Nuclear Pore Complex 62 Promotes Metastasis of Gastric Cancer by Regulating Wnt/β-Catenin and TGF-β Signaling Pathways

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ABSTRACT: Gastric cancer (GC) is the third leading cause of cancer-related deaths in the world. Tumor metastasis is considered one of the main factors for GC development. Nup62 is a member of the nuclear pore complex (NPC). It bridges the nuclear envelope, is important in nucleocytoplasmic exchange, and is associated with cancer. This study aimed to investigate the role of Nup62 in GC metastasis. The relationship between the expression level of Nup62 in GC and patient survival was evaluated using Kaplan-Meier analysis. Then Nup62 expression in GC tissues and matched normal gastric tissues was analyzed by immunohistochemistry and that in cell lines by Western blot analysis. Furthermore, clonogenic and Transwell migration assays were performed, and the expression of epithelial-mesenchymal transition (EMT) proteins was detected to determine the metastatic functional roles of Nup62 in GC. Compared with the adjacent normal tissues, Nup62 was found to be upregulated in GC tissues using software prediction and detecting clinical specimens and cell lines. Moreover, the downregulation of Nup62 suppressed colony formation and decreased the number of migrated cells. In contrast, Nup62 overexpression promoted colony formation and increased the number of migrated cells. Further functional studies showed that the abnormal expression of Nup62 influenced cell migration and EMT through wingless/β-catenin (Wnt/β-catenin) and transforming growth factor (TGF)-β signaling pathways. In summary, the findings indicate that Nup62 regulates cell migration by interfering with Wnt/β-catenin and TGF-β signaling pathways in GC.

KEY WORDS: gastric cancer, Nup62, migration, Wnt/β-catenin, TGF-β

I. INTRODUCTION

Gastric cancer (GC) is the most common cause of gastrointestinal malignant tumor and has high morbidity and mortality globally.[1,2] In 2018, more than 1,000,000 new cases were diagnosed and almost 783,000 deaths occurred.[3] Various factors are responsible for the development of malignancy. One of the most important is GC metastasis.[4] Therefore, the molecular mechanism underlying the metastasis in GC cells needs to be explored.

The nuclear pore complex (NPC) is a large protein channel for transport into and out of the nucleus.[5,6] NPC bridges the nuclear envelope and contains 30 proteins, called nucleoporins (Nups). Besides regulating nucleo-cytoplasmic trafficking, Nups are essential in many cellular processes such as growth and differentiation and gene expression.[7,8] Furthermore, the expression of several Nups significantly correlates with cancer. Mullan et al. showed that high Nup98 expression is associated with poor outcomes in triple-negative breast cancer and predicts response to anthracycline-based chemotherapy as a novel marker.[9] Moreover, Nup153 correlates with the proliferation of colorectal cancer cells and tumor growth.[10] In a recent study, Nup107 expression increased in cervical tissues and Nup107 abnormal expression

influenced oxidative insult.[11] Nup62 is located in the central avenue of the NPC. In squamous cell carcinomas (SCCs), Nup62 expression increases in normal versus tumor samples and its knockdown strongly inhibits SCC cell proliferation.[12] In ovarian cancer, the silencing of Nup62 results in growth retardation.[13]

In our study, Nup62 expression in GC tissues and adjacent nontumor tissues and cell lines was investigated and its relationship with GC clinical characteristics was evaluated. It was revealed that Nup62 expression is significantly associated with cell migration and epithelial-mesenchymal transition (EMT) as mediated by wingless/β-catenin (Wnt/β-catenin) and transforming growth factor (TGF)-β signaling.

II. MATERIALS AND METHODS

A. Tissue Specimens, Cell Lines, and Cultures

Clinical specimens of GC tissues and adjacent nontumor tissues were collected from 45 patients admitted to the China-Japan Union Hospital of Jilin University, Jilin, China. They were embedded in paraffin and analyzed via immunohistochemistry (IHC). All procedures were approved by the Institutional Ethics Committee of Jiangsu University (No. UJS-IACUC-AP-20190307087). The six human GC cell lines (AGS, SGC-7901, MKN-45, BGC-823, MGC-803, and HGC-27) and gastric epithelial cells (GES-1) used in this study were preserved in the laboratory. GES-1, SGC-7901, MKN-45, BGC-823, MGC-803, and HGC-27 cells were cultured in Roswell Park Memorial Institute (RPMI, Buffalo, NY, USA) 1640 (Gibco, Gaithersburg, MD, USA), while the AGS cell was propagated in Ham's F (F12; Gibco). All media were supplemented with 10% fetal bovine serum (FBS; Wisent Bio Products, Quebec, Canada). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

B. Immunohistochemistry

Immunohistochemistry was performed as described in a previous study.[14] Briefly, the tissues were fixed with 4% paraformaldehyde solution and embedded in paraffin. The paraffin-embedded tissues were then cut into 4-μm-thick sections and blocked after antigen retrieval. Thereafter, the tissue sections were incubated with primary (Nup62, 1:1,000) and secondary antibodies. Finally, the sections were counterstained with hematoxylin after visualizing with DAB.

C. Nup62 Gene Transfection and siRNA Knockdown Assay

The Nup62 expression vector pcDNA3.1-3FLAG-Nup62 was kindly provided by Professor Decheng Yang (Department of Pathology and Laboratory Medicine, University of British Columbia). The HGC-27 cells were cultured on six-well plates and transfected with pcDNA3.1-Nup62 and a control vector using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), respectively. The SGC-7901 cells were transfected with siRNA-Nup62 purchased from Santa Cruz Biotechnology (Dallas, TX, USA) (sc-36107). After 48 h, the transfected cells were extracted for later use.

D. Clonogenic Assay

The transfected cells were seeded into six-well plates (1,000 cells/well) and then incubated for 14 days at 37°C in a humidified atmosphere with 5% CO_2. The visible colonies were fixed with 4% paraformaldehyde and stained with crystal violet.

E. Transwell Migration Assay

The transfected cells were harvested and seeded in the upper chambers of the wells (Merck Millipore, Burlington, MA, USA). Every upper chamber of the Transwell was filled with 200-μL serum-free medium; the lower compartment of the chamber was filled with 600-μL medium containing 10% FBS. After incubating for 24 h at 37°C in a humidified atmosphere with 5% CO_2, the cells on the upper surface were removed to the lower surface, fixed with 4% paraformaldehyde, and stained with crystal violet. Finally, images were obtained and the invaded cells were counted under a microscope.

F. Western Blot Analysis

Total protein was extracted with a radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Beijing, China), separated via 10% or 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to polyvinylidene difluoride (PVDF) membranes for 90 min, and blocked in 5% skimmed milk for 1 h at room temperature. Finally, the protein was probed with appropriate antibodies: anti-Nup62 (1:5,000; Thermo Fisher, Waltham, MA, USA); antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-β-catenin, anti-E-cadherin, antiproliferating cell nuclear antigen (PCNA), antiphosphorylated glycogen synthase kinase 3β (p-GSKβ), anti-Snail, anti-Timp1, anti-Timp2 (1:1,000; Cell Signaling Technology, Danvers, MA, USA); antimatrix metalloprotein 2 (MMP2), anticellular myelocytomatosis oncogene (c-Myc), anti-Cyclin D1, anti-N-cadherin, anti-TGF-β, anti-Smad2/3, and anti-p-Smad2/3 (1:200; Wanlei). Next, appropriate goat antirabbit and horse antimouse antibodies (Fcmacs,) were used at 1:2,000 dilutions. The bands were visualized using an enhanced chemiluminescence (ECL) kit (Merck Millipore, Burlington, MA, USA) following manufacturer instructions, and then the relative density of the bands was quantified using ImageJ software.

G. Statistical Analysis

One-way ANOVA was used to analyze the data. The data were expressed as means ± standard deviation (SD) of three independent experiments. All statistical analysis was performed using SPSS19 (IBM), and $P < 0.05$ indicated a statistically significant difference.

III. RESULTS

A. Relationship between Nup62 Expression and Overall Patient Survival

Kaplan-Meier analysis was performed to evaluate the relationship between Nup62 expression in GC tissues and patient survival. The results showed that high Nup62 expression correlated with poor overall survival and progression-free survival in patients with GC (Fig. 1A and 1B). Gene expression profiling interactive analysis (GEPIA) and UALCAN were used to evaluate Nup62 expression in all types of tumor tissues and adjacent normal tissues. The results showed that Nup62 was expressed in most tumor and adjacent normal tissues, including gastric tissues. Expression was higher in tumor tissues than in adjacent normal tissues, excluding a few tumors. In GC, Nup62 expression was higher in GC tissues than in adjacent normal tissues (Fig. 1C and 1D).

B. Nup62 Protein Expression in GC Tissues and Human GC Cell Lines

Nup62 expression was investigated in 45 paired GC tissues and adjacent normal tissues by IHC analysis to confirm the results of the software prediction. As shown in Fig. 2A and 2B, Nup62 expression was

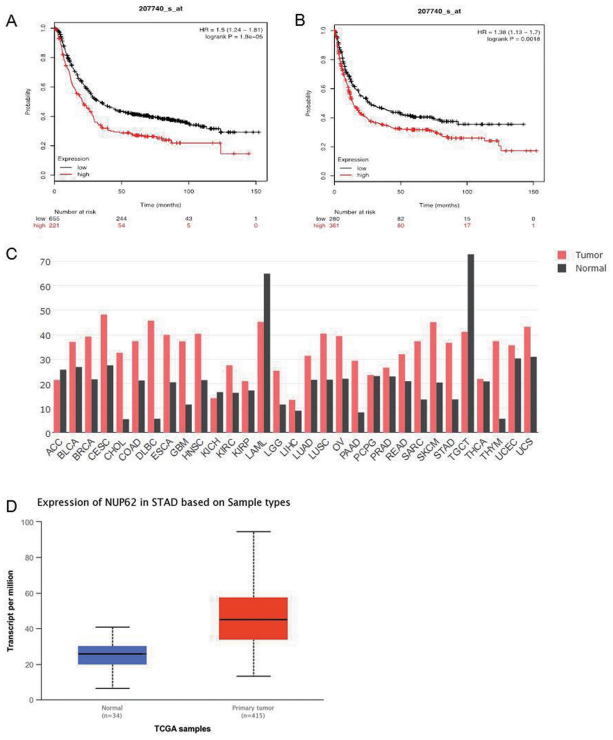

FIG. 1: Relationship between Nup62 expression and poor overall survival and progression-free survival reflected in GC tissues and adjacent normal tissues. (A and B) Kaplan-Meier analysis. (C and D) ($P < 0.01$) GEPIA and UALCAN analysis.

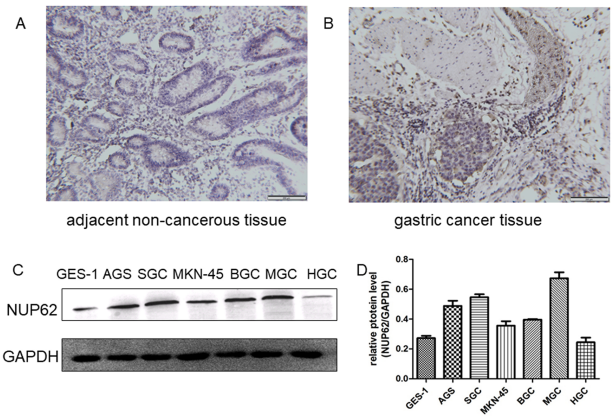

FIG. 2: IHC analysis showing high Nup62 expression in GC tissues and cell lines. (A and B) Matched normal gastric tissue (left) and GC tissue (right) (magnification ×100). (C and D) Cell lines.

found to be significantly higher in GC tissues than in adjacent normal tissues. Among the 45 paired tissue specimens, Nup62 expression increased in 29 GC tissues compared with the paired adjacent nontumor tissue. Furthermore, Nup62 was expressed in all seven human gastric cell lines (GES-1, AGS, SGC-7901, MKN-45, BGC-823, MGC-803, and HGC-27). Compared with GES-1, Nup62 expression was found to be higher in GC cell lines, especially in SGC-7901 and MGC-803. Moreover, of the six GC cell lines, Nup62 expression was lowest in HGC-27 (Fig. 2C and 2D).

C. Influence of Abnormal Nup62 Expression on Cell Migration and EMT *In Vitro*

Nup62 was silenced in SGC-7901 using siRNA-Nup62 or control siRNA, and overexpressed in HGC-27 by the transfection of pcDNA3.1-3FLAG-Nup62 or control pcDNA3.1 to explore the role of Nup62 in GC. Nup62 expression was confirmed by Western blot analysis. Colony formation and Transwell migration assays were then performed. The results showed that Nup62 silencing decreased the proliferation of SGC cells and the formation of fewer and smaller clones compared with that in control cells (Fig. 3C and 3D). Transwell migration assay showed that migrated cells were fewer in the siRNA-Nup62 cells compared with the control cells (Fig. 3G and 3H). In contrast, Nup62 overexpression

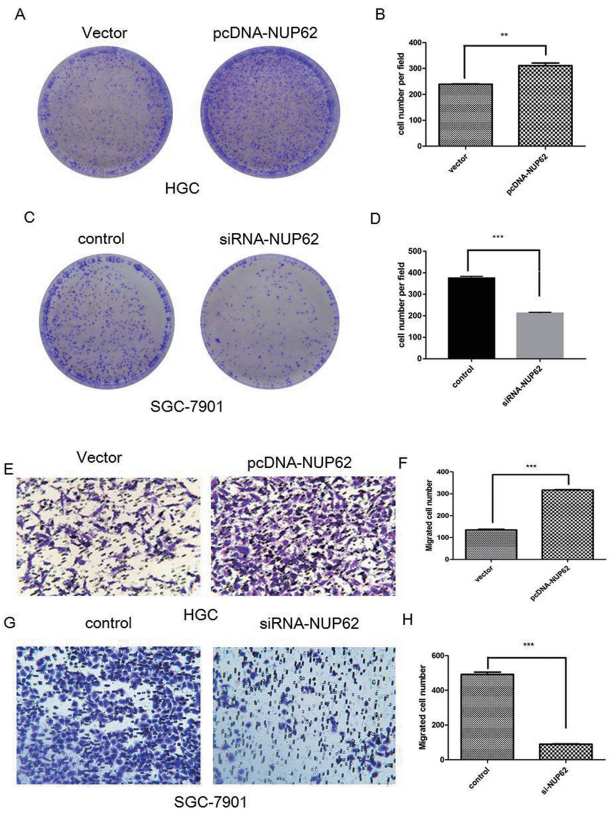

FIG. 3: Transwell migration assay showing influence of abnormal Nup62 expression on cell migration. (A–D) Clonogenic assay showingNup62-overexpressed HGC cells and Nup62-silenced SGC cells, respectively. (E–H) Transwell migration assay showing Nup62-overexpressed HGC cells and Nup62-silenced SGC cells, respectively. $^{**}P < 0.01$; $^{***}P < 0.001$.

led to the formation of many more clones, and the number of cells that passed through the membrane into the lower chamber was more than the number of control cells (Fig. 3A, 3B, 3E, and 3F).

Several indicators of migration and EMT were detected using Western blot analysis. As shown in Fig. 4A, Nup62 overexpression induced a decrease in the expression of E-cadherin (an epithelial marker) and Timp1, whereas the expression of N-cadherin (an epithelial marker), MMP2 and MMP9 (mesenchymal markers), and Snail (the key regulator of EMT) increased. Following Nup62 knockdown, the results for the expression of the series of molecular markers were contrary to those for the overexpression of Nup62.

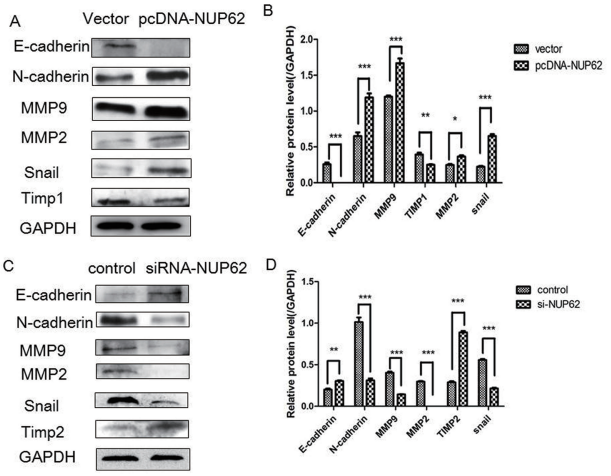

FIG. 4: Western blot analysis showing loss of cellular adhesive molecules in EMT. (A and B) Nup62 knockout cells and control cells. (C and D) Nup62-overexpressed cells and control cells. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

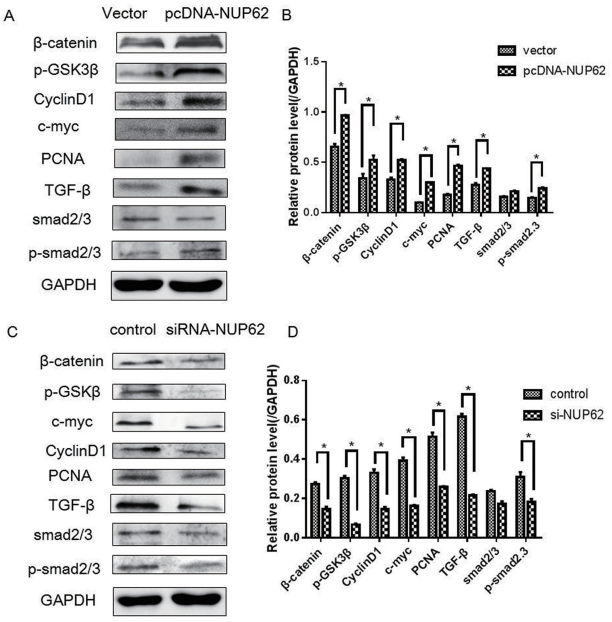

FIG. 5: Western blot analysis showing influence of Nup62 expression on Wnt/β-catenin and TGF-β signaling pathways. (A and B) Wnt and TGF-β signaling canonical gene targets in Nup62 knockout cells and control cells. (C and D) Wnt and TGF-β signaling canonical gene targets in Nup62-overexpressed cells and control cells. *$P < 0.05$.

D. Mediation of Nup62-Induced Cell Migration and EMT by Wnt/β-Catenin and TGF-β Signaling Pathways

Wnt and TGF-β signaling canonical gene targets—β-catenin, PCNA, Myc, Cyclin D1, phosphorylation of GSK-3β (p-GSK3β), TGF-β, Smad1/2, and phosphorylation of Smad1/2 (p-Smad1/2)—were measured by Western blot analysis to investigate the possible signaling pathway in Nup62-induced cell migration and EMT. The results showed that all markers of the Wnt signaling pathway were increased in the overexpression cells in contrast to the control cells (Fig. 5A and 5B). Moreover, levels of TGF-β and p-Smad1/2 in Nup62-transfected cells were remarkably higher than those in control cells; however, Nup62 downregulation in the SGC-7901 cell decreased these markers (Fig. 5C and 5D).

IV. DISCUSSION

Nup62, containing phenylalanine-glycine (FG) repeats, has been reported in SCCs and ovarian carcinoma. Using RNA sequencing and cDNA microarray analysis in normal versus tumor samples, Hazawa et al. found that Nup62 expression was higher in SCC tumor samples than in normal samples.[12] In our study, Kaplan-Meier analysis revealed that Nup62 was highly expressed in GC tumors.

Tissues from patients with GC were collected and matched with nontumor tissues, and IHC analysis was conducted to determine Nup62 expression. The overexpression of Nup62 in GC tissues was found to be significantly higher than that in adjacent normal tissues. Among human gastric cell lines, Nup62 expression was increased.

EMT is believed to be a driving force in cancer progression and metastasis. Progression involves diminishing cell-cell junctions and cell polarity, downregulation of epithelial markers, upregulation of mesenchymal markers, and increasing cell mobility.[15–18] During EMT, cells lose some cellular adhesive molecules, such as E-cadherin—the most important factor in mediating cell-cell adhesion—and acquire some characteristics of mesenchymal cells, such as N-cadherin upregulation, leading to mesenchymal cell migration.[15,19] Moreover, several transcription factors participate in EMT development, one of

which is the Snail family, including Snail, Slug, and Smus. Snail inhibits the expression of two integral membrane proteins (occluding and claudins) and is associated with lymph node metastasis.[20,21]

In our study, Transwell migration and clonogenic assays were performed to examine the role of Nup62 in EMT. Nup62 downregulation decreased the number of migrated cells and colony formation. Conversely, Nup62 overexpression in HGC increased cell migration. E-cadherin, N-cadherin, Snail, and other molecular markers, such as MMP9 and MMP2 as mesenchymal markers, were detected following the overexpression vector and si-Nup62 transfection. E-cadherin expression significantly decreased and N-cadherin, MMP9, MMP2, and Snail expression dramatically increased in the HGC overexpression group compared with cells transfected with the empty vector. Nup62 silencing, in contrast, decreased levels of the four markers (N-cadherin, MMP9, MMP2, and Snail) compared with levels in the control group (except for E-cadherin).

TGF-β is crucial in regulating tumor cell adhesion and EMT.[22] In typical Smad and non-Smad pathways, TGF-β participates in EMT.[23] In the classic Smad pathway, TGF-β regulates target gene transcription by activating the type I receptor, then phosphorylating Smad2/3 and interacting with Smad4, and finally translocating from the cytoplasm into the nucleus. Moreover, Wnt/β-catenin is pivotal in EMT in cancer cells.[24] Many essential genes, such as chromobox protein homolog 7 (CBX7) and fermitin family homolog 1 (FERMT1), regulate the Wnt/β-catenin signaling pathway and modulate EMT.[25–27] In our study, Nup62 overexpression increased TGF-β and p-Smad2/3 compared with their levels in cells treated with the control vector. Moreover, in the absence of Nup62, TGF-β and p-Smad2/3 levels were attenuated.

The effects of Nup62 knockdown or overexpression on the activation of the Wnt/β-catenin signal pathway were investigated. Expression of β-catenin and p-GSK3β, as core components of the Wnt/β-catenin signaling pathway, and expression of PCNA, Myc, and Cyclin D1, as indicators of Wnt/β-catenin signaling, were higher in Nup62-overexpressed cells than in control cells. Nup62 knockdown dramatically downregulated these markers.

V. CONCLUSIONS

We showed that Nup62 is highly expressed in GC tumor tissues and cell lines. Moreover, Nup62 downregulation decreases the number of migrated cells and colony formation whereas its overexpression increases cell migration and colony formation. Furthermore, abnormal Nup62 expression influences cell migration and EMT in GC *in vitro* through the Wnt/β-catenin and TGF-β signaling pathways. The role of Nup62 in patient survival should be further investigated.

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