High expression of transforming acidic coiled coiled-containing protein 3 strongly correlates with aggressive characteristics and poor prognosis of gastric cancer

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Abstract. Transforming acidic coiled coiled-containing protein 3 (TACC3) is well understood to regulate mitotic spindle dynamics and centrosome integrity during mitosis. TACC3 has been suggested to be deregulated in a variety of human malignancies and may be involved in the process of cancer progression. The aim of the present study was to determine the status of TACC3 expression in gastric cancer (GC) and to clarify its clinical/prognostic significance. In the present study, we applied quantitative PCR (qPCR) and western blotting to examine TACC3 mRNA/protein expression in paired GC tissues and matched adjacent non-malignant tissues. Immunohistochemistry (IHC) was performed on a large cohort of 186 postoperative GC samples. Chi-square test, Kaplan-Meier analysis and Cox regression modelling were used to analyse the data. Upregulated mRNA and protein expression levels of TACC3 were observed in the majority of the GC tissues based on qPCR and western blotting compared to the adjacent non-cancerous gastric tissues. Specific IHC staining for TACC3 was predominantly identified in the cytoplasm of the cancer cells. A high expression of TACC3 was detected in 102 of the 186 (54.8%) tissue samples and was significantly associated with the extracapsular extension of the tumour (P<0.001), tumour relapse (P<0.001) and shortened overall survival in GC (P<0.001). Further analysis demonstrated that the TACC3 expression level stratified the patient outcome in stage II (P=0.040), stage III (P<0.001), T3/4 (P<0.001), N positive (P<0.001) and poorly differentiated/undifferentiated tumour subgroups (P<0.001). The Cox regression analysis suggested that a high expression of TACC3 was an independent prognostic factor for GC patients. The measurement of TACC3 protein expression may be beneficial for predicting clinical outcomes for GC patients.

Introduction

Although the morbidity and mortality of gastric cancer (GC) have been on the decrease over the past few decades, GC remains a major public health issue worldwide. The prognosis of GC remains poor, with a 5-year survival rate of <30% (1). Approximately 70% of new cases occur in East Asia, including a higher rate of locoregionally advanced disease and a younger age at diagnosis (2). The standard of treatment for GC has been surgery and multimodal treatment (3). However, traditional methods used may not precisely predict the prognosis of GC patients after surgical excision. Thus, there is a need for novel biomarkers that distinguish between patients experiencing different clinical outcomes, facilitating the individualisation of treatments for GC patients.

Centrosomes are crucial microtubule organising centres in animal cells (4). The physiological function of centrosomes is responsible for organising the poles of the mitotic spindle and for equally segregating chromosomes into two daughter cells during mitosis. Previous findings have shown that centrosomal abnormalities may lead to aneuploidy and genomic instability, further contributing to neoplastic transformation and
tumourigenesis (5,6). Moreover, a previous study suggested that chromosomal aneuploidy is associated with the formation and progression of GC (7).

Members of the transforming acidic coiled-coil (TACC) family of proteins are centrosome- and microtubule-binding proteins that play an important role in the regulation of centrosome integrity, spindle stability and microtubule assembly during cell division (8-14). TACC3, the third member in the TACC family, contains a highly conserved C-terminal coiled coil domain that interacts with microtubules (15). TACC3 is expressed in certain human proliferative tissues, such as testis, bone marrow, lung, spleen, thymus and peripheral blood leukocytes (10). Regarding its function in mitosis, TACC3 was suggested to be involved in the process of tumourigenesis in various types of cancer through the regulation of the transcriptional activities and the proliferative properties of tumour cells (16). TACC3 was found to be upregulated in multiple malignancies, including ovarian and invasive breast cancer (17,18). It has been suggested that a high expression of TACC3 is a prognostic indicator of poor clinical outcome in non-small cell lung cancer (NSCLC) (19). The elevated expression of TACC3 was associated with tumour metastasis and chemoresistance in cervical cancer (20). Moreover, TACC3 induced EGF-mediated epithelial mesenchymal transition (EMT) by activating the PI3K/Akt and ERK signalling pathways in cervical cancer (21,22). Alternatively, the depletion of TACC3 induced tumour regression in vivo (23). From a gene mapping perspective, TACC3 is located on chromosome 4p16.3, which is vulnerable to genetic variation, leading to an increased risk for bladder cancer, multiple myeloma and ovarian adenocarcinoma (24-26). Furthermore, TACC3 is closely mapped to the fibroblast growth factor receptor 3 (FGFR3) gene, and this region is susceptible to forming an FGFR3-TACC3 fusion in a subset of glioblastoma multiforme (27). This fusion gene escapes microRNA regulation via 3'-UTR depletion and encodes an oncogenic protein that induces mitotic and chromosomal segregation defects, possibly leading to aneuploidy in glioblastoma (28). Furthermore, cells exhibiting high levels of TACC3 expression exhibited defective cell cycle checkpoints and repair systems, leading to genomic instability (29). These results indicated that TACC3 may be an important factor that contributes to tumour development and progression, providing a novel potential target for various cancer therapies.

Although previous studies have investigated TACC3 expression in many human cancers, little is known regarding its role in GC. In the present study, we examined the expression pattern of TACC3 in human GC samples. The correlation between TACC3 expression and the clinicopathological characteristics and prognosis of GC patients was evaluated.

Materials and methods

Patients and tissue specimens. This retrospective study was conducted on a cohort of 186 GC patients receiving surgical resection of the primary tumour at the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, between January 2007 and May 2009. Subjects with R1/R2 margins, with distant metastasis and who died during the perioperative period were excluded from the present study. Age, gender, intraoperative examination, tumour location, WHO classification, histological differentiation, pathologic TNM (pTNM) staging classification of the stomach [according to the American Joint Committee on Cancer (AJCC), 7th edition, 2010], extracapsular extension and tumour emboli in the vasculature were assessed by reviewing the medical records, the pathological records and glass slides. The clinical data for these samples are described in detail in Table 1. None of the patients received preoperative chemotherapy or radiotherapy. The patients with advanced disease received postoperative chemotherapy (5-FU alone, 5-FU and platinum or paclitaxel and platinum) with or without radiotherapy. Follow-up assessments were performed every 3 months postoperatively for the first 2 years, followed by every 6 months until the patient succumbed. The survival status of the patients was ascertained by April, 2014. Moreover, 30 paired GC and adjacent macroscopically normal gastric mucosal tissues were collected from the patients using the same criteria at the same hospital between October, 2013 and March, 2014. The tissue samples were rapidly frozen following collection and were stored in a -80°C freezer until use for quantitative polymerase chain reaction (qPCR) and western blotting. Written informed consent was obtained from all the patients. The present study was approved by the Ethics Committee of Sun Yat-sen University, Guangzhou, China.

Western blotting. Eight pairs of GC tissues and matched adjacent non-malignant tissues were lysed in sample buffer containing complete protease inhibitors and PhosSTOP phosphatase inhibitor. The protein levels were quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). Then, 40 µg of tissue protein lysates were mixed with loading buffer containing complete protease inhibitors and PhosSTOP phosphatase inhibitor. The protein lysates were then separated via 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to an Immuno-Blot PVDF membrane (Pall Corp., Port Washington, NY, USA). The membrane was blocked with 5% skim milk for 1 h. Primary rabbit monoclonal antibodies against TACC3 (Abcam, Cambridge, MA, USA) and α-tubulin (Proteintech Group, Chicago, IL, USA) were then applied at dilutions of 1:1,000 and 1:3,000, respectively.

RNA extraction and real-time fluorescent quantitative PCR. Total RNA from the surgically obtained paired tissues (n=30) was extracted using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA was pretreated with DNase, and 2-µg aliquots were used for cDNA synthesis using random hexamers. The primers used to amplify TACC3 were: 5'-CTCTTTCAAGCGTTTGTGAGAAAC-3' (sense) and 5'-GCCCTCCTGGGTGATCTCTT-3' (antisense). β-actin was amplified as an internal control using the primers: 5'-CGGGAG AAGAGCAGCCAGATG-3' (sense) and 5'-GGGCATACCCCT CGTAGATG-3' (antisense). The amplification procedure was performed as follows: initial step at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The instrumental software (SDS 2.0) was used to calculate the relative quantity of the amplified samples. Relative quantification was used to analyze TACC3 gene expression using the 2^ΔΔCt method.

Immunohistochemistry (IHC). IHC analysis was performed to examine the TACC3 expression levels in 186 GC tissue specimens. The paraffin-embedded GC specimens were sliced into 4-µm sections and placed in an oven at 65°C for...
2 h. The sections were deparaffinised in xylene and rehydrated using an ethanol alcohol gradient followed by distilled water. Subsequently, the sections were immersed in 3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity. The sections were boiled in citrate antigen retrieval solution (pH 6.5) for 2.5 min in a high-pressure cooker for antigen retrieval. When the temperature of the antigen retrieval solution returned to room temperature, the sections were incubated in a primary antibody against TACC3 (1:800 dilution; Abcam, Cambridge, MA, USA) at 4˚C overnight. As a negative control, the primary antibody was replaced with normal rabbit IgG at 4˚C overnight while maintaining all the other treatment conditions. After washing three times for 5 min in 0.1% phosphate-buffered saline containing Tween-20 (PBST), the tissue sections were treated with a secondary antibody at 37°C for 30 min. After three further washes in PBST, the tissue sections were stained with 3,3-diaminobenzidine (DAB) for 10 min, counterstained with 10% Mayer's haematoxylin, dehydrated and mounted. Negative controls were performed concurrently by replacing the primary antibody with PBST. Positive control tissue was used as per the manufacturer's recommendation.

**IHC analysis.** The degree of immunostaining of the sections was scored by two independent pathologists who were blinded to the clinical parameters. For TACC3, only cytoplasmic staining was scored, while negligible nuclear or membrane staining was observed. Cytoplasmic immunoreactivity was scored in a semi-quantitative manner by calculating the ratio of positively stained tumour cells to the total tumour cells in 5% increments at a range of 0-100 (P0, P1-3). A combined score was derived from the product of the percentage of positively stained tumour cells and the staining intensity. The staining intensity was classified as 0 (no staining), 1+ (weak), 2+ (moderate) or 3+ (strong) (I0, I1-3). Each IHC score was

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### Table I. Correlation of the TACC3 IHC score with the clinicopathological parameters.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All patients</th>
<th>Low expression</th>
<th>High expression</th>
<th>P-value</th>
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<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
<td></td>
</tr>
<tr>
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<td>Female</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>&lt;65</td>
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<td>60 (50.4)</td>
<td>59 (49.6)</td>
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<tr>
<td>≥65</td>
<td>67 (36.0)</td>
<td>24 (35.8)</td>
<td>43 (64.2)</td>
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<tr>
<td>T1+T2</td>
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<td>26 (51.0)</td>
<td>25 (49.0)</td>
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<tr>
<td>T3+T4</td>
<td>135 (72.6)</td>
<td>58 (43.0)</td>
<td>77 (57.0)</td>
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<tr>
<td>Nodal invasion (N)</td>
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<tr>
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<td>24 (44.4)</td>
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<tr>
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<td>24 (27.6)</td>
<td>63 (72.4)</td>
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<td>61 (56.5)</td>
<td>47 (43.5)</td>
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<td>78 (41.9)</td>
<td>23 (29.5)</td>
<td>55 (70.5)</td>
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*Significant difference. IHC, immunohistochemistry.
calculated based on the staining intensity and percentage scores according to the equation: IHC score = I1 x P1 + I2 x P2 + I3 x P3 (ranging from 0 to 300).

Selection of the threshold IHC score for a high TACC3 expression. The threshold IHC score for a high expression of TACC3 was determined using the receiver operating characteristic (ROC) curve analysis. A final IHC score for each tumour was derived by averaging the replicated scores. The sensitivity and specificity for discriminating between death and survival in this study was plotted, followed by the generation of a ROC curve. The IHC score that was closest to the point of maximal sensitivity and specificity was selected as the threshold value (30).

Statistical analysis. Statistical analyses were performed using SPSS v19.0 (IBM) and Prism software (GraphPad). P<0.05 was considered to be significant. The Chi-square test or Fisher's exact test was used to evaluate the correlation between TACC3 expression and the clinicopathological variables. Disease-free survival (DFS) was defined as the time from the initial treatment to cancer progression, metastasis, or death. Overall survival (OS) was defined as the time from the initial treatment to death. Estimates of the cumulative survival distributions were calculated using the Kaplan-Meier method, and differences between the groups were compared using the log-rank test. The significance of various clinicopathological characteristics was assessed using a univariate Cox regression model and multivariate Cox proportional hazard regression analysis followed by the log-rank test. The variables that were found to be significant based on the univariate analysis were selected for inclusion in the multivariate Cox model.

Results

Clinicopathological characteristics of the GC patients. The clinicopathological characteristics of the 186 GC patients in this study are shown in Table I. The median follow-up duration was 62.3 (range, 1.2-87.2) months. The patients underwent total or subtotal gastrectomy with or without lymph node dissection, depending on the tumour location. A total of 112 males and 74 females were included in the present study, with a mean age of 59.2±12.5 years. At the time of surgery, the percentage of patients classified as T1, T2, T3 and T4 was 16.7, 10.8, 2.2 and 70.3%, respectively. A total of 109 (58.6%) patients exhibited lymph node involvement. The percentage of patients with stage I, II and III disease was 22.0, 25.3 and 52.7%, respectively. Any patients exhibiting distant metastases were not included in the present study. As for the pathological type, 159 (85.5%) patients were diagnosed with adenocarcinoma, and other pathological types included signet ring cell carcinoma (4.8%), tubular adenocarcinoma (3.8%), mucinous adenocarcinoma (3.2%), papillary carcinoma (1.1%), neuroendocrine carcinoma (1.1%), and undifferentiated carcinoma (0.5%). Regarding the tumour location, 52 (28.0%) cases were located in the esophagogastric junction or the fundus of the stomach, 39 (21.0%) cases were located in the middle of the stomach, 82 (44.1%) cases were located in the lower portion of the stomach, 1 (0.5%) case exhibited a diffuse lesion, and 12 (6.5%) cases exhibited a tumour in an unspecified location.

Expression level of TACC3 based on western blotting and qPCR. We used western blotting to examine the expression of TACC3 in 8 paired GC and adjacent non-cancerous tissues. The paired samples exhibited a higher TACC3 expression in the tumour tissues than in the adjacent non-cancerous tissues (Fig. 1a). We also employed qPCR to detect the TACC3 mRNA expression level in 30 paired tissues. The results of qPCR revealed a consistent trend of higher mRNA expression of TACC3 in the GC tissues (P=0.0081) (Fig. 1b).

IHC analysis of TACC3 in clinical GC cases. Based on further investigation of the TACC3 expression level in GC tissues via IHC, TACC3 immunoreactivity was predominantly found in the cytoplasm of carcinoma cells and was also detected in specific areas of intestinal metaplasia tissue and in certain highly proliferative lymphocytes in the form of clusters of yellow-brown granules (Fig. 2b). Weak or negative TACC3 staining was observed in adjacent normal gastric epithelial cells (Fig. 2a) and in surrounding mesenchymal cells. The four categories of the intensity of TACC3 immunostaining are shown in Fig. 3. According to ROC analysis, the patients were separated into two groups: low TACC3 expression (IHC score ≤50) and high TACC3 expression (>50). TACC3 expression

Figure 1. The protein and mRNA expression of TACC3 in GC and adjacent non-malignant gastric tissues. (a) Upregulated expression of TACC3 was detected via western blotting in 8 GC tissues compared with the matched non-cancerous gastric tissues. The expression levels were normalised to those of α-tubulin. (b) The mRNA expression of TACC3 was significantly elevated in 30 pairs of GC tissues based on qPCR. The expression levels were normalised to those of β-actin. N, adjacent normal gastric tissue; T, GC tissue.
(AUC=0.672, P<0.001) demonstrated a significant association with the survival status. Furthermore, 102 out of 186 (54.8%) cases exhibited a high expression of TACC3 in the tissue.

**TACC3 expression and its association with the clinicopathological characteristics.** We analysed the association between TACC3 protein expression and the clinicopathological characteristics using the Chi-square test or Fisher’s exact test. This analysis revealed that a high expression of TACC3 positively correlated with extracapsular extension (P<0.001) and tumour relapse (P<0.001). However, we failed to detect a correlation between TACC3 expression and other standard clinicopathological variables, including age, gender, pathological T stage, N stage, TNM staging status, tumour grade and the status of tumour emboli in the vasculature (P>0.05) (Table I).

*High TACC3 expression based on IHC is associated with reduced survival in postoperative GC.* A total of 78 deaths...
YUN et al: HIGH EXPRESSION OF TACC3 STRONGLY CORRELATES WITH POOR PROGNOSIS OF GASTRIC CANCER

Figure 4. Survival curves for 186 GC patients according to TACC3 expression (log-rank test). (a) Probability of DFS for all GC patients: low TACC3 expression, n=84; high TACC3 expression, n=102. (b) Probability of OS for all GC patients: low TACC3 expression, n=84; high TACC3 expression, n=102.

Figure 5. Survival analysis according to TACC3 expression in subsets of GC patients with different stages, pT/pN status and histological grade (log-rank test). Probability of OS for: (a) the stage I GC subgroup: low TACC3 expression, n=21; high TACC3 expression, n=22; (b) the stage II GC subgroup: low TACC3 expression, n=38; high TACC3 expression, n=60; (c) the pT1 and pT2 GC subgroup; low TACC3 expression, n=26; high TACC3 expression, n=25; (e) the pN(-) GC subgroup, low TACC3 expression, n=41; high TACC3 expression, n=56; (f) the G1/G2 GC subgroup, low TACC3 expression, n=24; high TACC3 expression, n=30; (g) the pT 3 and pT4 GC subgroup, low TACC3 expression, n=58; high TACC3 expression, n=77; (h) the pN(+) GC subgroup: low TACC3 expression, n=43; high TACC3 expression, n=66; and (I) the G3/G4 GC subgroup, low TACC3 expression, n=60; high TACC3 expression, n=72.
(41.9%) had occurred at the time of our analysis, with a median follow-up duration of 62.1 (range, 1.2-87.2) months for the surviving patients. The patients exhibiting a high TACC3 expression exhibited a significantly shorter DFS (49.1 vs. 69.6 months, log-rank test P<0.001) and OS (50.9 vs. 72.0 months, log-rank test P<0.001) than the patients exhibiting a low expression of TACC3 (Fig. 4). Moreover, survival analysis was performed according to the level of TACC3 expression in subsets of GC patients at different clinical stages, pT statuses, pN statuses and histological grades. Our results demonstrated that a high expression of TACC3 was a prognostic factor for the stage II (P=0.04, Fig. 5b), stage III (P<0.001, Fig. 5c), pT3/T4 (P<0.001, Fig. 5g), pN-positive (P<0.001, Fig. 5h), and poorly differentiated/undifferentiated tumour subgroups of GC patients (P<0.001, Fig. 5i). However, the level of TACC3 expression did not differentiate the outcomes of stage I (P=0.329, Fig. 5a), pT1/T2 (P=0.273, Fig. 5d), pN0 (P=0.247, Fig. 5e) or well- to moderately differentiated tumour subgroups (P=0.142, Fig. 5f).

Univariate and multivariate analysis of the prognostic factors. The prognostic value of TACC3 expression was confirmed by Cox regression analysis. Clinical characteristics, including age, gender, histological grade, tumour emboli in the vasculature, T status, N status, extracapsular extension, and TACC3 expression were included in the univariate analysis to assess their impact on the survival of GC patients. Based on this analysis, T status, N status, extracapsular extension, and TACC3 expression were found to impact survival. Therefore, these factors were further examined using a multivariate analysis model. Ultimately, T status, N status, extracapsular extension, and TACC3 expression were identified as independent prognostic factors (Table II). Thus, the results indicated that the TACC3 expression level significantly correlates with the prognosis of GC.

Discussion

The present study has provided, to the best of our knowledge, the first evidence that high TACC3 expression correlates with shorter survival, tumour recurrence and extracapsular extension in GC patients. In the present study, we first performed western blotting and qPCR to examine the expression level of TACC3 in several paired GC and adjacent non-neoplastic tissues. TACC3 was upregulated at the protein and mRNA levels in the tumour tissues compared with the normal tissues. We then evaluated the protein expression of TACC3 using IHC in a cohort of 186 GC cases for whom follow-up data were available. We observed that the TACC3 protein was predominantly detected in the cytoplasm of gastric malignancies and was increased in 54.8% of the GC tissue samples. By contrast, the normal gastric mucosal tissue exhibited an absent or low expression of TACC3. A further correlation analysis revealed that a high level of TACC3 protein expression significantly correlated with extracapsular extension and tumour recurrence. Moreover, the patients with a high TACC3 expression exhibited shortened OS and DFS. Taken together, these findings suggest that TACC3 is associated with GC progression and is a potential independent prognostic indicator of survival for GC patients.

TACC3 is a microtubule- and centrosome-associated protein that regulates mitotic spindle dynamics and centrosome integrity...
It is well known that the dynamic instability of microtubules is responsible for inaccurate chromosomal segregation during mitosis, and this process has been shown to be an effective target for cancer chemotherapy. Novel agents targeting non-microtubule mitotic proteins are currently under development as chemotherapeutic drugs (37). The study by Yao et al (23) demonstrated that depletion of TACC3 causes growth retardation of mouse thymic lymphoma and caused embryonic lethality to mice due to increased apoptosis, suggesting that TACC3 may serve as a promising anticancer target. The induction of TACC3 knockdown may accelerate the senescence of cancer cells in the presence of non-toxic paclitaxel concentrations. The combination of drugs targeting TACC3 and drugs interfering with microtubule dynamics may efficiently improve the chemosensitivity of cancer cells (38). Yim et al (20) also reported that paclitaxel treatment and TACC3 knockdown using siRNA led to a significant synergistic enhancement of G2/M phase arrest and apoptosis in HeLa cells. In addition, TACC3 depletion sensitised cells to paclitaxel-induced death and superseded p21 WAF-mediated cell cycle arrest (39). From a therapeutic perspective, TACC3 may serve as a potential target for cancer therapy and as a synergistic target in combination with paclitaxel.

In summary, to the best of our knowledge, the present study has shown for the first time that TACC3 expression was closely associated with extracapsular invasion of the tumour and tumour recurrence in GC. The expression of TACC3, as detected by IHC, may serve as a useful predictor of clinical outcome for GC patients. Thus, the expression level of TACC3 may be useful for assessing risk and optimising therapy.

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References