Downregulation of the novel tumor suppressor DIRAS1 predicts poor prognosis in esophageal squamous cell carcinoma

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Analysis and interpretation of data: Y Zhu, J Li, Y Li, Y Dai

Writing, review, and/or revision of the manuscript: Y Zhu, L Fu, XY Guan

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Study supervision: XY Guan

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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 ESCC 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 ESCC where it was associated significantly with advanced clinical stage, lymph nodes metastasis and poor overall survival. Loss of heterozygosity 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 ERK1/2 (MAPK3/1) and p38 MAPK (MAPK14) signaling to trigger BAD Ser112 dephosphorylation and MMP2/9 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 utility as a biomarker and intervention point for new therapeutic strategies.
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 histological 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 ESCC (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 be more significant in high-incidence areas of northern China, suggesting that genetic susceptibility may play a crucial role in esophageal carcinogenesis (6). Like other solid tumors, inactivation of tumor-suppressor genes (TSGs) 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 ESCC patients 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 ESCC detected by comparative genomic 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 tumor suppressor genes (TSGs) on 19p. In esophageal cancer, chromosome 19p13.3 is a frequently deleted region. We detected the mRNA levels of nine genes (C19orf6, CIRBP, DAPK3, DIRAS1, FEM1A, MADCAM1, MATK, MBD3 and WDR18) in this region in 50 pairs of primary ESCC tumors and their corresponding non-tumor tissues by qPCR. As a result, only DIRAS1 expression differs between non-tumor and ESCC samples, which thereby brought up our attention (Supplementary Fig. S1). DIRAS1 belongs to the small GTPase Ras superfamily 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 PI-3 kinase and members of the RalGEF family (RalGDS, Rlf and Rgl) (20). Ras superfamily proteins are activated by various 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 oncoproteins, such as Ras-, Ral-, Rit-, and Rho-subfamily proteins (21, 22). Meanwhile, more and more tumor suppressors have also been identified in this superfamily, such as RERG (23), ARHI (24, 25), and DIRAS1 (26). The DIRAS family, including DIRAS1, DIRAS2, and ARHI (DIRAS3), contain a highly conserved
GTP-binding domain and a membrane localizing CAAX motif at the carboxyl terminus. 

*DIRAS1* gene comprises two exons and encodes a protein of 198 amino acids. Downregulation 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 ESCC are still unclear.

In the present study, we studied *DIRAS1* expression status and its clinical significance in ESCC. Both *in vitro* and *in vivo* functional assays were performed to characterize the biological 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 (Braunschweig, Germany), the German Resource Centre for Biological Material.(27) One Chinese ESCC cell line HKESC1 was kindly provided by Professor Srivastava (Department of Pathology, The University of Hong Kong, Hong Kong, China), and two 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% fetal bovine serum. All cell lines used in this study were regularly authenticated by morphological observation and tested for absence of
Mycoplasma contamination (MycoAlert, Lonza Rockland, Rockland, ME). 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, Sun Yat-Sen University and The University of Hong Kong.

Quantitative real-time PCR (qPCR)

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 performed to detect levels of the corresponding GAPDH, DIRAS1, and several MMPs using a SYBR Green PCR Kit (Applied Biosystems) and an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The 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 ($ΔCt$) and related to the amount of target gene in control sample, which was defined as the calibrator at 1.0. Three independent experiments were performed to analyze the relative
gene expression and each sample was tested in triplicate. Supplemental Table S1 provides a list of the primer sequence used to amplify  **DIRAS1** in the qPCR assay.

**ESCC tissue microarray (TMA) and immunohistochemical (IHC) staining**

A total of 300 formalin-fixed, paraffin-embedded ESCC tumor specimens and the corresponding normal epithelia were selected from Linzhou Cancer Hospital (Henan, China). The ESCC TMA was constructed as described previously. Briefly, tissue sections with 5 µm thick were cut from the tissue microarray blocks and mounted on microscope slides. For 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 plc. UK) 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 min 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**

Genomic DNA 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, Valencia, CA). Bisulfite genomic sequencing (BGS)
and methylation specific-PCR (MSP) were done as previously described(29) using primers listed in Supplemental Table S1.

Results

**DIRAS1 is frequently downregulated in ESCC**

The mRNA expression of *DIRAS1* was initially tested in 75 pairs of primary ESCC tumors and their corresponding non-tumor tissues by qPCR. Downregulation of *DIRAS1* was detected in 34/75 (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 non-tumor counterparts (*P* < 0.0001, paired Student’s *t*-test; Fig. 1A). Western blotting showed that downregulation of DIRAS1 protein was detected in 23/50 (46%) of randomly selected ESCC (Fig. 1A). Downregulation of *DIRAS1* was also investigated in 7/9 (HKESC1, KYSE30, 140, 180, 410, 510 and 520) of 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 tissue microarray. Informative IHC results were obtained from 216 pairs of ESCCs. Non-informative 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 non-tumor 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 107/216 (49.5%) of informative ESCC tissues compared with their adjacent non-tumor 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).

Kaplan-Meier analysis was used to study the survival curves in 216 ESCC patients with survival data. The result showed that the overall 3-year survival rate was significantly lower in ESCC patients with DIRAS1 downregulation ($n=109$, with a median of 19 months) than that in ESCC patients with normal DIRAS1 expression ($n=107$, with a median of 31 months, $P < 0.001$; Fig. 1D). By univariable analysis, downregulation of DIRAS1 ($P = 0.001$), presence of lymph node metastasis ($P < 0.001$) and advanced clinical stage ($P < 0.001$) were significant negative prognostic factors for overall survival in ESCC patients (Supplementary Table S3). Nevertheless, multivariate analysis showed that downregulation of DIRAS1 ($P = 0.007$) and advanced clinical stage ($P = 0.027$) were two independent prognostic predictors for ESCC patients.
enrolled in this study (Supplementary Table S3).

**Downregulation of DIRAS1 is associated with DNA copy number loss**

It has been reported that *DIRAS1* resides within a loss of heterozygosity (LOH) hotspot, LOH of *DIRAS1* region was studied by Single Nucleotide Polymorphism (SNP) markers. We identified two genotyping available SNP sites (rs14713 and rs8737) spanning *DIRAS1* region. The LOH status of 50 primary ESCC specimens were investigated by PCR amplification of the genomic regions containing these two SNP sites followed by Sanger sequencing. LOH was identified in 27 of 50 (54%) of the ESCC cases (Fig. 2A). Downregulation of DIRAS1 was observed in 16 out of 27 (59.3%) ESCCs with LOH at *DIRAS1* region, which was significantly higher than those without LOH (7/23, 30.4%, *P* = 0.042, Pearson Chi-square test, Supplementary Table S4).

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

To explore the role of aberrant promoter hypermethylation involving the *DIRAS1* downregulation in ESCC, we performed BGS and MSP to investigate the methylation status of the *DIRAS1* promoter region. The methylation levels of 11 CpG sites within the upstream region (-513 to -281) was analyzed in NE1 and three 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 line (Fig. 2B). To identify whether DIRAS1 promoter methylation was a common event, we performed MSP to investigate the methylation status of DIRAS1 in all 10 esophageal cell lines as well as in the cohort of 50 human ESCCs and their corresponding non-tumor esophageal tissues. The result 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 at promoter region was associated with its transcriptional repression. Methylation of DIRAS1 was detected in 20 of 50 (40%) of the primary ESCCs. The frequency of methylation in ESCC with DIRAS1 downregulation (13 out of 23, 56.5%) was obviously higher than that in ESCC with normal DIRAS1 expression (7 out of 27, 25.9%). In contrast, methylation was only found in 4 out of 50 (8%) of the paired non-tumor tissues (Fig. 2C and Supplementary Table S5). To further determine whether the methylation of DIRAS1 directly mediates its repression, KYSE30 and 510 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 demonstrating downregulation of DIRAS1, inactivation of DIRAS1 in 19 (82.6%) cases was correlated with either LOH \((n=6)\) or methylation \((n=3)\), or both \((n=10, \text{Fig. 2E})\). Statistical analysis showed that the downregulation of DIRAS1 was
significantly associated with LOH ($P = 0.042$, chi-square test, Supplementary Table S4) and methylation of $DIRAS1$ ($P = 0.028$, chi-square test, Supplementary Table S5).

**$DIRAS1$ has tumor suppressive ability**

To study the tumor suppressive ability of $DIRAS1$, $DIRAS1$ was stably transfected into two ESCC cell lines, KYSE30 and 180 ($DIR-30$ and $DIR-180$) cells. 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 were 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’s $t$-test; Fig. 3B) and reduction in foci formation frequency ($P < 0.001$, Student’s $t$-test; Fig. 3C).

To further explore the *in vivo* tumor suppressive ability of $DIRAS1$, tumor formation in nude mice was performed 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’s $t$-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 ESCC.
Ectopic expression of \textit{DIRAS1} promotes apoptosis

The potential role of \textit{DIRAS1} in apoptosis was studied by the treatment of \textit{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 cytometric was used to analyze cells stained with Annexin-V and the necrotic indicator propidium iodide (PI). Prior to STS treatment, the apoptotic index was found to be similar between \textit{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 \textit{DIRAS1}-expressing cells, compared with control cells ($P < 0.001$, Student’s $t$-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 caspases activation. The result showed that cleavages of caspase-9, caspase-3 and PARP were dramatically increased in \textit{DIRAS1}-expressing cells after STS treatment, compared with control cells (Fig. 4B). However, no obvious difference of caspase-8 was detected between \textit{DIRAS1}-expressing cells and control cells.

\textbf{DIRAS1} dephosphorylates Bad at Ser-112 by ERK1/2 and p38 MAPK Pathways

To elucidate the molecular basis of apoptosis promoted by \textit{DIRAS1}, we analyzed the effects of \textit{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 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, p38 MAPK were markedly decreased in DIRAS1-expressing cells compared with control 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 demonstrated that cell motility was dramatically reduced in DIRAS1-expressing cells, compared with control cells \( (P < 0.001, \text{Student’s } t\text{-test}; \text{Fig. 5A}) \). Similarly, the Matrigel invasion assay revealed that the invasiveness of the DIRAS1-expressing cells was significantly decreased than control cells \( (P < 0.001, \text{Student’s } t\text{-test}; \text{Fig. 5B}) \).

**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 control. Compared with Scr siRNA, treatment with specific siRNA 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’s $t$ 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’s $t$-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’s $t$-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 is 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 performed 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 \( (P < 0.001, \text{Student’s } t\text{-test}; \text{Fig. 6D}). \) 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. MAPK 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 biological function is largely unrevealed in ESCC. To our knowledge, this is the first study to report a role of DIRAS1 in ESCC. Downregulation of DIRAS1 in mRNA and protein level was detected in ESCC tumors and cell lines, compared with non-tumor 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 ESCC patients, 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 ESCC.

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 DIRASI was closely associated with its pro-apoptotic effect, which could be triggered by the stimulation of apoptotic agents such as STS. The apoptosis promoted by DIRASI 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 demonstrated that DIRASI could dephosphorylate the pro-apoptotic 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 pro-apoptotic 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 DIRASI 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 pro-apoptotic effect of DIRASI 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 pro-apoptotic effect, we also found that DIRASI could mediate
ESCC cell motility and invasiveness via deregulating matrix metalloproteinase (MMP)-2 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 MAPKs 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 >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 to 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 demonstrate that DIRAS1 is a novel TSG and plays an important role in the pathogenesis of ESCC. A better understanding of the molecular mechanism of DIRAS1 in promoting tumor cell apoptosis and metastasis would provide a novel therapeutic strategy to ESCC patients.
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Table 1. Association of DIRAS1 downregulation with clinicopathological features in ESCCs

<table>
<thead>
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<th>Clinical features</th>
<th>Number</th>
<th>DIRAS1 downregulation</th>
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<td>≤60</td>
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<tr>
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<tr>
<td>T₃</td>
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<tr>
<td>T₄</td>
<td>148</td>
<td>77 (52.0%)</td>
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<tr>
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<tr>
<td>N₁</td>
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<tr>
<td>Clinical stage</td>
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<td>Advanced (III-IV)</td>
<td>76</td>
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* Statistical significance (P < 0.05) is shown in bold
**Figure Legends**

**Figure 1.** Downregulation and clinical significance of DIRAS1 in ESCC. A, Downregulation of *DIRAS1* was frequently detected in primary ESCCs by qPCR (left) and western blot analysis (right). N, non-tumor tissue; T, tumor tissue; **, *P* < 0.001, paired Student’s *t*-test. B, Absent or downregulation of *DIRAS1* was detected in 6 out of 9 ESCC cell lines by qPCR (left) and western blot analysis (right). Immortalized esophageal epithelial cell line (NE1) was used as control. C, Representative of DIRAS1 expression in a pair of ESCC (lower) and adjacent non-tumor tissue (upper) detected by immunostaining with anti-DIRAS1 antibody (brown). The slide was counterstained with hematoxylin (original magnification ×200). D, Kaplan-Meier curves for overall survival rate of patients with ESCC according to the expression status of DIRAS1. Green, patients with normal expression of DIRAS1 (*n*=109, median survival 31 months); Blue, patients with downregulation of DIRAS1 (*n*=107, median survival 19 months; *P* < 0.001, log-rank test).

**Figure 2.** LOH at *DIRAS1* region and promoter hypermethylation of *DIRAS1* in ESCC. A, Representative chromas of rs14713 and rs8737 in tumor tissues and paired nontumor tissues. The SNP sites (rs14713 and rs8737) is indicated by arrows. LOH was found in case E9 and E23. N, non-tumor tissue; T, tumor tissue. B, High-resolution mapping of the methylation status of individual CpG site in the *DIRAS1* promoter by BGS in ESCC cell lines (EC18, KYSE30, and KYSE510) and immortalized esophageal epithelial cell line (NE1). A 233-bp region spanning the CpG island with 11 CpG sites was analyzed.
Each CpG site analyzed is shown at the top row as a short vertical bar. Percentage methylation was determined as percentage of methylated CpGs from 9 randomly sequenced clones. Black and gray pie, completely methylated or completely unmethylated CpGs, respectively. Concentric pies, partially methylated CpGs. C, Detection of promoter methylation of DIRAS1 in all 10 esophageal cell lines as well as in ESCC tumor tissues and paired non-tumor tissues by MSP. M, methylated allele; U, unmethylated allele. N, non-tumor tissue; T, tumor tissue. D, Restoration of DIRAS1 expression in KYSE30 and KYSE510 cells after 5-aza-dC (Aza) treatment. +, Aza treated; -, untreated. E, Association study indicates that the downregulation of DIRAS1 was significantly associated with the LOH at DIRAS1 site (P = 0.042, chi-square test) and hypermethylation in the promoter region of the DIRAS1 gene (P = 0.028, chi-square test).

Figure 3. Tumor-suppressive function of DIRAS1 in ESCC cells. A, Expression of DIRAS1 in DIRAS1- transfected ESCC cells was confirmed by qPCR and western blot analysis. Empty vector-transfected ESCC cells were used as controls. B, Growth curves of DIRAS1-expressing cells were compared with control cells by XTT assay. Points, mean of at least three independent experiments; bars, SD. *, P < 0.01, Student’s t-test. C, Representative inhibition of foci formation in monolayer culture by DIRAS1 and quantitative analyses of foci numbers. Columns, mean of at least three independent experiments; bars, SD. **, P < 0.001 versus controls by using the Student’s t-test. D, Tumor growth curves of DIRAS1-expressing cells in nude mice were compared with
vector-transfected ESCC cells by tumor xenograft experiments. Points, mean of 5 mice; bars, SD. *, $P < 0.01$, Student’s $t$-test. Representatives of tumors formed in nude mice induced by vector-transfected cells and $DIRASI$-expressing cells. E, IHC staining was performed to confirm the expression of $DIRASI$ in $DIR$-30 generated xenografts (lower) but not in the Vec-30 generated xenografts (upper). (magnification, ×200).

**Figure 4.** $DIRASI$ has pro-apoptotic effect. A, Representative images of Annexin-V and propidium iodide double staining. Before STS-treatment the apoptotic index were similar between $DIRASI$-expressing cells and vector-transfected cells. After STS treatment the $DIRASI$-expressing cells revealed a higher apoptotic index compared to control cells (***, $P < 0.001$, Student’s $t$-test). B, The cleavages of caspase-9, caspase-8, caspase-3, and PARP were compared between $DIRASI$- 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 (Ser 112), P-BAD (Ser 136), 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 $DIRASI$- and vector-transfected cells by western blot analysis. β-Tubulin was used as a loading control.

**Figure 5.** $DIRASI$ inhibits cell motility and invasiveness. A, Wound healing assay showed that cell motility was inhibited by $DIRASI$. Representative of images were photographed at time 0, 24, and 48h after scratching. B, Transwell cell migration and invasion assays were used to compare cell migration and invasion between $DIRASI$-
and vector-transfected cells. The cells that migrated through the PET-membrane or invaded through the Matrigel were fixed and stained with crystal violet (magnification, ×200). The results were expressed as mean ± SD of three independent experiments. **, \( P < 0.001 \), Student’s \( t \)-test. C, \( DIRAS1 \) was efficiently silenced in EC109 by the treatment of \( DIR-1 \) or \( DIR-2 \) siRNA, as determined by qPCR and western blotting, whereas no such silencing was observed in Scr siRNA-transfected cell. GPADH and β-Tubulin were used as loading controls. **, \( P < 0.001 \), Student’s \( t \)-test. D, Transwell cell migration and invasion assays were used to compare cell migration and invasion between EC109 cells treated with Scr, \( DIR-1 \) and \( DIR-2 \) siRNA. The cells that migrated through the PET-membrane or invaded through the matrigel were fixed and stained with crystal violet (magnification, ×200). The results are expressed as mean ± SD of three independent experiments. **, \( P < 0.001 \), Student’s \( t \)-test.

**Figure 6.** ERK1/2 and p38 MAPK are likely the downstream targets of \( DIRAS1 \)-mediated signaling that regulate MMP2 and MMP9 expression. A, mRNA levels of \( MMP1, MMP2, MMP3, MMP9, MMP10, \) and \( MMP13 \) were quantified by real-time PCR between \( DIRAS1 \)- and vector-transfected cells or between EC109 cells treated with Scr, \( DIR-1 \) and \( DIR-2 \) siRNA. Levels of \( GAPDH \) mRNA were used as internal standards. **, \( P < 0.001 \), Student’s \( t \)-test. B, MMP2 and MMP9 levels in the culture media between \( DIRAS1 \)- and vector-transfected cells or between EC109 cells treated with Scr, \( DIR-1 \) and \( DIR-2 \) siRNA were evaluated by gelatin zymography. The volume of conditioned medium loaded on the zymogram gel was normalized to the
β-Tubulin protein levels in the cell lysate. C, Expression of MMP2 and MMP9 was compared between xenograft generated from Vec-30 and DIRAS1-30 cells by IHC using anti-MMP2 or MMP9 antibody. Positive staining (brown) was observed in Vec-30 generated xenograft (magnification, ×200). D, EC109 cells were co-transfected with Scr, or DIR-1 or DIR-2 siRNA and MMP2-Luc or MMP9-Luc. Cells were harvested 48h later and analyzed for luciferase expression. pRL-TK vector was co-transfected for normalization of transfection efficiency. Values are expressed as mean ± SD of three independent experiments. **, P < 0.001, Student’s t-test. E, P-ERK1/2, ERK1/2, P-p38 MAPK, p38 MAPK, P-JNK, JNK, P-Akt, and Akt were compared between DIRAS1- and vector-transfected cells or between EC109 cells treated with Scr, DIR-1 and DIR-2 siRNA by western blot analysis. β-Tubulin was used as a loading control.
Zhu et al. Figure 1

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A

Chr19:2714666 (rs14713)

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Chr19:2714666 (rs14713)

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Chr19:2714720 (rs8737)

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B

BGS region

NE1

EC18

KYSE30

KYSE510

NE1

EC18

EC109

HKESC1

KYSE30

KYSE40

KYSE180

KYSE410

KYSE510

KYSE520

C

D

E

Zhu et al. Figure 2
Author Manuscript Published OnlineFirst on February 22, 2013; DOI: 10.1158/0008-5472.CAN-12-2663
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Zhu et al. Figure 4
Downregulation of the novel tumor suppressor DIRAS1 predicts poor prognosis in esophageal squamous cell carcinoma

Yinghui Zhu, Li Fu, Leilei Chen, et al.

Cancer Res Published OnlineFirst February 22, 2013.

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