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**Original Research Article**

# Riccardin D, a drug candidate, inhibits gastric cancer progression by targeting RAD54L

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## *Abstract*

***Purpose:*** *To investigate the beneficial function of RAD54L in gastric cancer.*
***Methods:*** *Batch correction, metaboanalyst, volcano plot and heatmap were used for analyzing different expression genes. Metascape and strings were used for functional enrichment and protein-protein interaction network analysis, respectively. Expression of RAD54L was analyzed using online platform-timer, ualcan and GEPIA. Correlation between RAD54L and poor prognosis was analyzed using Kaplan-Meier curve. Expression of RAD54L was evaluated by Real Time-quantitative PCR and western blotting, while cell proliferation, cell apoptosis and cell cycle were determined by CCK8 and flow cytometry, respectively.*
***Results:*** *A total of 57 upregulated DEGs and 33 downregulated DEGs were involved in gastric cancer. Among these, RAD54L was a hub gene which is highly expressed, and is related to poor survival in gastric cancer. Moreover, knockdown of RAD54L inhibited cell viability and facilitated cell apoptosis in gastric cancer cells ($p < 0.05$). On the other hand, overexpression of RAD54L promoted cell proliferation and reduced cell apoptosis in gastric cancer ($p < 0.05$). In addition, RAD54L positively regulated cell cycle in gastric cancer cell lines. Furthermore, riccardin D negatively regulated cell cycle in gastric cancer cell lines by targeting RAD54L.*
***Conclusion:*** *Riccardin DRAD54L is a potential drug for the treatment of gastric cancer. However, developmental work, including in vivo studies, are required to ascertain this.*

***Keywords:*** *Riccardin, RAD54L, Gastric carcinoma, Tumor progression, Cell apoptosis, Poor prognosis*



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## INTRODUCTION

Gastric carcinoma (GC) is a malignant tumor originating from gastric mucosa epithelium, and its incidence ranks first among various malignant tumors in China. Currently, GC is mainly treated clinically by surgical resection [1]. Early symptoms of gastric cancer are generally difficult to be detected and diagnosed at an early stage, thus losing the opportunity for curative resection. Currently, chemotherapy is the clinical treatment for gastric cancer. However, gastric cancer has a strong ability to metastasize, which leads to a poor prognosis for patients with gastric cancer [2]. Therefore, elucidating the pathogenesis and specific processes of GC can better contribute to the clinical treatment and diagnosis of GC [3]. Located at 1p32, RAD54L has regulatory

functions in mitotic and meiotic recombination, it is a member of the SNF2/SWI2 family of DNA-dependent ATPases [4]. Studies have shown that RAD54L is increasingly expressed in bladder cancer, and its high expression correlates with the poor prognosis of bladder cancer [5]. RAD54L promoter activity regulates the expression of DNA repair/recombinant protein 54L (RAD54L), thereby increasing the proliferation and malignant phenotype of glioma [6]. Expression of RAD54L is also associated with increased glioma resistance to radiotherapy [7]. In addition, studies have found that RAD54L is highly expressed in gastric cancer tissues [8].

In this study, we provide evidence that RAD54L is overexpression in gastric cancer and correlated with poor survival. In addition, we identify an important function of RAD54L in regulation of  in human CRC. These results demonstrate that SMURF2 plays a critical role in the regulation of SIRT1 stability and function.

## METHODS

### Gene expression analysis

The expression of RAD54L in gastric cancer was analyzed using an online platform, i.e. ACLBI (https://www.aclbi.com/static/index.html#/), The PLSDA analysis was measured using the metaboanalyst platform (https://www.metaboanalyst.ca/), and data using GSE118897. The prediction of potential small molecule compounds for RAD54L targets was measured using a network analyst analysis platform (https://www.networkanalyst.ca/).

### Kaplan-Meier analysis

This was based on TCGA-gastric cancer dataset, which was used to draw the survival prognosis curve. For survival analysis, the prognosis of patient is measured by online software Kaplan-Meier curve tool (https://kmplot.com/analysis).

### GO-KEGG enrichment analysis

The genes with expression correlation to RAD54L in GC were selected from ualcan dataset. Then genes with Pearson score ≥ 0.4 were screened for GO-KEGG enrichment analysis.

### Correlation analysis

Correlation analysis was performed as previously described [9]. The correlation between RAD54L and genes associated with immune infiltration were analyzed using Pearson score. The data in the correlation analysis were obtained from the TEMER platform.

### Cell cycle analysis

Cell cycle analysis was performed as previously reported [10]. Cell lines were washed with PBS, fixed with 70 % ethanol for 12h, and stained with PI, and the cell cycle was analyzed using flow cytometry.

### Quantitative real time-polymerase chain reaction (qRT-PCR) and western blotting analysis

RNA isolation and protein extraction were performed as previously described [11]. Cells were collected and total RNA was extracted using the Trizol method. cDNA was obtained by reverse transcription. quantitative real-time RT-qPCR was then performed using SYBR GREEN qPCR Mix (Biorad, USA). The primers used PCR are listed in Table 1.

| Gene | Primer |
|---|---|
| RAD54L forward | 5'-TTGAGTCAGCTAACCAATCAACC-3' |
| RAD54L reverse | 5'-GGAGGCTCATACAGAACCAAGG-3' |
| GAPDH forward | 5'-AGGTCGGTGTGAACGGATTTG-3' |
| GAPDH reverse | 5'-GGGGTCGTTGATGGCAACA-3' |

For western blotting, cells were collected, then total protein was isolated by RIPA lysis buffer. And immunoblotted with the following antibodies:

RAD54L (1:1000, Santa, sc-55584, USA), β-actin (1:1000, Santa, sc-8432, USA). Then, the PVDF membrane was washed and a 1:5000 secondary antibody was applied for 1 hour at room temperature to visualize the immunoreactivity with chemiluminescent ECL reagents.

### Cell viability and apoptosis analysis

Determination of the survival analysis of CRC cell lines was performed as previously reported [12]. Cell Counting Kit 8 (CCK8) assay was used to analyze cell viability. Cell apoptosis was analyzed by flow cytometry.

### Statistical analysis

Statistical analyses were performed using the SPSS 22 Statistical Software. $p \leq 0.05$ was

considered statistically significant. The results are shown as mean + SD from three independent experiments as indicated in Figure legends. Differences between control and experimental conditions were evaluated by Student's t test.

## RESULTS

### Differentially-expressed gene in gastric cancer tissue and normal tissue based on GEO data

To determine the role of RAD54L in gastric cancer, batch correction was used to analyze both the tumor and normal groups (Figure 1 A). When these data were analyzed using PLSDA on the metaboanalyst platform, there was a significant difference between the tumor and non-tumor groups ($p < 0.05$, Figure 1 B). The different expression genes were assessed by volcano plot, and there were 57 upregulated DEGs and 33 downregulated DEGs in the dataset (Figure 1 C). Similarly, the heat map showed that gene expression is well differentiated between tumor group and normal group (Figure 1 D).

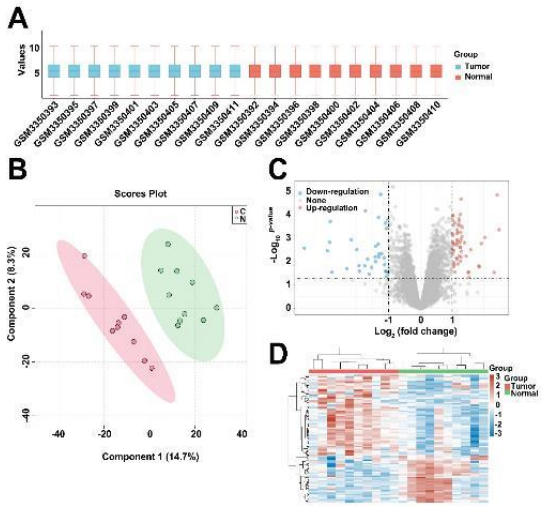


**Figure 1:** Differential gene expression between gastric cancer tissue and normal tissue based on GEO data. (A): Batch correction analysis of DEGs in GSE118897, including 10 tumor groups and 10 normal groups; (B): PLSDA analysis of tumor/non-tumor group was measured using metaboanalyst platform. Red dot: cancer, green dot, normal. (C): volcano map was used to visualize the differential genes expression between gastric cancer group and normal group. (Red: up-regulation DEGs, grey: no difference, blue: down-regulation DEGs; (D): Heatmap data showed the significantly differential expression of between tumor group and normal group

### Up-regulated differential gene enrichment analysis

The up-regulated differential genes with fold change ≥ 2 were selected for enrichment analysis. As shown in Figures 2 A - C, differential genes were mainly involved in chromosome segregation, regulation of protein serine/threonine kinase activity, chemokine-mediated signaling pathway, ERK1 and ERK2 cascade, negative regulation of phosphate metabolic process, epithelial cell proliferation, G0, and early G1 phase.

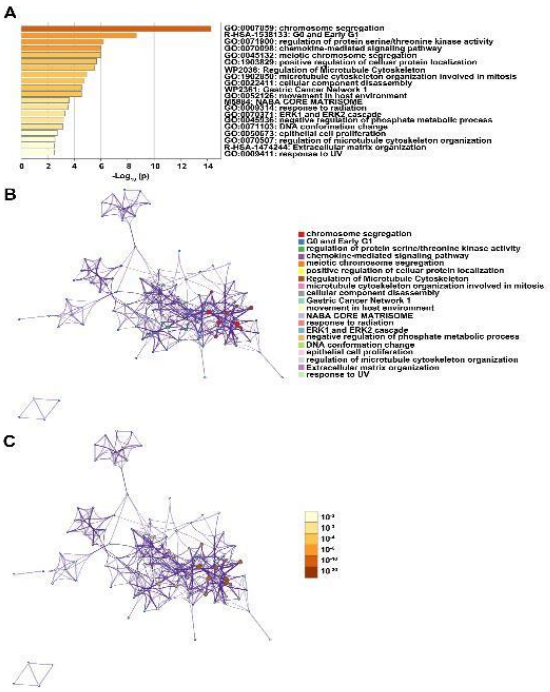


**Figure 2:** Enrichment analysis of up-regulated differential gene. (A): Metascape was used to analyze functional enrichment. Bar graph of enriched terms across input gene lists. (B&C): Network of enriched terms: (B): colored with respect to cluster ID, where nodes that share the same cluster ID are typically close to each other; (C): colored by p-value, where terms containing more genes tend to have a more significant *p*-value

### RAD54L was highly expressed in gastric cancer, and its high expression was associated with poor prognosis

In addition, 48 upregulated DEGs were analyzed by STRING online platform, and most of these genes interacted with RAD54L, which is a pivotal gene in the PPI network (Figure 3 A). The expression of RAD54L in gastric cancer tissues was then analyzed by western blotting. As shown in Figure 3 B, analysis of the Ualcan and GEPIA datasets revealed that RAD54L is highly expressed in tissues from gastric cancer patients

compared to normal tissues. Moreover, RAD54L also showed up-regulated expression in the tumor group compared to the normal group in GSE118897 (Figure 3 B). In addition, we also measured the survival rate of gastric cancer patients. As shown in Figure 3 C, patients with low RAD54L expression (n = 654) performed well compared with the RAD54L high expression group (n = 221), and the pre-progression survival and post-progression survival rates of gastric cancer patients with high RAD54L expression were similar to the overall survival rates. Collectively, these data suggest that RAD54 expression is increased in gastric cancer and positively correlates with poor survival in GC.

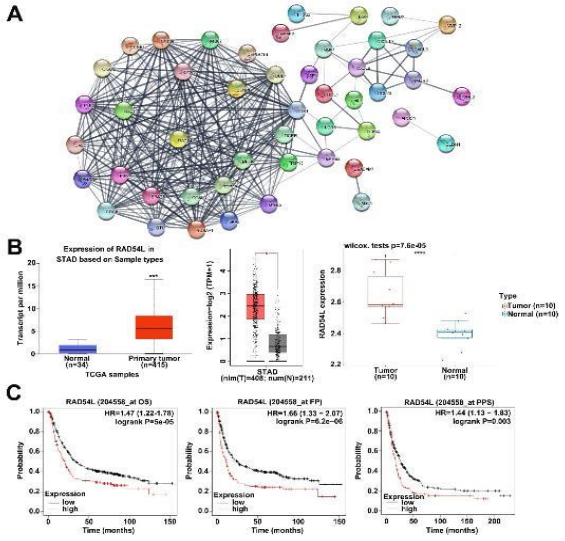


**Figure 3:** RAD54L was highly expressed in gastric cancer, and its high expression was associated with poor prognosis. (A): STRING analysis of protein-protein interaction network of RAD54L and related upregulated DEGs; (B): Analysis of RAD54L expression in colorectal cancer using Timer, ualcan, and GEPIA online platforms, respectively; (C): Correlation between RAD54L expression and patient survival measured by Kaplan-Meier plots. (OS: overall survival. FP: first progression, PPS: post progression survival)

## RAD54L enhanced gastric cancer cell proliferation and inhibited apoptosis

Based on the analysis of bioinformatics-related data, IFI6 showed a high expression in GC tissues, and correlated with poor survival. First, the expressions of RAD54L in human gastric mucosa cells Ges-1 and GC cell lines: NCI-N87, HGC-27, AGS, SNU-1, and HS-746T were measured. As shown in Figures 4 A and 4 B, RAD54L was upregulated expression in GC cells NCI-N87, HGC-27, AGS, SNU-1, and HS-746T compared with normal gastric mucosa Ges-1 cells. In order to clarify the critical role of RAD54L in cell level, AGS and NCI-N87 cells were treated with the indicated plasmids (Figure 4 C). As shown in Figure 4 D, knockdown of RAD54L reduced cell proliferation in AGS and NCI-N87 cells when compared to the sh-control cells. Moreover, overexpression of RAD54L facilitated cell proliferation in AGS and NCI-N87 cells compared with the control cells. As shown in Figure 4 E, RAD54L increased the expression of inhibited cell apoptosis in AGS and NCI-N87 cells, while RAD54L deficiency induced apoptosis in the cell lines. In addition, cell cycle progression was inhibited by RAD54L in AGS and NCI-N87 cells, and overexpression of RAD54L promoted cell cycle in these cell lines (Figure 4 F). These data suggest that RAD54L positively regulated cell proliferation and cell cycle in GC cells.

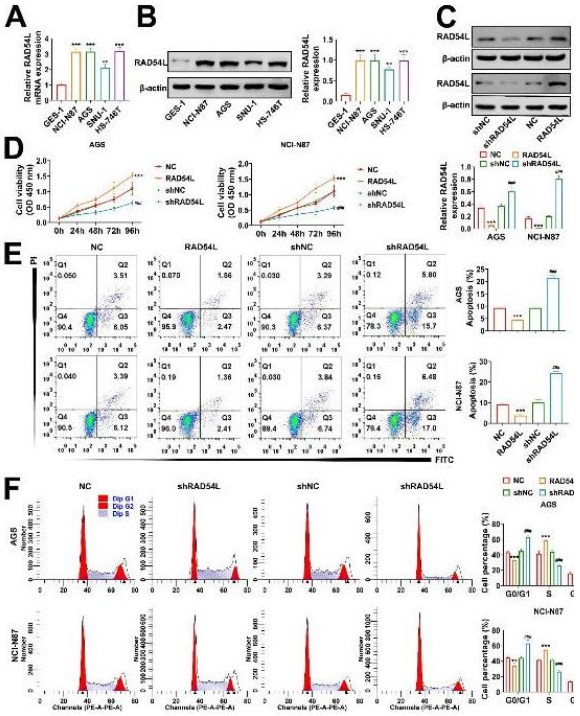


**Figure 4:** RAD54L promotes gastric cancer cell proliferation and inhibited apoptosis (A&B): The expressions of RAD54L in human gastric mucosa cells Ges-1 and GC cell lines: NCI-N87, HGC-27, AGS, SNU-1, HS-746T were measured using RT-qPCR and western blotting; (C): NCI-N87 and AGS cells were transfected with shNC, shRAD54L, mock, and RAD54L plasmids, the expression of RAD54L in the cell lines was analyzed by western blotting; (D): NCI-N87 and AGS cells were transfected with the indicated plasmids, and cell proliferation of these cell lines was measured by CCK8 assay; (E&F): Flow cytometry was used to measured cell apoptosis and cell cycle in the cell lines. Data are mean ± SD, n =3). ∗∗∗*P* < 0.001. ###*p* < 0.001. ∗represented RAD54L overexpression group compared to negative control group, # represented RAD54L knockdown group compared to related control group

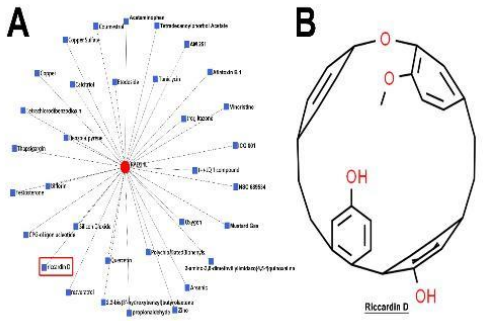


**Figure 5:** Prediction of potential therapeutic agents targeting RAD54L. (A): Prediction of potential small molecule compounds as RAD54L targets by network analyst analysis platform (https://www.networkanalyst.ca/); (B): Structure of riccardin D

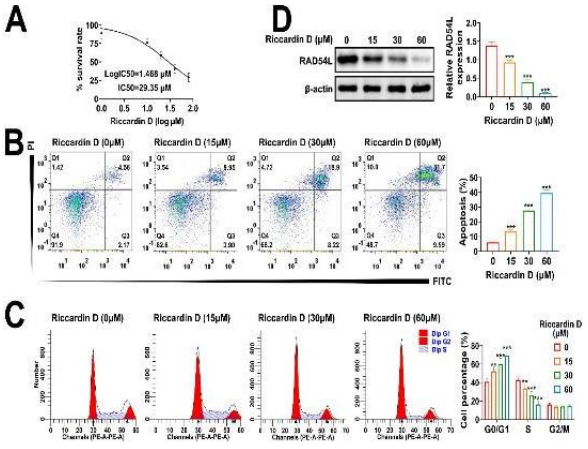


**Figure 6:** Riccardin D inhibited gastric cancer cell viability. (A): CCK8 assay was used to measure the survival rate of NCI-N87 cells with riccardin D treatment; (B&C): Cell apoptosis and cell cycle of NCI-N87 cells with riccardin D treatment were analyzed by flow cytometry; (D): Western blotting was used to measure the expression of RAD54L in NCI-N87 cells with riccardin D treatment. $***P < 0.001$. * represented the experimental group with riccardin D treatment compared to the control group without riccardin D treatment.

### Riccardin D enhanced gastric cancer cell apoptosis and inhibited cell growth by targeting RAD54L

Using an online platform (networkanalyst) to select the compounds targeting RAD54L, riccardin D was found to be a candidate targeting RAD54L (Figure 5 A and B). Then, the $IC_{50}$ of riccardin D in NCI-N87 cells was measured using CCK8 assay. As shown in Figure 6 A, the $IC_{50}$ of riccardin was 29.35 μM. Furthermore, cell apoptosis increased with increasing concentration by riccardin D treatment in NCI-N87 cells (Figure 6 B). Cell cycle was inhibited by riccardin D treatment in NCI-N87 cells (Figure 6 C), and cell cycle progression was arrested in the G0/G1 and S phase. The expression of RAD54L was also inhibited by riccardin D treatment, and a higher concentration of riccardin D led to a lower RAD54L expression in NCI-N87 cell lines (Figure 6 D).

## DISCUSSION

Gastric cancer, is one of the most common digestive tract malignancies, ranks fifth in incidence and third in mortality among all cancers, and accounts for 5.7 and 8.2 % respectively, of the incidence and mortality of various tumors [13]. Gastric cancer has a poor prognosis, low survival rate, and drug resistance due to its complexity, histopathological diversity, and clinical features. In recent decades, increasing number of studies have reported that the occurrence and development of gastric cancer is related to various signaling pathways. However, the molecular mechanism related to the progression and development of gastric cancer remains unravelled.

In recent, many studies reported that RAD54L plays an important role in different types of cancer, including lung cancer, bladder cancer, prostate cancer, glioblastoma, pancreatic cancer, leukemia and ovarian cancer. Previous studies have demonstrated from GEO data analysis that RAD54L expression is an upregulated in gastric cancer [8]. In this study, RAD54L was highly expressed in gastric cancer when compared to normal matched specimens. Moreover, recent studies have also demonstrated that the high expression of RAD54L is associated with poor survival rate of gastric cancer patients [8]. In the present study, RAD54L exerted a positive association with the survival rate of patients with gastric cancer. Furthermore, riccardin D as a prediction drug targeting RAD54L, inhibited cell cycle progression and promoted cell apoptosis dose-dependently.

Moreover, previous studies also demonstrated that RAD54L functions as an oncogene in the development and progression of choroid plexus carcinoma [14]. RAD54L facilitated cell proliferation in choroid plexus. cell division cycle 7-related protein kinase, also known as CDC7, exerts a critical role in tumor cells, including the promotion of cell proliferation. Li *et al* have reported that the expression of RAD54L is regulated by CDC7 through the regulation of its promoter activity. Therefore, RAD54L also plays an important role in the regulation of cell proliferation [6]. Apart from this, Bai *et al* demonstrated that RAD54L is regulated by

CHEK1 with a similar signal in the regulation of cell proliferation in glioblastoma [7]. In this study, RAD54L deficiency inhibits the cell proliferation and overexpression of RAD54L, but increases cell proliferation in gastric cancer. However, the underlying regulatory mechanism of RAD54L in cell proliferation needs further investigation.

Fischer *et al* reported that RAD54L is a cell cycle related gene, which is expected to be mediated through p53/p21/DREAM/CDE/CHR signaling pathway [15]. Furthermore, previous studies have reported that RAD54L is highly expressed in the G1/S or S phase [16]. Here, overexpression of RAD54L induces cell cycle progression from G1/S to S phase, and RAD54L deficiency reduced the percentage of S-phase cells and increased the number of Go/G1 phase cells. In addition, CDC7 also participated in the regulation of cell apoptosis by positively regulating RAD54L in glioblastoma [6].

CHEK1 also regulated cell apoptosis by mediating over the promoter activity of RAD54L in glioblastoma [7]. Similarly, the overexpression of RAD54L reduces cell apoptosis in gastric cancer cell lines, while the knockdown of RAD54L increases cell apoptosis in gastric cancer cells. Whether the function of RAD54L is regulated by CDC7 or CHEK1 or not will require further research. Moreover, the related signaling pathway of RAD54L in the regulation of cell proliferation, cell apoptosis and cell cycle requires further investigation.

## CONCLUSION

RAD54L is highly expressed in gastric cancer, and is related to the poor survival rate in GC. It also positively regulates cell growth and cell cycle, but negatively regulates cell apoptosis. Riccardin D negatively regulates cell growth and cell cycle, while positively regulating cell apoptosis dose-dependently. Thus, riccardin D RAD54L is a potential candidate drug for the treatment of gastric cancer.

## DECLARATIONS

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### *Funding*

None provided.

### *Ethical approval*

None provided.

### *Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### *Conflict of Interest*

No conflict of interest associated with this work.

### *Contribution of Authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Qi Pan designed the experiments, and Weimin Zhu carried them out. Pei Xiang analyzed and interpreted the data, and prepared the manuscript with contributions from all co-authors.



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