Prognosis of gastric adenocarcinoma associated with girdin, Akt, and cortactin

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BACKGROUND: The actin-binding protein girdin regulates tumor cell migration and invasion by maintaining actin structure. PI3K/Akt signaling is an important actin-remodeling pathway. The protein cortactin acts directly on microfilaments and promotes tumor invasion and metastasis by rearranging the cytoskeleton. However, there are few reports on the co-expression of girdin, Akt, and cortactin in gastric adenocarcinoma (GAC).

OBJECTIVES: Evaluate girdin, Akt, and cortactin expression in GAC tissues and assess their relationship to the prognosis of GAC patients.

DESIGN: Survival analysis

SETTING: Medical college in China

PATIENTS AND METHODS: We compared survival in 110 paraffin-preserved GAC with corresponding normal gastric mucosa tissues in relationship to girdin, Akt, and cortactin expression levels.

MAIN OUTCOME MEASURE: Expression levels of the proteins.

SAMPLE SIZE: 110

RESULTS: The expression of girdin, Akt, and cortactin were all upregulated in GAC tissues compared with corresponding normal tissues (66.4% vs 36.3%, 57.3% vs 28.2% and 69.1% vs 22.7%, respectively; *P*<.05) and expression was mutually positive (all *P*<.05). Overall survival in the girdin, Akt, and cortactin high expression groups was reduced. Multivariate analysis showed that girdin, Akt, cortactin, lymph node metastasis (LNM) and TNM stages were independent factors affecting GAC patients prognosis (*P*<.05).

CONCLUSIONS: Girdin and cortactin may promote GAC invasion and metastasis via the PI3-K/Akt signaling pathway. Girdin, Akt, and cortactin co-expression might serve as a novel molecular target for GAC therapy and improve the prognosis of patients with this disease.

LIMITATIONS: A small sample size and lack of related research on molecular mechanisms.

CONFLICT OF INTEREST: None.
Gastric cancer (GC) is one of the most common malignant tumors worldwide with the fifth and third highest incidence and mortality rates, respectively. Gastric adenocarcinoma (GAC) comprises about 95% of all histopathological types of GC. At the time of diagnosis, most patients with GAC are already in the mid- to late stages of the disease and their 5-year preoperative survival rate is <30%. However, in the earlier stages of the disease, the 5-year postoperative survival rate is >90%. Hence, early diagnosis and treatment is critical in GAC prognosis.

Girdin is an actin-binding protein also known as the “girder of actin filaments” because it recruits, binds, and regulates intracellular microfilaments. Girdin controls angiogenesis and autophagy by participating in tumor cell migration and invasion. It plays important roles in tumorigenesis and progression. Girdin is upregulated in breast, colon, cervical, and esophageal cancers, glioblastoma, and other malignant tumors.

Akt is an important mediator of the PI3K/Akt signaling pathway. It activates the signaling pathway by phosphorylation and regulates tumor cell proliferation and apoptosis. Moreover, PI3K/Akt signaling plays a key role in actin remodeling. Cortactin is a protein that directly interacts with microfilaments. It is considered a promising molecular prognostic factor in various types of cancer and is associated with cancer aggressiveness. It is also an important tumor regulatory molecule and is highly expressed in breast, bladder, head and neck squamous cell and other cancers. The aim of this study was to evaluate girdin, Akt, and cortactin expression in GAC and determine their relationships with patient pathology and prognosis.

### METHODS

**Patients and tissue specimens**

We collected a convenient sample of 110 paraffin-preserved GAC specimens from the Department of Pathology of the First Affiliated Hospital of Bengbu Medical College, Bengbu, Anhui Province, China. Corresponding normal gastric mucosa tissues located >5 cm from the GAC tumors were collected between January 2015 and December 2016 and designated as the control group. All cases had complete clinical data and pathological diagnosis (Table 1). Patients who had received preoperative treatment such as radiation and chemotherapy were excluded. Follow-up ranged from a minimum of 4 months to a maximum of 62 months. Patients were followed until March 2021 or

| Table 1. Characteristics of patients with gastric adenocarcinoma (n=110). |
|---------------------|---------------------|
| Gender | 87 (79.1) |
| Male | 23 (20.9) |
| Female | 38 (34.5) |
| Ages | 72 (65.5) |
| ≤60 | 59 (53.6) |
| >60 | 51 (46.4) |
| Size | 80 (72.7) |
| Lauren classification | 30 (27.3) |
| Intestinal | 25 (22.7) |
| Diffuse | 32 (29.1) |
| Location | 53 (48.2) |
| Cardiac | 15 (13.6) |
| Pylorus | 90 (81.8) |
| Gross type | 5 (4.5) |
| Invasive | 17 (15.5) |
| Ulcerative | 85 (77.3) |
| Polypoid | 8 (7.3) |
| Invasion | 9 (8.2) |
| Submucosa | 54 (49.1) |
| Muscularis | 47 (42.7) |
| Grade | 35 (31.8) |
| Well | 75 (68.2) |
| Moderate | 50 (45.5) |
| Poor | 60 (54.5) |
| LNM | 29 (26.4) |
| Yes | 81 (73.6) |
| No | 60 (54.5) |
| TNM stage | 35 (31.8) |
| I + II | 29 (26.4) |
| III + IV | 81 (73.6) |

Data are n (%).
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death. Tumors were classified by TNM Classification of Malignant Tumors (T category describes the primary tumor site and size, N category describes the regional lymph node involvement, M category describes the presence or otherwise of distant metastatic spread).

Immunohistochemistry
Tissues were fixed overnight in 10% (v/v) buffered formalin, embedded in paraffin, and sectioned to a thickness of 4 μm. The sections were heated to 65 °C for 60 minutes, de waxed with xylene, dehydrated with an alcohol gradient, subjected to antigen repair, and washed with phosphate-buffered saline (PBS) for 15 minutes. Elivision Plus immunostaining was performed according to the kit instructions. All sections were re-stained with hematoxylin and fixed with gum. Rabbit anti-human girdin polyclonal antibody (AB113890), rabbit anti-human Akt polyclonal antibody (AB8805), and rabbit anti-human cortactin monoclonal antibody (AB81208) were acquired from Abcam, Cambridge, MA, USA. Max vision and DAB color development kits were purchased from Fuzhou Maixin Biological Co., Fuzhou, China.

Immunostaining evaluation
IHC staining results were independently assessed by two experienced pathologists blinded to the treatments. Ten representative high-power field (HPF; 400×) staining regions were randomly selected per section for comprehensive interpretation and were selected based on the percentage of staining cells and the staining intensity. Fewer than 10% positively stained cells=one point; 11–50% positively stained cells=two points; 51–75% positively stained cells=three points; and >75% positively stained cells=four points. The staining intensity scores were zero for unstained tumor cells, one for pale yellow tumor cells, two for brownish yellow tumor cells, and three for tan tumor cells. The percentages of staining cell and staining intensity scores were multiplied to derive a comprehensive score (range: 0–12 points). Scores >3 indicated positive results.

Statistical analysis
Data were analyzed with SPSS v. 26.0 (IBM Corp., Armonk, NY, USA). Survival analyses of the girdin, Akt, and cortactin-positive and -negative groups were performed by the Kaplan-Meier method and log-rank tests. Comparisons among groups were performed by χ² tests. Multivariate analyses were conducted using a Cox multivariate regression model. P<.05 was considered statistically significant.

RESULTS

Correlations among girdin, Akt, and cortactin expression levels in GAC and clinicopathological parameters
Positive girdin expression was observed mainly in the cytoplasm and cell membrane (Figures 1A and 1B). The positive girdin expression rates were 66.4% (73/110) in GAC and 36.3% (40/110) in normal gastric mucosa (P<.05). IHC staining showed that positive girdin expression in GAC was positively correlated with the Lauren classification (P=.006), depth of invasion (P=.022), degree of differentiation (P=.016), lymph node metastasis (LNM) (P=.024) and TNM stage (P=.004), but not with sex, age, tumor size, or distant metastasis (Table 2). Akt expression was localized mainly to the cytoplasm (Figures 1C and 1D). The positive Akt expression rates were 57.3% (63/110) in the GAC and 28.2% (31/110) in normal gastric mucosa tissues (P<.001). Moreover, positive Akt expression in GAC was correlated with TNM stage (P=.001) and LNM (P=.037) and distant metastases (P=.018). Furthermore, there were no significant differences in Akt expression among age groups, sexes, tumor sizes, gross tumor types, tumor locations, or tumor invasion depths (all P>.05; Table 2). Positive cortactin expression was located mainly in the cytoplasm (Figures 1E and 1F). The positive cortactin expression rates were 69.1% (76/110) in the GAC tissues and 22.7% (25/110) in normal gastric mucosa tissues (P<.001). Moreover, cortactin expression in the tumor cells was strongly correlated with tumor invasion depth (P=.018), gross tumor type (P=.042), LNM (P=.006) and TNM stage (P<.001), and distant metastasis (P=.020; Table 2). A Spearman’s correlation analysis showed that girdin expression was positively correlated with both Akt (r=0.202; P<.05) and cortactin expression (r=0.357; P<.001). In addition, the correlation between Akt and cortactin expression was positive (r=0.496; P<.001) (Table 3a, 3b, 3c).

Survival analysis
Due to the loss of postoperative follow-up of five patients, survival data on only 105 patients were analyzed. The Kaplan-Meier analysis assessed OS for the positive and negative girdin, Akt, and cortactin groups in GAC. Overall survival (OS) in the positive girdin expression group (28.91 [18.07] month) was significantly lower than that for the negative girdin expression group (48.43 [14.91] month) (Figure 2A). Patients with negative Akt expression had longer OS than those with positive Akt expression (47.20 [16.14] month vs. 26.24 [16.55] month, respectively) (Figure 2B).
Table 2. Correlations between girdin, AKT and cortactin with clinicopathological characteristics of gastric adenocarcinoma.

| Variable                | Girdin | | | AKT | | | Cortactin | | |
|-------------------------|-------|---|---|-----|---|---|-------|---|---|---|
|                         | Negative | Positive | P | Negative | Positive | P | Negative | Positive | P |
| Gender                  |         |         |   |         |         |   |         |         |   |
| Male                    | 28      | 59      | .531 | 41      | 46      | .070 | 30      | 57      | .115 |
| Female                  | 9       | 14      |     | 6       | 17      |     | 4       | 19      |     |
| Ages                    |         |         |   |         |         |   |         |         |   |
| ≤60                     | 12      | 26      | .740 | 14      | 24      | .365 | 13      | 25      | .586 |
| >60                     | 25      | 47      | .640 | 33      | 39      | .281 | 21      | 51      | .466 |
| Size                    |         |         |   |         |         |   |         |         |   |
| <5.0                    | 21      | 38      | .006 | 29      | 31      | .098 | 20      | 39      | .555 |
| ≥5.0                    | 16      | 35      | .302 | 19      | 32      | .111 | 14      | 37      | .800 |
| Lauren classification   |         |         |   |         |         |   |         |         |   |
| Intestinal              | 33      | 47      |     | 38      | 42      |     | 26      | 54      |     |
| Diffuse                 | 4       | 26      |     | 9       | 21      |     | 8       | 22      |     |
| Location                |         |         |   |         |         |   |         |         |   |
| Cardiac                 | 10      | 15      |     | 9       | 16      |     | 7       | 18      |     |
| Body                    | 13      | 19      |     | 10      | 22      |     | 9       | 23      |     |
| Pylorus                 | 14      | 39      |     | 28      | 25      |     | 18      | 35      |     |
| Gross type              |         |         |   |         |         |   |         |         |   |
| Invasive                | 4       | 11      |     | 4       | 11      |     | 1       | 14      |     |
| Ulcerative              | 31      | 59      | .801 | 41      | 49      | .389 | 30      | 60      | .042 |
| Polypoid                | 2       | 3       |     | 2       | 3       |     | 3       | 2       |     |

Figure 1. Immunostaining for Girdin, AKT, and Cortactin in gastric adenocarcinoma and control tissues.
Table 2 (cont.). Correlations between girdin, AKT and cortactin with clinicopathological characteristics of gastric adenocarcinoma.

| Variable           | Girdin Negative | Girdin Positive | P    | AKT Negative | AKT Positive | P     | Cortactin Negative | Cortactin Positive | P      |
|--------------------|-----------------|-----------------|------|--------------|--------------|-------|--------------------|---------------------|--------|
| Invasion           |                 |                 |      |              |              |       |                    |                     |        |
| Submucosa          | 6               | 2               |      | 6            | 2            |       | 6                  | 2                   | .018   |
| Muscularis         | 7               | 10              | .022 | 8            | 9            | .131  | 4                  | 13                  |        |
| Subserosa          | 24              | 61              |      | 33           | 52           |       | 24                 | 61                  |        |
| Grade              |                 |                 |      |              |              |       |                    |                     |        |
| Well               | 6               | 3               |      | 6            | 3            |       | 4                  | 5                   | .603   |
| Moderate           | 21              | 33              | .016 | 21           | 33           | .296  | 17                 | 37                  |        |
| Poor               | 10              | 37              |      | 19           | 27           |       | 13                 | 34                  |        |
| LNM                |                 |                 |      |              |              |       |                    |                     |        |
| Yes                | 20              | 55              | .024 | 27           | 48           | .037  | 17                 | 58                  | .006   |
| No                 | 17              | 18              |      | 20           | 15           |       | 17                 | 18                  |        |
| TNM stage          |                 |                 |      |              |              |       |                    |                     |        |
| I + II             | 24              | 26              | .004 | 30           | 20           | .001  | 27                 | 23                  | <.001  |
| III + IV           | 13              | 47              |      | 17           | 43           |       | 7                  | 53                  |        |
| Distant metastasis |                 |                 |      |              |              |       |                    |                     |        |
| Yes                | 7               | 22              | .207 | 7            | 22           | .018  | 4                  | 25                  |        |
| No                 | 30              | 51              |      | 40           | 41           |       | 30                 | 51                  | .020   |

2B). The OS of the negative cortactin expression group (52.56 [10.79] month) was longer than that of the positive cortactin expression group (27.21 [17.05] month) (Figure 2C). Patients in TNM stage III + IV had shorter OS than those in TNM stage I + II (P<.001; Figure 2D). The OS of patients with LNM was significantly lower than that of patients without LNM (P<.05; Figure 2E). Distant metastasis (P<.05; Figure 2F) and depth of invasion (P<.001; Figure 2G) were closely correlated with shorter OS. In addition, patients with diffuse GAC had shorter OS than those with intestinal GAC (P<.05; Figure 2H) (Table 4).

Cox multivariate analysis
A Cox multivariate analysis was performed on age, sex, tumor size, Lauren classification, tumor location, gross tumor type, depth of invasion, degree of differentiation, TNM, LNM, and girdin, Akt, and cortactin expression in GAC patients. Positive girdin, Akt, and cortactin expression and LNM and TNM stages were independent factors affecting patients prognosis (P<.05) (Table 5).

Table 2a. Correlation between contactin (blue) vs girdin (red).

|                  | Negative | Positive |
|------------------|----------|----------|
| Negative         | 20       | 14       |
| Positive         | 17       | 59       |

r=0.357, P<.001

Table 2b. Correlation between girdin (blue) vs AKT (red).

|                  | Negative | Positive |
|------------------|----------|----------|
| Negative         | 21       | 16       |
| Positive         | 26       | 47       |

r=0.202, P=.034

Table 2c. Correlation between AKT (blue) vs contactin (red).

|                  | Negative | Positive |
|------------------|----------|----------|
| Negative         | 27       | 20       |
| Positive         | 7        | 56       |

r=0.496, P<.001
Figure 2A. Kaplan-Meier analysis curve of the survival rate of patients with gastric adenocarcinoma. The y-axis means the percentage of patients; the x-axis means their survival in months. A: Overall survival analysis of all patients in relation to Girdin (log-rank=24.997, $P<.001$)

Figure 2B. Overall survival analysis of all patients in relation to AKT expression (log-rank=26.113, $P<.001$)

Figure 2C. Overall survival analysis of all patients in relation to Cortactin expression (log-rank=24.997, $P<.001$)

Figure 2D. Overall survival analysis of all patients in relation to TNM stages (log-rank=33.317, $P<.001$).

Figure 2E. Overall survival analysis of all patients in relation to lymph node metastasis (LNM) stages (log-rank=12.847, $P<.001$)

Figure 2F. Overall survival analysis of all patients in relation to tumor Distant metastasis (log-rank=21.164, $P<.001$).
DISCUSSION

Girdin is an actin-binding protein first identified by Japanese researchers in 2005. Girdin is also called Akt-phosphorylation enhancer (APE) and is a macromolecular protein composed of 1870 amino acid residues. Akt-phosphorylation enhancer (APE), Gα-interacting vesicle-associated protein (GIV), and hook-related protein 1 (HKRP1) are members of the girdin, DAPLE (dishevelled-associating protein with a high frequency of leucine residues), and HKRP3 families, respectively.\textsuperscript{16-19} Girdin regulates cancer cell migration by controlling cell adhesion and cytoskeletal tissue.\textsuperscript{19} Several girdin-mediated signaling pathways such as PI3K/Akt participate in tumor genesis and development.\textsuperscript{20} Wang et al found that girdin regulates pancreatic cancer (PC) cell proliferation and apoptosis.

Table 4. Univariate analyses of overall survival (OS) time.

| Variable                  | n   | Mean OS (months) | Log-Rank | P value |
|---------------------------|-----|------------------|----------|---------|
| Girdin                    |     |                  |          |         |
| Negative                  | 35  | 48.43 (14.91)    | 24.997   | <.001   |
| Positive                  | 70  | 28.91 (18.07)    |          |         |
| AKT                       |     |                  |          |         |
| Negative                  | 46  | 47.20 (16.14)    | 26.113   | <.001   |
| Positive                  | 59  | 26.24 (16.55)    |          |         |
| Cortactin                 |     |                  |          |         |
| Negative                  | 34  | 52.56 (10.79)    | 24.997   | <.001   |
| Positive                  | 71  | 27.21 (17.05)    |          |         |
| Gender                    |     |                  |          |         |
| Male                      | 83  | 36.01 (20.03)    | 0.932    | .334    |
| Female                    | 22  | 33.18 (16.80)    |          |         |
| Ages                       |     |                  |          |         |
| ≤60                       | 36  | 35.08 (20.22)    | 0.172    | .678    |
| >60                       | 69  | 35.59 (19.04)    |          |         |
| Size                       |     |                  |          |         |
| <5.0                      | 56  | 38.52 (19.66)    | 3.419    | .064    |
| ≥5.0                      | 49  | 31.88 (18.57)    |          |         |
| Lauren classification      |     |                  |          |         |
| Intestinal                | 76  | 38.34 (19.83)    | 8.336    | .004    |
| Diffuse                   | 29  | 27.76 (15.94)    |          |         |
| Location                  |     |                  |          |         |
| Cardiac                   | 24  | 34.42 (19.90)    |          |         |
| Boby                      | 30  | 32.40 (18.39)    | 1.394    | .498    |
| Pylorus                   | 51  | 37.67 (19.75)    |          |         |
| Gross type                |     |                  |          |         |
| Invasive                  | 15  | 27.73 (14.47)    |          |         |
| Ulcerative                | 85  | 36.51 (20.10)    | 3.316    | .190    |
| Polypoid                  | 5   | 40.00 (15.86)    |          |         |
| Invasion                  |     |                  |          |         |
| Submucosa                 | 8   | 57.37 (6.28)     |          |         |
| Muscularis                | 17  | 39.53 (19.91)    | 9.568    | .008    |
| Subserosa                 | 80  | 32.35 (18.64)    |          |         |
| Grade                     |     |                  |          |         |
| Well                      | 9   | 44.00 (22.78)    |          |         |
| Moderate                  | 50  | 37.38 (19.94)    | 3.154    | .207    |
| Poor                      | 46  | 31.61 (17.52)    |          |         |
increase with positive girdin expression. Positive girdin expression was greater in GAC patients with LNM than in those without it, and the difference was statistically significant. A Kaplan-Meier survival analysis showed that patients with positive girdin expression had shorter OS than those with negative girdin expression. Hence, abnormal increases in girdin expression are associated with disease progression, tumor aggressiveness, and prognosis in patients with GAC. Akt is a serine/threonine protein kinase. As it has high homology with PKA and PKC, it is also referred to as protein kinase B. Akt is a major downstream effector molecule of the PI3K/Akt signal transduction pathway and regulates cell apoptosis/survival. In cell proliferation and differentiation, activated Akt accelerates cell cycle transformation and promotes tumor occurrence and development. In normal physiological cell function, excessive cell proliferation is controlled by apoptosis. Thus, blocking PI3K/Akt signaling can induce apoptosis, interfere with breast cancer cell activity, and inhibit the proliferation of tumor cell. PI3K/Akt signaling is closely related to colorectal cancer cell proliferation, invasion, apoptosis, and the cell cycle. PI3K/Akt signaling participates in tumor cell migration via several regulatory pathways and girdin phosphorylation is mediated by Akt. In the present study, we used IHC methods to detect Akt protein expression in GAC and its adjacent normal tissues. Akt was upregulated in GAC tissues (positive rate=57.3%). In contrast, the positive Akt rate was 28.2% in adjacent normal tissues. Akt expression intensity was significantly associated with tumor invasion depth, lymph node stage, distant metastasis, and TNM stage (P<.05). The OS rate was significantly higher in GAC patients with low Akt expression than in those with high Akt expression. A pairwise correlation analysis showed a significant positive correlation between girdin and Akt. Therefore, girdin and Akt play important roles in tumor occurrence and development and collaborate in tumor invasion and metastasis. For these reasons,
girdin and Akt share close upstream and downstream relationships. Moreover, girdin and Akt could serve as molecular markers of malignant tumor prognosis.

Cortactin or cortical actin was first identified in 1993. It is a regulatory protein in the microfilament cytoskeleton that aggregates in the subcellular cortex, binds actin filaments, and regulates microfilament aggregation and cytoskeleton rearrangement in cellular cortical regions. Cortactin undergoes tyrosine and serine/threonine phosphorylation, promotes the maturation of invasive tumor cells pseudopodia, participates in extracellular matrix degradation, and enhances tumor cell invasion and metastasis. Wu et al demonstrated through mutation studies that Akt is vital to cortactin phosphorylation during PI3K-enhanced cell migration and invasion. They speculated that Akt is a potential pathway activator or therapeutic target. Studies have shown Akt promotes pancreatic cancer development by regulating cortactin acetylation and phosphorylation. Meran et al reported that *Helicobacter pylori* infection is a major risk factor in GC development. *H. pylori* manipulates actin-cytoskeletal rearrangement and cell motility by inhibiting cortactin phosphorylation and changing the molecular interactions and activities of various important proteins. In the present study, the positive cortactin expression rates were 22.7% and 69.1% in normal gastric mucosal and GAC tissues, respectively, and the difference was statistically significant. Furthermore, pTNM stage, differentiation, LNM risk, and distant metastasis all increased with positive cortactin expression. There were no significant differences in cortactin expression among age groups, sexes, tumor sizes and locations. We speculate that the expression of cortactin is not related to the above factors. Of course, due to the small sample size of the current experiment, the possibility of type II error cannot be ruled out. A survival analysis showed that the OS in the cortactin-positive group was significantly shorter than that in the cortactin-negative group. Therefore, cortactin upregulation may be closely associated with GAC development, metastasis, and prognosis. Cortactin and Akt were positively correlated and could, therefore, provide guidance for the clinical diagnosis of malignant tumor progression, invasion, and metastasis as well as cancer prognosis.

In conclusion, we showed that abnormal expression levels of girdin, Akt, and cortactin were associated with decreased overall survival in patients with GAC. Both girdin and cortactin were positively correlated with Akt. Hence, we speculated that girdin and cortactin may influence GAC infiltration, metastasis, and prognosis by acting on Akt. The latter is an important downstream factor in PI3K/Akt signaling. Thus, we propose that girdin, Akt, and cortactin are potentially valuable GAC biomarkers.
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