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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

DNA Repair Protein Rad51 Induces Tumor Growth and Metastasis in Esophageal Squamous Cell Carcinoma via a p38/Akt-Dependent Pathway

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ABSTRACT

Background. Rad51 is a protein which plays a vital role in DNA double-strand break repair and maintenance of telomeres. However, the underlying mechanism for its action in esophageal squamous cell carcinoma (ESCC) remains unclear.

Patients and Methods. Eighty-seven patients with ESCC were enrolled in this study. Expression of Rad51 in ESCC was determined by immunohistochemistry and correlated with clinicopathological variables by Chi square test. The role of Rad51 in patient survival was determined by Kaplan–Meier estimates. The effects of Rad51 knockdown and overexpression on esophageal cancer growth, migration, and invasion were examined using TE8, CE81T, and KYSE70 cells. The mechanisms involved were also analyzed. Nude mice models were used for assessment of tumor growth.

Results. Rad51 staining was predominantly observed in ESCC patients. ESCC patients with high Rad51 expression had significantly decreased survival ($P < 0.001$) combined with increased tumor size ($P = 0.034$) and lymph node metastasis ($P = 0.039$). Rad51 overexpression promoted, while its knockdown attenuated, esophageal cancer cell viability through cell cycle entry and migration/invasion via epithelial–mesenchymal transition. Moreover, Rad51 overexpression increased colony formation in vitro and tumor growth in vivo. In addition, high Rad51 expression

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increased cancer progression through the p38/Akt/Snail signaling pathway.

Conclusions. This study indicates a new biological role for Rad51 in ESCC progression. Rad51 may serve as a potential prognostic biomarker and therapeutic target for ESCC patients.

Esophageal cancer is a common malignancy worldwide, including in Eastern Asia. It has an overall five-year survival rate of $< 25\%$.[1,2] Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) are the two major subtypes of esophageal cancer. ESCC accounts for more than 90% of all esophageal cancer cases and is characterized by metastatic capability.[3] Complete surgical resection is the best management in terms of local control, although recurrence and metastasis remain common after surgery. Despite treatment with concurrent chemoradiation therapy (CCRT) alone or as an adjunct to surgery,[4] esophageal cancer remains a major challenge and the delineation of new targets for clinical therapy is of great clinical importance.

The DNA repair process is essential for maintenance of genomic stability in somatic mammalian cells.[5,6] Presence of DNA double-strand breaks (DSBs) is a major threat to genomic integrity by causing defects in DNA that are frequently observed in tumor cells.[7] DSBs are repaired by either one of two distinct repair pathways depending on the cell cycle status.[8] Homologous recombination is the preferred mechanism for cells in S and G2/M phases, while nonhomologous end-joining is preferred for cells in G1 phase.[9] Repair proteins of the Rad51/Rad52 epistatic group are mainly involved in homologous recombination repair.[10]

Rad51 is a crucial element in DNA repair. Disruption of the *Rad51* gene in mice results in early embryonic lethality, suggesting that *Rad51* plays an essential role in cell proliferation and early embryonic development.[11] Additionally, Rad51 protein interacts with a variety of tumor suppressor proteins such as p53, BLM, BRCA1, and BRCA2, suggesting a possible role for Rad51 in tumorigenesis.[12] Overexpression of RAD51 in cancer cells with initial homologous recombination repair defects induced these cells to regain genomic stability during carcinogenic progression.[13] RAD51 overexpression also contributes to cancer development and drug resistance in pancreatic cancer.[14] However, whether Rad51 expression correlates with ESCC progression and its influence on esophageal cancer cell behavior in vitro and in vivo are mostly unclear. In this study, we explore the involvement of Rad51, a key player in homologous recombination DSB repair, in ESCC using in vitro, animal, and clinical approaches.

PATIENTS AND METHODS

Patient Samples

ESCC tissues and adjacent noncancer tissues were obtained from 87 patients undergoing surgical treatment at the Division of Thoracic Surgery, Department of Surgery, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, from January 2009 to December 2014. Diagnosis of ESCC was confirmed according to clinical and histological reports, which were retrospectively reviewed in this study. This study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital [KMUH-IRB-20130269, KMUHIRB-E(II)-20150192], and informed consent was obtained from each patient. Overall survival was defined as the interval between date of diagnosis and death. The resected specimens were processed for immunohistochemical analysis. All patients received regular postoperative follow-up in our outpatient clinic for a median period of 24 months (range 3–75 months).

Immunohistochemical Analysis

Immunohistochemical (IHC) staining was performed using a Leica Bond-Max autostainer (Leica Microsystems, Bannockburn, IL). Details are provided in the Supplementary Methods. The results for Rad51 staining were determined by two independent experts under the same conditions, and stratified into quartiles: score 1, $\leq 25\%$ positive cells; score 2, 26–50% positive cells; score 3, 51–75% positive cells; and score 4, $\geq 76\%$ positive cells. For statistical analysis, scores 1 and 2 were categorized as low expression, whereas scores 3 and 4 were categorized as high expression. For tissues from mice, staining of Rad51, Ki-67, phosphorylated Akt, and Snail was scored by the method of histochemical score (H-score), calculated as the product of the percentage of stained cells and the intensity of staining.[15]

Cell Culture

The human ESCC cell line CE81T was obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). The esophageal cancer cell line KYSE70 was kindly provided by Dr. Yi-Ching Wang (Department of Pharmacology, National Cheng Kung University, Tainan, Taiwan). The human ESCC cell line TE8 was kindly provided by Dr. Mien-Chie Hung (MD Anderson Cancer Center, Houston, TX). CE81T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA). KYSE70 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen). TE8 cells were cultured in DMEM/F12

medium (Invitrogen). For all cell lines, the media were supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (Biological Industries, Haemek, Israel). All cells were grown in a 5% CO_2 incubator at 37 °C.

Infection with Rad51-Specific shRNA for Rad51 Knockdown

Rad51 short hairpin RNAs (shRNAs) were purchased from the National RNAi Core Facility in the Institute of Molecular Biology (Academia Sinica, Taipei, Taiwan). Details are provided in the Supplementary Methods.

Stable Infection with Lentiviral Constructs for Rad51 Overexpression

Expression plasmid pReceiver-Lv105 (GeneCopoeia, Rockville, MD) was used for lentiviral vector production. Details are provided in the Supplementary Methods.

Statistical Analysis

All statistical analyses were performed using the SPSS v19 statistical package for PC (SPSS, Chicago, IL). Statistical comparison between control and Rad51 knockdown or overexpression groups was performed using the two-sided Student's *t* test. One-way analysis of variance (ANOVA), combined with Tukey's multiple-comparison test, was used to evaluate the statistical significance of differences between three or more groups. The correlation between Rad51 expression and clinical characteristics was calculated by the Chi square test. Survival curves were generated using Kaplan–Meier curve analysis, and the significance of differences between curves was evaluated by the two-sided log-rank test. Furthermore, hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated by univariate and multivariable Cox regression models and used to investigate the relationship between clinicopathological characteristics and survival. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

High Rad51 Expression Is Correlated with Poor Outcomes in ESCC Patients

To investigate the clinical significance of Rad51, tumor tissue specimens from 87 ESCC patients were used to determine the expression pattern by IHC analysis. Typical staining patterns for Rad51 in ESCC tissues and in adjacent normal esophageal tissues are shown in Fig. 1a. Rad51 expression levels (H score) in ESCC tissues were higher than in normal esophageal tissues (Fig. 1b). Next, the correlation between Rad51 expression and clinicopathological characteristics in ESCC patients was examined. Patients in the high Rad51 expression group had statistically significantly poorer overall survival than patients in the low Rad51 expression group (Fig. 1c). In addition, high Rad51 expression in ESCC tissues was positively correlated with clinical characteristics including tumor size and lymph node (LN) metastasis (Supplementary Table 1).

To further investigate the association between Rad51 expression and clinical parameters with overall survival, univariate and multivariable Cox regression analyses were applied. On univariate analysis, significant associations with overall survival were observed for four parameters: tumor size, LN metastasis, recurrence, and Rad51 status. On multivariable analysis (adjusted for age, tumor size, LN metastasis, recurrence, and Rad51 expression), age and Rad51 status showed statistically significant associations with overall survival (Table 1). These clinical findings suggest that Rad51 may play a crucial role in ESCC progression and may be a significant predictor of ESCC patient outcomes.

Downregulation of Rad51 Expression Decreases Esophageal Cancer Cell Viability and Cell Migration/Invasion

The association between Rad51 and ESCC behavior was addressed in vitro by knockdown or overexpression of Rad51 in esophageal cancer cells. To knockdown expression of Rad51, TE8 and CE81T cells were infected with lentivirus-based shRNA constructs. The efficacy of Rad51 shRNAs in gene knockdown is shown in Fig. 2a. In addition, the morphology of TE8 and CE81T cells with Rad51 knockdown did not change. The effects of Rad51 knockdown on cell viability were examined by XTT assay. Significantly reduced cell viability at 48 and 72 h was observed when Rad51 expression was suppressed by shRNA knockdown by both clone 1 and clone 2 in TE8 cells (sh-Luc vs. Rad51 knockdown clone 1, 48 h, 260.2% vs. 204.2%, $P < 0.01$; 72 h, 338.0% vs. 310.9%, $P < 0.05$) (Fig. 2b). Similar results were observed in CE81T shRNA51-1 and shRNA51-2 cells at 72 h. In addition, the effect of Rad51 on in vitro anchorage-independent esophageal tumor growth was evaluated. Rad51 knockdown by both clone 1 and clone 2 in CE81T cells suppressed colony formation compared with sh-Luc control (Fig. 2c). Since cell viability is regulated through the control of cell cycle progression, the effect of Rad51 knockdown on cell cycle distribution was examined by fluorescence-activated cell sorting (FACS) analysis. Knockdown of Rad51 (both

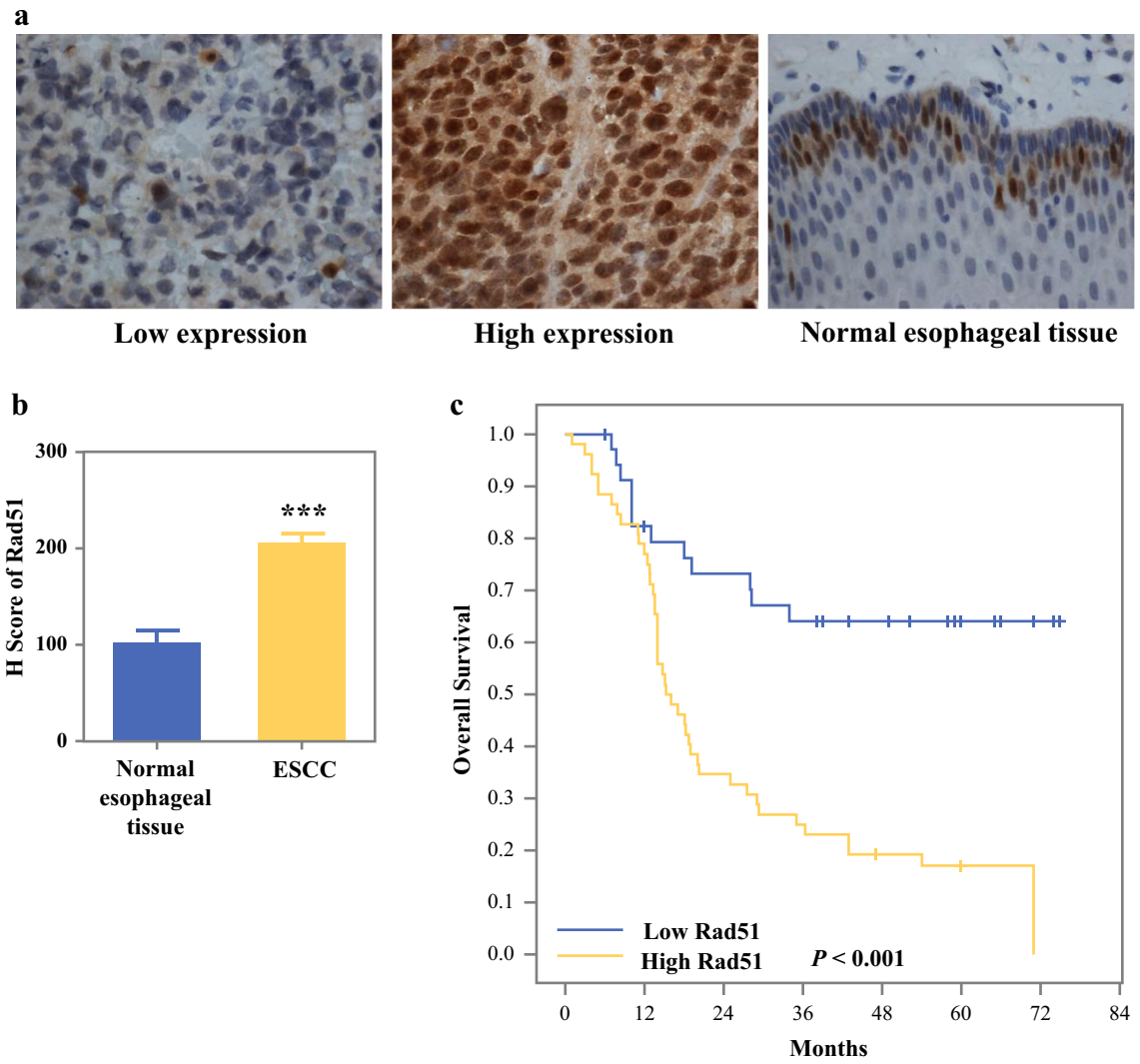


FIG. 1 Clinical significance of Rad51 expression levels in esophageal squamous cell carcinoma (ESCC) patients. **a** Rad51 staining in ESCC tissues detected by immunohistochemistry (IHC) analysis. The expression of Rad51 in adjacent normal esophageal tissues is also shown (400× magnification). **b** Histogram showing quantification of Rad51 (H-score) by IHC analysis of ESCC tissues and adjacent normal esophageal tissues. Data expressed as mean ± standard error on the mean (SEM). ***$P < 0.001$. **c** Overall survival analysis ($P < 0.001$) in 87 ESCC patients by low and high Rad51 expression level

clone 1 and clone 2) led to an increase of cell cycle arrest at the G0/G1 phases and decreased the proportion of cells in the S and G2/M phases compared with sh-Luc control (Fig. 2d).

Next, directed migration of cells in vitro was measured using the modified Boyden chamber assay. Lentiviral vectors expressing shRNAs for Rad51 knockdown by both clone 1 and clone 2 decreased TE8 and cell migration (sh-Luc vs. Rad51 knockdown clone 1: TE8, 1.10 vs. 0.82, $P = 0.048$; CE81T, 1.14 vs. 0.76, $P = 0.036$; sh-Luc vs. Rad51 knockdown clone 2: TE8, 1.10 vs. 0.68, $P = 0.020$; CE81T, 1.14 vs. 0.71, $P = 0.027$) as well as cell invasion (sh-Luc vs. Rad51 knockdown clone 1: TE8, 1.30 vs. 0.35, $P = 0.0028$; CE81T, 1.18 vs. 0.54, $P = 0.009$; sh-Luc vs. Rad51 knockdown clone 2: TE8, 1.30 vs. 0.46, $P = 0.008$; CE81T, 1.18 vs. 0.65, $P = 0.023$; Fig. 2e). Since epithelial to mesenchymal transition (EMT) markers are associated with cancer metastasis,[16] we examined the association between Rad51 expression and EMT markers. We found that the expression of epithelial maker (ZO-1) was increased, while the expression of mesenchymal markers (Slug, Snail, and Vimentin) were decreased, in Rad51-silenced TE8 and CE81T cells (both clone 1 and clone 2) compared with sh-Luc control cells (Fig. 2f).

TABLE 1 Univariate and multivariable analyses of overall survival for ESCC

Variable	Item	Univariate			Multivariable[a]		
		HR	95% CI	*P* value	HR	95% CI	*P* value
Age (years)	≥ 50	1.72	(0.98, 3.03)	0.059	1.96	(1.10, 3.51)	0.024
	< 50	1.00			1.00		
T status	T4/T3	2.25	(1.22, 4.12)	0.009	1.28	(0.66, 2.49)	0.464
	T2/T1	1.00			1.00		
LN metastasis	Yes	1.74	(1.02, 2.96)	0.042	1.63	(0.94, 2.85)	0.083
	No	1.00			1.00		
Recurrence	Yes	2.02	(1.18, 3.48)	0.011	1.51	(0.85, 2.67)	0.160
	No	1.00			1.00		
Grade	II/III	0.85	(0.38, 1.88)	0.681			
	I	1.00					
Alcohol	Yes	1.36	(0.77, 2.39)	0.284			
	No	1.00					
Betal nut	Yes	1.54	(0.83, 2.87)	0.170			
	No	1.00					
Rad51	High	3.61	(1.89, 6.88)	< 0.001	2.94	(1.46, 5.94)	0.003
	Low	1.00			1.00		

HR hazard ratio, *CI* confidence interval, *LN* lymph node

[a]Variables with $P < 0.1$ on univariate analysis were included in multivariable analysis

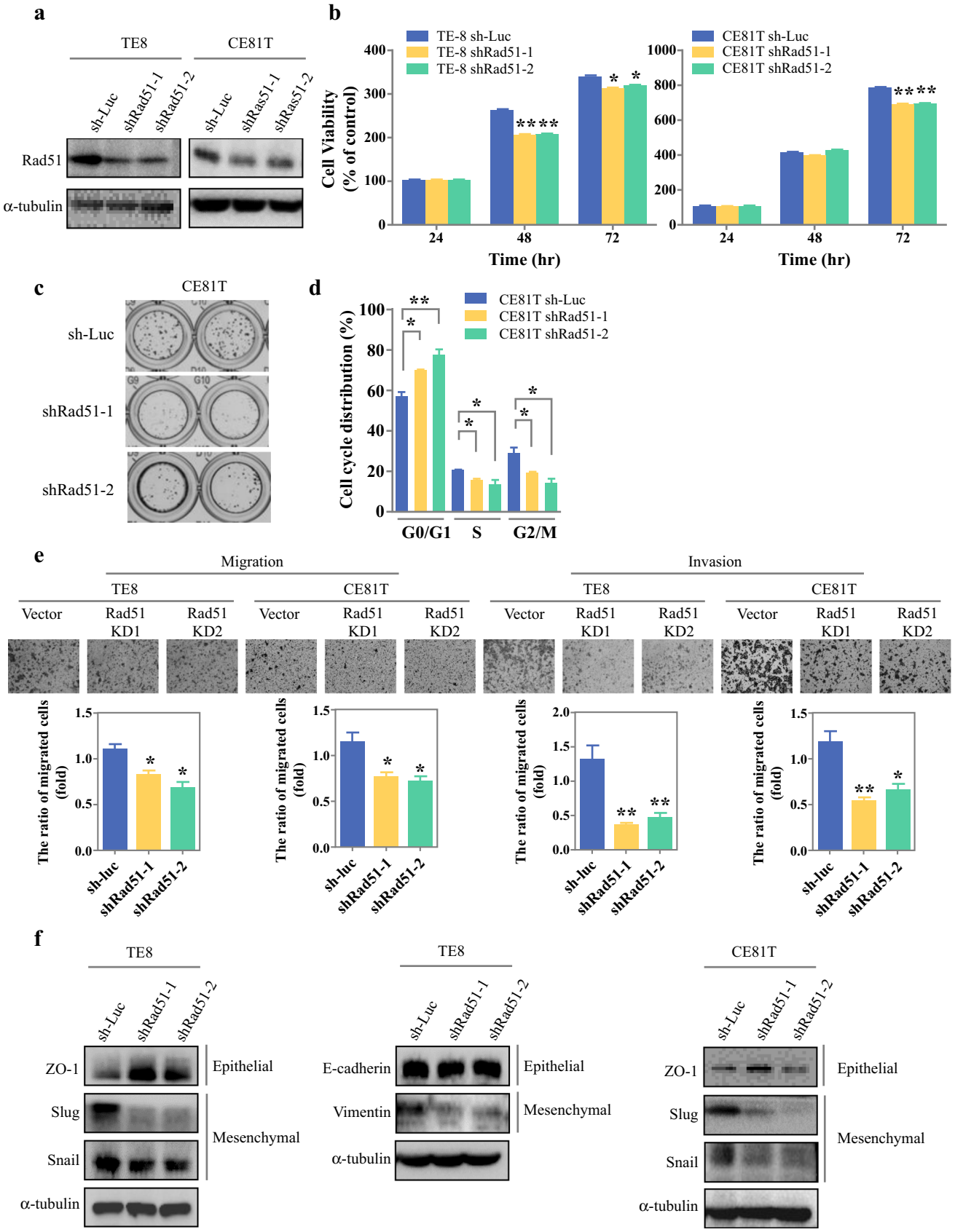
Upregulation of Rad51 Expression Promotes Esophageal Cancer Cell Viability, Cell Migration/Invasion, and Tumor Growth In Vivo

Stably overexpressed Rad51 constructs in CE81T and KYSE70 cells were established (Fig. 3a), and the morphology of these cells did not change. Quantitative assessment of cell viability by XTT assay and cell cycle distribution by FACS assay showed that Rad51 overexpression significantly increased cell viability in Rad51-overexpressed cells (vector vs. Rad51 overexpression, 48 h: CE81T, 171.9% vs. 210.2%, $P = 0.013$; KYSE70, 154.7% vs. 197.7%, $P < 0.01$; Fig. 3b) and increased the proportion of cells in the G2/M phases compared with vector control (Fig. 2c). Moreover, overexpression of Rad51 increased cell migration (vector vs. Rad51 overexpression: KYSE70, 1.0 vs. 1.71, $P = 0.026$; CE81T, 0.99 vs. 1.77, $P = 0.002$) and invasion (vector vs. Rad51 overexpression: KYSE70, 1.0 vs. 7.28, $P < 0.001$; CE81T, 1.25 vs. 6.16, $P < 0.01$; Fig. 2d). In addition, the expression of epithelial maker (ZO-1) was decreased, while the expression of mesenchymal markers (Twist, Slug, Snail, N-cadherin, and β-catenin) was markedly increased, in Rad51-overexpressing CE81T and KYSE70 cells compared with vector control (Fig. 2e). Moreover, Rad51 overexpression in CE81T cells increased colony formation compared with vector control (Fig. 3f). Taken together, these results show that the level of Rad51 expression affected cell viability via regulation of cell cycle and migration/invasion through expression of EMT markers in esophageal cancer cells.

To further confirm the association between Rad51 expression and tumor formation in vivo, KYSE70 cells with Rad51 overexpression or vector control were injected subcutaneously into nude mice ($n = 8$ per group). After 8 weeks, the average tumor volumes for the control and Rad51 overexpression groups were 215.60 ± 88.62 and 564.50 ± 121.90 mm^3, respectively (Fig. 3g), and the average tumor weights were 0.12 ± 0.03 and 0.28 ± 0.06 g, respectively (Fig. 3i). However, the mice did not show significant differences in body weight (Fig. 3h).

Involvement of p38/Akt/Snail Signaling Pathway in Rad51-Mediated ESCC Progression

To identify the signaling pathways regulated by Rad51 in esophageal cancer cells, we performed immunoblotting to compare the differentially expressed signaling pathways in control cells and in esophageal cancer cells with knockdown or overexpression of Rad51. Rad51 knockdown in TE8 cells decreased the levels of phosphorylated p38, PI3K, and Akt, while Rad51 overexpression in KYSE70 cells increased the levels of phosphorylated p38, PI3K, and Akt compared with vector control (Fig. 4a). Similar results were observed in CE81T esophageal cancer cells (Fig. 4a). To evaluate the involvement of the p38/Akt signaling pathway in cell viability and migration abilities,


a
TE8
CE81T
sh-Luc
shRad51-1
shRad51-2
sh-Luc
shRas51-1
shRas51-2
Rad51
α-tubulin
b
TE-8 sh-Luc
TE-8 shRad51-1
TE-8 shRad51-2
Cell Viability (% of control)
Time (hr)
CE81T sh-Luc
CE81T shRad51-1
CE81T shRad51-2
Time (hr)
c
CE81T
sh-Luc
shRad51-1
shRad51-2
d
CE81T sh-Luc
CE81T shRad51-1
CE81T shRad51-2
Cell cycle distribution (%)
G0/G1
S
G2/M
e
Migration
Invasion
TE8
CE81T
Vector
Rad51 KD1
Rad51 KD2
The ratio of migrated cells (fold)
sh-luc
shRad51-1
shRad51-2
f
TE8
CE81T
sh-Luc
shRad51-1
shRad51-2
ZO-1
Slug
Snail
α-tubulin
Epithelial
Mesenchymal
E-cadherin
Vimentin

◀**FIG. 2** Effects of Rad51 knockdown on cell viability, cell cycle distribution, and cell migration/invasion in TE8 and CE81T cells. **a** Representative blots of Rad51 expression for TE8 and CE81T cells infected with Rad51 shRNAs. Expression of α-tubulin used as internal control. **b** After seeding, cell viability of TE8 and CE81T cells with Rad51 knockdown determined by XTT assay at 24, 48, and 72 h and normalized to sh-Luc control. **c** CE81T cells with Rad51 knockdown grown for 21 days using anchorage-independent soft agar assay. **d** Cell cycle distribution detected by FACS analysis. All values expressed as percentage of sh-Luc group, which is set as 100%. **e** TE8 and CE81T cell migration and invasion ability after Rad51 knockdown. Representative photographs at 40× magnification. Histograms represent quantification of cell migration and invasion. All values expressed as ratio to sh-Luc group, which is set as 1. **f** EMT proteins analyzed by immunoblotting analysis in Rad51-silenced TE8 and CE81T cells at 24 h. All data presented as mean ± SEM (n = 3–8). *$P < 0.05$; **$P < 0.01$ compared with sh-Luc group

p38 inhibitor SB203580 was applied to block the expression of p38 and its downstream signaling Akt pathway. The effect of p38 inhibitor SB203580 on p38 phosphorylation and its downstream Akt phosphorylation is shown in Fig. 4b. Rad51 overexpression-induced phosphorylation of Akt in CE81T cells was abolished by p38 inhibitor SB203580. As expected, cell viability analysis and transwell migration assay demonstrated that blocking the p38/Akt pathway inhibited cell viability and migration in Rad51-overexpressing CE81T cells (Fig. 4c, d). Activation of the Akt signaling pathway has been shown to correlate with EMT.[17] We next investigated whether Rad51 upregulated expression of EMT markers through the Akt pathway. The results showed that the expression of Snail was significantly upregulated in Rad51-overexpressing CE81T cells but was decreased in SB203580-treated cells (Fig. 4b). These data suggested that Rad51 enhanced esophageal cancer cell migration through the p38/Akt/Snail signaling pathway.

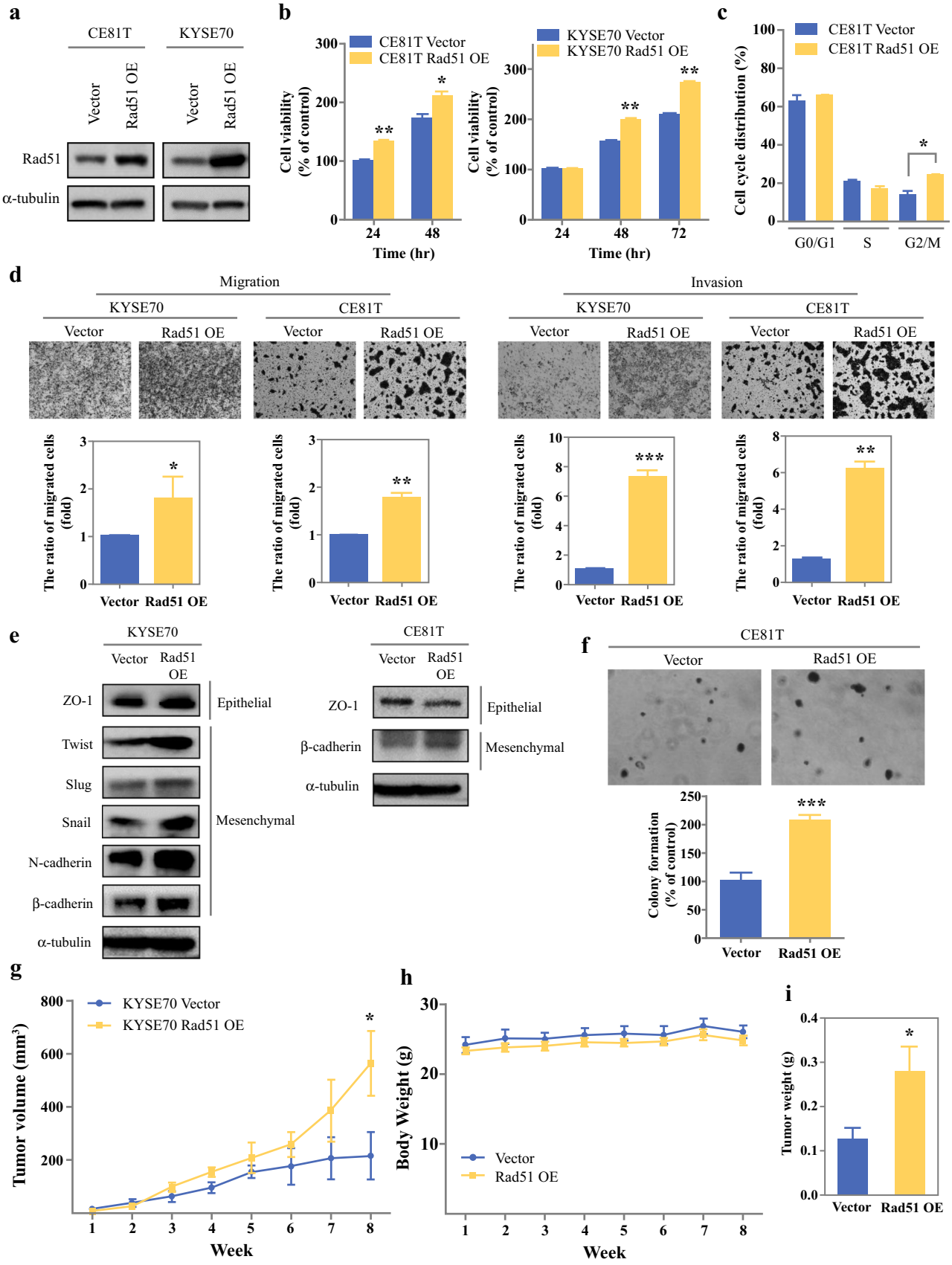
We further assessed p38/Akt/Snail signaling and cell proliferation by Ki67 staining in xenografted tumor tissues. IHC analysis revealed that the expression levels of Ki-67, phosphorylated Akt, Snail, and Rad51 in xenografted tumors were significantly increased in the Rad51 overexpression group compared with vector control (Fig. 4e).

DISCUSSION

Patients with esophageal cancer have poor prognosis. Randomized trials demonstrate that concurrent chemoradiotherapy (CCRT) using cisplatin-based regimens followed by surgery decreases local relapse as compared with surgery alone; however, the effect on overall survival remains uncertain.[18,19] Therefore, understanding the molecular pathogenesis behind malignant cancer behavior is essential for formulating therapeutic approaches for esophageal cancer. Moreover, the prognostic value of Rad51 remains insufficiently assessed, except for previous studies which reported that high Rad51 level was associated with poorer survival of esophageal cancer patients.[20,21] While a similarly unfavorable outcome for the survival of esophageal cancer patients with high Rad51 expression was observed in the present study, we further analyzed important clinical factors that have not been previously elucidated. First, analysis of clinical samples showed that higher Rad51 expression in esophageal cancer tissues was associated with malignant behaviors including larger tumor size, lymph node metastasis, and decreased overall survival in ESCC patients, presumably due to Rad51 activity in repairing DNA DSBs arising during DNA replication. Then, we aimed to clarify the phenotypic associations of altered Rad51 expression in esophageal cancer, first by demonstrating the effects of Rad51 expression levels on phenotypic changes of esophageal cancer cells in vitro and second by investigating the underlying molecular mechanisms. The distinct role of Rad51 found in esophageal tumor cells in this study provides the first evidence for the involvement of p38/Akt/Snail in Rad51-induced ESCC progression. These results provide a molecular explanation for the involvement of Rad51 in ESCC. Of note, these findings may have therapeutic implications in terms of prognostic markers, treatment stratification, and molecular targets for inhibition of tumor progression.

Oncogene addiction is a phenomenon regarding the dependence of tumor cells on an activated oncogenic protein or pathway for survival;[22] For example, treatment of c-MYC-expressing myeloma cells with compound 10058-F4, an inhibitor of MYC-MAX heterodimerization, resulted in rapid apoptosis, suggesting that there is an addiction to c-MYC for survival in these cancer cells.[23] In another study, malignant melanoma cells were addicted to ^{V600E}BRAF-driven glycolysis for efficient adenosine triphosphate (ATP) production.[24] Also, Bcr-Abl inhibitor imatinib selectively induced cytotoxicity in Bcr-Abl-transfected leukemic cells but not Bcr-Abl-negative parental cells.[25] Our data indicate that, while overexpression of Rad51 promotes esophageal cancer cell growth, treatment with p38 inhibitor SB203580 caused a greater reduction of cancer cell growth in Rad51-overexpressing cells, suggesting that esophageal cancer cells with high Rad51 levels may depend on p38 for survival.

Activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway is associated with a variety of cellular functions including cell cycle regulation, cell contact, and cell invasion.[26] Accumulating evidence indicates that inhibition of p38 activation is a potential method for suppression of cancer progression.[27] In this study, the specific p38 inhibitor SB203580 reduced cell viability and

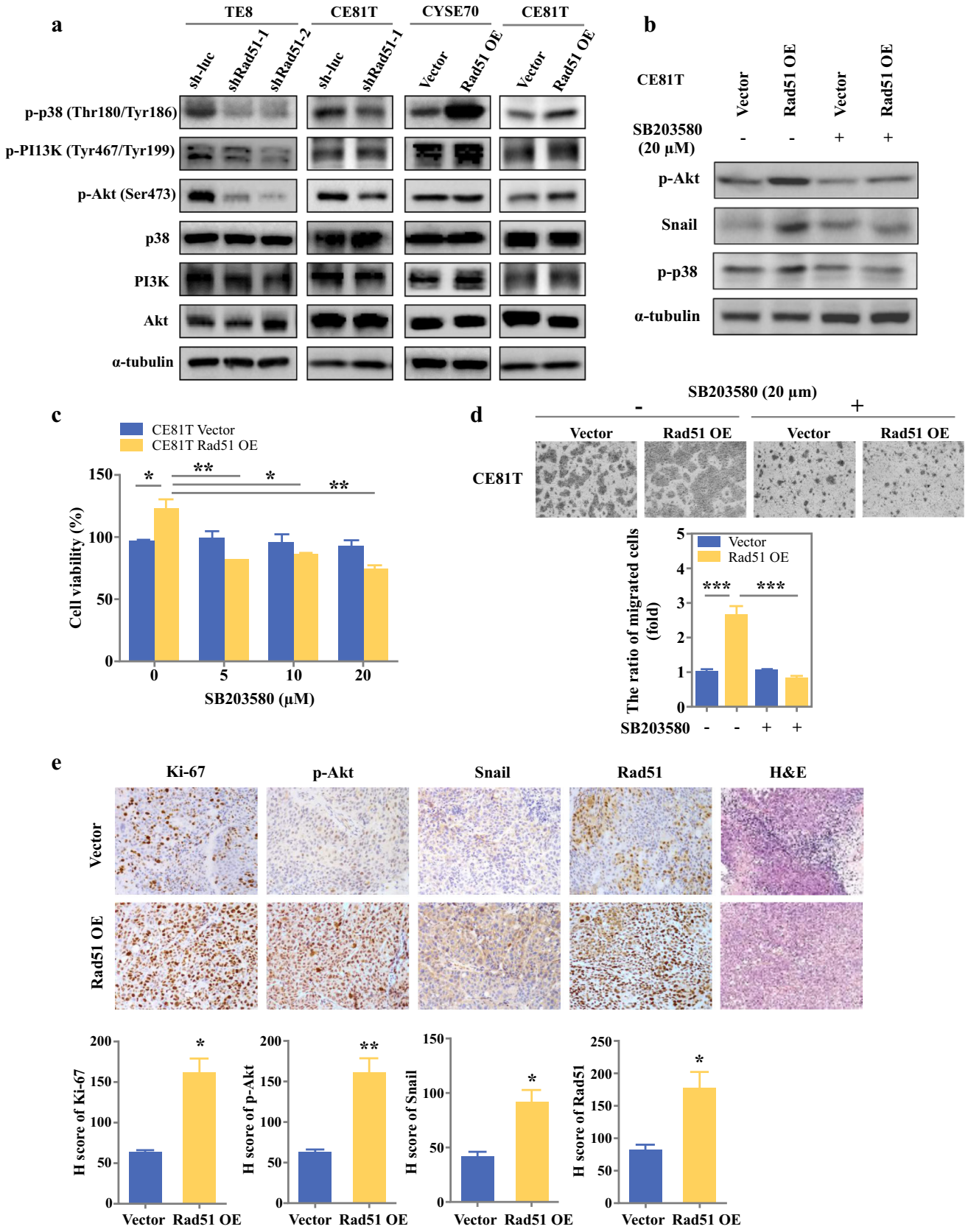

a
CE81T
KYSE70
Vector
Rad51 OE
Vector
Rad51 OE
Rad51
α-tubulin
b
CE81T Vector
CE81T Rad51 OE
Cell viability (% of control)
Time (hr)
KYSE70 Vector
KYSE70 Rad51 OE
Cell viability (% of control)
Time (hr)
c
CE81T Vector
CE81T Rad51 OE
Cell cycle distribution (%)
G0/G1
S
G2/M
d
Migration
Invasion
KYSE70
CE81T
Vector
Rad51 OE
The ratio of migrated cells (fold)
Vector
Rad51 OE
e
KYSE70
CE81T
Vector
Rad51 OE
ZO-1
Epithelial
Twist
Slug
Snail
N-cadherin
β-cadherin
Mesenchymal
α-tubulin
f
CE81T
Vector
Rad51 OE
Colony formation (% of control)
Vector
Rad51 OE
g
KYSE70 Vector
KYSE70 Rad51 OE
Tumor volume (mm³)
Week
h
Vector
Rad51 OE
Body Weight (g)
Week
i
Tumor weight (g)
Vector
Rad51 OE

◀**FIG. 3** Effects of Rad51 overexpression on cell viability, cell cycle distribution, cell migration/invasion, and tumor growth in vivo. **a** Rad51 overexpression (OE) in CE81T and KYSE70 cells assessed by immunoblotting analysis. Expression of α-tubulin used as internal control. **b** Cell viability of Rad51-overexpressing CE81T and KYSE70 cells determined by XTT assay. **c** After seeding, cell cycle distribution evaluated by FACS analysis. **d** KYSE70 and CE81T cell migration and invasion abilities after Rad51 overexpression. Representative photographs shown at 40× magnification. Histograms quantifying cell migration and invasion. **e** EMT proteins analyzed by immunoblotting analysis in Rad51-overexpressing KYSE70 and CE81T cells at 24 h. **f** CE81T cells with Rad51 overexpression grown for 21 days using anchorage-independent soft agar assay. Histogram quantifying colony formation, calculated as percentage of control group, which is set as 100%. **g**, **h** Male nude mice were subcutaneously xenografted with Rad51-overexpressing KYSE70 cells ($n = 8$ per group). The tumor volume (**g**) and body weight (**h**) of mice were measured weekly for each group. **i** Tumor weight measured after sacrifice of mice at end of 8-week period. All data presented as mean ± SEM ($n = 3$–8). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vector group

migration of esophageal cancer cells, particularly in high Rad51 expression groups, and further reduced the expression of downstream effectors including Snail. EMT is a reversible, multistep process defined by the loss of epithelial characteristic and the acquisition of mesenchymal phenotype.[28] Alterations in EMT and production of matrix metalloproteinases are associated with diverse pathologic changes, such as angiogenesis, restenosis in vessels, and tumors.[29,30] We found that the migration and invasion patterns were different between KYSE70 and CE81T cells (Fig. 3d). Many tumor cells have been noted to migrate as clusters utilizing the alternative (protein internalization) EMT program, in contrast to the single-cell migration pattern associated with classically defined (transcription-dependent) EMT mechanisms.[31] Additionally, we examined the expression of E-cadherin in Rad51 knockdown and overexpression cells using immunoblotting analysis, and E-cadherin expression was not significantly up- and downregulated, respectively. The evidence may provide some alternative pathways for Snail-mediated EMT activity other than regulation of E-cadherin expression.[32] According to previous studies, activation of Akt signaling promotes tumor cell proliferation and prevents apoptosis.[33,34] Other studies indicate that activated Akt is tightly correlated with cellular DNA DSB repair function in tumor cells.[35,36] Moreover, Akt-EMT axis inactivation has been shown to inhibit cancer cell migration.[37] In agreement with these reports, we observed that phosphorylation of Akt is induced by Rad51 overexpression and inactivation of Akt/EMT by p38 inhibitor SB203580 is responsible for the inhibition of migration in Rad51-overexpresssing cells. Therefore, our results indicate the possibility for treatment of Rad51-overexpressing esophageal cancer cells by targeting p38.

High DNA repair gene expression within cancer cells may be hypothesized to allow increased DSB repair, leading to poorer patient outcome.[38,39] Our findings support this hypothesis and clearly show that ESCC patients with high Rad51 expression in esophageal cancer tissues had increased tumor size and lymph node metastasis. Moreover, Rad51 plays an important role in the repair of DSBs through homologous recombination. Our data suggest that Rad51 might be essential for survival in esophageal cancer cells, probably because it helps to keep high DNA repair capacity for cell survival. The importance of Rad51 expression is further highlighted by the fact that, after adjusting for age, tumor size, lymph node metastasis, recurrence, and Rad51 expression in multivariable Cox regression analysis, only age and Rad51 expression were significantly correlated with patient survival. Variations in ESCC patient survival are frequently affected by a single factor. However, we found that tumor size, LN metastasis, or recurrence may be rarely affected after adjusting for potential covariates, such as Rad51 or age. We found that the adjusted HR for overall survival in ESCC patients with high Rad51 expression was 2.94 (Table 1). Furthermore, the adjusted HR for overall survival in the age ≥ 50 years group and patients with recurrence were 1.96 and 1.51, respectively. The cut-off point of age was estimated according to previous work which listed the risk factors for ESCC patients at diagnosis over age 50 years.[40] Therefore, identification of prognostic factors in old ESCC patients, including Rad51 in this study, may provide useful information for physicians to design better schemes for prevention of ESCC progression and recurrence.

There are a number of limitations to this study, including the size and geographic localization of the study population. In addition, there is no standard approach for clinical assessment of Rad51 expression, and care should be taken in interpreting clinical analyses. Also, no adequate information on preoperative and postoperative adjuvant treatments of the patients was available in this retrospective study. Therefore, no conclusion can be reached regarding the potential role of Rad51 expression as a predictive factor of response to treatments. However, it is tempting to speculate that Rad51 expression may also predict the treatment effects on the basis of in vitro data, indicating the influence of Rad51 expression in mechanisms of treatment resistance after exposure of cells to DNA-damaging agents or irradiation.[12] Moreover, although we investigated the effects of Rad51 on esophageal cancer cells using both in vitro and in vivo approaches, this may not fully represent human esophageal cancer behaviors in the clinical setting, and further large-scale clinical studies including recurrence-free survival in ESCC patients are required.


a
TE8
CE81T
CYSE70
CE81T
sh-luc
shRad51-1
shRad51-2
sh-luc
shRad51-1
Vector
Rad51 OE
Vector
Rad51 OE
p-p38 (Thr180/Tyr186)
p-PI13K (Tyr467/Tyr199)
p-Akt (Ser473)
p38
PI3K
Akt
α-tubulin
b
CE81T
Vector
Rad51 OE
Vector
Rad51 OE
SB203580 (20 μM)
- - + +
p-Akt
Snail
p-p38
α-tubulin
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CE81T Vector
CE81T Rad51 OE
Cell viability (%)
150
100
50
0
* ** * **
0 5 10 20
SB203580 (μM)
d
SB203580 (20 μm)
- +
Vector
Rad51 OE
Vector
Rad51 OE
CE81T
Vector
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The ratio of migrated cells (fold)
5 4 3 2 1 0
*** ***
SB203580 - - + +
e
Ki-67
p-Akt
Snail
Rad51
H&E
Vector
Rad51 OE
H score of Ki-67
H score of p-Akt
H score of Snail
H score of Rad51
200 150 100 50 0
250 200 150 100 50 0
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Vector Rad51 OE

◀**FIG. 4** Involvement of p38/Akt signaling in esophageal cancer cell progression after Rad51 knockdown and overexpression. **a** Cell lysates from TE8, CE81T, and KYSE70 cells with Rad51 knockdown and overexpression (OE) analyzed for phosphorylation levels of p38, PI3K, and Akt by immunoblotting analysis. Expression of α-tubulin used as internal control. **b** Immunoblotting analysis of phosphorylated Akt, Snail, and phosphorylated p38 in Rad51-overexpressing CE81T cells after treatment with p38 inhibitor SB203580. **c** Cell viability of Rad51-overexpressing CE81T cells treated with SB203580 determined by XTT assay and normalized to vector control. **d** Effect of p38 inhibitor on cell migration in Rad51-overexpressing CE81T cells. Representative photographs shown at 40× magnification. **e** Representative images of IHC analysis for Ki-67, phosphorylated Akt, Snail, and Rad51 in mice xenografted tumor tissues. Representative photographs shown at 200× (IHC) and 200× (H&E) magnification. Histograms show H-score calculated as product of percentage of stained cells and intensity of staining. All data presented as mean ± SEM ($n = 3$–6). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vector group

In conclusion, high Rad51 expression in esophageal cancer cells correlated with more malignant cancer cell behaviors in vitro and in vivo and worse survival in ESCC patients. The underlying molecular mechanisms of Rad51 in esophageal cancer demonstrated in this study may have a number of important clinical implications and offer a number of novel therapeutic targets.

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DISCLOSURE The authors declare no conflicts of interest.

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