Downregulation of the Novel Tumor Suppressor DIRAS1 Predicts Poor Prognosis in Esophageal Squamous Cell Carcinoma

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Abstract

Loss of chromosome 19p is one of the most frequent allelic imbalances in esophageal squamous cell carcinoma (ESCC), suggesting the existence of one or more tumor suppressor genes within this region. In this study, we investigated a role in ESCCs for a candidate tumor suppressor gene located at 19p13.3, the Ras-like small GTPase DIRAS1. Downregulation of DIRAS1 occurred in approximately 50% of primary ESCCs where it was associated significantly with advanced clinical stage, lymph node metastasis, and poor overall survival. LOH and promoter methylation analyses suggested that loss of DIRAS1 expression was mediated by epigenetic mechanisms. Functional studies established that ectopic re-expression of DIRAS1 in ESCC cells inhibited cell proliferation, clonogenicity, cell motility, and tumor formation. Mechanistic investigations suggested that DIRAS1 acted through extracellular signal-regulated kinase (ERK1/2; MAPK3/1) and p38 mitogen-activated protein kinase (MAPK: MAPK14) signaling to trigger BAD Ser112 dephosphorylation and matrix metalloproteinase (MMP)2/9 transcriptional inactivation to promote apoptosis and inhibit metastasis, respectively. Taken together, our results revealed that DIRAS1 has a pivotal function in ESCC pathogenesis, with possible use as a biomarker and intervention point for new therapeutic strategies.

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Introduction

Esophageal cancer is one of the most aggressive malignancies and has been ranked as the sixth leading cause of cancer deaths worldwide (1). Esophageal squamous cell carcinoma (ESCC), the major histologic form of esophageal cancer, dominates in most parts of the world, especially in the northern China with a particularly high incidence rate (2). Epidemiologic studies have revealed that tobacco smoking, alcohol consumption, and micronutrient deficiency are related to the etiology of ESCCs (3, 4). However, in high-risk areas such as Linzhou city (Henan, China), these risk factors play a less significant role (5). Studies also show that familial aggregation may play a crucial role in esophageal carcinogenesis (6). Like other solid tumors, inactivation of tumor suppressor genes (TSG) and activation of oncogenes occur during ESCC development and progression. Although tremendous progress in diagnosis and therapeutic options has been achieved in the past decades, the prognosis for patients with ESCCs remains grim (7), mainly because of the advanced stage at initial diagnosis and the deficiency of efficacious therapies. Therefore, it is essential for identification of new sensitive and specific molecular markers for early detection and therapeutic targets (8, 9).

Deletion of chromosome 19p is one of the most frequent allelic imbalances in ESCCs detected by comparative genominc hybridization (CGH) and genome-wide genotyping (10–12). Deletion of 19p is also a common event in many other cancers including lung cancer (13), hepatocellular carcinoma (14), and ovarian cancer (15), suggesting the existence of multiple TSGs on 19p. In esophageal cancer, chromosome 19p13.3 is a frequently deleted region. We detected the mRNA levels of 9 genes (C19orf6, CIRBP, DAPK3, DIRAS1, FEM1A, MADCAM1, MAFK, MBD3, and WD18) in this region in 50 pairs of primary ESCC tumors and their corresponding non-tumor tissues by quantitative PCR (qPCR). As a result, only DIRAS1 expression differs between nontumor and ESCC samples, which thereby brought up our attention (Supplementary Fig. S1). DIRAS1 belongs to the small GTPase Ras superfamily and consists of more than 170 monomeric GTPases, sharing an essential biochemical activity, GTP binding and hydrolysis (16). They are conserved in primary structures bound to a function of GDP
or GTP binding and GTPase activity (17–19). Furthermore, they have a similar effect domain, a region binding to a group of downstream effector proteins specifically, including the members of the Raf family (A-Raf, B-Raf, and c-Raf), the phosphoinositide-3-kinase (PI3K) kinase, and members of the Rap family (RapGDS, Rit, and Rgl; ref. 20). Ras superfamily proteins are activated by extracellular stimuli and function as regulators of varying intracellular signaling pathways that touch on diverse cellular activities running the gamut from gene expression, mitosis, and metabolism to motility, survival and apoptosis, and differentiation. Most members in this superfamily have been widely studied and identified as oncogenes, such as Ras, Rap, Rit, and Rho subfamily proteins (21, 22). Meanwhile, more and more tumor suppressors have also been identified in this superfamily, such as RERG (23), ARHII (24, 25), and DIRAS1 (26). The DIRAS family, including DIRAS1, DIRAS2, and ARHII (DIRAS3), contains a highly conserved GTP-binding domain and a membrane-localizing CAAX motif at the carboxyl terminus. DIRAS1 gene comprises 2 exons and encodes a protein of 198 amino acids. Down-regulation of DIRAS1 has been reported in primary human neural tumors, and ectopic expression of DIRAS1 could suppress neural tumor cell growth by blocking Ras-mediated transformation (26). However, the molecular expression and the function of DIRAS1 in ESCCs are still unclear.

In the present study, we studied DIRAS1 expression status and its clinical significance in ESCCs. Both in vitro and in vivo functional assays were conducted to characterize the biologic effects of DIRAS1 in ESCC tumorigenicity. The tumor-suppressive mechanism of DIRAS1 was also investigated.

Materials and Methods

Cell lines and clinical samples

Six Japanese ESCC cell lines (KYSE30, KYSE140, KYSE180, KYSE410, KYSE510, and KYSE520) were acquired from DSMZ, the German Resource Centre for Biological Material (27). One Chinese ESCC cell line HKESEC1 was kindly provided by Professor Srivastava (Department of Pathology, The University of Hong Kong, Hong Kong, China), and 2 Chinese ESCC cell lines EC18 and EC109 were kindly provided by Professor Tao (Department of Anatomy, The University of Hong Kong). All 9 human ESCC cells were cultured in RPMI-1640 supplemented with 10% FBS. All cell lines used in this study were regularly authenticated by morphologic observation and tested for absence of mycoplasma contamination (MycoAlert, Lonza). The primary ESCC tumor tissues and their matched nontumorous tissues from surgically resection obtained from Linzhou Cancer Hospital (Henan, China). No patients recruited in this study have received any preoperative treatment. All clinical samples used in this study were approved by the committee for ethical review of research involving human subjects at Zhengzhou University (Zhengzhou, China), Sun Yat-Sen University (Guangzhou, China), and The University of Hong Kong.

Quantitative real-time PCR

Total RNA was extracted from clinical samples and cultured cell lines using TRIzol reagent (Invitrogen) and was reverse-transcribed with random primers using an Advantage RT-for-PCR Kit (Clontech Laboratories) according to the manufacturer’s instructions. Real-time qPCR was carried out to detect levels of the corresponding GAPDH, DIRAS1, and several matrix metalloproteinase (MMP) using a SYBR Green PCR Kit (Applied Biosystems) and an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for each specific gene. The relative levels of expression were quantified and analyzed by using SDS 2.3 software (Applied Biosystems). The real-time value for each sample was averaged and compared using the Ct method. The relative expression level (defined as fold change) of target gene (2–ΔΔCt) was normalized to the endogenous GAPDH reference (∆Ct) and related to the amount of target gene in control sample, which was defined as the calibrator at 1.0. Three independent experiments were carried out to analyze the relative gene expression and each sample was tested in triplicate. Supplementary Table S1 provides a list of the primer sequence used to amplify DIRAS1 in the qPCR assay.

ESCC tissue microarray and immunohistochemical staining

A total of 300 formalin-fixed, paraffin-embedded ESCC tumor specimens and the corresponding normal epithelia were selected from Linzhou Cancer Hospital. The ESCC tissue microarray (TMA) was constructed as described previously (28). Briefly, tissue sections with 5-μm thickness were cut from the TMA blocks and mounted on microscope slides. For immunohistochemical (IHC) analysis, the slides were deparaffinized, rehydrated, and blocked by 10% normal goat serum at room temperature for 30 minutes. The slides were then incubated with rabbit polyclonal antibody against DIRAS1 (Abcam) at a dilution of 1:100 at 4°C overnight and subsequently incubated with biotinylated goat anti-rabbit immunoglobulin at a concentration of 1:100 for 30 minutes at 37°C. A staining index (values 0–7) was calculated by adding the scores for the intensity of DIRAS1-positive staining (negative, 0; weak, 1; moderate, 2; or strong, 3) and the percentage of DIRAS1-positive cells (<25%, 1; 25%–50%, 2; >50%–75%, 3; ≥75%, 4 scores).

Bisulfite treatment and promoter methylation analysis

gDNA was extracted from normal and tumor esophageal tissues and cell lines by phenol–chloroform method followed by bisulfite modification using the Epitect Bisulfite Kit (Qiagen). Bisulfite genomic sequencing (BGS) and methylation-specific PCR (MSP) were done as previously described (29) using primers listed in Supplementary Table S1.

Results

DIRAS1 is frequently downregulated in ESCCs

The mRNA expression of DIRAS1 was initially tested in 75 pairs of primary ESCC tumors and their corresponding nontumor tissues by qPCR. Downregulation of DIRAS1 was detected in 34 of 75 (45.33%) of ESCC tumors compared with their normal counterparts (defined as a 2-fold decrease
of DIRAS1 expression in tumors). The relative expression level of DIRAS1 was significantly downregulated in tumor tissues compared with their nontumor counterparts ($P < 0.0001$, paired Student’s t test; Fig. 1A). Western blotting showed that downregulation of DIRAS1 protein was detected in 23 of 50 (46%) randomly selected ESCCs (Fig. 1A). Downregulation of DIRAS1 was also investigated in 7 of 9 (HKESC1, KYSE30, 140, 180, 410, 510, and 520) ESCC cell lines compared with immortalized esophageal epithelial cell line NE1 by using both qPCR and Western blot analysis (Fig. 1B).

**DIRAS1 downregulation is associated with ESCC metastasis and poor prognosis**

DIRAS1 expression in protein level was further studied in 300 primary ESCCs by IHC using a TMA. Informative IHC results were obtained from 216 pairs of ESCCs. Noninformative samples included lost samples, unrepresentative samples, and samples with too few tumor cells; such were not used in data complication. The staining index of DIRAS1 in each informative nontumor tissue was equal or greater than 5; therefore, staining index 5–7 was counted as normal expression of DIRAS1 whereas 0–4 was counted as downregulation of DIRAS1. Using this designation, downregulation of DIRAS1 was detected in 107 of 216 (49.5%) informative ESCC tissues compared with their adjacent nontumor tissues (Fig. 1C).

The correlation between DIRAS1 expression status and clinicopathologic features of ESCC was further evaluated, which was summarized in Table 1. The results showed that DIRAS1 downregulation was significantly associated with advanced clinical stage (Pearson $\chi^2$ test, $P = 0.01$) and lymph node metastasis (Pearson $\chi^2$ test, $P = 0.00$). No correlation was observed between DIRAS1 downregulation and patient’s age (Pearson $\chi^2$ test, $P = 1.00$), gender (Pearson $\chi^2$ test, $P = 0.89$), tumor cell differentiation (Pearson $\chi^2$ test, $P = 0.21$), and tumor invasion (Pearson $\chi^2$ test, $P = 0.15$; Table 1).
containing these 2 SNP sites followed by Sanger sequencing.

The LOH status of 50 primary ESCC specimens was investigated by PCR amplification of the genomic regions containing available SNP sites (rs14713 and rs8737) spanning the hotspot, LOH of DIRAS1 promoter region. To investigate the methylation status of DIRAS1 promoter region, we conducted BGS and MSP to detect methylation of 11 CpG sites within the upstream region (~513 to ~281) in NE1 and 3 ESCC cell lines using BGS. Results showed a high density of methylation in KYSE30 and KYSE510 cells with downregulated or absent expression of DIRAS1, whereas methylation was rarely detected in the same CpG sites in the DIRAS1 expressing NE1 and EC18 cell lines (Fig. 2B). To identify whether DIRAS1 promoter methylation was a common event and correlated with its transcriptional repression, we conducted MSP analysis in NE1 and 3 ESCC cell lines using BGS. The results showed that the methylated allele of DIRAS1 could be detected in almost all ESCC cell lines except NE1 and EC109 (Fig. 2C), indicating that methylation of the DIRAS1 promoter region was associated with its transcriptional repression. Methylation of DIRAS1 was detected in 20 of 50 (40%) primary ESCCs. The frequency of methylation in ESCC with DIRAS1 downregulation (15 of 23, 65.3%) was obviously higher than that in ESCC with normal DIRAS1 expression (7 of 27, 25.9%). In contrast, methylation was only found in 4 of 50 (8%) paired nontumor tissues (Fig. 2C and Supplementary Table S5). To further determine whether the methylation of DIRAS1 directly mediates its repression, KYSE30 and S10 cells were treated with demethylating agent 5-aza-dC. The result found that 5-aza-dC treatment could restore DIRAS1 expression in both cell lines (Fig. 2D).

**The DIRAS1 promoter region is frequently hypermethylated in ESCC**

To explore the role of aberrant promoter hypermethylation involving the DIRAS1 downregulation in ESCCs, we conducted BGS and MSP to detect methylation of 11 CpG sites within the upstream region (~513 to ~281) in NE1 and 3 ESCC cell lines using BGS. The results showed a high density of methylation in KYSE30 and KYSE510 cells with downregulated or absent expression of DIRAS1, whereas methylation was rarely detected in the same CpG sites in the DIRAS1 expressing NE1 and EC18 cell lines (Fig. 2B). To identify whether DIRAS1 promoter methylation was a common event, we conducted MSP analysis in NE1 and 3 ESCC cell lines using BGS. The results showed that the methylated allele of DIRAS1 could be detected in almost all ESCC cell lines except NE1 and EC109 (Fig. 2C), indicating that methylation of the DIRAS1 promoter region was associated with its transcriptional repression. Methylation of DIRAS1 was detected in 20 of 50 (40%) primary ESCCs. The frequency of methylation in ESCC with DIRAS1 downregulation (15 of 23, 65.3%) was obviously higher than that in ESCC with normal DIRAS1 expression (7 of 27, 25.9%). In contrast, methylation was only found in 4 of 50 (8%) paired nontumor tissues (Fig. 2C and Supplementary Table S5). To further determine whether the methylation of DIRAS1 directly mediates its repression, KYSE30 and S10 cells were treated with demethylating agent 5-aza-dC. The result found that 5-aza-dC treatment could restore DIRAS1 expression in both cell lines (Fig. 2D).

**Downregulation of DIRAS1 is associated with LOH and hypermethylation**

In the present study, downregulation of DIRAS1, LOH, and hypermethylation of DIRAS1 promoter region were detected in 23, 27, and 20 cases, respectively. Among 23 ESCCs showing downregulation of DIRAS1, inactivation of DIRAS1 in 19 (82.6%) cases was correlated with either LOH (n = 6) or methylation (n = 3) or both (n = 10, Fig. 2E). Statistical analysis showed that the downregulation of DIRAS1 was significantly associated with LOH (P = 0.042, χ² test, Supplementary Table S4) and methylation of DIRAS1 (P = 0.028, χ² test, Supplementary Table S5).

**DIRAS1 has tumor-suppressive ability**

To study the tumor-suppressive ability of DIRAS1, KYSE30 and 180 cells were stably transduced into 2 ESCC cell lines, KYSE30 and 180. Empty vector–transfected KYSE30 and 180 (Vec-30 and Vec-180) cells were used as controls. Expression of DIRAS1 gene and protein in these transfectants was studied by single-nucleotide polymorphism (SNP) markers. We identified 2 genotyping available SNP sites (rs14713 and rs8737) spanning the hotspot, LOH of DIRAS1 promoter region. To investigate the methylation status of DIRAS1 promoter region, we conducted BGS and MSP to detect methylation of 11 CpG sites within the upstream region (~513 to ~281) in NE1 and 3 ESCC cell lines using BGS. The results showed a high density of methylation in KYSE30 and KYSE510 cells with downregulated or absent expression of DIRAS1, whereas methylation was rarely detected in the same CpG sites in the DIRAS1 expressing NE1 and EC18 cell lines (Fig. 2B). To identify whether DIRAS1 promoter methylation was a common event, we conducted MSP analysis in NE1 and 3 ESCC cell lines using BGS. The results showed that the methylated allele of DIRAS1 could be detected in almost all ESCC cell lines except NE1 and EC109 (Fig. 2C), indicating that methylation of the DIRAS1 promoter region was associated with its transcriptional repression. Methylation of DIRAS1 was detected in 20 of 50 (40%) primary ESCCs. The frequency of methylation in ESCC with DIRAS1 downregulation (15 of 23, 65.3%) was obviously higher than that in ESCC with normal DIRAS1 expression (7 of 27, 25.9%). In contrast, methylation was only found in 4 of 50 (8%) paired nontumor tissues (Fig. 2C and Supplementary Table S5). To further determine whether the methylation of DIRAS1 directly mediates its repression, KYSE30 and S10 cells were treated with demethylating agent 5-aza-dC. The result found that 5-aza-dC treatment could restore DIRAS1 expression in both cell lines (Fig. 2D).

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<tr>
<td>&gt;60</td>
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</tr>
<tr>
<td>Poor</td>
<td>51</td>
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<td>15</td>
<td>4 (26.7%)</td>
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<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>22 (45.8%)</td>
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<tr>
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<td>Advanced (III-IV)</td>
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<td>47 (61.8%)</td>
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<sup>a</sup> Statistical significance (P < 0.05) is shown.
was confirmed by qPCR and Western blot analysis (Fig. 3A). Compared with control cells, in vitro assays found that ectopic expression of DIRAS1 could effectively inhibit tumorigenic ability in its transfected cells, including a significant inhibition of cell growth rate ($P < 0.01$, Student $t$ test, Fig. 3B) and reduction in foci formation frequency ($P < 0.001$, Student $t$ test, Fig. 3C).

To further explore the in vivo tumor-suppressive ability of DIRAS1, tumor formation in nude mice was conducted by injecting DIR-30 or DIR-180 cells subcutaneously into 5 nude mice. Vec-30 or Vec-180 cells were used as controls. The results showed that tumor formation in nude mice was significantly suppressed in DIRAS1-expressing cells ($P < 0.01$, Student $t$ test, Fig. 3D). With IHC staining using anti-DIRAS1 antibody, we confirmed that DIRAS1 was expressed in DIR-30-derived tumors (Fig. 3E). These results strongly suggest that DIRAS1 plays a tumor suppressive role in the development of ESCCs.

**Ectopic expression of DIRAS1 promotes apoptosis**

The potential role of DIRAS1 in apoptosis was studied by the treatment of DIRAS1-expressing and vector-transfected KYSE30 and 180 cells with STS, a broad-spectrum kinase inhibitor that can induce apoptosis in a wide variety of cells. Because Annexin-V binds to externalized phosphatidylserine on membranes of early apoptotic cells, flow cytometry was used to analyze cells stained with Annexin-V and the necrotic indicator propidium iodide (PI). Before STS treatment, the apoptotic index was found to be similar between DIRAS1-expressing cells and vector-transfected cells. After STS treatment, both early apoptotic (Annexin-V–positive/PI-negative) and late apoptotic (Annexin-V–positive/PI-positive) indexes were significantly increased in DIRAS1-expressing cells, compared with control cells ($P < 0.001$, Student $t$ test, Fig. 4A).

Because apoptosis is often mediated by the activation of caspases that lead to PARP binding to fragmented DNA, Western blot analysis was then used to detect caspase activity.
activation. The result showed that cleavages of caspase-9, caspase-3, and PARP was dramatically increased in DIRAS1-expressing cells after STS treatment, compared with control cells (Fig. 4B). However, no obvious difference of caspase-8 was detected between DIRAS1-expressing cells and control cells.

DIRAS1 dephosphorylates Bad at Ser-112 by ERK1/2 and p38 MAPK pathways

To elucidate the molecular basis of apoptosis promoted by DIRAS1, we analyzed the effects of DIRAS1 on the phosphorylation status of Bad, which is critical in the activation of caspase-9 and -3. Bad was significantly dephosphorylated at Ser-112 in DIRAS1-expressing cells compared with control cells but not at Ser-136 (Fig. 4C). However, DIRAS1 did not affect the protein expression level of Bcl-2 and Bax (Fig. 4C). We next examined the signaling pathways involved in the phosphorylation of Bad by Western blotting. It has been reported that Bad phosphorylation at Ser-112 is promoted by the Ras/MEK/ERK/p90RSK and p38 mitogen-activated protein kinase (MAPK) pathways (30–32). On the other hand, Bad phosphorylation at Ser-136 is promoted by the PI3K/Akt pathway (33, 34). The results showed that the phosphorylation levels of c-Raf, MEK, ERK1/2, p90RSK, and p38 MAPK were markedly decreased in DIRAS1-expressing cells compared with control cells.
cells. However, *DIRAS1* did not affect the phosphorylation status of PI3K and Akt (Fig. 4C).

**DIRAS1 inhibits cell motility and invasiveness**

As the TMA result indicated that downregulation of *DIRAS1* was significantly associated with ESCC metastasis, the effects of *DIRAS1* on cell migratory and invasive capabilities were further tested by wound healing, cell migration, and invasion assays. Wound-healing and migration assays showed that cell motility was dramatically reduced in *DIRAS1*-expressing cells, compared with control cells (*P* < 0.001, Student *t* test, Fig. 5A). Similarly, the Matrigel invasion assay revealed that the invasiveness of the *DIRAS1*-expressing cells was significantly decreased than control cells (*P* < 0.001, Student *t* test, Fig. 5B).

![Figure 4](image_url)

**Figure 4.** *DIRAS1* has proapoptotic effect. A, representative images of Annexin-V and PI double staining. Before STS treatment, the apoptotic indexes were similar between *DIRAS1*-expressing cells and vector-transfected cells. After STS treatment, the *DIRAS1*-expressing cells revealed a higher apoptotic index than control cells (**P** < 0.001, Student *t* test). B, the cleavages of caspase-9, caspase-8, caspase-3, and PARP were compared between *DIRAS1*- and vector-transfected cells by Western blotting at the indicated time points after STS treatment. β-Tubulin was used as a loading control. C, Bcl-2, Bax, p-BAD (Ser112), p-BAD (Ser136), BAD, p-c-Raf, p-MEK, p-ERK1/2, ERK1/2, p-p90RSK, p-p38 MAPK, p38 MAPK, p-PI3K, p-Akt (Ser 473), and Akt were compared between *DIRAS1*- and vector-transfected cells by Western blot analysis. β-Tubulin was used as a loading control.
Silencing DIRAS1 expression by siRNA promotes cell motility

To investigate whether endogenous DIRAS1 is important for cancer cell migration and invasion, RNA interference (RNAi) was used to knockdown DIRAS1 expression. EC109 cells were treated with siRNAs targeting DIRAS1 (DIR-1 or DIR-2 siRNA) or scramble siRNA (Scr siRNA) as a negative. Compared with Scr siRNA, treatment with specific siRNAs against DIRAS1 could dramatically reduce DIRAS1 expression in EC109 cells at both mRNA and protein levels (Fig. 5C). Matrigel migration and invasion assays showed that DIRAS1 knockdown could significantly increase cell migration and invasive abilities ($P < 0.001$, Student $t$ test, Fig. 5D).
**DIRAS1 modulates cell motility by regulating MMP2 and MMP9 expression**

To explore whether the effect of **DIRAS1** on cell motility is associated with downregulation of MMPs, expression levels of MMPs (MMP1, 2, 3, 9, 10, and 13) were compared between **DIRAS1**- and empty vector-transfected cells by qPCR. The results showed that the mRNA levels of MMP2 and MMP9 were significantly decreased in **DIRAS1**-expressing cells compared with controls cells ($P < 0.001$, Student t test, Fig. 6A). No obvious difference was detected in the expression of MMP1, MMP3, MMP10, and MMP13. Further study confirmed that **DIRAS1** knockdown could significantly increase the expression of MMP2 and MMP9 by qPCR ($P < 0.001$, Student t test, Fig. 6A). In addition, we used the gelatin zymography assay to measure the activity of MMP2 and MMP9 as a function of **DIRAS1** overexpression or knockdown. **DIRAS1**-transfected KYSE30 and 180 cells markedly inhibited the secretion of MMP2 and MMP9, whereas **DIRAS1** knockdown in EC109 cells substantially enhanced the activity of MMP2 and MMP9 (Fig. 6B). This result is interesting in light of the fact that MMP2 and MMP9...
are considered to be an indicator of tumor progression and metastasis (35). Xenograft tissue sections were also examined for MMP2 and MMP9 expression by IHC analysis. As compared with DIRAS1-30, xenografts generated with Vec-30 cells displayed an enhanced MMP2 and MMP9 expression (Fig. 6C). To confirm that DIRAS1 regulates MMP2 and MMP9 expression at the transcriptional level, we conducted a luciferase reporter assay using constructs containing promoter regions of MMP2 or MMP9. Knockdown of DIRAS1 could enhance the transcriptional activity of both MMP2 and MMP9 promoters ($P < 0.001$, Student $t$ test, Fig. 6D). These results indicate that DIRAS1 is involved in transcriptional regulation of MMP2 and MMP9 in ESCC cells.

**DIRAS1 downregulates MMP2 and MMP9 via ERK1/2 and p38 MAPK signaling pathways**

Signal transduction pathways that modulate the activity of MMP transcription are highly diverse. MMP signaling pathway, including well-known mediators p38 MAPK, ERK1/2, and JNK, can stimulate or inhibit MMP expression depending on cell types (36–38). To further gain insight into signaling pathways by which DIRAS1 regulates MMP2 and MMP9 expression, we analyzed the effects of DIRAS1 expressing or silencing on the activation of several potential signaling pathways. We found that the expression levels of phosphorylated ERK1/2 and p38 MAPK were dramatically decreased in DIRAS1-expressing cells but were increased in DIRAS1-silenced EC109 cells (Fig. 6E). However, expression of phosphorylated JNK and Akt remained unchanged in DIRAS1-expressing or silenced cells. These results suggest that ERK1/2 and p38 MAPK are likely the downstream targets of DIRAS1-mediated signaling that influence cell migration and invasion via regulating MMP2 and MMP9 expression.

**Discussion**

DIRAS1 belongs to Ras superfamily of monomeric GTPases and is predicted to act as a tumor suppressor in neural tumor (26). Although DIRAS1 has been identified as an inhibitor of cell proliferation and Ras-mediated transformation, its biologic function is largely unrevealed in ESCCs. To our knowledge, this is the first study to report a role of DIRAS1 in ESCCs. Downregulation of DIRAS1 in mRNA and protein level was detected in ESCC tumors and cell lines, compared with nontumor counterparts or immortalized normal esophageal cell line. Furthermore, the genetic–clinicopathologic correlation analysis found that downregulation of DIRAS1 was significantly associated with poor outcome of patients with ESCCs, suggesting that DIRAS1 might be an important tumor suppressor in ESCC development and progression. This is also the first comprehensive study to identify LOH at DIRAS1 region and promoter hypermethylation as possible mechanisms for inactivation of DIRAS1 during the tumorigenesis of ESCCs.

The tumor-suppressive function of DIRAS1 was addressed by both in vitro and in vivo assays. The results showed that DIRAS1 could effectively suppress cell growth rate, foci formation, and tumor formation in nude mice. Molecular studies revealed that the tumor-suppressive role of DIRAS1 was closely associated with its proapoptotic effect, which could be triggered by the stimulation of apoptotic agents such as STS. The apoptosis promoted by DIRAS1 is mainly mediated by the mitochondrial pathway but not the death receptor pathway because there was detectable activation of caspase-9, but not caspase-8. Our study also showed that DIRAS1 could dephosphorylate the proapoptotic protein Bad at Ser-112 but not at Ser-136 without affecting the expression of Bcl-2 and Bax. It has been shown that 14-3-3 proteins seclude phosphorylated Bad from mitochondria where it exerts the proapoptotic actions. In contrast, when Bad is dephosphorylated, unleashed Bad can interact with Bcl-xL or Bcl-2 in the mitochondria, thereby inactivating these anti-apoptotic proteins and inducing apoptosis (39). Bad phosphorylation is known to be regulated by diverse signaling pathways, in which protein tyrosine phosphorylation plays crucial roles. It has been clarified that the Ras/MEK/ERK/p90RSK and p38 MAPK pathways are involved in phosphorylation at Ser-112 (30–32), whereas the PI3K/Akt pathway is involved in phosphorylation at Ser-136 (33, 34). Our study revealed that the apoptosis promoted by DIRAS1 was closely connected with the inactivation of several signal transduction molecules, including ERK1/2, p90RSK, and p38 MAPK, which led to Bad dephosphorylation at Ser-112. We hypothesized that the proapoptotic effect of DIRAS1 might be exerted through maintaining the signal transduction molecules in the inactive state, thereby disabling phosphorylation of Bad and its sequestration from mitochondria.

In addition to its proapoptotic effect, we also found that DIRAS1 could mediate ESCC cell motility and invasiveness via deregulating MMP2 and MMP9, which are reported to be involved in tumor cell migration, spreading, invasion and metastasis (40–44). Luciferase reporter assay further confirmed that DIRAS1 could modulate MMP2 and MMP9 expression at the transcriptional level, suggesting that MMP2 and MMP9 are indeed downstream targets of DIRAS1-mediated signaling. It is well-known that the MAPK pathways (i.e., ERK1/2, JNK, and p38) participate in numerous signaling cascades that play critical regulatory roles in cell growth, apoptosis, differentiation, and metastasis (45). In line with these findings, we did identify DIRAS1 as a tumor modulator in ESCC tumorigenicity and metastasis through deregulated ERK1/2 and p38 MAPK pathways. It has also been reported that both ERK1/2 and p38 MAPK could regulate expression levels of MMP2 and MMP9 in cancer cells (46–49). Collectively, our results implied that DIRAS1 could mediate the malignant behavior of ESCC cells by regulating the transcriptional levels of MMP2 and MMP9 through ERK1/2 and p38 MAPK pathways.

DIRAS1 belongs to a distinct branch of the functionally diverse Ras superfamily, sharing more than 50% sequence similarity with the Ras and Rap subfamilies (26). DIRAS1 and Ras have similar effector domains, indicating that it can interact with c-Raf, a typical downstream effector of Ras, but this interaction was nonproductive (26). Furthermore, DIRAS1 shows reduced GTPase activity compared with that
of most RAS superfamily GTPases, and DIRAS1 maintains predominantly in the GTP-bound state. Therefore, DIRAS1 could serve as a competitive inhibitor of Ras and antagonize Ras-mediated ERK1/2 signaling, consequently, promote cell apoptosis, and prohibit cell invasion. In this study, we also revealed that DIRAS1 could resist p38 MAPK pathway, implicating that it could activate unique, non-Ras effectors to suppress ESCC cells. Further studies will be necessary to elucidate the tumor-suppressive mechanism of DIRAS1 on ERK1/2 and p38 MAPK signaling pathways. In summary, our findings show that DIRAS1 is a novel TSG and plays an important role in the pathogenesis of ESCCs. A better understanding of the molecular mechanism of DIRAS1 in promoting tumor cell apoptosis and metastasis would provide a novel therapeutic strategy to patients with ESCCs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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