2 expression. The Kaplan-Meier curve (**Figure 2A, 2B**) demonstrated that ESCC patients without IDH2 upregulation showed longer OS and progression-free survival (PFS) compared to patients with IDH2 upregulation (OS: $P=0.003$, PFS: $P=0.002$). The univariate survival analysis (**Table 2**) revealed that IDH2 expression had a negative correlation with OS and

PFS ($P=0.003$ and $P=0.002$, respectively). Multivariate analysis (**Table 2**) further verified that IDH2 may be an independent prognostic marker for OS and PFS in ESCC patients (both $P<0.001$). T stage and N stage, as well as adjuvant therapy were also found to be independent predictive markers for ESCC patients (OS: $P=0.016$, $P<0.001$ and $P=0.002$, respectively; PFS: $P=0.008$, $P<0.001$ and $P<0.001$, respectively). The ROC curves of IDH2 in the OS and PFS models are shown in **Figure 2C, 2D**, respectively.

Downregulation of IDH2 expression in ESCC cell lines

In order to determine whether the sh-IDH2 plasmids were successfully transfected or not, qRT-

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Table 2. Univariate and multivariate analyses of prognostic variables

Variable	OS		OS		PFS		PFS	
	Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis			
	P value	P value	HR	95% CI	P value	P value	HR	95% CI
Gender (Female VS. Male)	0.792	0.751	1.086	0.652-1.811	0.971	0.901	1.030	0.647-1.638
Age (<65 vs. ≥65)	0.760	0.483	0.828	0.489-1.403	0.908	0.638	0.892	0.554-1.436
Smoking (Yes vs. No)	0.765	0.138	1.624	0.855-3.084	0.835	0.213	1.457	0.806-2.635
Drinking (Yes vs. No)	0.671	0.200	1.555	0.792-3.051	0.460	0.083	1.735	0.931-3.236
T stage	0.001*	0.016*			<0.001*	0.008*		
T1			1.000	Ref.			1.000	Ref.
T2		0.026*	3.207	1.148-8.958		0.028*	2.798	1.118-7.003
T3		0.002*	5.075	1.845-13.96		0.001*	4.701	1.971-11.53
T4		0.062	2.638	0.951-7.314		0.045*	2.533	1.022-6.278
N stage	<0.001*	<0.001*			<0.001*	<0.001*		
N0			1.000	Ref.			1.000	Ref.
N1		0.002*	3.108	1.531-6.312		<0.001*	3.542	1.847-6.793
N2		0.004*	2.804	1.383-5.686		0.009*	2.392	1.241-4.610
N3		<0.001*	6.988	3.004-16.26		<0.001*	6.771	3.069-14.94
Differentiation	0.020*	0.272			0.140	0.447		
Well			1.000	Ref.			1.000	Ref.
Moderate		0.107	1.713	0.890-3.296		0.275	1.393	0.768-2.527
Poor		0.475	1.253	0.675-2.325		0.907	0.967	0.548-1.704
Adjuvant therapy (Yes vs. No)	0.034*	0.002	2.301	1.368-3.870	0.012*	<0.001*	2.577	1.584-4.192
IDH2	0.003*	<0.001*	3.107	1.735-5.565	0.002*	<0.001*	3.473	2.040-5.912

(Overexpression VS. nonoverexpression)

Abbreviations: OS, overall survival; PFS, progression-free survival; CI: confidence interval. *P<0.05.

PCR analysis (**Figure 3A**) and Western blot analysis (**Figure 3B, 3C**) were used to detect IDH2 expression at mRNA and protein levels, respectively, in Eca109 and Eca9706 cells. The result indicated that IDH2 mRNA was successfully downregulated by sh-IDH2-1 and sh-IDH2-2 compared to sh-NC (Eca109: 1.02 ± 0.13 vs. 0.42 ± 0.09 and 0.47 ± 0.08 , $P < 0.001$, respectively; Eca9706: 1.01 ± 0.15 vs. 0.45 ± 0.05 and 0.40 ± 0.05 , $P < 0.001$, respectively). In addition, the result also indicated that IDH2 protein expression was effectively downregulated by sh-IDH2-1 and sh-IDH2-2 compared to sh-NC (Eca109: 0.87 ± 0.09 vs. 0.40 ± 0.08 and 0.37 ± 0.11 , $P < 0.01$ and $P < 0.001$ respectively; Eca9706: 0.84 ± 0.07 vs. 0.44 ± 0.04 and 0.44 ± 0.10 , $P < 0.001$ respectively).

Downregulation of IDH2 inhibited ESCC cells proliferation and invasion

To ascertain whether IDH2 affects the proliferation of Eca109 and Eca9706 cells, we performed the CCK8 and colony formation assays. Compared with the sh-NC group, the OD₄₅₀ values of the Eca109 and Eca9706 cells transfected with sh-IDH2-1 and sh-IDH2-2 were

smaller (**Figure 4A**). At 144 h, the OD₄₅₀ values in Eca109 transfected with sh-NC vs. sh-IDH2-1 or sh-IDH2-2 were: 1.02 ± 0.09 vs. 0.56 ± 0.06 and 0.66 ± 0.06 , respectively, (all $P < 0.001$) and in Eca9706 transfected with sh-NC vs. sh-IDH2-1 and sh-IDH2-2 were: 1.10 ± 0.11 vs. 0.63 ± 0.04 and 0.67 ± 0.07 , respectively, (all $P < 0.001$). Similarly, sh-IDH2 transfection groups of ESCC cells showed less colony formation than sh-NC groups (**Figure 4B, 4C**). The clone number in Eca109 transfected with sh-NC vs. sh-IDH2-1 and sh-IDH2-2 were: 124 ± 5 vs. 55 ± 7 and 60 ± 6 , respectively, (all $P < 0.001$) and in Eca9706 transfected with sh-NC vs. sh-IDH2-1 and sh-IDH2-2 were: 129 ± 7 vs. 70 ± 8 and 74 ± 9 respectively, (all $P < 0.001$). To detect the effect IDH2 on the biological behaviors of Eca109 and Eca9706 cells, the transwell invasion assays were performed (**Figure 4D, 4E**). The number of invasive Eca109 cells of the sh-NC group vs. the sh-IDH2-1 and sh-IDH2-2 groups were: 431 ± 13 vs. 242 ± 18 and 230 ± 12 , respectively, (all $P < 0.001$), while the number of invasive Eca9706 cells of the sh-NC group vs. the sh-IDH2-1 and sh-IDH2-2 groups were: 447 ± 9 vs. 249 ± 12 and 258 ± 12 , respectively, (all $P < 0.001$). These results indi-

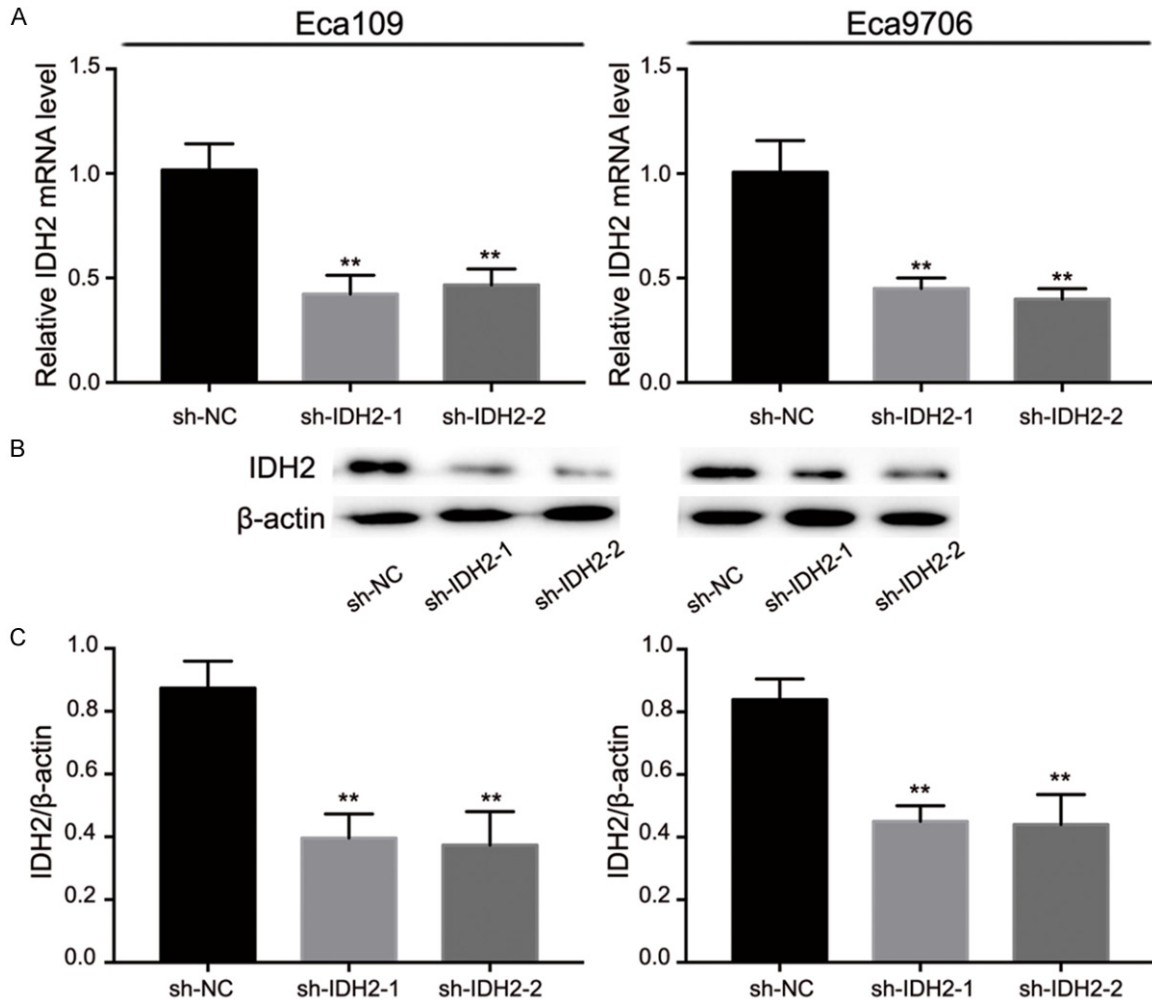


Figure 3. The downregulation effect of sh-IDH2-1 and sh-IDH2-2. A: sh-IDH2-1 and sh-IDH2-2 decreased mRNA expression of IDH2. B and C: Expression of IDH2 protein in Eca109 and Eca9706 was downregulated after sh-IDH2-1 and sh-IDH2-2 transfection.

cate that ESCC cells with higher level of IDH2 have stronger capability for invasion.

Overexpression of IDH2 promoted ESCC cells proliferation and invasion

To detect the gene expression level of IDH2 in control lentiviruses groups, IDH2W groups, and IDH2R groups. qRT-PCR and Western blot analysis were performed (Figure 5). IDH2 mRNA was successfully upregulated by IDH2W and IDH2R compared to control groups (Eca109: 0.96 ± 0.03 vs. 3.37 ± 0.22 and 3.33 ± 0.20 , $P < 0.001$, respectively; Eca9706: 0.96 ± 0.03 vs. 3.32 ± 0.31 and 3.40 ± 0.11 , $P < 0.001$, respectively). Western blot analysis confirmed the elevation of IDH2 expression level in IDH2W and IDH2R groups (Eca109: 0.79 ± 0.03 vs.

1.01 ± 0.02 and 1.02 ± 0.06 , $P < 0.01$ and 0.001 , respectively; Eca9706: 0.81 ± 0.03 vs. 1.11 ± 0.04 and 1.13 ± 0.07 , $P < 0.001$, respectively). The effect of IDH2 upregulation on ESCC cells were showed in Figure 6. The OD_{450} values at 144 h were higher in IDH2W and IDH2R groups than in control groups (Eca109: 1.00 ± 0.10 vs. 1.22 ± 0.06 and 1.26 ± 0.09 , $P < 0.05$, respectively; Eca9706: 1.08 ± 0.07 vs. 1.27 ± 0.05 and 1.30 ± 0.06 , $P < 0.05$ and 0.01 , respectively). IDH2W and IDH2R groups had more clone formation than control groups (Eca109: 579 ± 26 vs. 673 ± 40 and 671 ± 29 , $P < 0.05$, respectively; Eca9706: 572 ± 35 vs. 691 ± 39 and 682 ± 32 , $P < 0.05$, respectively). The invasive cell numbers were higher in IDH2W groups (Eca109: 352 ± 18 vs. 435 ± 15 and

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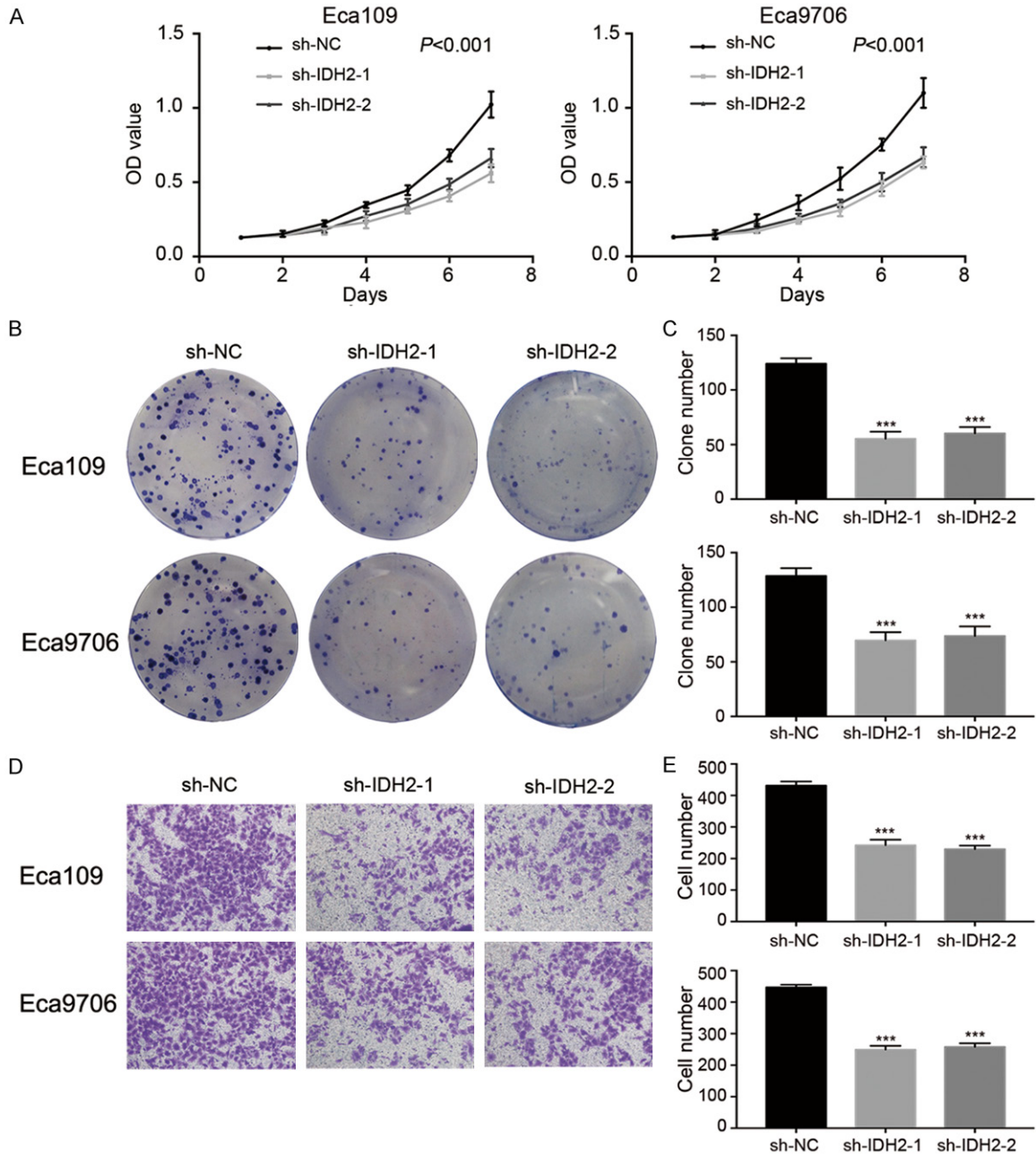


Figure 4. The effects of sh-IDH2-1 and sh-IDH2-2 on ESCC cells. A: The results of the CCK8 assays in Eca109 and Eca9706. B and C: The results of clonal efficiency assays in Eca109 and Eca9706. D and E: The results of cell invasion in Eca109 and Eca9706.

425 ± 12, $P < 0.01$, respectively; Eca9706: 335 ± 17 vs. 424 ± 19 and 422 ± 11, $P < 0.001$ and 0.01, respectively) as well. These results demonstrated that the elevated expression of IDH2 promoted the proliferation and invasion of transduced cells compared with the control cells.

Rescue of IDH2 knockdown phenotype by expression of shRNA-resistant IDH2

To further confirm the specificity of shRNA manipulations, rescue experiments using lentiviruses were carried out (**Figure 7**). ESCC cells with sh-IDH2-1 plasmids stably transfected

The clinical significance of IDH2 in ESCC

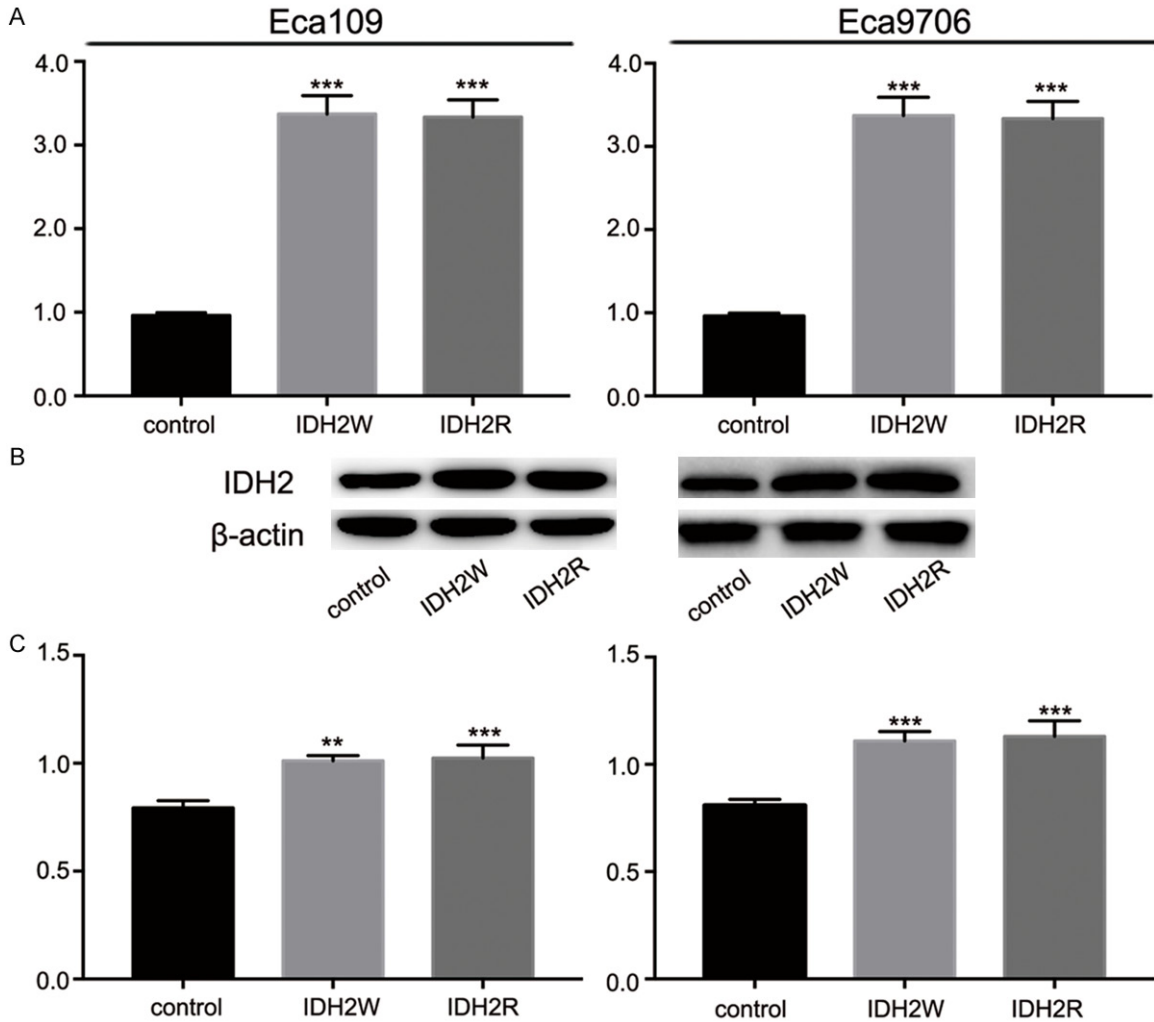


Figure 5. The upregulation effect of IDH2W and IDH2R. A: IDH2W and IDH2R increased IDH2 expression at mRNA level. B and C: IDH2W and IDH2R increased IDH2 expression at protein level.

were infected by IDH2W lentiviruses and IDH2R lentiviruses. The OD_{450} values at 144 h were higher in sh-IDH2-1 + IDH2W and sh-IDH2-1 + IDH2R groups than sh-IDH2-1 groups (0.65 ± 0.05 vs. 0.99 ± 0.11 and 1.33 ± 0.08 in Eca109 and 0.67 ± 0.06 vs. 1.04 ± 0.07 and 1.29 ± 0.07 in Eca9706, $P < 0.001$, respectively). The clone numbers (281 ± 19 vs. 454 ± 28 and 580 ± 23 in Eca109 and 297 ± 14 vs. 475 ± 21 and 598 ± 23 in Eca9706, $P < 0.001$, respectively) and invasive cell numbers (161 ± 18 vs. 291 ± 15 and 390 ± 15 in Eca109 and 134 ± 11 vs. 308 ± 19 and 397 ± 11 in Eca9706, $P < 0.001$, respectively) showed the similar trend. Additionally, these results indicated that IDH2R had stronger ability of rescuing IDH2 knockdown phenotype than IDH2W (all $P < 0.05$).

Discussion

In the present study, the result of qRT-PCR showed no difference of mRNA expression between these two groups ($P > 0.05$). mRNA level of one gene is not always interrelated with protein level. In addition to transcription and mRNA stability, translation and protein degradation are also essential factors for protein expression [24]. The protein expression levels of IDH2 in cancerous tissues were higher than those in paracancerous tissues (IHC: $P < 0.001$; Western: $P < 0.001$), despite the range of expression levels from patient to patient. The study suggests that IDH2 protein may be a good biomarker for ESCC patients. The biological behavior of carcinoma and molecular pathogenesis

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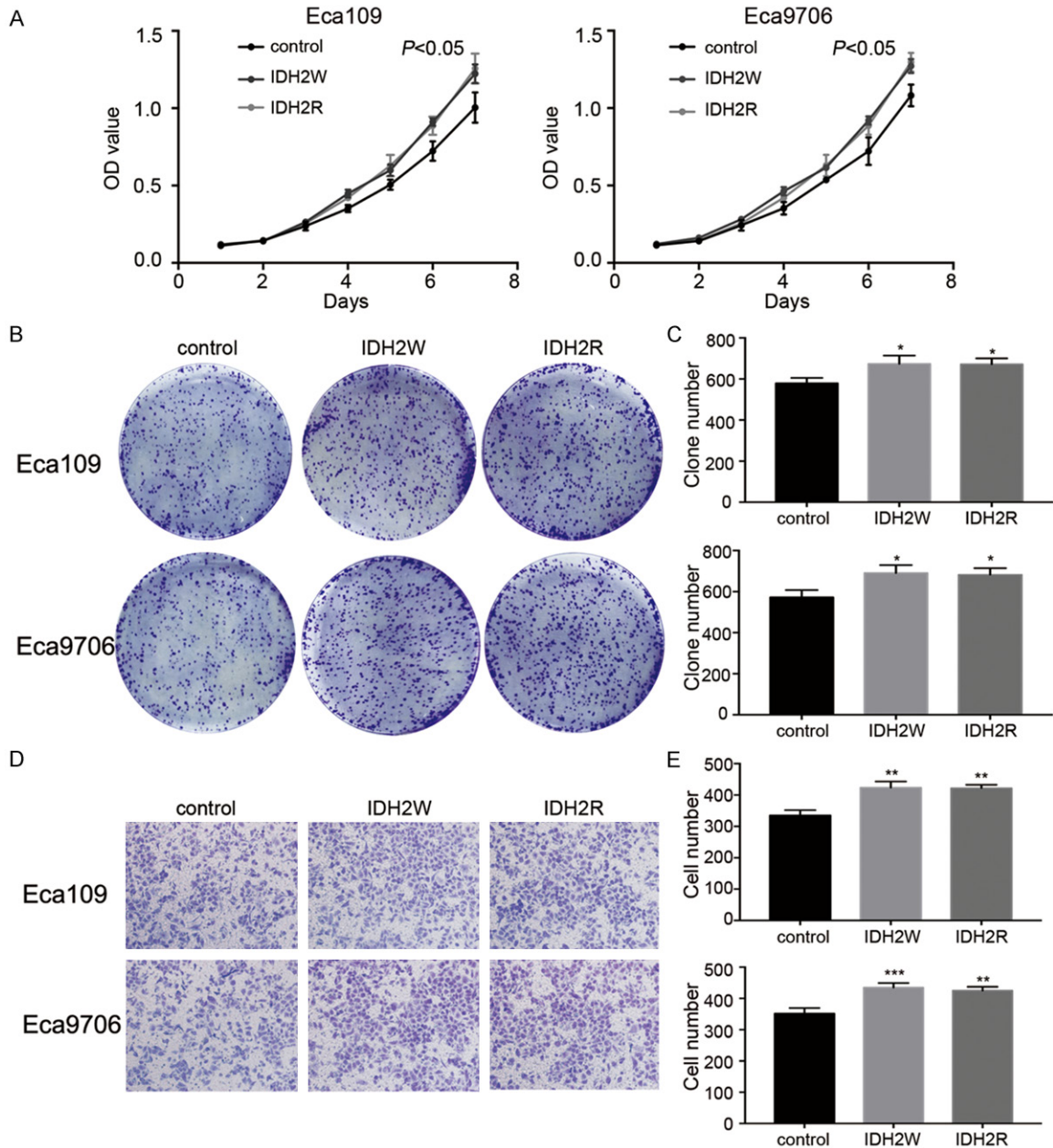


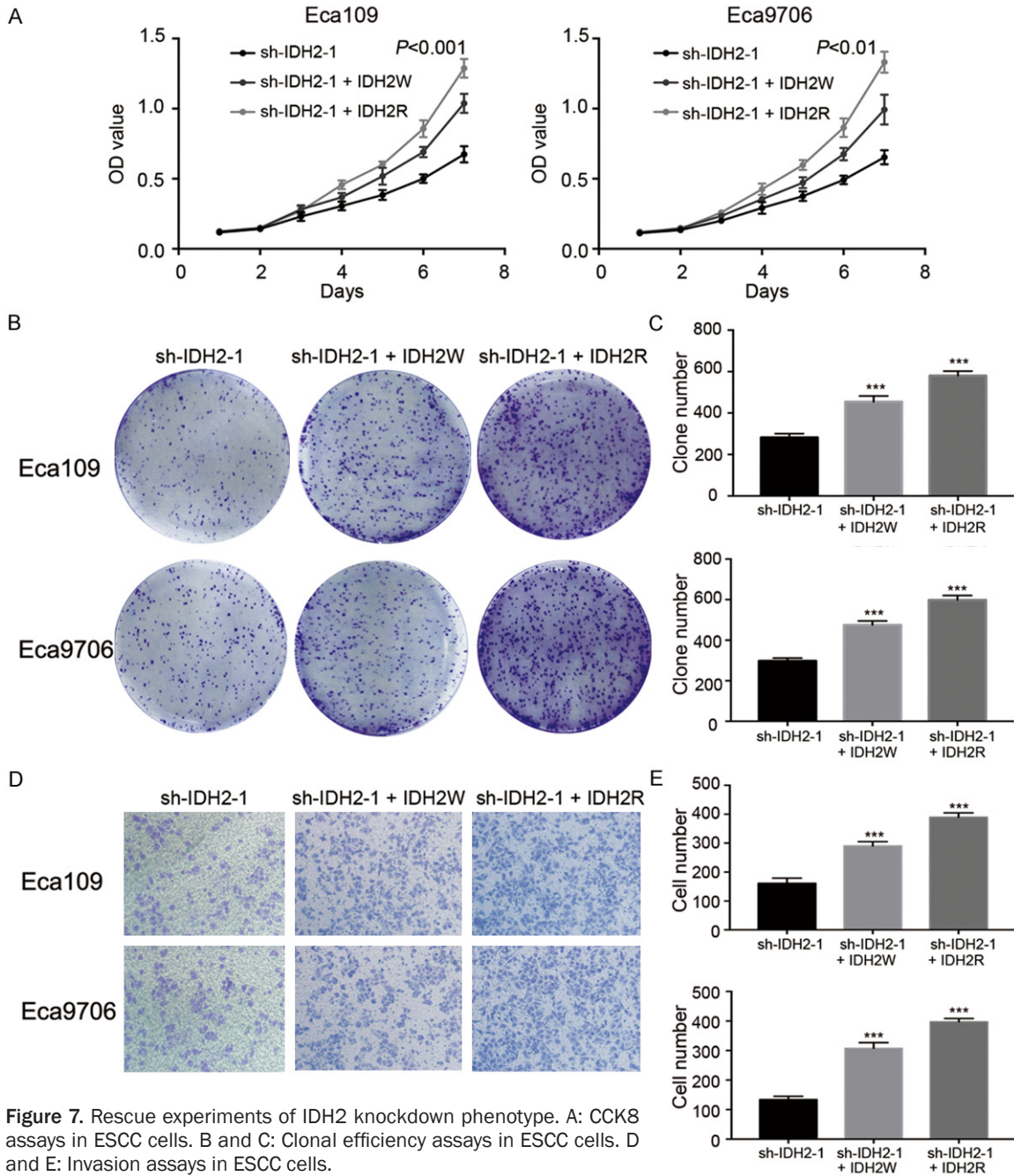
Figure 6. The effects of IDH2W and IDH2R on ESCC cells. A: CCK8 assays in Eca109 and Eca9706. B and C: Clonal efficiency assays in Eca109 and Eca9706. D and E: Invasion assays in Eca109 and Eca9706.

differ with respect to different sites of origin and histological types. Unlike the results in this study on ESCC, IDH2 is downregulated in HCC cells, GC cells and the early phase of colon cancer [19, 20, 23]. The upregulation of IDH2 in endometrial cancer, prostate and testicular cancer, as well as advanced phase of colon cancer is in line with the IDH2 overexpression in ESCC [21-23]. Here, the analysis of the correlations of IDH2 expression with clinicopatho-

logical characteristics revealed that the expression level of IDH2 was related to the T stage, but not to other factors, which may be due to the heterogeneity and insufficient number of samples evaluated.

Additionally, the prognostic value of IDH2 was investigated in ESCC. The Kaplan-Meier curve revealed that IDH2 overexpression was correlated to poor outcomes in patients with ESCC,

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including worse OS and PFS. Univariate analysis showed that IDH2 may be an independent prognostic marker for OS and PFS. Multivariate analysis further confirmed this result. The ROC curve was used for judging the prognostic value of IDH2 and the AUC was 0.638 ($P=0.014$) for OS and 0.638 ($P=0.030$) for PFS. This indicated that IDH2 had highly sensitive and specific prognostic value. Furthermore, the T stage, N stage and adjuvant therapy were relevant to

the outcomes of ESCC patients as well. These outcomes raised the possibility of IDH2 as an oncogene in ESCC.

To verify the cancer promotion effect of IDH2 in vitro, we performed CCK8, clonal efficiency, and invasion assays. The OD values, number of colonies formed and the number of invasive cells were lower in the sh-IDH2 transfected groups. These results indicated that IDH2 inhi-

hibition decreased the proliferation and invasion abilities of ESCC cells. The overexpression of IDH2 showed an opposite effect of sh-IDH2 on ESCC cells. Furthermore, the specificity of sh-IDH2-1 was verified by rescue experiment. Thus, IDH2 can act as an adverse prognostic biomarker for the postoperative outcomes of ESCC patients.

The mechanisms of regulation of IDH2 and its effects on cancer have not been elucidated. IDH2 plays an effective role in producing GSH reductase and regenerating thioredoxin (Trx) by supplying NADPH [25]. GSH and Trx are critically important antioxidative systems for protecting cells against oxidative damage and xenobiotic toxicity by scavenging the reactive oxygen species (ROS) [26, 27]. Furthermore, GSH and Trx systems also promote cancer cells growth and cause immune response suppression [28]. The redox status changes the survival and proliferation of cancer cells by modifying the transcriptional and posttranscriptional status of proteins involved in cell cycle control, such as apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase [29, 30].

In addition to the significant role of IDH2 in the antioxidative system, the effect of 2-HG on epigenetic modification is another important mechanism. Mutations in the active site's arginine residues IDH2 R172 and IDH2 R140 have been shown to result in the conversion of NADPH and α -KG to NADP⁺ and 2-HG [31, 32]. 2-HG inhibits differentiation and facilitates oncogenesis by promoting hypermethylation of global DNA and histone [33]. Numerous kinds of cancer cells are characterized by ectopic DNA methylation [34], and epigenetic silencing is regarded as a third pathway satisfying the hypothesis of two-hit that at least two classes of gene mutations are responsible for the silencing of tumor-suppressor genes (TSGs) [35]. Several TSGs, such as EDNRB, TIP30, TPEF and ECRG4 have been reported to be silenced through promoter hypermethylation during the development of ESCC [36-39]. Interestingly, high levels of 2-HG occur not only in cancer cells with mutant IDH but even in cancer cells with wild-type IDH in hypoxic microenvironment. In hypoxic cells, upregulated reductive metabolism of wild-type mitochondrial IDH2 could also increase the reductive carboxylation of α -KG to citrate and lead to the accumulation of 2-HG [40].

Our in vitro assays confirmed the effects of IDH2 on ESCC. By obtaining more information about IDH2, we will better understand how it affects the biological behaviors of ESCC. Further studies will be conducted to clarify in more details the mechanisms of action of IDH2 and its mutation status in ESCC patients. Additionally, ROS are important mediators of radiotherapy and chemotherapy for damaging DNA of cancer cells. Accordingly, the expression level of IDH2 may be an effective predictive marker for chemoradiotherapy, and targeting IDH2 may sensitize their effect in addition to interfering with other biological behaviors of ESCC.

Conclusion

In summary, IDH2 is significantly upregulated in cancerous tissues of patients with ESCC. IDH2 could act as a promising biomarker for identifying ESCC patients with poor outcomes.

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Disclosure of conflict of interest

None.

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