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# Significant association of DIRC1 overexpression with tumor progression and poor prognosis in gastric cancer

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**Abstract. – OBJECTIVE: DIRC1, Disrupted in Renal Cancer 1, was identified as a breakpoint-spanning gene in a chromosomal translocation, which was associated with the onset and progression of some cancers. However, the expression in human gastric cancer (GC) and the role of DIRC1 in human gastric tumorigenesis are unknown. Thereby, the main purpose of this study was to unearth the association of DIRC1 with GC.**

**MATERIALS AND METHODS: By analyzing The Cancer Genome Atlas (TCGA) datasets, the expression of DIRC1 in GC and normal gastric tissues were compared. Besides, its association with clinicopathological significance, overall survival (OS) and independent prognosis were analyzed by Pearson's Chi-square$^2$ test, Kaplan-Meier method and Cox proportional hazards model, respectively. Functionally, the knockdown of DIRC1 was performed by siRNA method; moreover, its effects on the proliferation and metastasis of GC cells were examined by CCK-8 and transwell assays. Furthermore, the key markers of protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling were tested by Western blot assay.**

**RESULTS: The results showed that high expression of DIRC1 was found in GC tissues compared with normal gastric tissues. High expression of DIRC1 was associated with more cases of severer tumor malignancy and shorter OS; besides, high-level of DIRC1 was suggested to be an independent prognostic factor for GC. Furthermore, the knockdown of DIRC1 inhibited SGC-7901 GC cells proliferation, migration and invasion. Mechanically, the activity of AKT/mTOR signaling was suppressed by the knockdown of DIRC1.**

**CONCLUSIONS: These findings offer clinical associations and an *in vitro* evidence showing that the knockdown of DIRC1 impeded the GC carcinogenicity possibly via suppression of AKT/mTOR signaling. This work might provide a potential therapeutic target for GC treatment.**

*Key Words:*
Gastric cancer, DIRC1, Prognosis, Proliferation, Migration, Invasion, AKT/mTOR signaling.

## Introduction

Gastric cancer (GC), known as a common gastrointestinal tumor, is the fourth most frequent cancer and the third leading cause of cancer-related mortality worldwide[1,2]. Particularly, according to the National Central Cancer Registry of China, GC has become the second highest morbidity and the second highest lethality of all cancers in China with estimated 679,000 new cases and 498,000 deaths annually[3]. Despite remarkable advances in cancer treatment, namely surgical resection, chemotherapy and adjuvant therapy, the 5-year survival rate remains relatively low[4,5]. The management of early detection and availability of appropriate biomarkers may aid to the effective monitor and therapy of GC[6]. However, the molecular mechanisms underlying GC have not been properly understood. It is well-established that chromosomal translocation is known as one of the crucial molecular genetic variations of a variety of malignant tumors, largely attributed to emergences of aberrant oncogenes and new fusion genes eliciting abnormal cell growth and differentiation caused by chromosomal translocation[7,8]. Therefore, the identification of genes at or near the chromosomal breakpoints would contribute


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to figure out characteristic biomarkers of tumors. Several chromosomal translocations mainly in chromosome 7, 11, 12, 14, 18 etc. were involved in some of the breakpoint-spanning genes reported in GC initiation and progression[9-11]. Whether other chromosomal translocations or novel breakpoint-spanning genes are involved in GC still needs to be investigated. Disrupted in Renal Cancer 1 (DIRC1), initially characterized as a chromosome 2q33 breakpoint-spanning gene in a chromosomal translocation, has been closely associated with the development of renal cancer previously. Beyond that, low-level of DIRC1 has been found in various tissues, including adult placenta, testis, ovary, prostate and fetal kidney, spleen and skeletal muscle[12]. However, the detailed study of DIRC1 on GC remains limited. In view of this, we downloaded and analyzed The Cancer Genome Atlas (TCGA, public datasets containing expression data) data available of DIRC1 expression level in GC and normal gastric issues, showing that elevated DIRC1 level was identified in BC issues. Aside from this, high-level of DIRC1 was associated with clinicopathological implications and poor prognosis in GC patients. Furthermore, the upregulation of DIRC1 was detected in GC cell lines. The knockdown of DIRC1 through siRNA approach inhibited GC proliferation, migration and invasiveness. This work offers clinical correlation and potential mechanism *in vitro* supporting that the DIRC1 overexpression promotes GC progression.

## Materials and Methods

### TCGA Data Acquisition and Analysis

First, we downloaded the RNA-seq data of GC and normal gastric tissues in the TCGA database; the DIRC1 expressions of them were analyzed by limma package in R and prognostic analysis was evaluated by survival function.

### Cell Lines

Human GC cell lines, MGC-803, SGC-7901 and immortalized normal human gastric epithelial cell line (GES-1) were obtained from the Shanghai Institute for Life Science, Chinese Academy of Sciences (Shanghai, China). All of these cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), containing 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated in a humidified incubator containing 5% at 37°C.

### RNA Interference and Transfection

DIRC1 siRNA (si-DIRC1#1, 5'-AACAGGGCAGAAAGUUGUA -3'; si-DIRC1#2: 5'- AUGAAAUGGGAUCAAGUGG -3') and their negative control siRNA (si-NC) were purchased from RuiBo (Guangzhou, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used to transfect cells according to manufacturer's protocols. Cells were incubated for 72 h and then harvested for correlation analysis.

### RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells and tissue specimens using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. cDNA was synthesized using PrimeScript™ RT reagent kit (TaKaRa Bio, Otsu, Shiga, Japan) and following the manufacturer's protocols. The mRNA levels were determined by qRT-PCR using SYBR Premix Ex Taq™ kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's protocol. GAPDH was used as the internal control. Primers were as follows: DIRC1, F: 5'-CAGGGACCTCGTTAATGGCT-3'; R: 5'-CCGTTCAGACAGCTGACGTT-3'. GAPDH, F: 5'-TCACACCAAGTGTCAGGACG-3'; R: 5'-TCAAGAAAGCAGCACGGGTC -3'. The amplification reaction was performed following the reaction procedure: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Experiments were repeated in triplicate. The relative expression levels of DIRC1 were calculated using the $2^{-\Delta\Delta Ct}$ method.

### Cell Counting Kit-8 (CCK-8)

CCK-8 assay was performed to determine the cells viabilities according to the instructions provided by the manufacturer. Cells were seeded in 96-well plates and cultivated for 24, 48, 72 and 96 h, and finally assessed using the Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Shanghai, China). The optical density (OD) was measured using a spectrophotometer (Bio-Rad, Hercules, CA, USA) at 450 nm. Each experiment was performed in triplicate.

### Transwell Assays

The cell migration assay was performed using a transwell chamber (8-μm pore size; Corning Inc.,

Lowell, MA, USA). At 48 h after transfection, cells ($1 \times 10^5$/ml) were plated to the upper chamber in 100 µl medium supplemented with no FBS, and 500 µl culture medium containing 10% FBS was added into the bottom chamber. After 24-h incubation, the cells that didn't migrate into the upper chamber were removed with cotton-tipped swabs, and those on the lower chamber were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 20 min. Finally, migrated cells were counted by photographing 5 random fields at 200 × magnification. For the cell invasion assay, the steps were similar to the cell migration assay, except that the transwell chambers were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Each experiment was performed in triplicate.

### Western Blot Analysis

After 48 h of transfection, total proteins were extracted from cells using Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentrations were examined by BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). An equal amount of protein (20 µg) was separated on 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. After blocking with 5% non-fat milk in Tris-Buffered Saline with Tween-20 (TBST) for 1.5 h at room temperature, membranes were incubated with primary antibodies, including DIRC1 (Thermo Fisher Scientific, Waltham, MA, USA, dilution 1: 1000), protein kinase B (AKT), phosphorylated-(p-) AKT, mammalian target of rapamycin (mTOR), p-mTOR (Cell Signaling Technology, Inc., Danvers, MA, USA, dilution 1: 1000), GAPDH (Beyotime, Shanghai, China, dilution 1: 1000) at 4°C overnight and with the anti-mouse/rabbit horseradish peroxidase-conjugated secondary antibodies (Beyotime, Shanghai, China, dilution 1: 1000) at room temperature for 2 h. Target proteins were visualized by enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology, Inc., Waltham, MA, USA). The protein band density was measured with Image J software (National Institutes of Health, Stapleton, NY, USA).

### Statistical Analysis

Relationships between the DIRC1 expression and clinicopathological parameters were evaluated using Pearson's chi-squared test. Overall survival was analyzed using the Kaplan-Meier method. Significant differences between the groups were calculated using a log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazards model. The statistical significance of the difference between the groups in remaining experiments was analyzed using Student's *t*-test. All data were presented by the mean ± standard deviation (SD). Statistical analysis was conducted with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) and a *p*-value less than 0.05 or 0.01 was considered statistically significant.

## Results

### DIRC1 is Highly Expressed in the GC and Associates With Clinicopathological Significance

By analyzing TCGA data, we found that DIRC1 was highly expressed in GC tissues compared with normal gastric tissues (Figure 1). Next, we analyzed its relation with clinicopathological parameters, namely age, gender, grade, pathologic-stage, pathologic-T (tumor status), pathologic-N (lymph node status), and pathologic-M (metastasis status). The data showed that high expression of DIRC1 was markedly associated with higher grade ($p = 0.03$) and higher pathologic-T ($p = 0.048$) (Table I). However, no significant difference was observed between the DIRC1 expression and other clinicopathologic characteristics (all $p>0.05$). These results indicated that high DIRC1 may be tightly correlated with tumor progression.

### High Expression of DIRC1 Independently Predicts Poor Overall Survival (OS) in GC

The Kaplan-Meier survival analysis and log-rank test revealed that high DIRC1 level in GC was associated with shorter OS (Figure 2, $p = 0.002$). Furthermore, as shown in Table II, univariate Cox regression analysis disclosed that high DIRC1 ($p = 0.003$), pathologic-stage (I+II/III+IV) ($p < 0.001$), pathologic-T (T1+T2/T3+T4) ($p = 0.012$), pathologic-M (M0/M1) ($p = 0.012$), pathologic-N (N0/N1+N2+N3) ($p = 0.002$) and age ($p = 0.029$) predicted poor survival in GC patients. Moreover, multivariate Cox regression analysis with the above clinicopathologic factors revealed that high DIRC1 and age were considered as the independent prognosticator of unfavorable prognosis in GC patients.

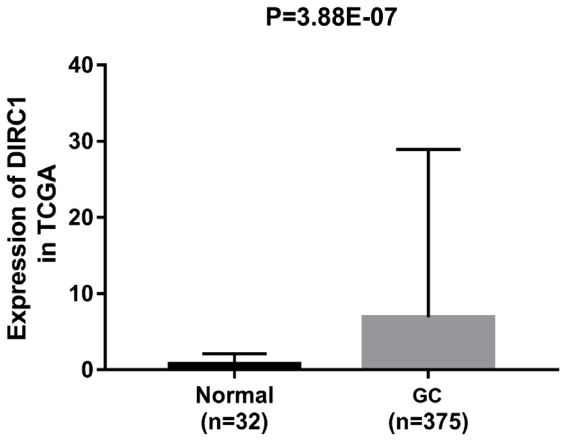


**Figure 1.** High level of DIRC1 was expressed in GC issues *vs.* normal gastric issues by analyzing the TCGA datasets.

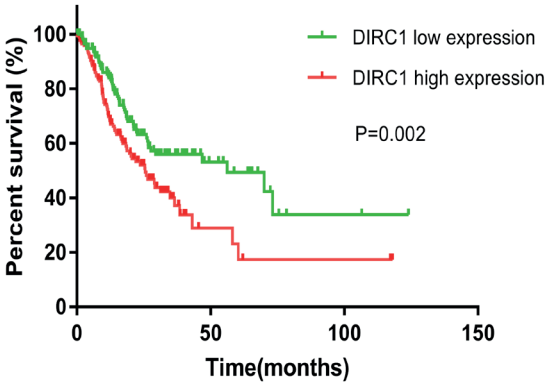


**Figure 2.** Highly expression of DIRC1 predicted a shorter OS in GC patients.

### *Overexpression of DIRC1 in GC Cells*

Using the qRT-PCR, DIRC1 mRNA level in some of GC cell lines was determined. The results showed that DIRC1 mRNA level in SGC-7901 cells was higher than either normal gastric GES-1 cells or other GC cells MGC-803 (Figure 3A, $p<0.01$). In light of this, the SGC-7901 cells were used in the following experiments. To examine the role of DIRC1 on GC progression, first, DIRC1 was silenced by siRNA method. The efficacy of the knockdown was identified using qRT-PCR and Western blot assay. The results showed that DIRC1 both in mRNA and protein was significantly reduced in si-DIRC1#2 group but not in the si-DIRC1#1 group in comparison with the si-NC group (Figure 3B and C, $p<0.01$). Therefore, si-DIRC1#2 group was applied in the following experiments and was expressed as the si-DIRC1 group.

### *DIRC1 Knockdown Inhibits the Growth of GC Cells*

To explore the effect of the DIRC1 knockdown on the GC cells proliferation, CCK-8 and colony formation assays were carried out. The CCK-8 as-

**Table I.** Relationship between DIRC1 expression and clinicopathological variables in GC patients.

| Characteristics | Expression of DIRC1 | | *p*-value |
|---|---|---|---|
| | Low | High | |
| Age | | | 0.886 |
| <60 | 54 | 54 | |
| ≥60 | 118 | 122 | |
| Gender | | | 0.943 |
| female | 62 | 63 | |
| male | 113 | 113 | |
| Grade | | | 0.030* |
| G1+G2 | 77 | 59 | |
| G3 | 92 | 114 | |
| Pathologic-Stage | | | 0.815 |
| I+II | 80 | 76 | |
| III+IV | 90 | 90 | |
| Pathologic-T | | | 0.048* |
| T1+T2 | 54 | 37 | |
| T3+T4 | 121 | 135 | |
| Pathologic-N | | | |
| N0 | 52 | 51 | 0.906 |
| N1 | 118 | 119 | |
| Pathologic-M | | | 0.899 |
| M0 | 154 | 159 | |
| M1 | 11 | 12 | |

*$p$ <0.05

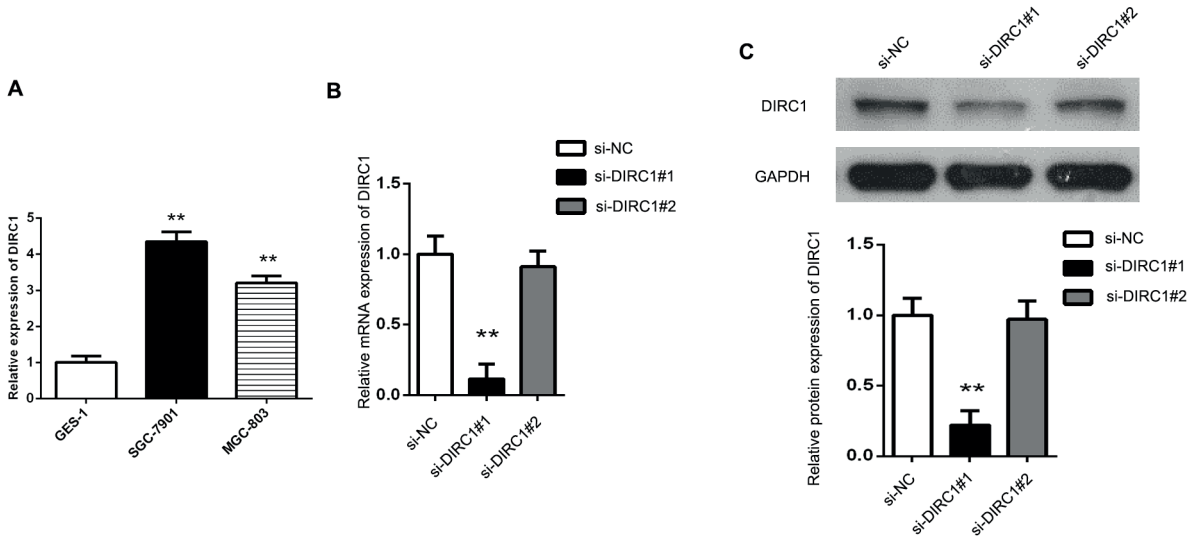


**Figure 3.** The mRNA level of DIRC1 in GC cells and the DIRC1 knockdown efficacy was identified. ***A,*** mRNA levels of DIRC1 in some of GC cell lines. ***B,*** mRNA levels of DIRC1 in SGC-7901 cells transfected with si-DIRC1#1, si-DIRC1#2 and si-NC. ***C,*** The protein levels of GC cells transfected with si-DIRC1#1, si-DIRC1#2 and si-NC. $^{**}p<0.01$ *vs.* normal gastric cells (GES-1) or si-NC group.

say showed that OD values in the si-DIRC1 group were decreased with a time-dependent manner compared with the si-NC group (Figure 4, $p<0.01$). The results indicated that the viability of GC cells was inhibited when DIRC1 was silenced.

### *DIRC1 Knockdown Represses the GC Cells Migration and Invasion*

To determine the effect of the DIRC1 knockdown on the abilities of migration and invasion in GC cells, transwell assay was implemented. As shown in Figure 5, the number of migrated and invaded cells was markedly reduced in the si-DIRC1 group compared with the si-NC group (Figure 5B and C, $p<0.01$). All these results indicated that the capabilities of migration and invasion of GC cells were repressed by the DIRC1 knockdown.

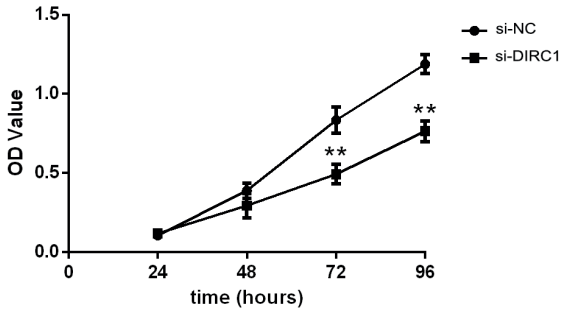


**Figure 4.** DIRC1 knockdown inhibited GC cell proliferation. CCK-8 assay was used to measure cell viability in GC cells. $^{**}p<0.01$ *vs.* si-NC group.

### *DIRC1 Knockdown Results in Inactivity of AKT/mTOR Signaling*

To address the suppressive function of DIRC1 knockdown on GC progression, we investigated the status of AKT/mTOR signaling which is known to regulate the development and progression in various types of cancer by measuring the levels of the signaling key markers including AKT, p-AKT, mTOR and p-mTOR. The Western blot results suggested that the p-AKT and p-mTOR, but not AKT and mTOR were upregulated in the si-DIRC1 group compared to the si-NC group (Figure 6, $p<0.01$). The data indicated that the knockdown of DIRC1 might suppress carcinogenesis and progression of GC through the inactivation of the AKT/mTOR signaling pathway.

## Discussion

In this work, the expression of DIRC1 was upregulated in GC tissues by analyzing the TCGA data and the upregulation of DIRC1 was associated with higher GC grade and severer pathologic tumor status. In addition, high DIRC1 was considered an

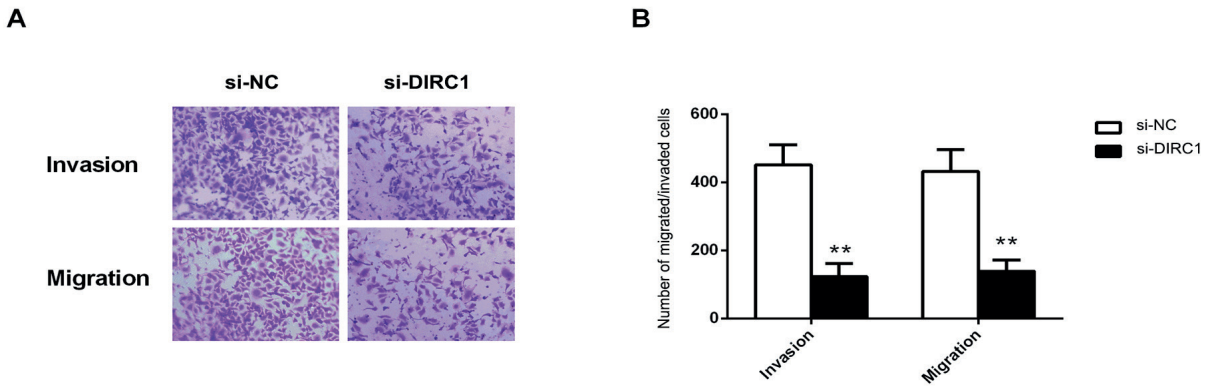


**Figure 5.** DIRC1 knockdown suppressed GC cell migration and invasion. ***A,*** The migration and invasion abilities of GC cells transfected with si-DIRC1 and si-NC was examined by transwell assay. ***B,*** The number of migrated and invaded of GC cells was shown. ***p*<0.01 *vs.* si-NC group.

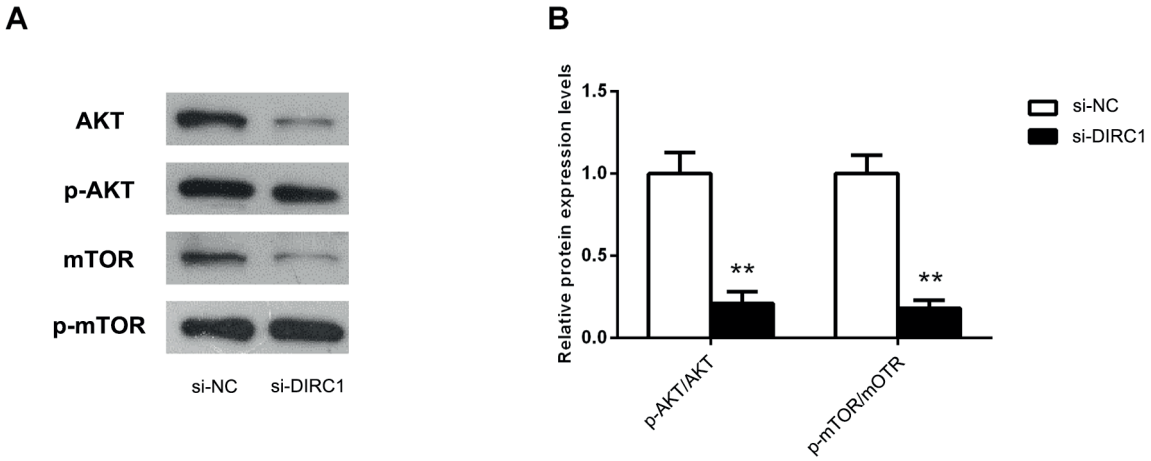


**Figure 6.** Effect of DIRC1 knockdown on markers associated with AKT/mTOR signaling. ***A,*** Proteins of AKT, p-AKT, mTOR and p-mTOR were tested using Western blot assay. ***B,*** Densitometric quantification of protein bands were measured by Image J software. ***p*<0.01 *vs.* si-NC group.

unfavorable prognostic predictive marker for GC. Furthermore, we performed the loss-of-function assay using siRNA approach on GC cells and found that the DIRC1 knockdown could inhibit the proliferation, migration and invasion of SGC-7901 cells. The AKT/mTOR signaling key markers were also suppressed by the knockdown of DIRC1. Overall, these findings implicate that DIRC1 might play a crucial role in the pathological process of GC and may be a potential biomarker target for GC treatment. DIRC1 belongs to a breakpoint-spanning gene in a chromosomal translocation. It was identified that many of the breakpoint-spanning genes were of importance in various types of cancers or disorders. For example, LSAMP and NORE1 as the breakpoint-spanning genes were involved in clear cell renal cell carcinomas[13]. Langer et al[14] found that MLL breakpoints were inducible by apoptotic triggers in acute myeloid leukemia. Brain-expressed Netrin G1 (NTNG1), as a breakpoint-spanning gene, was reported to impair central nervous system in Rett syndrome[15]. C11orf95-MKL2 fusion was identified as an oncogene in chondroid lipoma[16]. SLC44A1-PRKCA fusion gene was found as an oncogene in papillary glioneuronal tumor[17]. In this study, we analyzed the DIRC1 expression and its relation with clinicopathological features and OS through analyzing data from TCGA datasets. The data suggested that it was highly expressed in GC tissues and it related to higher tumor grade and

**Table II.** Univariate and multivariate analysis of the correlation between DIRC1 expression and prog-nostic significance of GC patients.

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| Variables | *p*-value | HR | 95% CI | *p*-value | HR | 95% CI |
| DIRC1 expression (high/low) | 0.003* | 1.670 | 1.196-2.331 | 0.042* | 1.448 | 1.013-2.071 |
| Pathologic-Stage (I+II/III+IV) | 0.000* | 1.923 | 1.340-2.760 | 0.234 | 1.390 | 0.808-2.390 |
| Pathologic-T (T1+T2/T3+T4) | 0.012* | 1.707 | 1.123-2.596 | 0.736 | 1.094 | 0.649-1.843 |
| Pathologic-M (M0/M1) | 0.012* | 2.093 | 1.180-3.712 | 0.055 | 1.852 | 0.988-3.470 |
| Pathologic-N (N0/N1+N2+N3) | 0.002* | 1.918 | 1.259-2.922 | 0.191 | 1.461 | 0.828-2.577 |
| Age (<60/≥60) | 0.029* | 1.525 | 1.044-2.229 | 0.010* | 1.713 | 1.135-2.588 |
| Gender (fe-male/male) | 0.153 | 1.296 | 0.908-1.849 | | | |
| Grade (G1+G2/G3+G4) | 0.095 | 1.345 | 0.950-1.904 | | | |

Abbreviations: CI, confidence interval; HR, hazard ratio. *Statistically significant.

worse tumor progress with shorter OS. In addition, using siRNA interference technology to down-regulate the expression of DIRC1 in GC cells, the proliferation and mobility properties of BC cancer cells were evidently reduced. The data above indicated that DIRC1 exerted a potential oncogenic action in GC. To gain insight into the underlying mechanism of action, the activity of AKT/mTOR was determined. It is well known that AKT/mTOR plays a critical role in numerous tumor development and progression[18-20]. Ying et al[21] demonstrated that PI3K/p-AKT/p-mTOR was highly expressed and its simultaneous expression was independent prognostic parameter in the expression of the PI3K/AKT/mTOR pathway in gastric cancer and its role in gastric cancer. It was investigated that p-AKT and p-mTOR were markedly reduced while no detectable changes of AKT and mTOR were observed in GC cell lines *vs.* normal gastric cells[22-24]. Cao et al[25] determined that p-mTOR expression, but not mTOR, was closely related with OS of GC patients. In line with these studies, the results of our work suggested that after interfering with DIRC1, the expression of p-AKT and p-mTOR, but not AKT and mTOR were remarkably decreased in GC cells. All of these findings support our result that AKT/mTOR signaling might be implicated in the tumorigenic effect of DIRC1 in GC. In spite of what has been investigated in this paper, some additional limitations still existed in this work. For instance, there was no clinical sample collected by our own; besides, this research merely focused on the study in cell level and confirmation in the animal model should be considered. Therefore, further investigations on the DIRC1 regulation in GC will be needed.

## Conclusions

The results of the current work revealed that high expression of DIRC1 was more likely to develop higher GC grade and severer tumor status with a worse prognosis. Functionally, our studies suggested that the DIRC1 knockdown significantly suppressed GC cells proliferation and mobility, which was regulated by the inactivity of AKT/mTOR signaling. This work implies that DIRC1 might serve as an oncogene in the development of GC, which provides a novel therapeutic target option for GC.

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### Conflict of Interest

The Authors declare that they have no conflict of interest.

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