Gastroenterology Research and Practice

# Systematic Therapy for Gastrointestinal Tumors

Lead Guest Editor: Zhihua Kang
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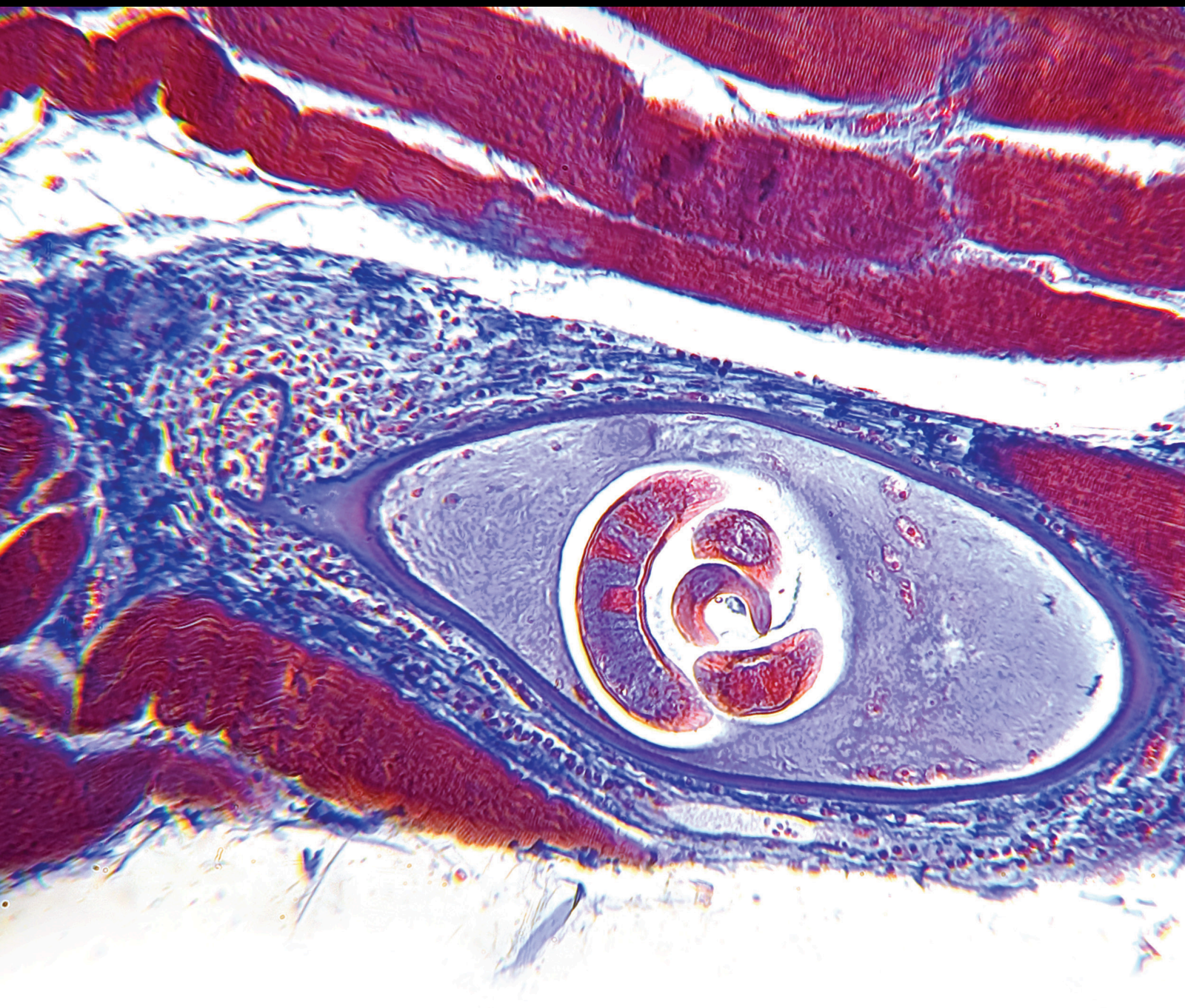

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# Contents

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*Editorial*

# Systematic Therapy for Gastrointestinal Tumors

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Systematic therapy for gastrointestinal tumor has revolved around radiation therapy, chemotherapy, targeted therapy, and immunotherapy over the last few decades. Among which, immunotherapy is undoubtedly the most prospective field for tumor treatment. In recent years, immunotherapy, especially immune checkpoint inhibitors (ICIs), has demonstrated promising and exciting results in various clinical trials. The Checkmate-648 and KEYNOTE-590 studies laid the foundation for PD-1 antibodies plus chemotherapy as first-line treatment in advanced esophageal squamous cell carcinoma. The KEYNOTE-181 and ATTRACTION-3 studies secured PD-1 antibodies as second-line treatment for advanced ESCC. In advanced gastric adenocarcinoma, the Checkmate-649 and ATTRACTION-4 studies established PD-1 antibodies plus chemotherapy as first-line treatment, while KEYNOTE-061 and ATTRACTION-2 studies recognized PD-1 antibodies as second- and third-line treatment. The KEYNOTE-177 study showed that pembrolizumab was superior to chemotherapy with respect to PFS (median, 16.5 vs. 8.2 months) in MSI-H/dMMR advanced or metastatic colorectal cancer. The REVONIVO and LEAP-005 studies advocated PD-1 antibodies plus targeted therapy as third-line treatment for metastatic colorectal cancer with MSS.

Immunotherapy as one of the most important part of systemic therapy for gastrointestinal tumor has gained rapidly recognition. A few promising research prospects for gastrointestinal tumors include the following: (1) ICIs combined with low-dose chemotherapy and/or radiation therapy. Low-dose chemotherapy or radiation therapy delivers tumor cytotoxicity, with limited impairment to the systemic immune system, providing a synergistic treatment effect. A low-dose, high-frequency administration continues to kill tumor cells, which releases tumor antigens to activating the antitumor immune response. In addition, the establishment of an optimal duration for ICIs and chemotherapy administration is of clinical significance. (2) ICIs combined with VEGF pathway inhibitors. Through increasing vessel permeability, VEGF pathway inhibitors can improve tumor perfusion, improve hypoxia, encourage immune cell migration, and finally augment the overall treatment efficacy of ICIs. (3) Establishment of a systematic immune evaluation criteria, such as calculating the proportion of inhibitory and activating immune cells. The individual immune system should be evaluated continuously during systematic therapy. If the immune system has been significantly impaired, treatment course should be shortened. (4) Tumor cytotoxicity can cause certain tumor cells to enter the G0 stage of mitosis or complete cell death, which causes the tumor tissue to become fibrotic, but can still be detected by CT or MIR. Continuous treatment will not provide additional antitumor effects, but instead cause systemic damage. Therefore, with the help of new surveillance methods such as PET-CT, a dynamic evaluation of tumor vitality can minimize systemic damage and improve the survival of patients through shortening the course of antitumor treatment. (5) Activation of tumor-specific immune responses may be the key for tumor patients to achieve complete remission. Neoantigen vaccine (including peptide vaccine and RNA vaccine) may be a promising direction in the treatment of gastrointestinal tumors. In conclusion, based on continuous monitoring of tumor vitality and evaluation of the immune system, application of neoantigen immunotherapy combined with PD-1 antibody,

along with chemotherapy and radiotherapy, can altogether provide a new paradigm for the systematic therapy of gastrointestinal tumors.

## Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this special issue.

## Acknowledgments

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*Zhihua Kang*
*Qingyuan Yang*
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*Research Article*

# Pleckstrin-2 as a Prognostic Factor and Mediator of Gastric Cancer Progression

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Pleckstrin-2 (PLEK2) is a crucial mediator of cytoskeletal reorganization. However, the potential roles of PLEK2 in gastric cancer are still unknown. PLEK2 expression in gastric cancer was examined by western blotting and real-time PCR. Survival analysis was utilized to test the clinical impacts of the levels of PLEK2 in gastric cancer patients. In vitro and in vivo studies were used to estimate the potential roles played by PLEK2 in modulating gastric cancer proliferation, self-renewal, and tumourigenicity. Bioinformatics approaches were used to monitor the effect of PLEK2 on epithelial-mesenchymal transition (EMT) signalling pathways. PLEK2 expression was significantly upregulated in gastric cancer as compared with nontumour samples. Kaplan-Meier plotter analysis revealed that gastric cancer patients with higher PLEK2 levels had substantially poorer overall survival compared with gastric cancer patients with lower PLEK2 levels. The upregulation or downregulation of PLEK2 in gastric cancer cell lines effectively enhanced or inhibited cell proliferation and proinvasive behaviour, respectively. Additionally, we also found that PLEK2 enhanced EMT through downregulating E-cadherin expression and upregulating Vimentin expression. Our findings demonstrated that PLEK2 plays a potential role in gastric cancer and may be a novel therapeutic target for gastric cancer.

## 1. Introduction

Gastric cancer remains a critical cancer; the incidence rate of gastric cancer ranks the fifth globally [1]. Although the diagnosis and management of gastric cancer have improved, the overall survival rate has not increased remarkably [2, 3]. The pronounced tendency towards recurrence and metastasis is the primary reason for the high rates of death and poor outcomes. The potential pathways and molecular mechanisms underlying gastric cancer development are still not fully elucidated [4, 5].

The human pleckstrin-2 (PLEK2) gene is located on chromosome 14q24.1 [6]. PLEK2 encompasses two conserved pleckstrin homology (PH) domains and an intervening disheveled-Egl-10-pleckstrin (DEP) domain. The expression of PLEK2 was found in various adherent cell lines [7]. PLEK2 promotes the shape changes associated with actin rearrangement. To date, the expression of PLEK2 and its role in gastric cancer progression and pathogenesis are still elusive.

In this study, PLEK2 expression was evaluated in The Cancer Genome Atlas (TCGA) datasets [8] and clinical gastric cancer samples using real-time PCR, western blotting, and immunohistochemistry (IHC). We detected that PLEK2 overexpression in gastric cancer was associated with shorter survival time. Our research also revealed the key functions of PLEK2 in promoting gastric cancer epithelial-mesenchymal transition (EMT), which may have implications for gastric cancer metastasis. Overall, our findings indicate that PLEK2

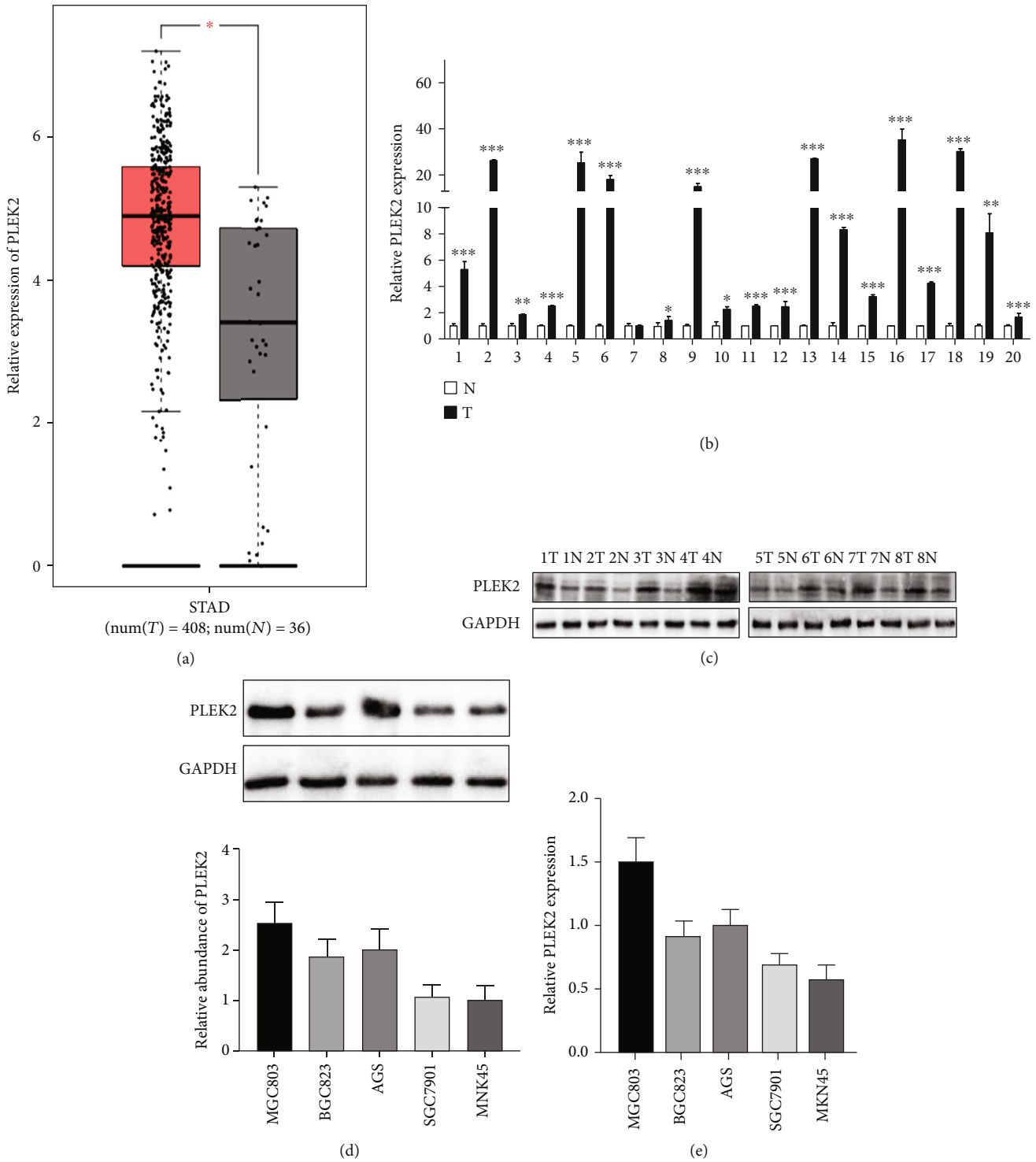
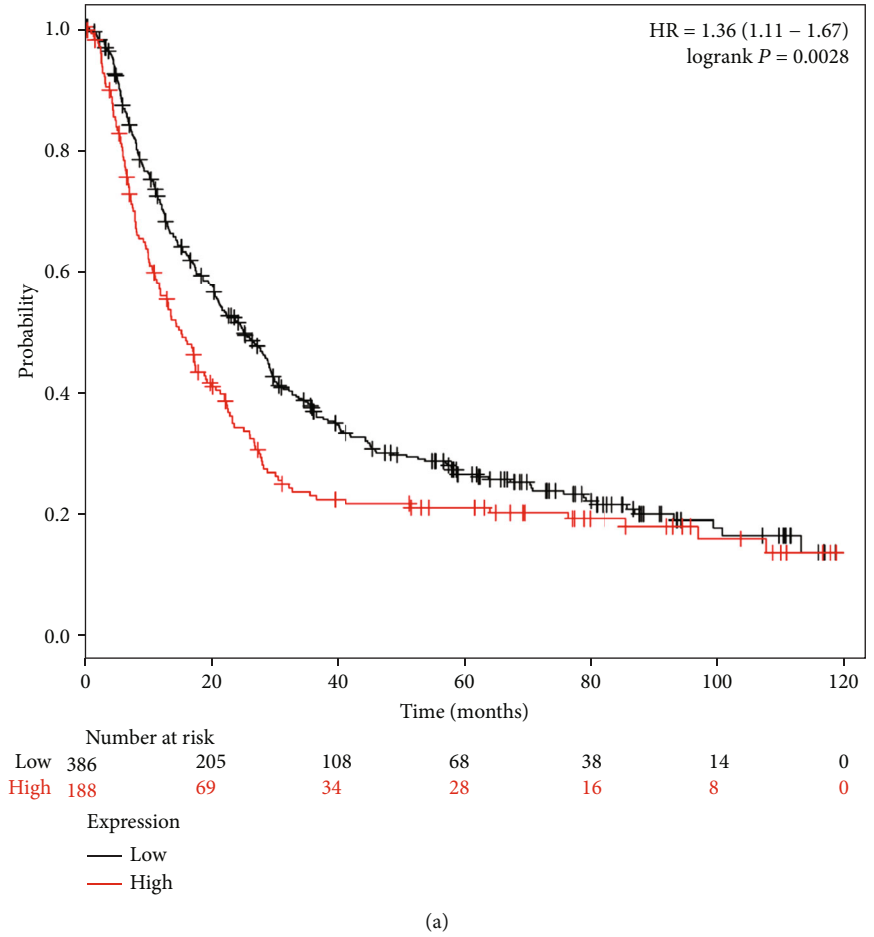
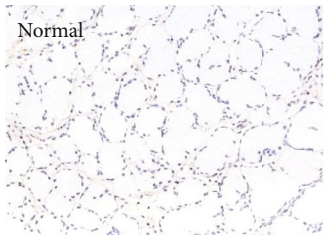
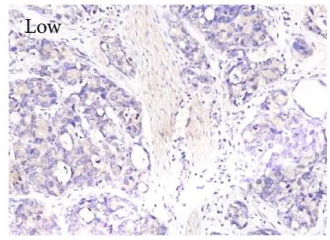
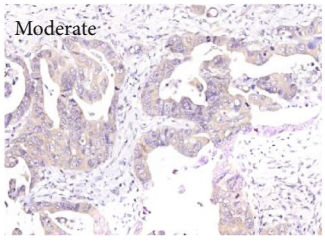


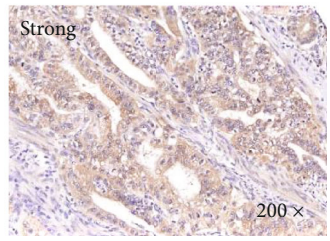

Figure 1: PLEK2 expression was elevated in gastric cancer. (a) The expression of PLEK2 mRNA in primary gastric cancer and adjacent noncancerous tissues in TCGA dataset. (b) The expression of PLEK2 mRNA in primary gastric cancer and adjacent noncancerous tissues by real-time PCR. The expression levels were normalized to those of GAPDH. (c) Representative western blots demonstrating the expression of PLEK2 protein in each of the primary gastric cancer and adjacent noncancerous tissues paired from the same patient. The expression levels were normalized to those of GAPDH. (d) The expression of PLEK2 protein in gastric cancer cell lines. GAPDH was the loading control. (e) Relative PLEK2 mRNA expression in gastric cancer cell lines. STAD: stomach adenocarcinoma; num: number; T: tumour; N: noncancerous tissues.



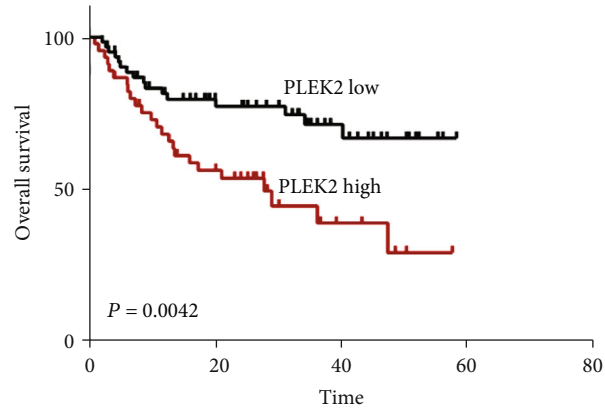




(b)




(c)

Figure 2: Survival analysis of patients with gastric cancer according to the expression of PLEK2. (a) The effect of PLEK2 on overall survival using 574 cancer samples based on the Kaplan-Meier plotter. (b) Examples of gastric cancer tissues immunostained for PLEK2. Different PLEK2 staining intensities are exemplified. (c) Kaplan-Meier analysis of overall survival in patients with gastric cancer ($n = 108$, $P = 0.0042$). HR: hazard ratio.

Table 1: Relationships between PLEK2 expression and clinicopathological characteristics.

| Characteristics | PLEK2 expression | | *P* value |
|---|---|---|---|
| | Low | High | |
| Age (years) | | | |
| <65 | 29 | 25 | |
| ≥65 | 33 | 21 | 0.436 |
| Gender | | | |
| Male | 24 | 20 | |
| Female | 38 | 26 | 0.618 |
| Grade | | | |
| Well or moderate | 54 | 32 | |
| Poor | 8 | 14 | **0.025** |
| Pathologic stage | | | |
| I-II | 34 | 16 | |
| III-IV | 28 | 30 | **0.039** |
| T stage | | | |
| T1-T2 | 24 | 9 | |
| T3-T4 | 38 | 37 | **0.033** |
| N stage | | | |
| N0 | 35 | 17 | |
| N1-N3 | 27 | 29 | **0.045** |
| Distant metastasis | | | |
| M0 | 57 | 34 | |
| M1 | 5 | 12 | **0.011** |

*P* values in bold indicate significant results.

is a potential prognostic indicator and employed by self-renewal and proliferation which may provide a better understanding of gastric cancer tumourigenesis.

## 2. Materials and Methods

*2.1. Patient Samples and Clinical Information.* Gastric cancer tissue samples and matched nontumour samples were collected from the Fudan University Affiliated Huashan Hospital (Shanghai, China). Data from one hundred eight patients with pathologically proven gastric cancer were collected between May 2011 and April 2016. All patients who gave informed consent underwent pathological and radiological examinations to confirm the diagnosis. Data on the clinical characteristics of all participants were collected and made available. The therapies that participants underwent after enrollment were assessed, and follow-ups were performed until April 30, 2016. This research was authorized by the Ethics Committee at Fudan University-Affiliated Huashan Hospital.

*2.2. Cell Cultures.* The human gastric cancer cell lines BGC-823, MGC-803, MKN45, SGC-7901, AGS, and human embryonic kidney (HEK) 293T cells were obtained from Cobioer Biosciences Company (Nanjing, China). Short Tandem Repeat (STR) analysis was performed to authenticate all the cell lines before starting the study. The human gastric adenocarcinoma cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin. HEK 293T cells were maintained in DMEM (Gibco).

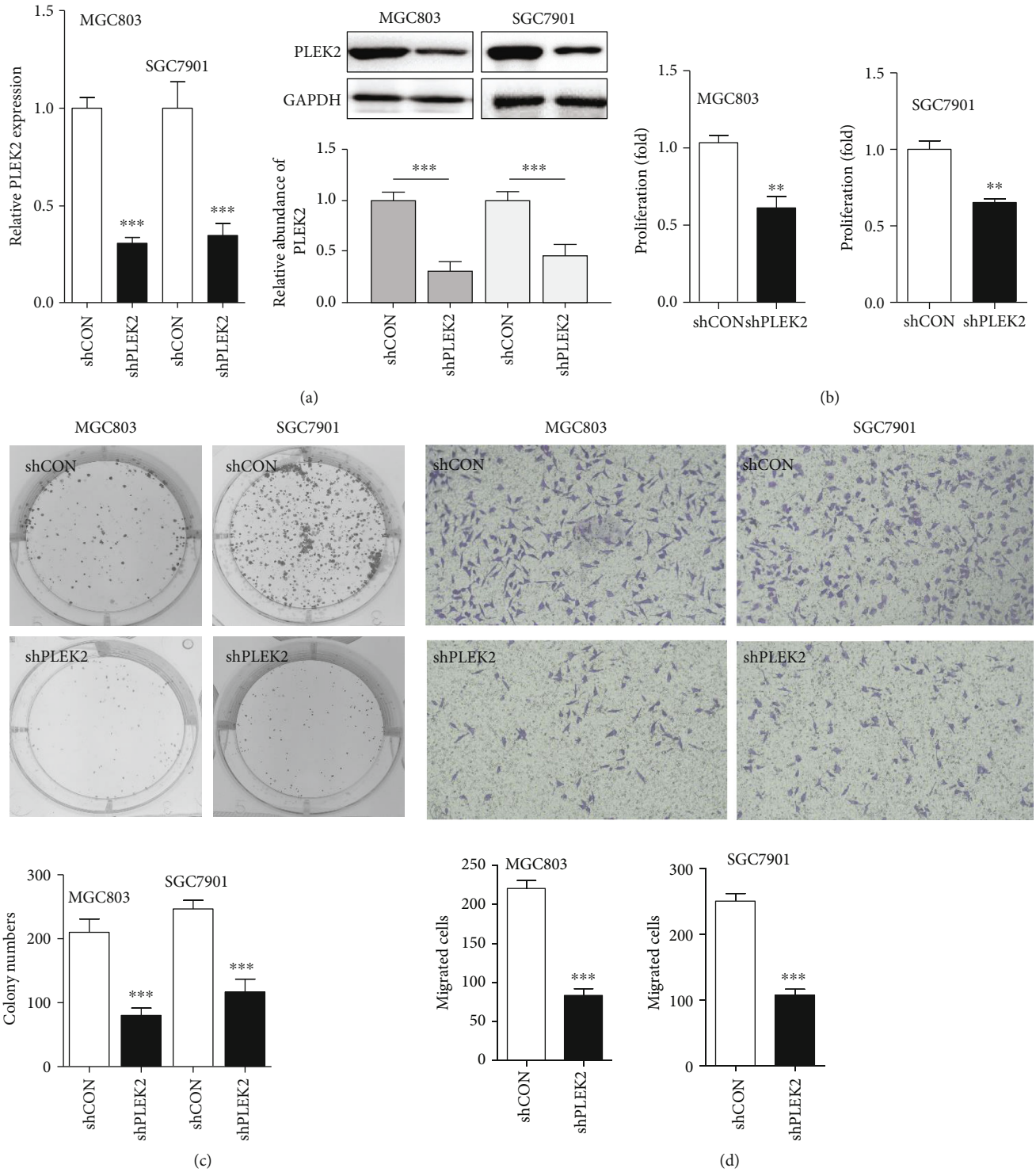
*2.3. Vector Construction and Retroviral Infection.* The wild-type PLEK2-CDS was PCR-amplified from HEK-293T cDNA using the following primers: forward 5′-TCGGAGCTGCT TCCTGGG-3′ and reverse 5′-CATGTTAGCTTTTTGATA GCTTCA-3′. Then, it was cloned into the pcDNA3.1/myc-His B vector (Invitrogen, Grand Island, NY, USA). The shRNAs targeting human PLEK2 (5′-AAGTGGCACGGT GGTGAAACA-3′) were cloned into pSilencer 4.1-CMV puro vectors (Ambion, Foster City, CA, USA). Retroviral production and infection were performed as the standard procedure. Stable cell lines expressing PLEK2 or shPLEK2 were selected for 10 days with 400 mg/mL G418 or 0.5 mg/mL puromycin, respectively.

*2.4. Real-Time PCR.* Total RNA from gastric cancer tissues and cells was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase reaction was then performed using M-MLV reverse transcriptase (Takara, Dalian, China). All experiments were conducted following the manufacturer's instructions. Quantitative real-time PCR was performed using the CFX Connect Real-time PCR System (Bio-Rad, Hercules, CA, USA). GAPDH was used as the internal standard gene. The primers used for PCR were PLEK2-forward 5′-AGCCTGAGCACTGTGGAGTT-3′ and PLEK2-reverse 5′-GCTGCTGGCCTGAATGTAAT-3′. The primers used for PCR were GAPDH-forward 5′-ATGGGG AAGGTGAAGGTCG-3′ and GAPDH-reverse 5′-CTCCAC GACGTACTCAGCG-3′.

*2.5. Western Blotting.* To prepare protein samples, cells washed with ice-cold phosphate-buffered saline (PBS) were lysed with a radioimmunoprecipitation assay (RIPA) buffer supplemented with Complete Protease Inhibitor. Protein concentration was determined using the BCA Protein Assay Kit (Beyotime, Haimen, China). Protein prepared from tissues and cells was loaded and separated with a 10% SDS polyacrylamide gel and electrotransferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5% skim milk for 2 h, the membrane was incubated with primary antibodies overnight at 4°C, including an anti-GAPDH antibody (Proteintech, Rosemont, IL, USA), anti-PLEK2 antibody (Proteintech), anti-E-cadherin antibody (Cell Signaling Technology, Danvers, MA, USA), and anti-Vimentin antibody (Cell Signaling Technology). After washing with PBS, the membrane was incubated with secondary antibodies. Signals were detected with the enhanced ECL western blotting reagent (Millipore).

*2.6. Immunohistochemistry.* IHC was performed on tissues and adjacent nontumour tissues in 108 cases of gastric cancer, as recently described [9].

*2.7. MTT Assays.* A total of $5 \times 10^3$ cells in 100 $\mu$l of culture medium were plated in 96-well plates. The experiments were performed in triplicate. After 72 h, the cells were stained with

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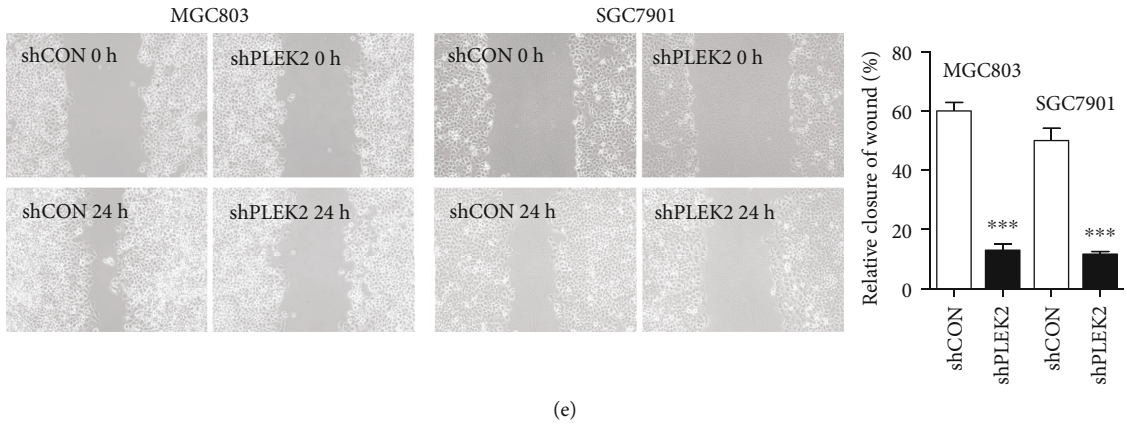



(e)

Figure 3: Knockdown of PLEK2 significantly suppressed gastric cancer cell proliferation, colony formation, and migration. (a) The stable knockdown of PLEK2 in MGC803 and SGC7901 cells by shRNA sequences (shPLEK2). The knockdown effect was verified at the mRNA and protein levels. (b) PLEK2 knockdown significantly reduced the proliferation rate of MGC803 and SGC7901 cells. (c) The knockdown of PLEK2 impaired the colony formation ability of gastric cancer cells. (d) The knockdown of PLEK2 suppressed gastric cancer cell migration. (e) The knockdown of PLEK2 significantly reduced the wound healing ability of gastric cancer cells. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ ($t$-test).

20 $\mu$l of MTT dye (5 mg/ml, Sigma-Aldrich) for an additional 3-4 h at 37°C before the removal of MTT and the addition of 200 $\mu$l of dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The absorbance was measured at 570 nm (with a reference at 630 nm).

*2.8. Colony Formation Assays.* Cells were seeded and cultured on 6-well plates (200 cells/well) for 3 weeks. First, colonies were stained with 4% paraformaldehyde for 10 minutes and then fixed with 1% crystal violet (Beyotime) for 5 minutes. Colonies with greater than 50 cells were counted randomly.

*2.9. Migration Assays.* A Transwell membrane (6.5 mm diameter with 8 $\mu$m pores, Corning) was used for the Transwell migration assay. The upper chambers were filled with RPMI 1640 medium with cells ($2.5 \times 10^5$ cells per well), and the lower chambers were loaded with RPMI 1640 containing 10% FBS to induce cell migration. After incubation for 16 h, cells on the top surface of the inserts were removed and cells that migrated to the bottom surface of the inserts were fixed and stained with 0.1% crystal violet for 30 minutes. Cells were counted in five random fields at ×200 magnification.

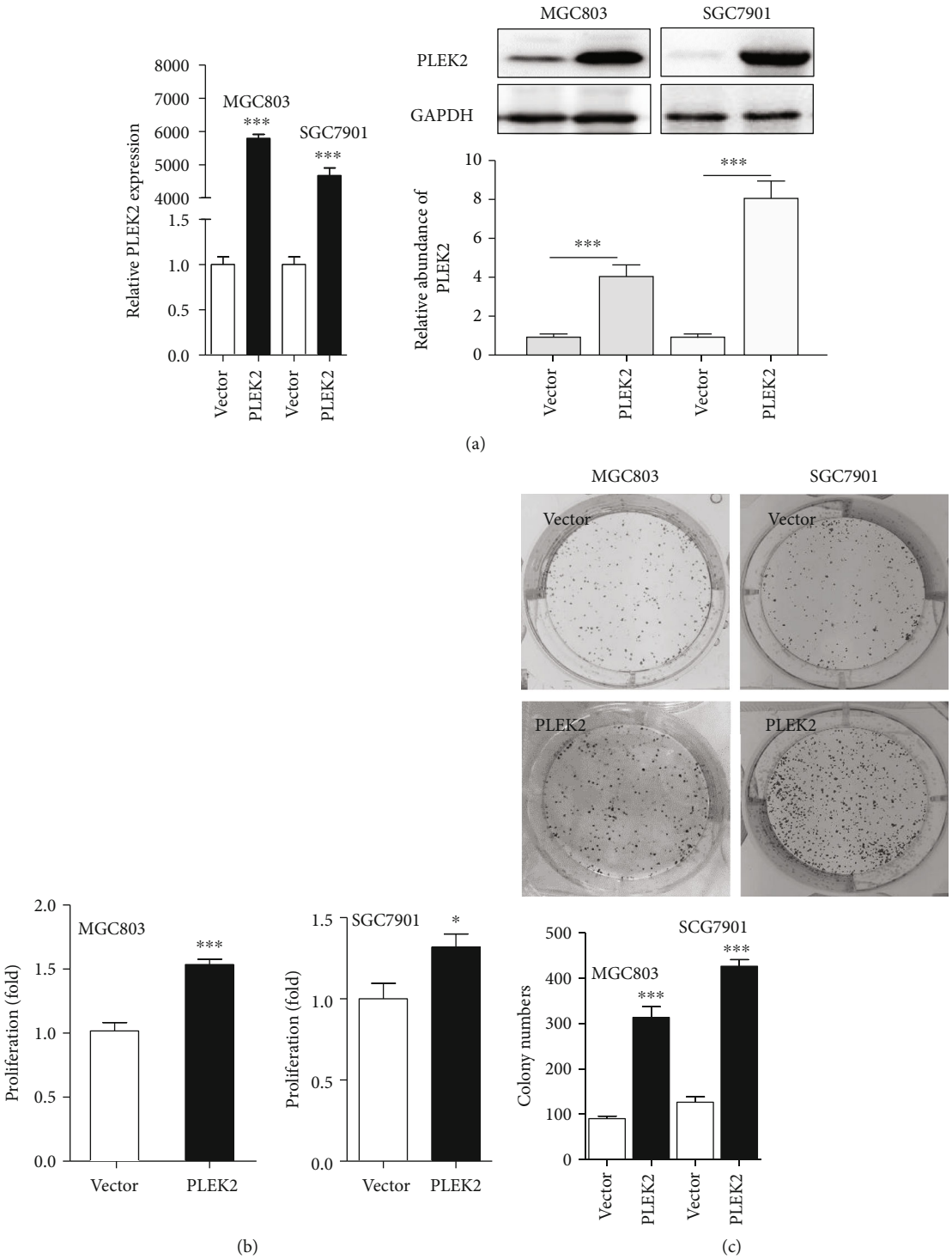
*2.10. Wound Healing Assays.* Cells seeded on 6-well plates were cultured until confluence. A homogeneous wound was scratched with a yellow pipette tip. Three wounds were made for each sample. Images of cells migrating into the wound were photographed and measured at zero time and after 24 h.

*2.11. Animal Model.* Mice were randomly assigned to experimental groups for the animal experiments. MGC803 cells ($2 \times 10^6$) transduced with lentiviruses expressing shPLEK2 or PLEK2 were harvested and resuspended in sterile PBS. Groups of 4-week-old BALB/c nu/nu female mice were subcutaneously injected with the above cell suspensions. Tumour development was detected after 5 days. The formula $(a \times b^2)/2$ was used to monitor the tumour volume ($a$ is the length and $b$ is the width of the tumour). Twenty-one days later, all mice and controls were humanely sacrificed, and xenograft tumours were excised and measured. The animal study was reviewed and approved by the Animal Care and Use Committee of Fudan University.

*2.12. Statistical Analysis.* All data are presented using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation. For experiments involving three or more groups, comparisons between groups for statistical significance were performed with 2-tailed paired Student's $t$-tests. The correlations of PLEK2 with clinicopathological characteristics were examined using the chi-square test. The Kaplan-Meier method was used to calculate survival. Kaplan-Meier survival curves were generated using GraphPad Prism 5, and the $P$ values were calculated using the log-rank test. A Cox proportional hazards model was used to calculate the adjusted hazard ratio (HR) with a 95% confidential interval (CI). All statistical tests were two-sided. A $P$ value of <0.05 was considered significant.

## 3. Results

*3.1. PLEK2 Was Upregulated in Human Gastric Cancer.* To investigate the expression patterns of PLEK2 in gastric cancer, we first monitored PLEK2 expression in TCGA database in GEPIA, TCGA database-driven web portal for gene expression profiling and interactive analysis (http://gepia.cancer-pku.cn/). We found that PLEK2 was remarkably upregulated in the RNA-seq dataset of 444 gastric cancer samples (Figure 1(a)). Real-time PCR analysis showed that PLEK2 mRNA expression was increased in 19 of 20 (95%) cases of cancer tissues. The relative abundance of PLEK2 mRNA expression was up to 35-fold higher in all examined samples (Figure 1(b)). Western blotting illustrated that the expression of PLEK2 was stronger in gastric cancer tissues than in the peritumoural gastric tissues (Figure 1(c)). Western blotting and real-time PCR analysis also revealed that

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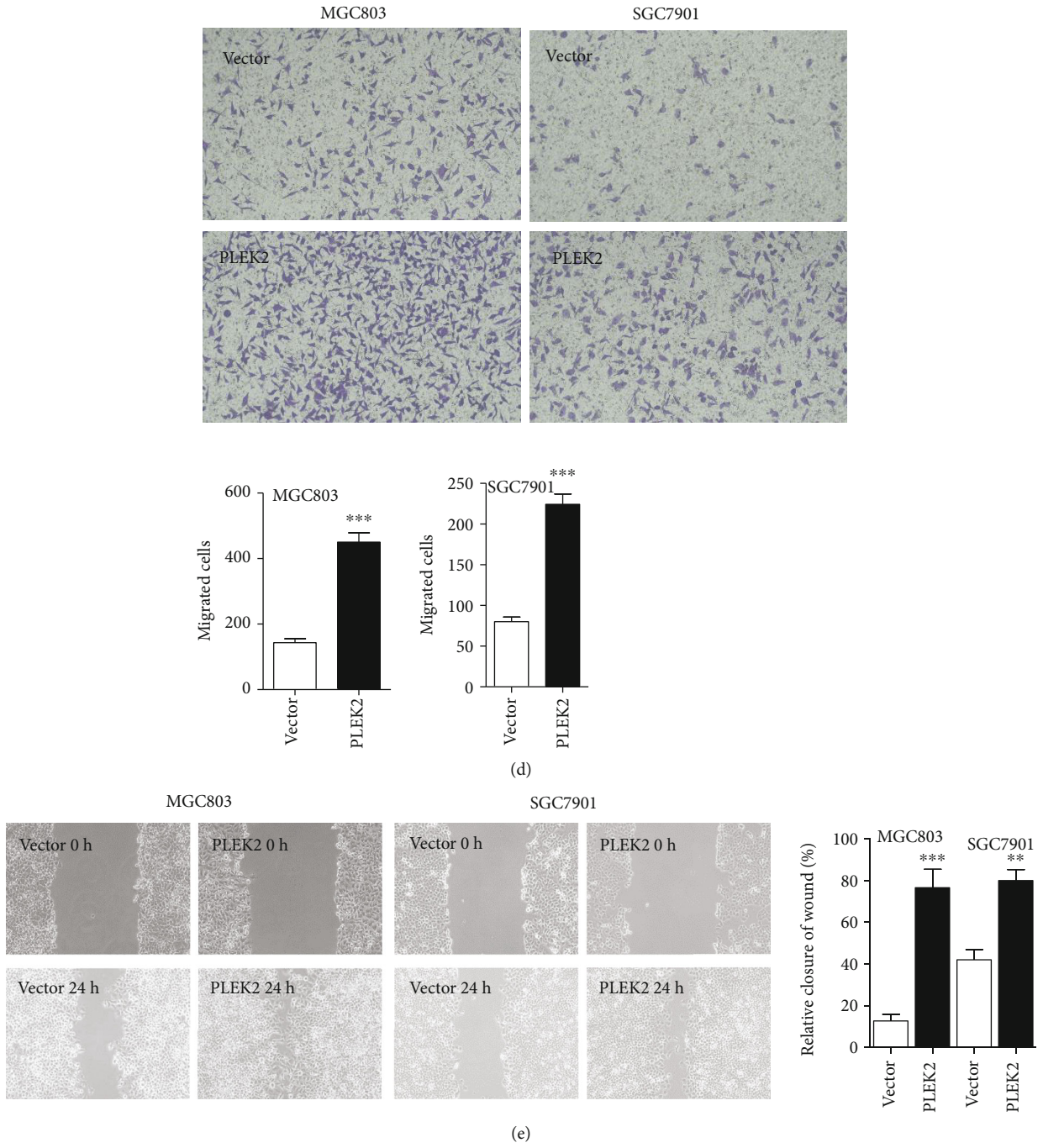



Figure 4: Overexpression of PLEK2 promoted gastric cancer proliferation, colony formation, and cell migration. (a) The overexpression of endogenous PLEK2 was confirmed at the mRNA and protein levels. (b) The overexpression of PLEK2 increased the cell proliferation rate in MGC803 and SGC7901 cells. (c) The overexpression of PLEK2 enhanced the colony formation ability of gastric cancer cells. (d) The overexpression of PLEK2 accelerated gastric cancer cell migration. (e) The overexpression of PLEK2 enhanced gastric cancer cell wound healing ability. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ ($t$-test).

gastric cancer cell lines, including BGC-823, MGC-803, MKN45, SGC-7901, and AGS, exhibited high PLEK2 protein and mRNA expression (Figures 1(d) and 1(e)).

*3.2. Increased PLEK2 Was Associated with Poor Survival in Gastric Cancer.* We further determined the relationship between PLEK2 mRNA and the overall survival of gastric cancer patients based on the web-based database Kaplan-Meier plotter. A total of 574 patients were included from the GSE14210, GSE15459, GSE22377, GSE29272, and GSE51105 datasets. Kaplan-Meier plotter analysis revealed that those with higher PLEK2 levels had substantially poorer overall survival (HR: 1.36, 95% CI: 1.11–1.67, $P = 0.0028$) compared with those with lower PLEK2 levels (Figure 2(a)).

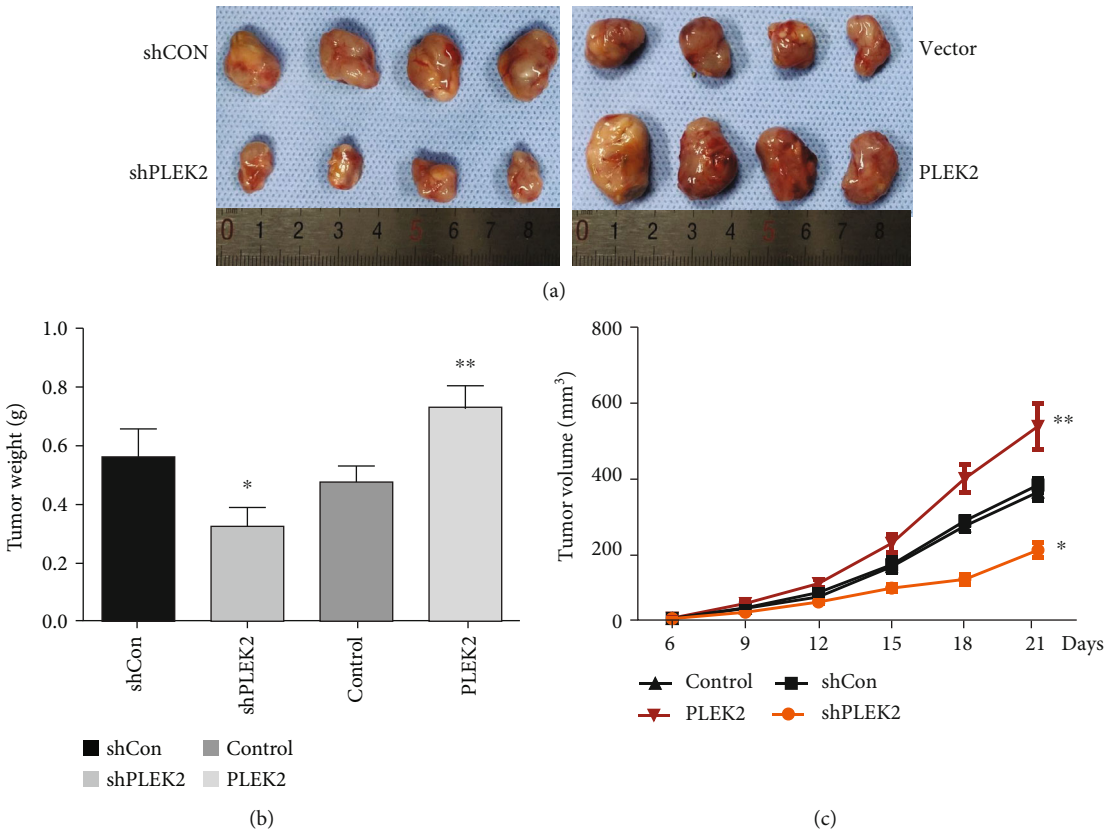


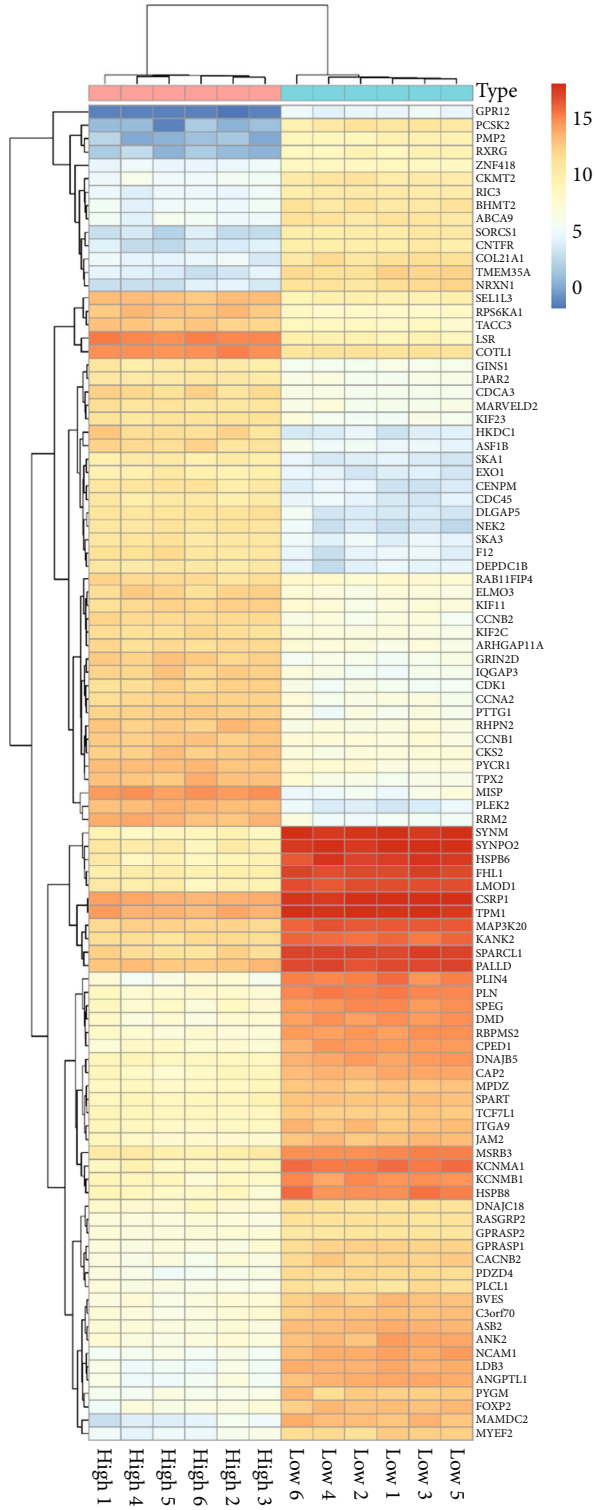

Figure 5: PLEK2 influenced gastric cancer tumour growth in a nude mouse model. (a) The inhibition of PLEK2 impaired gastric cancer tumour growth in a nude mouse model (left), while the overexpression of PLEK2 promoted gastric cancer tumour growth (right). The size of the tumour formed in the subcutaneous implantation mouse model was monitored every three days. (b) The mean tumour weights of each group. (c) Growth curves for tumour volumes at the indicated times.

Then, we evaluated the expression of PLEK2 by IHC staining in clinical gastric cancer tissues and found high expression in 46 cases (42.6%) and low expression in the other 62 cases (57.4%). PLEK2 showed no signal or a weak signal in peritumoural gastric tissues (Figure 2(b)). A chi-square test demonstrated that PLEK2 protein was prominently connected with the tumour grade ($P = 0.025$), tumour size ($P = 0.033$), lymph node invasion ($P = 0.045$), distant metastasis ($P = 0.011$), and advanced TNM stage ($P = 0.039$) (Table 1). Kaplan-Meier survival analysis also showed that high PLEK2 levels were closely related to poor overall survival (Figure 2(c)).

*3.3. Decreased PLEK2 Repressed the Proliferation and Migration of Gastric Cancer Cells.* To explore the effect of PLEK2 in human gastric cancer, we established stable PLEK2 knockdown models in MGC803 and SGC7901 cells by expressing short hairpin RNAs (shPLEK2). The knockdown of PLEK2 resulted in a significant reduction in the expression of PLEK2 RNA and protein levels, as revealed by real-time PCR and western blotting (Figure 3(a)). The downregulation of PLEK2 resulted in a remarkable decrease in cell proliferation, as shown by MTT assays (Figure 3(b)). The knockdown of PLEK2 significantly inhibited gastric cancer cell colony-forming abilities (Figure 3(c)). PLEK2 knockdown led to a significant decrease in cell migratory abilities (Figure 3(d)). Similarly, PLEK2 knockdown cells exhibited slower migration in the wound healing assays (Figure 3(e)). These results indicated that PLEK2 improves the self-renewal and migratory capacity of gastric cancer cells.

*3.4. Overexpression of PLEK2 Promoted Gastric Cancer Proliferation and Migration.* We upregulated PLEK2 expression in gastric cancer cells to determine its effects. The upregulation of endogenous PLEK2 in MGC803 and SGC7901 cells was confirmed at both the mRNA and protein levels (Figure 4(a)). As expected, the upregulation of PLEK2 accelerated gastric cancer cell proliferation in the MTT assay (Figure 4(b)). The overexpression of PLEK2 significantly promoted gastric cancer cell colony-forming abilities (Figure 4(c)). The overexpression of PLEK2 significantly improved gastric cancer cell migration in a Transwell assay (Figure 4(d)). Similarly, PLEK2-overexpressing cells more quickly migrated to fill in space in wound healing assays (Figure 4(e)).

*3.5. Inhibition of PLEK2 Effectively Suppresses Tumour Growth in Nude Mice.* We employed a nude mouse tumourigenicity assay to detect the functional roles of PLEK2 in gastric cancer tumourigenicity. Stable PLEK2-overexpressing




(a)

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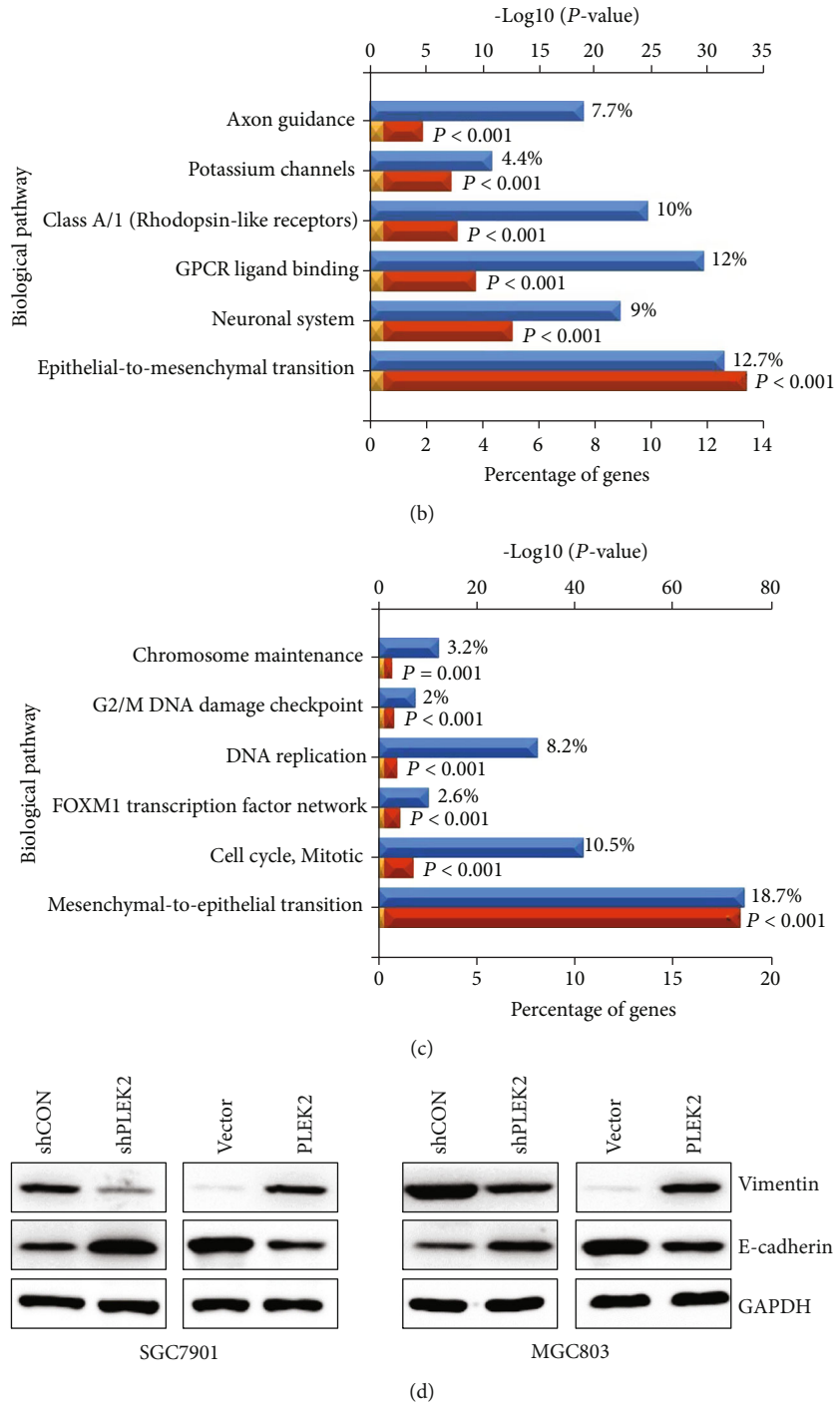


Figure 6: PLEK2 promotes EMT in gastric cancer cells. (a) The heat map shows the top 100 differentially expressed genes related to PLEK2. (b) Biological pathways enriched in genes that are more than 2-fold abundant in the PLEK2-high group compared to the PLEK2-low group. (c) Biological pathways enriched in genes that are more than 2-fold deficient in the PLEK2-high group compared to the PLEK2-low group. (d) PLEK2 overexpression induces hallmarks of EMT in gastric cancer cells, while PLEK2 knockdown inhibits EMT.

MGC803 cells showed remarkably improved tumour growth and tumour weight compared with those observed in the control groups. Conversely, the knockdown of PLEK2 significantly suppressed tumour growth and weight compared with the level of suppression in the nontarget shRNA control groups (Figure 5(a)). In addition, there was a difference in the size and weight of the tumours over time in the PLEK2 upregulation and downregulation groups (Figures 5(b) and 5(c)). Collectively, the results showed that PLEK2 may serve as an oncogene that enhances gastric cancer proliferation.

*3.6. PLEK2 Participates in EMT.* To reveal the potential mechanisms of PLEK2-mediated cell invasion and proliferation, we used TCGA database to compare the gene expression profiles

of the 6 PLEK2-highest and the 6 PLEK2-lowest databases. The top 100 differentially expressed genes are shown on the heat map in Figure 6(a). We further performed functional enrichment analysis using FunRich software. The biological pathways enriched in genes that are more than 2-fold abundant in the PLEK2-high group compared to the PLEK2-low group are displayed in Figure 6(b), and the biological pathways enriched in genes that are more than 2-fold deficient are shown in Figure 6(c). Most of the identified genes are already known to be involved in EMT. As the most significant pathways are related to EMT, we detected the level of Vimentin and E-cadherin in gastric cells after PLEK2 upregulation and downregulation. As shown in Figure 6(d), the knockdown of PLEK2 showed enhanced epithelial marker (E-cadherin) expression and decreased mesenchymal marker (Vimentin) expression. PLEK2 overexpression inhibited E-cadherin expression and induced Vimentin expression. The results showed that PLEK2 promoted EMT in gastric cancer cells.

## 4. Discussion

PLEK2 encompasses two PH domains and an intervening DEP domain, which control cytoskeletal reorganization in various cells. The DEP domain is in the center, and the two PH domains are in the amino- and carboxyl-terminals, which are important for PLEK2 colocalization with the actin cytoskeleton at the immune synapse and integrin clusters and involve a broad range of cellular functions [10–13]. Previous studies have shown that PLEK2 regulates actin dynamics and cofilin's mitochondrial localization during erythropoiesis [14, 15]. PLEK2 induces lamellipodia formation depending on its DEP domain. The DEP domain mutant disrupts the formation of lamellipodia and membrane ruffles [16, 17]. Unlike PLEK1, which is restricted to immune cells, PLEK2 has been identified in various adherent cell lines involved in cellular signalling and cytoskeleton organization [18, 19]. A study reported that PLEK2 is vital for the modulation of the actin cytoskeleton, cellular transdifferentiation, and prosurvival in the early stage of terminal erythroblasts and prevents early-stage terminal erythroblasts from oxidative damage [14, 20]. An important effect of PLEK2 in lineages of haematopoietic cells was discovered by modulating cytoskeleton organization and cell apoptosis through PLEK2's interplay with members of Rac1 signalling, such as cofilin. In another report, PLEK2 was found to be an effector of the JAK2/STAT5 pathway and an important regulator in the pathogenesis of JAK2V617F-induced myeloproliferative neoplasms, suggesting that PLEK2 is a feasible therapeutic target of myeloproliferative neoplasms [21]. Bach et al.'s group demonstrated that PLEK2, as an effector of PI3K, increases Jurkat cell spreading through both $\alpha 4\beta 1$ and the T-cell receptor [22]. However, the biological functions of PLEK2 in tumour progression have not been well defined, especially in gastric tumourigenesis.

Recent studies have found that pancreatic cancer tissues exhibited an enhancement of PLEK2 expression [23]. PLEK2 could promote the self-renewal and proliferation of pancreatic cancer stem cells. PLEK2 has been found to be overexpressed in the blood of melanoma patients in all stages of disease, suggesting its potential function as a liquid biopsy marker for early diagnosis [24]. PLEK2 also mediated vascular invasion and metastasis in non-small-cell lung cancer and gallbladder cancer [25, 26]. In this research, we studied the effect of PLEK2 in gastric cancer utilizing survival analysis, cell test in vitro, and nude mouse experiment in vivo. PLEK2 upregulation in gastric cancer cells increased cell migration, invasion, proliferation, and self-renewal in vitro and tumourigenesis in vivo, indicating that PLEK2 may have an oncogenic function in gastric cancer.

Now, bioinformatics tools are becoming important. Yang et al.'s group performed a comprehensive bioinformatics analysis on microarray data of myeloma cells. PLEK2 was identified as a potential therapeutic target [27]. In this study, we utilized FunRich to analyse the biological pathways related to PLEK2. The results revealed that the most significant biological pathway was EMT or mesenchymal-epithelial transition (MET). Further analysis confirmed the role of PLEK2 in promoting EMT. Previous studies reported that PLEK2 mRNA was upregulated in non-small-cell lung cancer cells with TGF-$\beta$-induced EMT, demonstrating the crucial role of PLEK2 in tumour invasion [25, 28]. We found that PLEK2 was preferentially accumulated in gastric cancer tissues and probably enhanced the EMT mechanism.

PLEK2 participates in actin reorganization in a PI3-kinase-dependent manner. PLEK2 has been previously reported to promote the migration of HCC2998 and COS-1 cells and T lymphocytes by binding with PI3K [20, 22]. Mounting evidences suggest that cytoskeleton reconstruction plays the crucial role in the EMT process. The dynamic reorganization of the actin cytoskeleton is a prerequisite for the morphology and invasive behaviour of cancer cells. In this study, we confirmed the role of PLEK2 in promoting EMT in gastric cancer cells. Further researches are needed to elucidate the mechanism underlying the role that PLEK2 plays in EMT regulation during cancer progression.

In summary, we discovered that PLEK2 was remarkably elevated in gastric cancer. Its upregulation functionally promoted the aggressiveness of gastric cancer cells by driving EMT. Our work suggests that targeting PLEK2 might be an effective therapeutic strategy to treat PLEK2-high gastric cancer.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Disclosure

The abstract of the study was published in Cancer Res 2020;80(16 Suppl):Abstract nr 361.

## Conflicts of Interest

The authors declare that there is no conflict of interests.

## Authors' Contributions

Jun Wang, Zhigang He, and Bo Sun contributed equally to this work.

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*Research Article*

# Clinical Outcomes of Ileostomy Closure before Adjuvant Chemotherapy after Rectal Cancer Surgery: An Observational Study from a Chinese Center

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*Background*. The optimal timing of temporary ileostomy closure with respect to the time of adjuvant chemotherapy following sphincter-saving surgery for rectal cancer remains unclear. The aim of this study is to investigate the clinical and oncological outcomes of ileostomy closure before, during, and after adjuvant chemotherapy following curative rectal cancer resection. *Methods*. Patients diagnosed with rectal adenocarcinoma who underwent low anterior resection and temporary loop ileostomy during May 2015 and September 2019 were retrospectively evaluated. Patients undergoing ileostomy closure before adjuvant chemotherapy (Group I) were compared to patients undergoing closure during (Group II) and after (Group III) adjuvant chemotherapy. *Results*. A total of 225 patients were evaluated for eligibility, and 132 were finally selected and divided into 3 groups (24 in Group I, 53 in Group II, and 55 in Group III). No significant differences were observed in operative time, postoperative hospital stay, postoperative complications, total adjuvant chemotherapy cycles, and low anterior resection syndrome scores among the three groups. There was no significant difference in disease-free survival ($p = 0.834$) and overall survival ($p = 0.462$) between the three groups. *Conclusion*. Temporary ileostomy closure before adjuvant chemotherapy following curative rectal cancer resection can achieve a clinical and oncological safety level equal to stoma closure during or after chemotherapy in selected patients.

## 1. Introduction

A temporary diverting ileostomy is frequently performed on patients who have had rectal cancer surgery to protect anastomosis, particularly for rectal cancer of the middle and lower third [1, 2]. Previous studies have demonstrated that patients with a temporary diverting ileostomy were at lower risk to suffer from anastomotic leakage, peritonitis, and their associated morbidity and mortality than those without it [3, 4].

There are some agreements among surgeons that ileostomy closure should not be performed earlier than 60-90 days [5, 6]. However, a diverting stoma has various adverse effects including stoma-related morbidities, physical stress, and psychological handicap, which affect the patients' quality of life [7, 8]. Therefore, patients suffering from considerable pressure are eager to close the diverting stoma after primary surgery; however, there are no strict recommendations for the optimal timing of ileostomy closure. Recently, several studies have addressed the safety and feasibility of early ileostomy closure (within 2 weeks after primary surgery) and reported promising results [9–11]. After rectal cancer surgery, the outcomes of early versus late closure of loop ileostomy have been studied; however, the outcomes remain controversial with respect to adjuvant chemotherapy. Studies showed that temporary ileostomy closure before [12, 13] or during [14–17]

adjuvant chemotherapy following rectal cancer surgery had similar outcomes to the closure of ileostomy after adjuvant chemotherapy. On the contrary, other studies showed that a shorter interval between primary surgery and ileostomy closure may negatively affect the completeness of chemotherapy resulting from stoma-related complications such as anastomotic leakage or incomplete anorectal function [18–20]. In addition, stoma closure before or during adjuvant chemotherapy may induce the delay or interruption of chemotherapy, which may alter the effects of chemotherapy [21, 22].

Considering the controversies regarding the optimal timing of temporary ileostomy closure with respect to the time of delivering adjuvant chemotherapy, our goal was to investigate the outcomes of ileostomy closure before, during, and after adjuvant chemotherapy following curative rectal cancer surgery.

## 2. Method

*2.1. Patients and Interventions.* Patient data was extracted from a prospectively collected colorectal cancer database retrospectively. Patients diagnosed with rectal adenocarcinoma who received low anterior resection with total mesorectal excision (TME) and temporary loop ileostomy closure between May 2015 and September 2019 were included. Informed consent had been obtained from individual patients, and the present study had been approved by the institutional review board of the hospital.

Exclusion criteria included patients with stage IV rectal cancer and postoperative radiotherapy or patients who suffered complications of anastomosis from the index surgery. Patients who did not undergo adjuvant chemotherapy were also excluded.

We divided our patients into three groups: Group I, Group II, and Group III, who underwent ileostomy closure before, during, and after chemotherapy, respectively. The demographics and clinical characteristics were compared between the groups.

The anastomosis site was assessed with contrast enema, abdominopelvic computed tomography, or/and colorectal endoscopy to ensure the safety of the ileostomy closure. The ileostomy closure technique included handsewn or stapled ileo-ileal anastomosis, which was left to the discretion of the surgeon. Eight different gastrointestinal surgeons working in the same institution performed all procedures.

*2.2. Outcomes.* The compared parameters included details of surgical procedures, demographics, clinical characteristics, stoma closure, length of hospital stay, information of adjuvant chemotherapy, complications related to the stoma formation, prevalence of low anterior resection syndrome (LARS), disease recurrence, and survival.

The stoma formation-related complications were extracted retrospectively by inspection of patient charts filled in by stoma care therapists. High volume output was defined as the combination of stoma content output of more than 1000 ml and electrolyte disturbance. Skin irritation included parastomal infection, rash, pain, or ecchymoma. Stoma closure-related complications were assessed within 30 days after ileostomy closure.

The overall survival (OS) was presented by the interval between index surgery and death, while the disease-free survival (DFS) was defined by the interval between index surgery and the date of the first recurrence (local and/or distant). Data on patients who were disease-free were censored from the date of the last follow-up until May 2020. Meanwhile, a LARS score questionnaire [23] was completed by the follow-up telephone calls to the surviving participants. The associated response categories were based on the frequency of symptom occurrence or number of bowel motions. A LARS score of 0–20 was interpreted as no LARS; 21–29, minor LARS; and 30–42, major LARS [23].

*2.3. Statistical Analysis.* Continuous parameters were presented as the mean and standard deviation and were further analyzed using one-way analysis of variance. The categorical parameters were described using percentages, the chi-squared test, or the Fisher exact test. The survival curve between groups regarding OS and DFS was calculated by Kaplan-Meier analysis and compared with the log-rank test. SPSS statistical software version 19.0 (SPSS Inc., Chicago, IL, USA) for Windows and GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) were applied for all data analyses. $p < 0.05$ was considered statistically significant.

## 3. Results

A total of 225 patients were evaluated for eligibility, and 132 were included and divided into three groups in the present study: Group I consisted of 24 patients and underwent ileostomy closure before chemotherapy; Group II consisted of 53 patients and underwent it during chemotherapy; and Group III consisted of 55 patients and underwent it after chemotherapy (Figure 1). The demographics and clinicopathologic characteristics among the three groups were comparable (Table 1). The preoperative BMI (body mass index) of patients in Group III was higher, and the difference nevertheless was not statistically significant.

The mean duration of the diverting stoma was $25.9 \pm 5.3$ days in Group I, $119.3 \pm 47.5$ days in Group II, and $202.3 \pm 93.8$ days in Group III ($p < 0.0001$) (Table 2). Intraoperative features such as the operative time and anastomosis method were comparable among the three groups. Notes about unexpected difficulties including mobilization of ileum adhesion or mobilization of ileostomy from the abdominal were described more often in Group I than the other two groups (29.2% in Group I vs. 11.3% in Group II and 9.1% in Group III, $p = 0.047$). No significant difference was observed in the interval for resuming diet, passing of gas, postoperative 30-day mortality, reoperation rate, and total adjuvant chemotherapy. The postoperative hospital stay was longer, but not statistically significant, in Group I than the others ($8.6 \pm 2.2$ days in Group I vs. $7.5 \pm 2.5$ days in Group II and $7.5 \pm 2.1$ days in Group III, $p = 0.121$). The mean interval between index surgery and first adjuvant chemotherapy was $51.5 \pm 7.0$ days in Group I, $33.8 \pm 12.4$ days in Group II, and $30.2 \pm 9.7$ days in Group III ($p < 0.0001$).

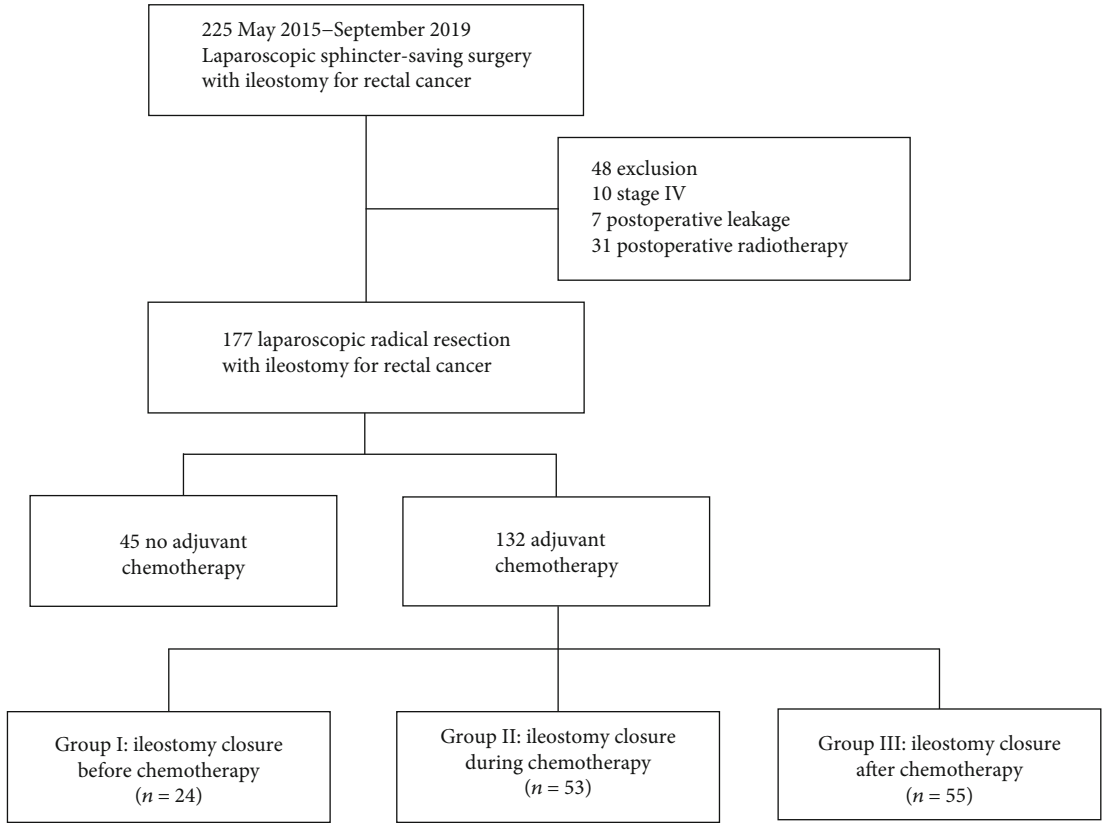


Figure 1: Flowchart of patients included in the study.

Overall, no significant difference was detected among the three groups regarding the incidence of stoma formation-related complications. Six (10.9%) patients in Group III had skin irritation after stoma formation, significantly higher than Group I (4.2%) and Group II (0.0%) ($p = 0.039$) (Table 3). One patient from Group I underwent stoma closure during the index surgery admission period for stoma prolapse and skin irritation. None of the patients required emergency surgery due to high volume output or postoperative ileus. Overall, the incidence of stoma closure-related complications was 20.8% in Group I, 13.2% in Group II, and 12.7% in Group III, which was not statistically different. No fistula and anastomotic leakage were observed among all three of the groups. The wound infection rate was higher but not significant in Group I (12.5%) than in Group II (7.5%) and Group III (9.1%) ($p = 0.783$). Two patients in Group III and one patient in Group II underwent reoperation due to incisional hernia on the stoma closure site 1 month after ileostomy closure. In addition, one patient in Group I underwent reoperation for postoperative ileus who did not succeed in conservative treatment.

126 of the original 132 participants were available in the survival analysis. The average follow-up period in Group I was $731 \pm 332$ days; in Group II, $977 \pm 399$ days; and in Group III, $897 \pm 389$ days. No differences were found in OS and DFS among the three groups ($p = 0.462$ for OS and $p = 0.834$ for DFS) (Figure 2).

The LARS score questionnaire was completed by all 106 surviving patients among the 126 participants for survival analysis. The demographics and clinicopathologic characteristics between the three groups among these participants were comparable. Overall, the incidence of LARS was 50.0% in Group I, 54.7% in Group II, and 47.7% in Group III. The median LARS scores for Groups I-III were 17 (interquartile range 6-29), 24 (14-32), and 20 (8-31), respectively ($p = 0.282$). No significant differences were noticed regarding incontinence to feces and flatus, increased stool frequency, clustering, and urgency among the three groups (Table 4).

## 4. Discussion

In this study, we compared the postoperative morbidity and mortality of ileostomy closure before, during, and after adjuvant chemotherapy after the curative rectal cancer resection and tried to find out whether survival rates and recurrence are associated with the time until closure. We found that in selected patients, temporary ileostomy closure before adjuvant chemotherapy could achieve a clinical safety level equal to stoma closure during or after chemotherapy in terms of postoperative complication, LARS, and oncological prognosis.

There is uncertainty about the timing of the closure of ileostomy since most patients with rectal cancer are likely to receive postoperative adjuvant chemotherapy. The interval between creation and closure of the temporary stoma is often delayed in rectal cancer patients who had received adjuvant chemotherapy [24–26]. These patients suffered more stoma-related complications before closure which negatively

Table 1: Demographics and clinicopathologic characteristics of the study groups.

| | Group I ($n = 24$) | Group II ($n = 53$) | Group III ($n = 55$) | $p$ value |
|---|---|---|---|---|
| Sex | | | | |
| Male | 16 (66.7%) | 41 (77.4%) | 32 (58.2%) | 0.104 |
| Female | 8 (33.3%) | 12 (22.6%) | 23 (41.18%) | |
| Age (years) | 57.2 ± 9.7 | 56.3 ± 10.0 | 53.7 ± 11.2 | 0.304 |
| BMI* ($kg/m^2$) | 21.1 ± 2.4 | 22.3 ± 3.4 | 22.8 ± 2.7 | 0.073 |
| Comorbidity | | | | |
| Cardiovascular diseases | 2 (8.3%) | 8 (15.1%) | 12 (21.8%) | 0.310 |
| Diabetes | 0 (0.0%) | 6 (4.5%) | 3 (2.3%) | 0.165 |
| Pulmonary diseases | 1 (4.2%) | 1 (1.9%) | 1 (1.8%) | 0.789 |
| Renal diseases | 0 (0.0%) | 1 (1.9%) | 2 (3.6%) | 0.590 |
| ASA class | | | | 0.961 |
| I | 3 (12.5%) | 5 (9.4%) | 4 (7.3%) | |
| II | 19 (79.2%) | 44 (83.0%) | 47 (85.5%) | |
| III | 2 (8.3%) | 4 (7.5%) | 4 (7.3%) | |
| Type of surgery | | | | 0.994 |
| Laparoscopic | 23 (95.8%) | 51 (96.2%) | 53 (96.4%) | |
| Conversion to open | 1 (4.2%) | 2 (3.8%) | 2 (3.6%) | |
| cTNM stage | | | | 0.706 |
| I | 1 (4.2%) | 5 (5.7%) | 18 (7.3%) | |
| II | 5 (20.8%) | 17 (32.1%) | 33 (21.8%) | |
| III | 18 (75.0%) | 33 (62.3%) | 39 (70.9%) | |
| ypTNM stage** | | | | 0.471 |
| 0 | 1 (4.2%) | 0 (0.0%) | 1 (1.8%) | |
| I | 2 (8.3%) | 4 (7.5%) | 9 (16.4%) | |
| II | 12 (50.0%) | 25 (47.2%) | 28 (50.9%) | |
| III | 9 (37.5%) | 24 (45.3%) | 17 (30.9%) | |
| Neoadjuvant CRT | 10 (41.7%) | 19 (35.8%) | 25 (45.5%) | 0.595 |
| Tumor location*** (cm) | 8.2 ± 3.8 | 7.5 ± 3.6 | 8.0 ± 3.7 | 0.698 |
| 3-5 | 9 (37.5%) | 22 (41.5%) | 16 (29.1%) | 0.597 |
| 6-10 | 9 (37.5%) | 23 (43.4%) | 27 (49.1%) | |
| 11-15 | 6 (25.0%) | 8 (15.1%) | 12 (21.8%) | |

Values are presented as the mean ± standard deviation or number of patients (%). *Measured before ileostomy closure. **Pathological stage according to UICC. ***Tumor lower border from the anal verge. BMI: body mass index; ASA: American Society of Anesthesiologists; UICC: Union Internationale Contre le Cancer; CRT: chemoradiotherapy.

impact their quality of life [7, 8, 27]. Whether the closure of a temporary stoma can be done before or during adjuvant chemotherapy instead of after needs clear guidelines. Thalheimer et al. [12] found that fewer complications happened in the cases of ileostomy closure performed before (12.5%) the start of adjuvant chemotherapy or radiochemotherapy, rather than during (42.9%) or after (21.2%). They speculated that the higher complication rates in the latter two groups might be because of the patients' compromised general physical condition during and after the adjuvant therapy lasting up to 6 months, such as decreased wound healing capacity. Furthermore, Lordan et al. [24] pointed out that it would be feasible to close the temporary stoma before starting adjuvant therapy because most times the postoperative therapy is not initiated in 2-3 weeks after the anterior resection. This would decrease the incidence of stoma-related complications and avoid a long delay in closure after adjuvant therapy. Recently, Kłęk et al. [13] reported that ileostomy closure performed in advance of adjuvant chemotherapy was safe and should be considered part of the enhanced recovery after surgery (ERAS) protocol. In our study, we found that stoma formation-related complication rates in Group III were higher than those in Group I or Group II. In particular, the patients in Group III suffered more skin irritation than those in the other two groups. Therefore, patients suffering from considerable pressure tend to choose to close the diverting stoma at an early stage.

In the present study, pairwise comparisons among all groups indicated that there were no significant differences between them in the stoma closure-related complications, reoperation rate, and mortality. Some notes about unexpected difficulties were described more often in Group I than

Table 2: Details of loop ileostomy closure.

| | Group I ($n = 24$) | Group II ($n = 53$) | Group III ($n = 55$) | $p$ value |
|---|---|---|---|---|
| Interval to ileostomy closure (day) | 25.9 ± 5.3 | 119.3 ± 47.5 | 202.3 ± 93.8 | <0.001 |
| Operative time (min) | 139.8 ± 30.8 | 132.2 ± 26.9 | 130.3 ± 25.0 | 0.344 |
| Anastomosis (ileo-ileal anastomosis) | | | | 0.613 |
| Handsewn | 14 (58.3%) | 37 (69.8%) | 36 (65.5%) | |
| Stapled | 10 (41.7%) | 16 (30.2%) | 19 (34.5%) | |
| Noted unexpected difficulties during surgery | 7 (29.2%) | 6 (11.3%) | 5 (9.1%) | 0.047 |
| Time until passing of gas (day) | 3.1 ± 0.7 | 3.0 ± 0.6 | 2.8 ± 0.6 | 0.069 |
| Time until fully oral nutrition (day) | 2.8 ± 1.1 | 2.6 ± 0.7 | 2.4 ± 0.7 | 0.108 |
| Stoma formation-related complications | 3 (13.0%) | 9 (17.0%) | 11 (20.0%) | 0.755 |
| Stoma closure-related complication | 5 (20.8%) | 7 (13.2%) | 7 (12.7%) | 0.609 |
| Postoperative 30-day mortality | 0 (0%) | 0 (0%) | 0 (0%) | NA |
| Reoperation | 1 (4.2%) | 1 (1.9%) | 2 (3.6%) | 0.815 |
| Hospital stay after closure (day) | 8.6 ± 2.2 | 7.5 ± 2.5 | 7.5 ± 2.1 | 0.121 |
| Total adjuvant chemotherapy (cycle) | 5.2 ± 2.0 | 5.7 ± 1.4 | 4.9 ± 2.0 | 0.060 |
| Interval between index surgery and 1st chemotherapy (day) | 51.5 ± 7.0 | 33.8 ± 12.4 | 30.2 ± 9.7 | <0.001 |

Values are presented as the mean ± standard deviation or number of patients (%). NA: not available.

Table 3: Stoma-related complications.

| | Group I ($n = 24$) | Group II ($n = 53$) | Group III ($n = 55$) | $p$ value |
|---|---|---|---|---|
| Stoma formation-related complications | 3 (13.0%) | 9 (17.0%) | 11 (20.0%) | 0.755 |
| High volume output | 1 (4.2%) | 5 (9.4%) | 5 (9.1%) | 0.715 |
| Adhesive ileus | 1 (4.2%) | 4 (7.5%) | 3 (5.5%) | 0.822 |
| Parastomal hernia | 1 (4.2%) | 0 (0%) | 2 (3.6%) | 0.353 |
| Prolapse | 1 (4.2%) | 0 (0%) | 0 (0%) | 0.104 |
| Skin irritation | 1 (4.2%) | 0 (0%) | 6 (10.9%) | 0.039 |
| Stoma closure-related complications | 5 (20.8%) | 7 (13.2%) | 7 (12.7%) | 0.609 |
| Wound infection | 3 (12.5%) | 4 (7.5%) | 5 (9.1%) | 0.783 |
| Adhesive ileus | 2 (8.3%) | 2 (3.8%) | 1 (1.8%) | 0.378 |
| Fistula/anastomotic leakage | 0 (0%) | 0 (0%) | 0 (0%) | NA |
| Incisional hernia | 0 (0%) | 1 (1.9%) | 2 (3.6%) | 0.590 |

Values are presented as the number of patients (%). NA: not available.

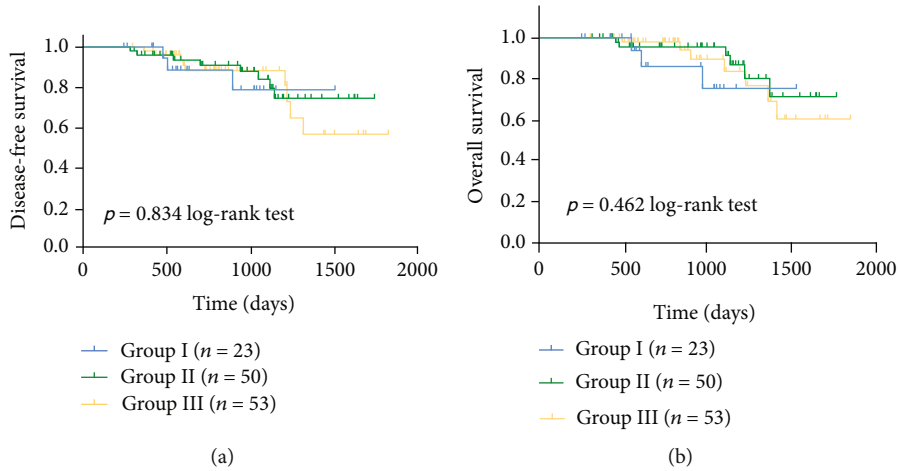


Figure 2: Kaplan-Meier curves of survival between the three groups: (a) disease-free survival and (b) overall survival.

Table 4: Details of LARS at follow-up 12 months after index surgery.

| | Group I ($n = 20$) | Group II ($n = 42$) | Group III ($n = 44$) | $p$ value |
|---|---|---|---|---|
| 12-month median LARS scores (IQR) | 17 (6-29) | 24 (14-32) | 20 (8-31) | 0.282 |
| Major LARS, $n$ (%) | 5 (25.0%) | 14 (33.3%) | 14 (31.8%) | 0.796 |
| Minor LARS, $n$ (%) | 5 (25.0%) | 9 (21.4%) | 7 (15.9%) | 0.660 |
| Incontinence to feces, $n$ (%) | 8 (40.0%) | 19 (45.2%) | 15 (31.4%) | 0.572 |
| Flatus, $n$ (%) | 1 (5.0%) | 3 (7.1%) | 5 (11.4%) | 0.644 |
| Increased stool frequency, $n$ (%) | 10 (50.0%) | 28 (66.7%) | 21 (47.7%) | 0.179 |
| Clustering, $n$ (%) | 14 (70.0%) | 36 (85.7%) | 30 (68.2%) | 0.138 |
| Urgency, $n$ (%) | 10 (50.0%) | 27 (64.3%) | 26 (59.1%) | 0.563 |

Values are presented as the number of patients (%). LARS: low anterior resection syndrome; IQR: interquartile range.

the other two groups. This could be because the collagen synthesis and the inflammatory process remain active until 4 weeks after index surgery [28]. In addition, preoperative radiotherapy induced inflammation and fibrosis needs time to regenerate and absorb [29] which could also explain a higher peristomal adhesion in Group I patients. Studies focusing on the timing of ileostomy closure after the neoadjuvant chemoradiotherapy (CRT) or total neoadjuvant treatment (TNT) in locally advanced rectal cancer need further investigation. In spite of this, the time of operation, passing of gas, fully oral nutrition, and postoperative hospital stay were similar among the three groups indicating that the peristomal adhesion is no longer an obstacle with the enhanced surgical technique.

Wound infection is a relatively common stoma closure-related complication. In the present study, we defined wound infection as the redness or tenderness of the surgical wound with the discharge of pus [30]. We found that wound infection after stoma closure was higher but not significant in Group I (12.5%) than in Group II (7.5%) and Group III (9.1%) ($p = 0.783$). One meta-analysis reported that the wound infection rate was 15.5% in patients who reversed their stoma within 2 weeks and 5.3% in patients who reversed their stoma at least 8 weeks after rectal surgery [11]. These results could be explained by the reduction of the recovery or immunity of patients in the immediate postoperative period, which leads the host susceptible to infectious complications through diverse cytokine activities [9, 31]. In addition, preoperative nutritional status is also one of the major risk factors for wound infection in patients undergoing abdominal surgery [30]. In the present study, the BMI and preoperative albumin level in Group I were lower than those in the other two groups which also might explain the higher frequency of wound infection in Group I. Therefore, to improve clinical outcomes, clinicians should reduce wound infection through preoperative nutrition support, accounting for the purse-string skin closure technique [14] in early stoma closure patients.

It is recommended to begin adjuvant chemotherapy up to 8 weeks from the date of colorectal cancer surgery [21]. Recently, surgeons [9–11] have claimed that early ileostomy closure within 2 weeks is safe, providing the patients with enough time to recover and undergo adjuvant therapy [13]. In the present study, the mean interval between index surgery and first adjuvant chemotherapy was $51.5 \pm 7.0$ days in Group I, suggesting that patients in the early ileostomy closure group did not significantly exceed recommended duration between radical surgery and the start of chemotherapy. In addition, our results showed that no significant differences were found in OS and DFS among the three groups, thus indicating that early stoma closure before adjuvant chemotherapy did not affect oncological outcomes [15, 17]. However, some surgeons and oncologists are reluctant to stoma closure before adjuvant chemotherapy because it is associated with a 17% postoperative morbidity rate [32], which may affect the initiation of adjuvant chemotherapy. Although the clinical and oncological outcomes are comparable among groups in the present study, the mean duration of the diverting stoma is $25.9 \pm 5.3$ days in Group I which is still relatively longer than other studies [9–11]. In order to start the adjuvant treatment in time, the ileostomy closure time needs to be shortened. In the future, our center will initiate a randomized controlled study in which the safety and feasibility of ileostomy closure will be evaluated 14 days after index surgery. In addition, a method to reduce postoperative morbidity should also be considered to shorten the interval of the ileostomy closure and the first chemotherapy. Two patients in Group I wanted more recovery time because of the postoperative ileus. The delay of adjuvant chemotherapy in these patients should be taken into account, and excessive delay should be prevented in adjuvant treatment. Among the 3 patients that required treatment for an ileostomy closure wound, the adjuvant chemotherapy was about 1 week delayed. In such cases, chemotherapy might not be delayed if the preoperative nutrition support or purse-string skin closure technique was taken into account. In addition, patients who underwent postoperative radiotherapy were not included in the present study because such treatment strategy may increase the risk of anastomotic leakage [33] and prolong the stoma closure time. In the future, ileostomy closure before chemotherapy should only be proposed for carefully selected patients without any signs of anastomotic leakage and uneventful postoperative outcomes. Therefore, in order to improve this selection process, another study to determine the risk factors for complications after loop ileostomy closure following sphincter-saving surgery with respect to adjuvant chemotherapy is ongoing in our center.

LARS is frequently reported in patients with rectal cancer who received TME with low colorectal anastomosis. The disordered defecatory function during LARS, such as increased stool frequency, clustering, urgency, and incontinence to feces and flatus after sphincter-saving procedures, negatively affects the patient's quality of life [34]. Opinions on the effect of a diverting stoma on the incidence and severity of anorectal functional alterations after anterior resection are controversial. In addition, the relationship between the timing of ileostomy closure and LARS is rarely reported. The pathophysiology leads us to believe that the disuse colitis and delayed restoration of bowel continuity may result in alterations in colonic nutrition, causing inflammation, changes in the bacterial flora and irreversible colon, and rectal atrophy of motility or sensory elements [23, 35, 36]. Recently, a prospective randomized controlled trial found earlier closure of ileostomy after anterior resection had a better LARS score than the later closure group, although the difference was not statistically significant. Several noncontrolled studies have also shown an association between the use of a diverting stoma and LARS in univariate analysis, indicating that the timely restoration of bowel continuity might avoid the irreversible colon and rectal atrophy and reduce the incidence of LARS [37–39]. However, confounding factors such as age, gender, tumor location, and perioperative radiotherapy need to be considered when interpreting these results. Notably, some other studies have found no difference in anorectal function between patients with and without a temporary defunctioning stoma [40, 41]. In the present study, the median LARS score in Group I was better than that in the other two groups, indicating that the early ileostomy closure might improve functional outcomes in these patients. Alternatively, a temporary loop ileostomy closure before adjuvant chemotherapy is comparable with the closure of ileostomy during or after adjuvant chemotherapy regarding the anorectal function. The controversial results mentioned above suggest that it is essential to apply powered prospective randomized studies to evaluate definitively whether early closure of an ileostomy could decrease the development of LARS.

Our study had some limitations, namely, because it is retrospective. Next, a lack of randomization to different groups creates bias. Surgeon preference and patient desire affect this selection. While some surgeons in this study were used to perform ileostomy closure after completion of chemotherapy, other surgeons choose to close an ileostomy during or before chemotherapy. Patient status such as systemic illness, variables during index surgery, or stoma formation-related complications may also play an important role in affecting this selection. In addition, a surgeon's decision is compromised due to a patient's strong desire to close the stoma. Nevertheless, our study has its advantages due to the sparse literature available reporting the safety of ileostomy closure before adjuvant chemotherapy regarding postoperative complications, which provides some hints for clinicians to make better clinical decisions on the optimal timing of ileostomy closure with respect to adjuvant chemotherapy. Furthermore, this is the first study comparing the effect of ileostomy closure before and during or after adjuvant chemotherapy on oncologic outcomes.

In conclusion, our findings suggest that after colorectal cancer resection, performing temporary loop ileostomy closure before adjuvant chemotherapy has comparable effects with the closure of ileostomy during or after adjuvant chemotherapy in terms of postoperative complication, LARS, and oncological prognosis in selected patients. A well-planned larger-scale, randomized, controlled trial with a long follow-up should be performed to accurately define which individuals stand to benefit from early closure of ileostomy before adjuvant chemotherapy and to assess this strategy with regard to the quality of life and compliance of adjuvant chemotherapy.

## Data Availability

Data are available on request from the authors.

## Ethical Approval

This trial was approved by the institutional ethical review board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

## Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

## Authors' Contributions

ZS and LL developed the conception and designed the study. ZS and YZ collected the data. ZS did the analysis and interpretation of data. ZS, LL, and JQ conceptualized and wrote the manuscript. The grant given to ZS financed this study. All the authors approved the version to be published.

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*Research Article*

# lncRNA GAU1 Induces GALNT8 Overexpression and Potentiates Colorectal Cancer Progression

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lncRNA is a key epigenetic regulator in biological processes. In the human cancer transcriptome library MiTranscriptome, we identified *GAU1* as the top upregulated lncRNA in colorectal cancer (CRC) by sample set enrichment analysis (overexpression ranking percentile = 99.75%, $P < 10^{-50}$), which is coexpressed with the potential oncogene *GALNT8* (Spearman rho = 0.67, $P = 2.44 \times 10^{-23}$, TCGA dataset $n = 184$). Experimental data revealed that *GAU1* regulates the expression of *GALNT8*. The overexpression of either *GAU1* or *GALNT8* significantly promotes the cell cycle and proliferation of CRC cell lines and correlates with poor prognosis in patients with CRC ($P = 3.04 \times 10^{-2}$), while silencing of *GAU1* or *GALNT8* suppressed the cancer cell proliferation and induced the CRC cell line resistance to oxaliplatin *in vitro* treatment. Our results suggested that the previously less studied *GAU1* and *GALNT8* may play as CRC prognosis markers and potential targets for chemotherapy treatment.

## 1. Introduction

Colorectal cancer (CRC) ranked the third common type of cancer, adding up 10% of all cases [1]. In 2018, there were over one million new cases and over half million deaths from the disease [2]. Genetic mutations in *APC* [3], *TP53* [4], and *K-RAS* [5] have been intensively studied as major contributors to the tumorigenesis of CRC. Besides, nongenetic risk factors like aging and lifestyle also induce the development of CRC cases. However, this nonmutational alteration in CRC was less studied [6]. Massive parallel sequencing facilitated the genome-wide characterization of the human cancer transcriptome and identified long noncoding RNA (lncRNA) expression as the most common transcriptional alteration in cancer [7]. Our previous reports revealed that lncRNAs are extensively involved in the CRC development [8] and drug resistance [9], indicating that more efforts should be encouraged to identify the CRC-specific lncRNA expression and to link the biological "operator" regulating these noncoding "regulators."

RNA-Seq technology empowered by sequence alignment and assembly provides a revolutionary approach for the prediction of full-length transcripts from both the intergenic "gene desert" and protein-coding loci [10, 11]. The MiTranscriptome database applied ab initio assembly to 7,256 curated RNA-Seq libraries from tumor, normal tissue, and cell lines so as to provide an unbiased method for gene discovery [12]. Here, by incorporating this ab initio assembly-based human cancer transcriptome database and experimental validation, we identified a colorectal cancer-related lncRNA *GAU1* from 12,382 cancer-associated lncRNA transcripts and verified its pro-cancer function as upregulating the mRNA expression of polypeptide N-acetylgalactosaminyl transferase GALNT8, whose overexpression correlates with the cancer cell proliferation and poor patient survival.

## 2. Materials and Methods

*2.1. Identification of GAU1 as the CRC-Related lncRNA.* The normalized counts of 12,382 ab initio-assembled lncRNA transcripts and library information of 6,476 RNA-Seq libraries (5,724 cancer-related samples and 752 normal samples) including 5,602 TCGA cases were downloaded from the MiTranscriptome website (http://MiTranscriptome.org/download/MiTranscriptome.expr.counts.tsv.gz).

Sample set enrichment analysis (SSEA) [12] was performed to test if a transcript is differentially expressed between the cancer and noncancer samples in an empirical ranking method. In brief, a weighted KS test was performed as gene set enrichment analysis (GSEA) [13] to generate the enrichment score (ES) describing the enrichment of the sample set among all tested samples. SSEA was further performed 1,000 times with random permutation of the ample labels for a set of null ES and the nominal $P$ value of relative rank of observed ES within the null ES. The hypothesis testing was performed by comparing the tested ES to the null normalized enrichment score (NES) for all transcripts in a sample set. SSEA percentile score was generated by ranking the transcripts in each analysis by their NES. The tissue-type information of each transcript was obtained from the MiTranscriptome browser (http://MiTranscriptome.org).

To perform *GAU1* coexpression analysis, the normalized RSEM-FPKM mRNA expression of 382 TCGA CRC samples was obtained from TCGA firehose legacy (https://gdac.broadinstitute.org/). After sample overlapping with the MiTranscriptome database, Spearman's rank correlation coefficient of *GAU1* and all 19,815 protein-coding gene mRNA expression was calculated in 184 TCGA CRC samples.

*2.2. Clinical Samples and Tissue Microarray.* Primary CRC tissues and paired adjacent tissues were collected from 66 CRC patients. All these samples were obtained between 2015 and 2017 and stored at -80°C.

Tissue microarrays (TMAs) with 55 paired cases of CRC and adjacent nontumorous tissues, plus 14 individual CRC tissues, were obtained from Shanghai Tenth Hospital (Shanghai, PR China). These CRC specimens were collected from CRC patients between 2010 and 2015 and followed until April 2019. No patient received chemotherapy or radiation before surgery, and no other concurrent cancer was observed in the patients. Both the Institutional Review Boards of Shanghai Tenth Hospital and Huashan Hospital, Fudan University, approved our study in compliance with Helsinki Declaration of 1975 as revised in 1996. All patients signed the informed consent before surgical operation. The clinical stages were classified by the American Joint Committee on Cancer and Union for International Cancer Control (AJCC/UICC) classification system [14]. Overall survival (OS) is defined as the time interval between the date of surgery and death.

*2.3. Cell Culture and Stable Cell Line Establishment.* Human embryonic kidney cell line HEK293T and human colon/rectum cancer cell lines LoVo, DLD1, SW620, and HCT116 were purchased from Shanghai Institute of Biological Sciences. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, CA, USA) with 10% FBS (Gibco) at 37°C in an atmosphere of 5% $CO_2$.

The *GALNT8* ORF sequence (NM_017417.2) and *GAU1* (NR_110112.1) were cloned by reverse-transcriptional PCR from human mRNA and were further integrated into the lentiviral expression vector pCDH (Addgene, #72265) to develop pCDH-GALNT8 and pCDH-GAU1 recombinant plasmid. Lentivirus with *GALNT8* or *GAU1* overexpression vector or pCDH control vehicle was packaged with packaging plasmid psPAX2 (Addgene, #12260) and envelope plasmid pMD2.G (Addgene, #12259) using Lipofectamine 2000 (Invitrogen, #11668019) in HEK293T. Stable cell lines overexpressing *GALNT8*, *GAU1*, or vehicle control were established with SW620 and HCT116 cell lines by lentivirus infection.

*2.4. siRNA Interference.* siRNAs targeting *GALNT8* (siGALNT8), *GAU1* (siGAU1), and nonsense scramble (siNS) were purchased from Tuoran Biotech (Shanghai, China). The siRNA sequence is as follows: siGAU1-1: 5′-CCAAGA ACUUCGGAAGCAUTT-3′, siGAU1-2: 5′-CCAGCUUAC ACGUCAGCUUTT-3′, siGALNT8-1: 5′-CUCGAUUGU UGAAGGAAAU-3′, siGALNT8-2: 5′-GCUCACAGAAU GUCUACUA-3′, and siNS: 5′-UCCTAAGGUUAAGUCG CCUC-3′. siRNA transfection of LoVo, DLD1, and their derived *GAU1*-overexpressing cells or vehicle control cells was undertaken with Lipofectamine RNAiMax (Invitrogen, #13778150). All the cancer cell line transfection was performed 48 hours before further experimental usage.

*2.5. Cell Proliferation and Cell Cycle Assay.* Cancer cell lines were seeded $1 \times 10^3$ per well in the 96-well plate. The cell proliferation was assessed by Cell Counting Kit-8 (CCK8, MCE, #HY-K0301) every 24 hours for 5 days. The colony formation ability of cancer cell lines was measured by 0.1% crystal violet/methanol staining 10 days after cell seeding in six-well plates at $1 \times 10^3$ per well density. Any colony that contains more than 50 cells was counted.

Cell cycle analysis was performed with Propidium Iodide (PI) staining. A total of $10^6$ cells were rinsed twice with cold PBS, then fixed with 75% ethanol overnight at -20°C, rinsed three times with PBS, and resuspended with 0.5 ml FxCycle™ PI/RNase Staining Solution (Life Technologies, #F10797). Keep the cell suspension for 15 min in the dark, and immediately subject to flow cytometry analysis on a FACSCanto system (BD Biosciences).

*2.6. Quantitative Real-Time PCR.* Trizol reagent (Invitrogen) was used for total RNA of tissues or cell extraction. Reverse transcription was performed with PrimeScript™ RT Reagent Kit (TaKaRa Biotechnology, #RR047A). Quantitative real-time PCR was conducted with TB Green Premix (TaKaRa Biotechnology, #RR820A) and gene-specific primers (Table 1) on an Applied Biosystems 7500 system (ABI); $\beta$-actin was used as a mRNA expression housekeeping gene (Table 1). Relative expression of *GALNT8* and *GAU1* was calculated with the $2^{-\Delta\Delta Ct}$ method.

Table 1: Primer sequences for gene amplification.

| Gene | Strand | Sequences (5′-3′) |
|---|---|---|
| GALNT8 | Forward | ACGCCCTCTCGATTGTTGAA |
| | Reverse | CTCTGCCCACCCAACATTGA |
| GAU1 | Forward | GCCCTTCCCAAAGCACAAAT |
| | Reverse | AGCACGTTAAGAGGCTTGGA |
| $\beta$-Actin | Forward | TTGTTACAGGAAGTCCCTTGCC |
| | Reverse | ATGCTATCACCTCCCCTGTGTG |

*2.7. Antibody and Regent Information.* The primary antibodies are GALNT8 (Abcam, #ab121374) and $\beta$-actin (Cell signaling technology, #3700). The secondary antibodies are HRP-labeled goat anti-rabbit IgG (Thermo Fisher, #31460) and HRP-labeled goat anti-mouse IgG (Thermo Fisher, #31430). Oxaliplatin was purchased from MCE (#HY-17371).

*2.8. Protein Isolation and Immunoblotting.* Cancer cell samples were suspended with 0.05% trypsin and washed twice with cold PBS and, after, homogenized with RIPA lysis buffer and protease inhibitor cocktail (Beyotime Biotechnology, #P0013D) on ice for 30 minutes. Cell lysates were harvested by 4°C centrifuge and diluted with 2× SDS sample buffer. The denaturized protein samples were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, #ISEQ00010). Blocked with 5% skimmed milk in PBST, the PVDF membranes were incubated with specific primary antibodies overnight at 4°C. After 3 times of 10-minute TBST buffer rinsing, the membranes were again incubated with secondary antibodies for 1 hour at room temperature and rinsed 3 times with TBST buffer for 10 minutes. Signals were detected with enhanced chemiluminescence (ECL) substrate (ThermoFisher, #32106) on a Las-3000 Luminescent Image Analyzer (Fujifilm, Japan).

*2.9. TMA Staining and Immunohistochemistry.* The TMA slide was air-dried at 60°C for an hour and treated with 0.01 M citric acid buffer solution for antigen retrieval. After cooling down to room temperature, the slide was further treated by 3% $H_2O_2$ solution in methanol for 10 minutes and rinsed 3 times with cold PBS before incubation with primary anti-GALNT8 antibody (1 : 100) at 4°C overnight. The slides were rinsed three times for 5 minutes and then incubated with ready-to-use biotinylated goat anti-rabbit IgG (Abcam, #ab64256) solution for 15 minutes at room temperature, followed by PBS rinsing for five times. Streptavidin peroxidase complex (Abcam, #ab64269) was applied to the TMA and incubated for 10 minutes at room temperature and rinsed by PBS for five times. After visualization with diaminobenzidine chromogen (Abcam, #ab64238) and hematoxylin counterstaining, the TMA was imagined using a Nikon Eclipse E-800 microscope. The stained TMA was then independently reviewed by two pathologists and rated for the grade of GALNT8 staining with scores of -, +/-, +, ++, and +++.

*2.10. Cytotoxic Assay.* For SW620 and DLD1, the cells with manipulated GAU1 expression or control were seeded in the 96-well plate at a density of $1 \times 10^3$ cells per well and incubated with low serum medium (1% *v*/*v* FBS) with or without oxaliplatin. Cells were replenished with fresh low serum medium with or without oxaliplatin on the third day. Cell Counting Kit-8 (CCK8, MCE, #HY-K0301) assay was used to estimate the cell viability at the end of the fifth day of treatment.

*2.11. Subcellular Isolation.* Subcellular isolation in LoVo and DLD1 cells was performed as described [15] with modification. Prepare isolation buffer (1.28 M sucrose; 40 mM Tris-HCl, pH 7.5; 20 mM $MgCl_2$; and 4% Triton-X 100) and diluted isolation buffer (cold $H_2O$ : cold PBS : isolation buffer = 3 : 1 : 1). $10^6$ cells were suspended in 200 $\mu$l diluted isolation buffer and incubated on ice for 10 min. 20 $\mu$l lysate was added to 1 ml Trizol for total RNA extraction. The rest of the lysate was rotated at 4°C for 20 min and centrifuged at 2500 × g for 15 min at 4°C. Add 1 ml Trizol to the supernatant for cytoplasmic RNA extraction. Wash the pellet once, resuspend with 160 $\mu$l isolation buffer, and add 1 ml Trizol for the nuclear RNA extraction. Fractionated RNAs were used for cDNA synthesis and qRT-PCR.

*2.12. Statistical and Survival Analysis.* GraphPad Prism 7 (La Jolla, CA, USA) and SPSS 20 (IBM, NY) were used for statistical analysis and graph preparation. All data are displayed as means ± SD. Two-tailed Student's *t*-test was used for assessment of differences between any two groups. Kaplan-Meier analysis was used to perform survival analysis, and the patients' survival comparison between subgroups was analyzed with log-rank test. Nonparametric Wilcoxon-Mann-Whitney test was performed for patients' clinical data analysis.

## 3. Results

*3.1. Identification of GAU1 as the Colorectal Cancer-Related lncRNA.* To identify the colorectal cancer-related lncRNA in the MiTranscriptome database, we first performed the differential expression analysis for all the 5,724 cancer libraries vs. 752 noncancer libraries by SSEA and annotated all the transcripts with tissue-type information (Figure 1(a)). After the empirical ranking test, two transcripts of *GAU1* (ENSG00000255474) were listed on the top CRC-related lncRNA (ranking percentile = 99.75% of 12,382) besides our previously reported CRC-specific lncRNA *PHiL* [9] (ranking percentile = 99.62%).

Then, we further experimentally quantified the *GAU1* mRNA overexpression in human colorectal cancer cell lines SW620, HCT116, DLD1, and LoVo versus human intestinal epithelial cell line NCM460 ($P < 0.05$, Figure 1(b)). Furthermore, qRT-PCR of *GAU1* mRNA in 66 pairs of CRC tissues and adjacent normal tissues also confirmed *GAU1* as the cancer-specific lncRNA in CRC ($P = 2.53 \times 10^{-2}$, Figure 1(c)). More importantly, the Kaplan-Meier analysis revealed that patients with higher *GAU1* expression had worse prognosis ($P = 3.04 \times 10^{-2}$, Figure 1(d)), indicating *GAU1* may play an oncogenic role in CRC. And the

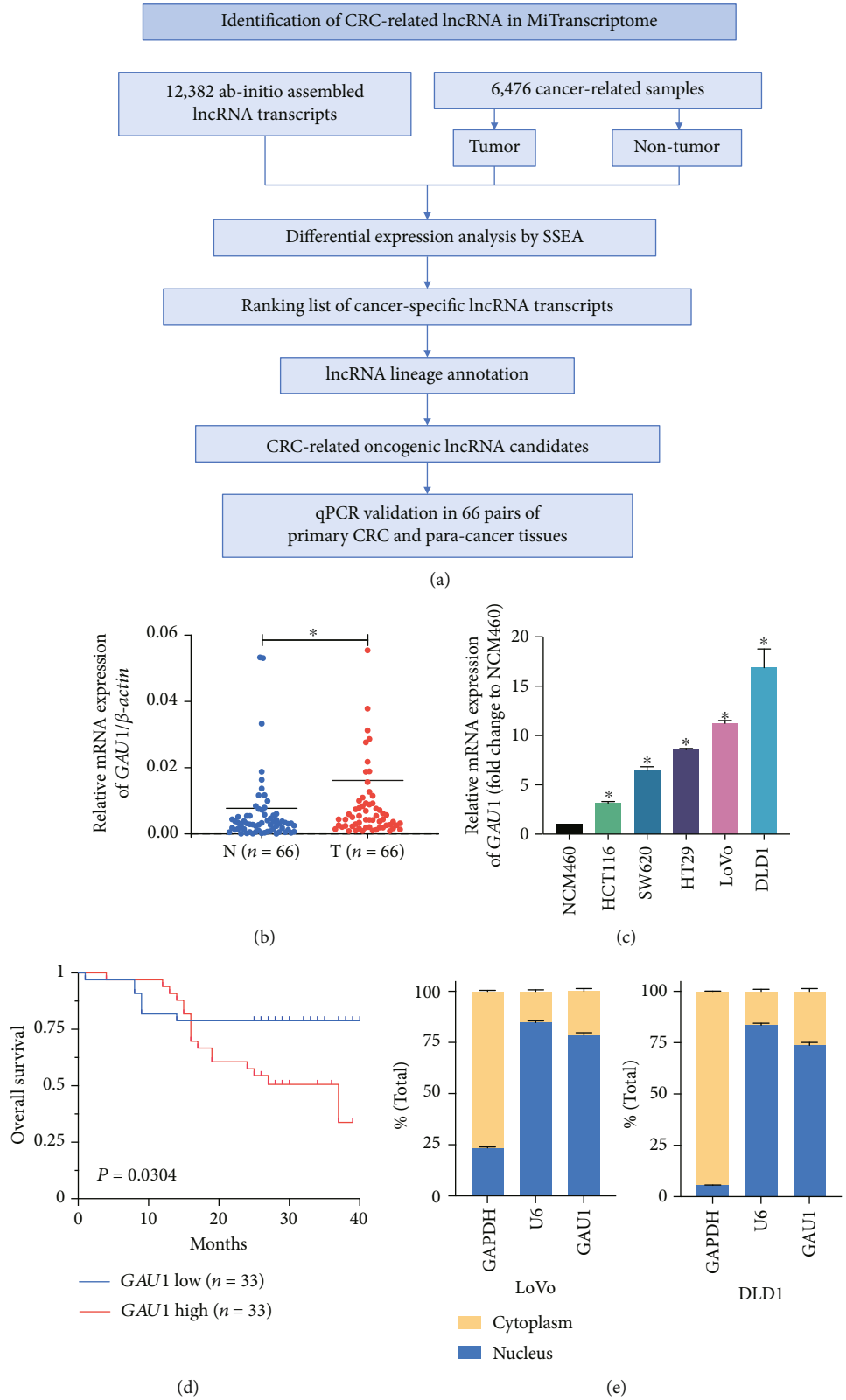
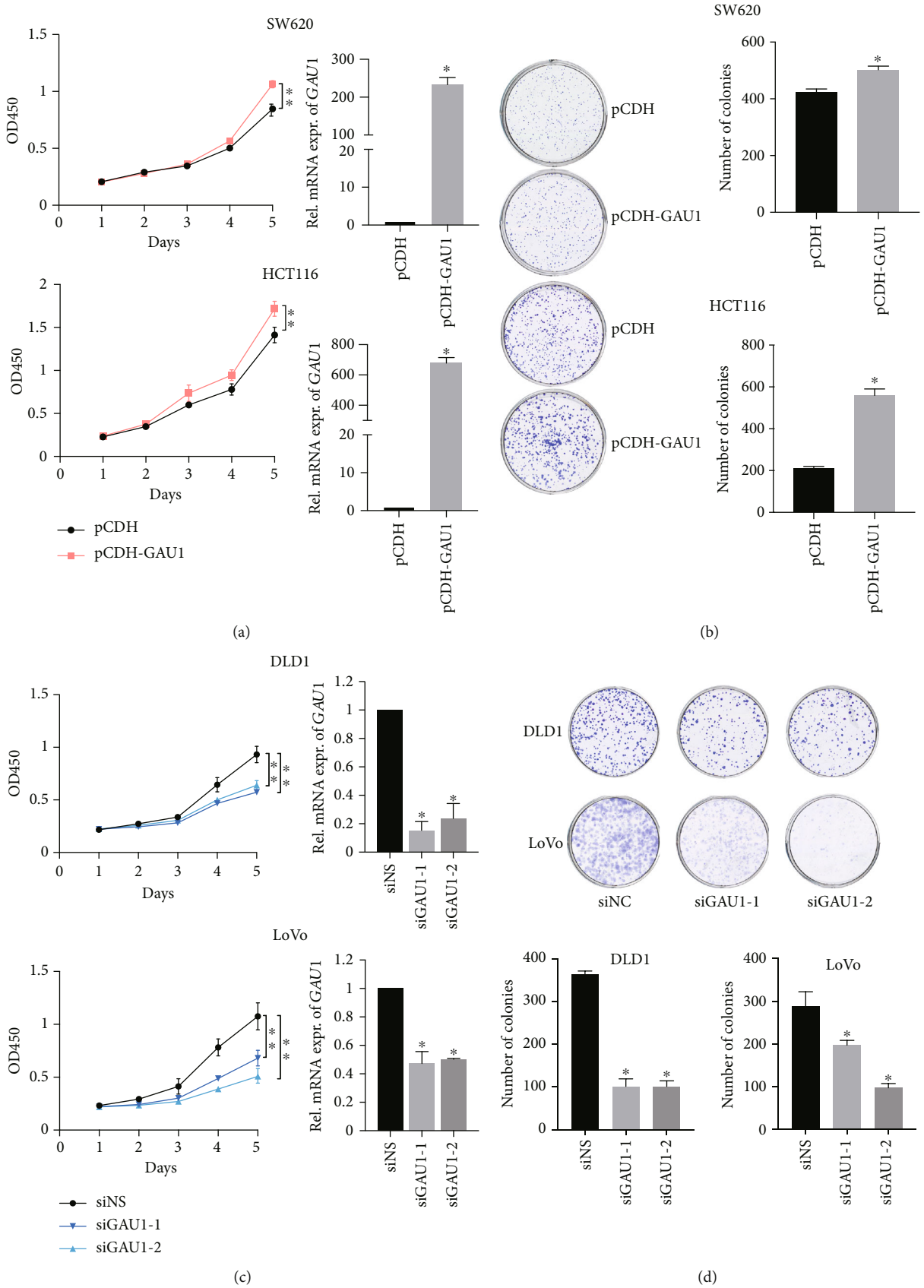



Figure 1: Identification of GAU1 as the colorectal cancer-related lncRNA. (a) The working pipeline of identifying CRC-related lncRNA in the MiTranscriptome database. (b) Relative expression level of *GAU1* detected by qRT-PCR in 66 paired colorectal cancer (CRC) tissues and adjacent normal tissues ($P = 2.53 \times 10^{-2}$). N: adjacent normal tissues; T: tumor tissues. (c) qRT-PCR analysis of *GAU1* expression in the five CRC cell lines and the normal NCM460 cells was tested. $^{*}P < 0.05$. (d) Kaplan-Meier analysis of the correlation between *GAU1* mRNA expression and overall survival in 66 CRC patients ($P = 3.04 \times 10^{-2}$). (e) Total RNA from LoVo and DLD1 cells was separated into cytoplasmic and nuclear fractions and analyzed by qRT-PCR. GAPDH serves as a positive control for cytoplasmic gene expression, and U6 as a positive control for nucleolus separation.

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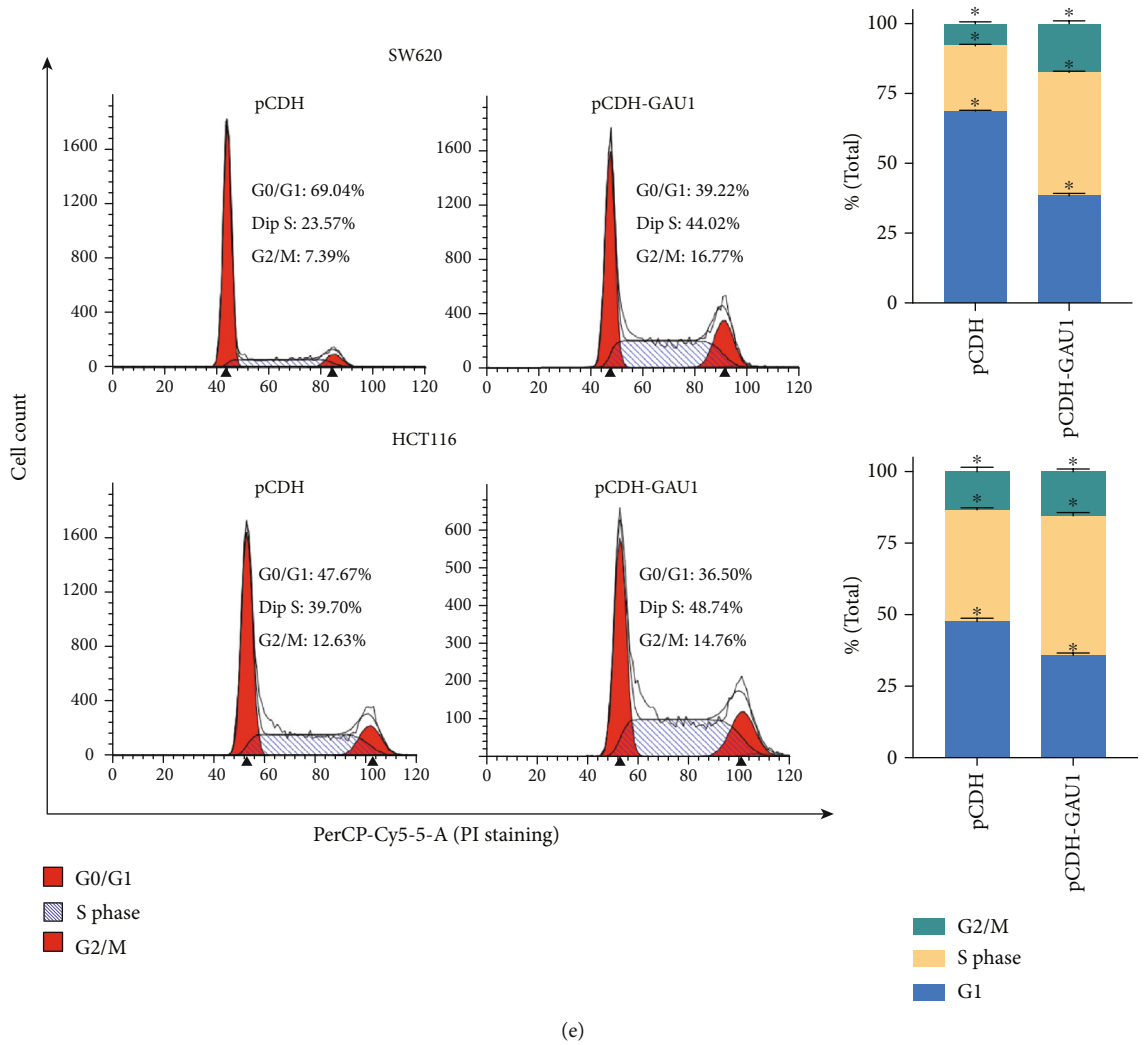
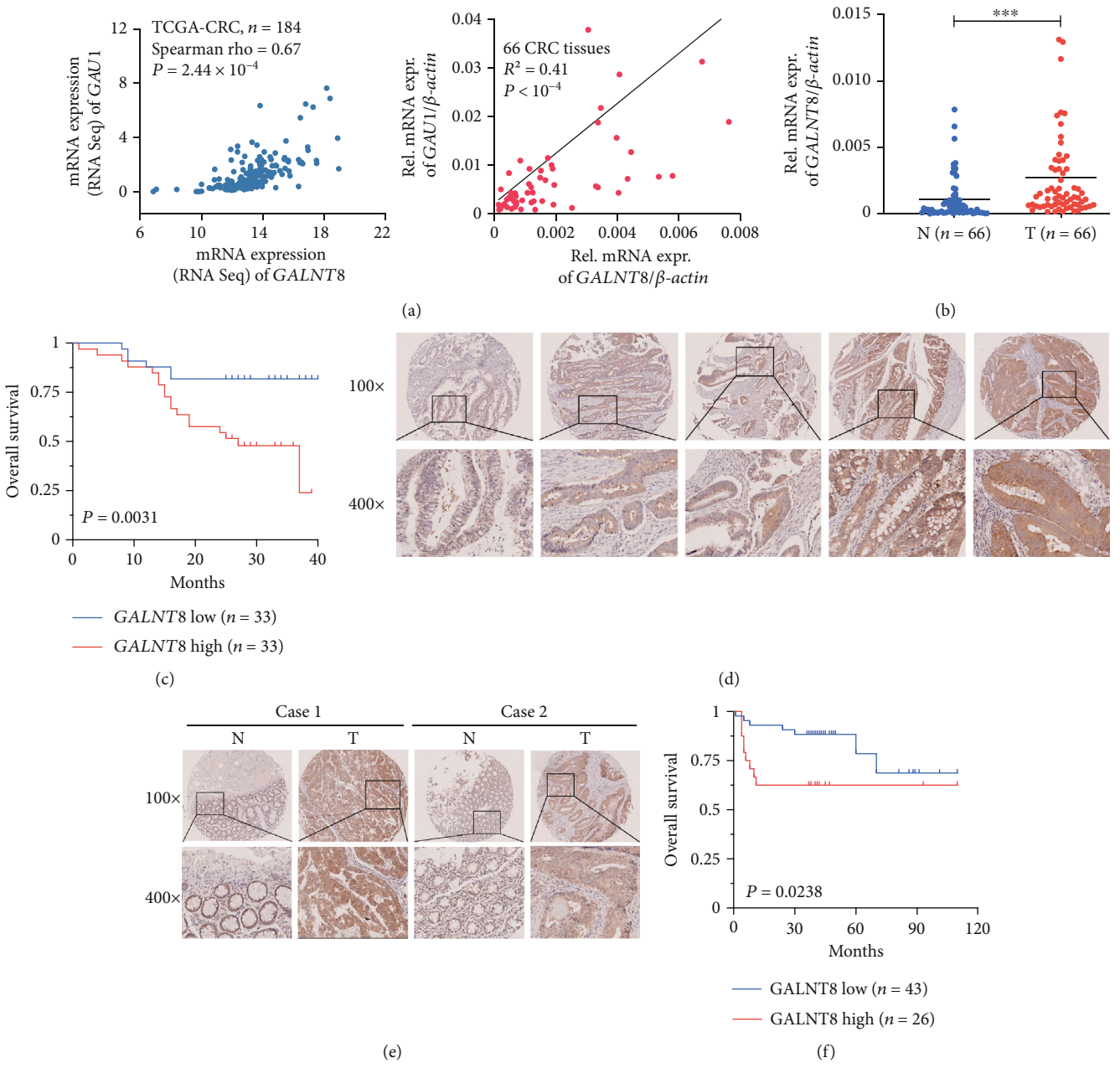



Figure 2: *GAU1* overexpression facilitates CRC cell proliferation. (a) CCK-8 assay and (b) colony formation assay were conducted in SW620 and HCT116 cells transfected with *GAU1* overexpression or control plasmids. $^{*}P < 0.05$; $^{**}P < 0.01$. (c) CCK-8 assay and (d) colony formation assay showed the proliferation of control siRNA (siNS) or siRNAs (siGAU1-1 and siGAU1-2) against GAU1-transfected DLD1 and LoVo cells. $^{*}P < 0.05$; $^{**}P < 0.01$. (e) Cell cycle analysis of GAU1-overexpressed HCT116, SW620, and their controls.

subcellular localization showed that GAU1 was mainly distributed in the nucleus (Figure 1(e)).

*3.2. GAU1 Overexpression Facilitates CRC Cell Proliferation by Promoting Cell Cycle.* To further determine if the *GAU1* overexpression can alter the biological phenotype of CRC, we first established the *GAU1*-overexpressing stable cell lines by lentiviral infection of pCDH-GAU1 in SW620 and HCT116 cell lines with intermediate *GAU1* expression. The CCK-8 and clonogenic assays both revealed that *GAU1* overexpression lead to a significantly increased cell proliferation in the CRC cell lines compared to the vehicle controls (Figures 2(a) and 2(b)). Consistently, *GAU1* knockdown in the *GAU1* high-expressing LoVo and DLD1 cell lines by short interfering RNA (siRNA) significantly reduced the cell proliferation and clonogenic ability of the CRC cells (Figures 2(c) and 2(d)). These data suggested that *GAU1* overexpression promotes CRC cell proliferation in vitro. Moreover, the cell cycle profile alteration after *GAU1* overexpression (increased S-phase commitment) (Figure 2(e)) also implied *GAU1* as a critical player in promoting S-phase entry.

*3.3. GALNT8 as the Oncogenic Operator of GAU1 in CRC.* To identify the biological "operator" of *GAU1* overexpression in CRC development, we performed coexpression analysis for *GAU1* in 184 TCGA CRC samples. Correlation analysis revealed *GALNT8*, located in the vicinal gene loci of *GAU1* on chromosome 12, as the most significantly coexpressed gene of *GAU1* among all the 19,815 protein-coding genes (Spearman rho = 0.67, $P = 2.44 \times 10^{-23}$, Figure 3(a)). The strong expression correlation between *GAU1* and *GALNT8* was further validated in our 66 pairs of clinical samples ($P < 10^{-4}$, Figure 3(a)), with a significant upregulation of *GALNT8* expression in the tumor tissues (T) compared with the adjacent nontumorous tissues (N) ($P < 10^{-4}$, Figure 3(b)). Clinically, the Kaplan-Meier analysis also revealed that patients with higher GALNT8 expression had worse overall survival ($P = 0.31 \times 10^{-2}$, Figure 3(c)).

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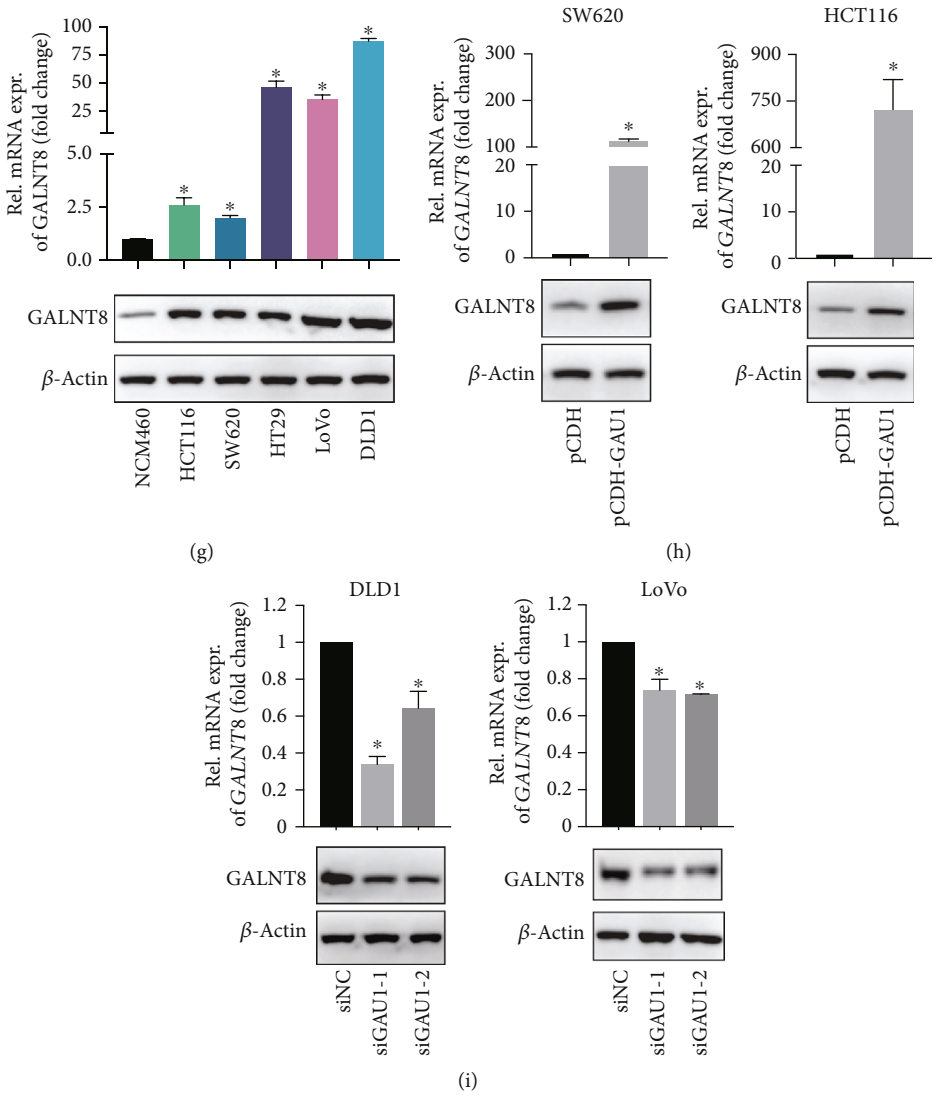


Figure 3: GALNT8 as the oncogenic operator of *GAU1* in CRC. (a) Correlation analysis of *GALNT8* and *GAU1* in 184 TCGA CRC samples (Spearman rho = 0.67, $P = 2.44 \times 10^{-23}$), and 66 frozen CRC tissues (qRT-PCR, $R^2 = 0.41$, $P < 10^{-4}$). (b) qRT-PCR analysis of *GALNT8* expression level in 66 paired CRC tissues and adjacent normal tissues ($P < 10^{-4}$). N: adjacent normal tissues; T: tumor tissues. (c) Kaplan-Meier analysis of *GALNT8* and overall survival in 66 CRC patients. (Kaplan-Meier $P = 0.31 \times 10^{-2}$). (d) Representative photomicrographs of GALNT8 in CRC specimen TMA (magnification: ×100, ×400). (e) Two paired tumor-adjacent control representative cases of GALNT8 expression in the TMA (magnification: ×100, ×400). (f) Kaplan-Meier analysis of *GALNT8* expression and overall survival in 69 TMA samples. (Kaplan-Meier $P = 2.38 \times 10^{-2}$). (g) qRT-PCR and western blot analysis of *GAU1* expression in the five CRC cell lines and the normal NCM460 cells was tested. $^*P < 0.05$. (h) qRT-PCR and western blot analysis of GALNT8 expression level in *GAU1*-overexpressed SW620 and HCT116 cells, as well as (i) *GAU1*-knockdown DLD1 and LoVo cells. $^*P < 0.05$.

The overexpression of GALNT8 in the CRC patients was further validated by the IHC staining of a TMA containing 55 paired cases of CRC and adjacent nontumorous tissues, plus 14 individual CRC tumors. According to the density of IHC staining (Figure 2(d)), GALNT8 protein expression in tumor tissues was classified as high expression (score ++, score +++) in 26 cases (26/69, 37.68%) and low expression (score +, score +/-, and score -) in 43 cases (43/69, 62.32%) (Figure 3(d)). Tumor tissues harbored a significantly increase GALNT8 expression compared to the adjacent nontumorous tissues (Fisher exact $P = 3.30 \times 10^{-8}$, Figure 3(e)). Further survival assay by Kaplan-Meier analysis also revealed that CRC patients with overexpressed GALNT8 suffered from poor overall survival ($P = 2.38 \times 10^{-2}$, Figure 3(f)).

Moreover, in contrast to human intestinal epithelial cell line, a higher expression of GALNT8 in CRC cells was observed in both mRNA and protein levels (Figure 3(g)). To further confirm the regulatory effect of *GAU1* on *GALNT8* expression, the effect of *GAU1* knockdown/overexpression on the expression levels of GALNT8 in CRC cells was determined. The mRNA and protein expression levels of GALNT8 were increased in the *GAU1* overexpression cell lines and decreased in the siGAU1 cell lines compared with the control group (Figures 3(h) and 3(i)). Altogether, the

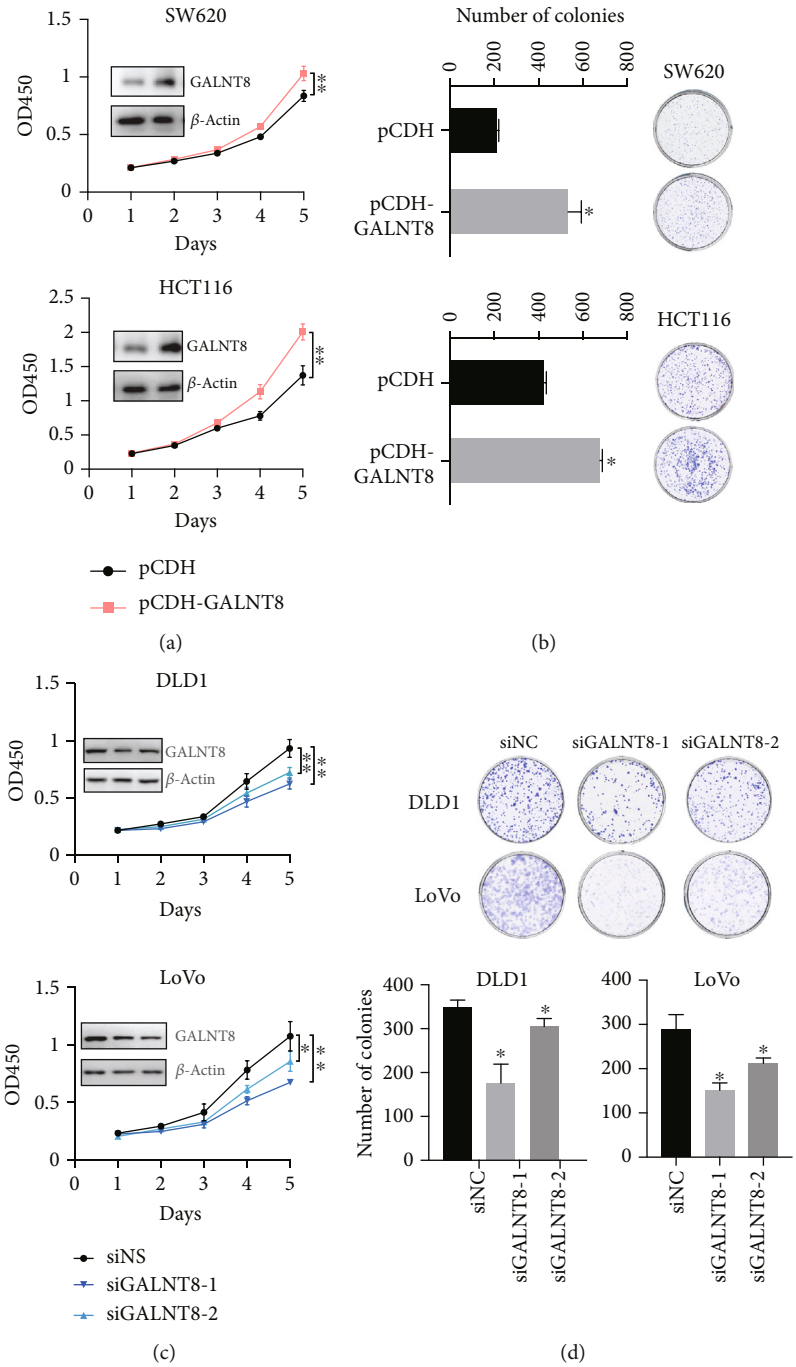


Figure 4: Continued.

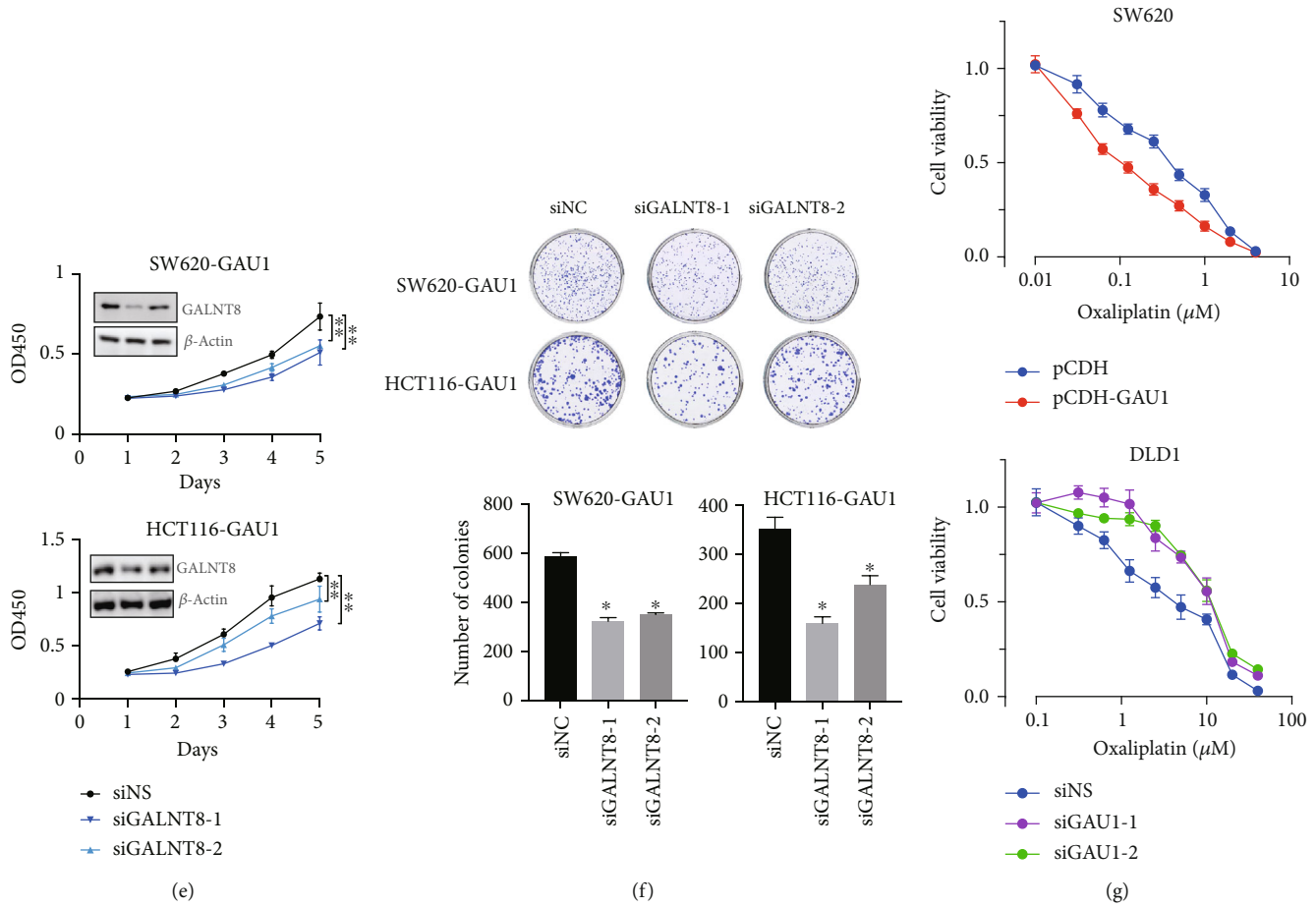


Figure 4: The oncogenic ability of *GAU1* is GALNT8 dependent, and overexpression of GAU1/GALNT8 axis sensitizes CRC cell lines to chemotherapy. (a) CCK-8 assay and (b) colony formation assay were used to measure proliferation in SW620 and HCT116 cells transfected with pCDH-GALNT8 or pCDH. $^{*}P < 0.05$; $^{**}P < 0.01$. (c) CCK-8 assay and (d) colony formation assay were conducted in control siRNA (siNS) or siRNAs (siGALNT8-1 and siGALNT8-2) against GALNT8-transfected DLD1 and LoVo cells. $^{*}P < 0.05$; $^{**}P < 0.01$. (e, f) Proliferation analysis by (e) CCK-8 assay and (f) colony formation assay in *GAU1*-overexpressed SW620 and HCT116 cells transfected with control siRNA (siNS) or siRNAs (siGALNT8-1 and siGALNT8-2) against GALNT8. $^{*}P < 0.05$; $^{**}P < 0.01$. (g) Viability of pCDH-GAU1- or pCDH-transfected SW620 and siGAU1-1/2- or siNS-transfected DLD1 cells incubated with multiple concentrations of oxaliplatin (0.01-40 $\mu$M) was monitored through CCK-8 assay.

computational and experimental evidence suggested GALNT8 as a regulatory downstream molecule of *GAU1* in CRC.

*3.4. The Oncogenic Ability of GAU1 Is GALNT8 Dependent.* Since the relationship between GALNT8 and cancer is limited, we experimentally manipulated the expression of *GALNT8* by lentiviral stable overexpression and siRNA interference. CCK-8 and colony forming assays demonstrated that the overexpression of *GALNT8* enhanced the proliferation and colony formation capacity of SW620 and HCT116 (Figures 4(a) and 4(b)), whereas the contrary results were observed in the GALNT8-suppressed DLD1 and LoVo cell lines (Figures 4(c) and 4(d)). With all these results, it is suggested that *GALNT8* contributes to CRC cell proliferation.

To further explore the oncogenic partnership of the *GAU1*/*GALNT8* cluster in CRC, siGALNT8 or negative control was transfected into *GAU1*-overexpressing cell lines to examine whether *GALNT8* silence could rescue *GAU1* overexpression-mediated enhanced proliferation of CRC. The CCK-8 and colony formation assay results demonstrated that the upregulated cell proliferation and colony formation in the *GAU1*-overexpressed SW620 and HCT116 cell lines were partially attenuated by siGALNT8 in Figures 4(e) and 4(f)), suggesting that *GALNT8* is a critical downstream operator of *GAU1* during the CRC proliferation.

*3.5. Overexpression of GAU1/GALNT8 Axis Sensitizes CRC Cell Lines to Chemotherapy.* Given the experimental evidence that *GAU1*/GALNT8 axis overexpression significantly promotes the cancer cell proliferation and *GAU1* boosts cell cycle by increasing S-phase entry, we further questioned if *GAU1*/GALNT8 axis can reshape the drug response of cancer cells to chemotherapy agents targeting DNA replication. The *in vitro* oxaliplatin drug response data revealed that cancer cells overexpressing *GAU1* or *GALNT8* are more vulnerable to chemotherapy agents causing replication fork collapse, indicating *GAU1*/GALNT8 axis as a potential actionable target for the personalized medicine of CRC. This finding was

further confirmed by the drug resistance phenotype in *GAU1*/*GALNT8* knockdown cell lines (Figure 4(g)).

## 4. Discussion

CRC is one of the most common and lethal types of cancer [16]. In the past decades, genetic alteration including *APC* and *K-RAS* somatic mutation has been identified to cause 70% of the CRC cases [17] and widely adapted into the diagnosis and drug response prediction during CRC management [18].

Recent studies attributed the transcriptional alteration of the lncRNAs as a hallmark of tumor development [19, 20]. The enormous efforts on the landscaping of lncRNA expression in cancer [21, 22] led to a number of fabulous investigations that improved the understanding of multiple major cancer types [7].

In this study, we identified *GAU1* as one of the major oncogenic lncRNAs for CRC by mining the ab initial strategy-based lncRNA database MiTranscriptome [10, 11]. According to our analysis, *GAU1* ranked one of the most differentially expressed lncRNAs between CRCs and normal tissues/cell lines (99.75% percentile of SSEA). Moreover, the overexpression of *GAU1* leads to a significant reduction in CRC patient survival ($P = 3.04 \times 10^{-2}$). After experimentally validating the procancerous ability of *GAU1* by the cell proliferation assay after *GAU1* expression manipulation in CRC cell lines, we further located *GALNT8* as the mostly coexpressed protein-coding gene for *GAU1*.

*GALNT8* encodes a 637-amino-acid type-II membrane protein (GalNAc-T8) [23]. The protein is a member of the UDP-GalNAc polypeptide N-acetylgalactosaminyl transferase (ppGaNTase) family, which initiates mucin-like O-linked protein glycosylation in the Golgi apparatus [24]. Previous research revealed that GALNT8 is expressed in the heart, placenta, skeletal muscle, liver, and kidney and plays a key role during embryonic development [23]. However, the oncogenic effect of GALNT8 is less characterized. Chai et al. reported *GALNT8* as the oncogene in retinoblastoma that potentially drives the cancer development and progression [25] by directly binding to the *GALNT8* promoter and boost the transcription of *GALNT8* through TCEA1 (Transcription Elongation Factor A1) recruitment, which mechanistically endorsed our experimental data in CRC.

Like *GAU1*, *GALNT8* is also associated with poor CRC prognosis ($P = 0.31 \times 10^{-2}$). Together with the experimental evidence (1) overexpression or silencing *GALNT8* mimicked the cancer cell line phenotypic alteration after *GAU1* overexpression or knockout. (2) *GALNT8* knockdown attenuated the *GAU1* overexpression-induced cell proliferation, and not vice versa; we confirmed GALNT8 as the downstream operator of *GAU1* in CRC.

Aside from the surgical operation, systemic chemotherapy with folinic acid, fluorouracil, and oxaliplatin (FOLFOX) is also a main treatment solution for CRC. Our result showed an oxaliplatin hypersensitivity in cancer cell lines overexpressing *GAU1*/GALNT8. This double-edge sword effect of *GAU1*/GALNT8 overexpression suggested the *GAU1*/-GALNT8 axis as a potential marker in the precision medicine of CRC, although more experimental evidence should be investigated in the future.

One limitation of this study is we did not provide the molecular interaction between *GAU1* and *GALNT8*. Although we have confirmed GALNT8 as the essential operator for the oncogenic ability of *GAU1*, further investigation on the regulatory mechanism between these bidirectionally transcribed lncRNA/protein-coding gene pairs needs to be clarified by protein-RNA interaction or DNA-RNA binding assay. According to the previous report that *GAU1* and *GALNT8* share a cisregulation relationship in retinoblastoma [25] and the mutual promoter region of the two genes, investigation on the mechanism behind the abnormal promoter activation in CRC should be conducted in our future studies.

To our best knowledge, this is the first study systematically reporting the oncogenic cascade of *GAU1*/*GALNT8* axis in CRC. By integrating the differential expression data from 7,256 curated RNA-Seq libraries in MiTranscriptome and experimental validation, we demonstrated that *GAU1*, together with its downstream protein GALNT8, is associated with cancer cell proliferation, poor patient survival, and chemotherapy response.

## Data Availability

The data used to support the findings of this study are included within the article.

## Conflicts of Interest

The author(s) declare(s) that they have no conflicts of interest.

## Authors' Contributions

Xuemei Tang and Haoyu Ruan contributed equally to this article.

## Acknowledgments

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*Research Article*

# Predicting Individual Survival after Curative Esophagectomy for Squamous Cell Carcinoma of Esophageal

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*Background*. Esophageal cancer is one of the leading causes of cancer-related death worldwide. Despite the significant progress in the overall treatment of esophageal cancer in recent years, the prognosis for patients who require surgery remains poor. *Methods*. The present study investigated the clinicopathological features of 503 patients who underwent radical esophagectomy at Huashan Hospital of Fudan University between January 2005 and January 2015. Nomograms that predicted the esophageal squamous cell carcinoma (ESCC) survival rates were established using the Cox proportional hazard regression model. Discrimination and calibration, which were calculated after bootstrapping, were used as a measure of accuracy. *Results*. Multivariate analyses were used to select five independent prognostic variables and build the nomogram. These variables were pathological T stage, pathological N factor, rate of positive LNs, history of chronic obstructive pulmonary disease (COPD) and postoperative sepsis. The nomogram was built to predict the rates for overall survival (OS) and disease-free survival (DFS). The concordance index for the nomogram prediction for OS and DFS was 0.720 and 0.707, respectively. Compared to the conventional TNM staging system, the nomogram had better predictive accuracy for survival (OS 0.720 vs. 0.672, $P < 0.001$; DFS 0.707 vs. 0.667; $P < 0.001$). *Conclusions*. The present study incorporated pathological T stage, pathological N factor, rate of positive LNs, history of COPD, and postoperative sepsis into a nomogram to predict the OS and DFS of ESCC patients. This practical system may help clinicians in both decision-making and clinical study design. The assessment of lung function for patients with COPD preoperative, and the control of disease progression are needed. Furthermore, the postoperative infection of patients should be controlled. Further studies may help to extend the validation of this method and improve the model through parameter optimization.

## 1. Introduction

Esophageal cancer is one of the leading causes of cancer-related death worldwide [1]. Despite the significant progress in the overall treatment of esophageal cancer in recent years, the prognosis for patients who require surgery remains poor. The establishment of an accurate cancer staging system would be valuable for both the provision of information and in guiding patient follow-up and subsequent treatments. The most commonly used staging system for esophageal squamous cell carcinoma (ESCC) is the tumor node metastasis (TNM) classification system from the 7$^{th}$ edition of the American Joint Committee on Cancer (AJCC). However, studies have demonstrated that other clinicopathological factors, such as lymph node ratio [2–4], comorbidities [5, 6], and postoperative complications [2], are also significant prognostic variables. Furthermore, there are no models that can concurrently take comorbidities and postoperative complications into account in constructing an accurate predictive model. Hence, the present study is aimed at assessing the comorbidities and postoperative complications in patients with esophageal cancer and designing a nomogram for the prediction of long-term survival in patients with resected ESCC. To the best of the knowledge of the authors, the

present study is the first to attempt to establish an ESCC nomogram based on comorbidities and postoperative complications using a relatively large cohort of patients.

## 2. Materials and Methods

A total of 503 patients participated in the present study. These patients underwent potential curative esophagectomy for squamous cell carcinoma of the esophagus between January 2005 and January 2015 in Huashan Hospital at Fudan University, which is a tertiary referral center with significant experience in esophageal surgery. The patients in the present study (1) underwent transthoracic esophagectomy with mediastinal and two-field abdominal lymphadenectomy with R0 resection, (2) had no in-hospital mortality, and (3) did not have other malignancies or distant metastases. The surgical methods used have been previously described [7].

The collected patient information included the demographic information such as age, gender, body mass index (BMI), tobacco use, alcohol use, preoperative albumin, preoperative platelet, preoperative white blood cell (WBC), and preoperative neutrophil to lymphocyte ratio (NLR). Additional variables included comorbidities, clinicopathological features, postoperative complications, and survival.

The comorbidities were identified during the preoperative evaluation of the physician or other healthcare professional notes and subsequently confirmed via appropriate medical tests. These comorbidities included history of cardiovascular disease (previous myocardial infarction, heart failure, peripheral arterial disease, or cerebrovascular disease), history of chronic obstructive pulmonary disease (COPD) [6], history of hepatitis, history of hypertension, and history of diabetes (with or without complications). Renal comorbidities were too rare to include in the statistical analyses.

The clinicopathological factors were evaluated in accordance to the guidelines for clinical and pathological studies on carcinoma of the esophagus. The tumor staging was based on the TNM classification specified by the International Union Against Cancer [8], and depth of invasion and lymph node metastasis were determined based on from the pathology of the surgically resected specimens. The postoperative pathological T (pT), N (pN), and Stage (pStage) factors were used for all cases. For patients who received preoperative therapy, the depth of invasion was determined through both the microscopic distribution of viable cancers, and the scar tissue and disappearance of normal structures, such as the lamina propria and proper muscular layer.

The $7^{th}$ edition of the AJCC recommends removing a sufficient number of LNs during the operation, and the detection of at least 12 nodes. However, in clinical practice, due to various factors such as individual physical condition, operating conditions, and pathological diagnosis, it remains difficult to ensure the removal of a sufficient number of LNs from each patient. Hence, this may result in the stage migration phenomenon. The metastatic lymph node ratio is the ratio of metastatic LNs to the number of total detected LNs, which may be affected by variability during detection. This variable was included in the present study.

The present study evaluated the postoperative complications that developed within 30 days after esophagectomy, which required either medication or surgical intervention. A postoperative pulmonary complication was defined as the presence of one or more of the following postoperative conditions: initial ventilator support for more than 48 hours or reintubation for respiratory failure, the need for tracheostomy, pneumonia, or acute respiratory distress syndrome (ARDS). Postoperative anastomotic leakage was defined in terms of the clinical signs of leaking, such as erythema, skin edema, emission of pus from a surgical wound or cervical drain, or a radiographically apparent leak confirmed by performing an esophagography or computed tomography, or both. Cardiovascular morbidity was defined as the presence of any cardiac disease or cerebrovascular disease, such as arrhythmia, ischemic heart disease, or pericardial fluid collection, which required pharmacological, electrical, or interventional treatment, or the presence of any thrombosis in line with the common terminology criteria for adverse events (CTCAE) version 4.03 [2]. Sepsis was defined as clinical signs of SIRS along with a culture or visually identified infection.

*2.1. Statistical Analysis.* The statistical analyses were performed using the statistical package R for Windows (version 3.4.2, http://www.r-project.org/). For the purpose of developing the nomograms, the outcome predictor was developed with the clinical experience of the authors, as well as through the search of prior literature. Quantitative data were expressed in median and interquartile range (IQR), and categorical data were expressed numerically and in percentage. The Kaplan-Meier method was used to estimate the OS and DFS. Cox regression analysis was used for the univariate and multivariate analyses. Variables with a $P$ value of $<0.05$ in the univariate analysis were subjected to the multivariable Cox regression analysis. A final model selection was performed using backward stepwise regression with Akaike's Information Criterion (AIC) [9]. Furthermore, the graphical assessment of proportional hazards assumptions and the test of nonlinear terms for significance using analysis of variance (ANOVA) were performed. A nomogram was formulated based on the results of the multivariate analysis using the rms statistical package [10].

Discrimination and calibration were used to test the accuracy of the nomograms. The discrimination of the nomogram was measured using a concordance index (C-index) and the bootstrap bias-corrected estimates of the C-index. Calibration curves, which measure the relationship between the outcomes predicted by the models and the observed outcomes in the patients, were used to assess calibration accuracy in predicting the probability of the overall survival probability and progression-free survival probability for 1, 3, and 5 years. These analyses were performed using a bootstrapping strategy with 200 replications. The nomogram and pathological staging systems were compared using the rcorrp.cens package.

The total points for each patient were calculated according to the established nomogram. Three groups of patients with high, moderate, and low risk of survival were delineated using maximally selected rank statistics, as implemented in

Table 1: Clinical, epidemiological, and pathological feature.

| | Median/*N* | IQR/percentage |
|---|---|---|
| Age, year | 62 | 56-67 |
| Sex | | |
| Male | 411 | 81.7% |
| Female | 92 | 18.3% |
| BMI, kg/m$^2$ | 22.23 | 20.07-24.19 |
| Tobacco use | | |
| No | 338 | 67.2% |
| Yes | 165 | 32.8% |
| Alcohol use | | |
| No | 381 | 75.7% |
| Yes | 122 | 24.3% |
| Comorbidities | | |
| History of hypertension | | |
| No | 330 | 65.6% |
| Yes | 173 | 34.4% |
| History of diabetes | | |
| No | 381 | 75.7% |
| Yes | 122 | 24.3% |
| History of COPD | | |
| No | 445 | 88.5% |
| Yes | 58 | 11.5% |
| History of hepatitis | | |
| No | 428 | 85.1% |
| Yes | 75 | 14.9% |
| History of cardiovascular disease | | |
| No | 422 | 83.9% |
| Yes | 81 | 16.1% |
| Preoperative albumin, g/L | 41.00 | 39.00-43.00 |
| Preoperative platelet, $*10^9$ | 194.00 | 157.00-239.00 |
| Preoperative WBC, $*10^9$ | 5.88 | 4.89-7.23 |
| Preoperative NLR | 2.25 | 1.67-3.17 |
| Length of tumor, cm | 3.00 | 2.00-4.50 |
| Location of tumor | | |
| Upper | 82 | 16.3% |
| Middle | 302 | 60.0% |
| Lower | 119 | 23.7% |
| Differentiation of tumor | | |
| Well | 65 | 12.9% |
| Moderate | 299 | 59.4% |
| Poor | 139 | 27.6% |
| Pathological T stage | | |
| T1 | 73 | 14.5% |
| T2 | 146 | 29.0% |
| T3 | 242 | 48.1% |
| T4 | 42 | 8.3% |
| Pathological N factor | | |
| N0 | 263 | 52.3% |
| N1 | 140 | 27.8% |
| N2 | 70 | 13.9% |
| N3 | 30 | 6.0% |
| Pathological stage | | |
| I | 72 | 14.3% |
| II | 227 | 45.1% |
| III | 135 | 26.8% |
| IV | 69 | 13.7% |
| Number of dissected LNs | 13 | 8-19 |
| Number of positive LNs | 0 | 0-2 |
| Rate of positive LNs | 0 | 0-0.17 |
| Postoperative complications | | |
| Postoperative pulmonary complications | 148 | 29.4% |
| Postoperative anastomotic leakage | 29 | 5.8% |
| Postoperative cardiovascular disease | 107 | 21.3% |
| Sepsis | 34 | 6.8% |

Data are expressed as median (interquartile range, IQR), *N* (percentage, %). BMI: body mass index; COPD: chronic obstructive pulmonary disease; NLR: neutrophil to lymphocyte ratio.

the Maxstat package [11]. The survival curves were drawn using the Kaplan-Meier method. Finally, with the risk group as a factor, these were compared using log-rank test.

All statistical tests were two-sided, and $P$ values of $<0.05$ were considered statistically significant.

## 3. Results

*3.1. Clinicopathologic Characteristics of Patients.* A total of 503 patients were enrolled in the present study. The patient characteristics are presented in Table 1. The median age of diagnosis was 62 years old. The median number of resected LNs was 13 (range: 8-19). The majority of patients were male (81.7%). The most common comorbidity was a history of hypertension (34.4%), and a total of 148 (29.4%) patients suffered from postoperative pulmonary complications.

*3.2. OS and DFS of Patients.* The median OS was four years (95% CI: 3.50-4.83 years), and the 1-, 3-, and 5-year OS rates were 82.5%, 57.5%, and 42.3%, respectively. The median DFS was 3.33 years (95% CI: 2.92-4.00 years), and the 1-, 3-, and 5-year disease free rate was 77.6%, 52.1%, and 40.9%, respectively. The median follow-up time was 4.62 years (range: 1.21-17.08 years).

*3.3. Independent Prognostic Factors.* In order to determine the factors that are independently prognostic of patient survival, the OS and DFS were analyzed using the Cox proportional hazards model. Tables 2 and 3 highlight all parameters identified to be of potential significance in the univariate analysis, and these were included in the multivariate analysis. The multivariate analyses indicated that history of COPD, pathological T stage, pathological N factor, rate of positive LNs, and postoperative sepsis were independent risk factors for OS and DFS.

Table 2: Univariable and multivariable Cox analysis of prognostic factors for overall survival in 503 patients with esophageal squamous cell carcinoma.

| | Univariable analysis | | Multivariable analysis | |
|---|---|---|---|---|
| | HR (95% CI) | *P* value | HR (95% CI) | *P* value |
| Age, year | 1.01 (0.99-1.03) | 0.11 | | |
| Sex | | | | |
| Male | Ref | | | |
| Female | 0.74 (0.54-1.02) | 0.07 | | |
| BMI, $kg/m^2$ | 0.97 (0.93-1.01) | 0.12 | | |
| Tobacco use | | | | |
| No | Ref | | | |
| Yes | 1.11 (0.86-1.42) | 0.42 | | |
| Alcohol use | | | | |
| No | Ref | | | |
| Yes | 1.14 (0.87-1.49) | 0.35 | | |
| Comorbidities history of hypertension | | | | |
| No | Ref | | | |
| Yes | 1.10 (0.85-1.41) | 0.48 | | |
| History of diabetes | | | | |
| No | Ref | | | |
| Yes | 1.18 (0.90-1.56) | 0.23 | | |
| History of COPD | | | | |
| No | Ref | | Ref | |
| Yes | 1.50 (1.08-2.10) | 0.02 | 1.79 (1.27-2.53) | <0.001 |
| History of hepatitis | | | | |
| No | Ref | | | |
| Yes | 1.03 (0.74-1.44) | 0.84 | | |
| History of cardiovascular disease | | | | |
| No | Ref | | | |
| Yes | 0.92 (0.66-1.29) | 0.62 | | |
| Preoperative albumin, g/L | 0.96 (0.93-0.99) | 0.03 | | |
| Preoperative platelet, $*10^9$ | 1 (0.998-1.002) | 0.63 | | |
| Preoperative WBC, $*10^9$ | 1.06 (1.004-1.129) | 0.04 | | |
| Preoperative NLR | 1.06 (1.006-1.117) | 0.03 | | |
| Length of tumor, cm | 1.18 (1.11-1.25) | <0.001 | | |
| Location of tumor | | | | |
| Upper | Ref | | | |
| Middle | 0.86 (0.62-1.19) | 0.35 | | |
| Lower | 0.91 (0.62-1.33) | 0.62 | | |
| Differentiation of tumor | | | | |
| Well | Ref | | | |
| Moderate | 1.69 (1.09-2.62) | 0.02 | | |
| Poor | 2.19 (1.37-3.49) | <0.001 | | |
| Pathological T stage | | | | |
| T1 | Ref | | Ref | |
| T2 | 2.51 (1.43-4.41) | 0.001 | 1.82 (1.02-3.22) | 0.004 |
| T3 | 4.76 (2.80-8.10) | <0.001 | 3.01 (1.74-5.20) | <0.001 |
| T4 | 6.33 (3.44-11.67) | <0.001 | 3.17 (1.66-6.07) | <0.001 |
| Pathological N factor | | | | |
| N0 | Ref | | Ref | |
| N1 | 2.24 (1.67-3.00) | <0.001 | 1.80 (1.30-2.49) | <0.001 |

Table 2: Continued.

| | Univariable analysis | | Multivariable analysis | |
|---|---|---|---|---|
| | HR (95% CI) | *P* value | HR (95% CI) | *P* value |
| N2 | 3.78 (2.34-6.10) | <0.001 | 2.26 (1.27-4.00) | 0.005 |
| N3 | 5.73 (4.10-8.01) | <0.001 | 3.63 (2.32-5.68) | <0.001 |
| Pathological stage | | | | |
| I | Ref | | | |
| II | 2.43 (1.41-4.18) | 0.001 | | |
| III | 6.82 (3.95-11.77) | <0.001 | | |
| IV | 6.61 (3.71-11.76) | <0.001 | | |
| Number of dissected LNs | 1.01 (0.99-1.02) | 0.37 | | |
| Number of positive LNs | 1.13 (1.10-1.17) | <0.001 | | |
| Rate of positive LNs | | | | |
| Postoperative complications | 10.06 (6.34-15.97) | <0.001 | 2.01 (0.91-4.43) | 0.008 |
| Postoperative pulmonary complications | 1.20 (0.92-1.55) | 0.18 | | |
| Postoperative anastomotic leakage | 1.59 (0.97-2.60) | 0.07 | | |
| Postoperative cardiovascular disease | 1.10 (0.82-1.46) | 0.54 | | |
| Sepsis | 1.82 (1.18-2.82) | 0.007 | 2.04 (1.31-3.18) | 0.002 |

BMI: body mass index; COPD: chronic obstructive pulmonary disease; NLR: neutrophil to lymphocyte ratio.

*3.4. Prognostic Nomogram for OS and DFS.* The prognostic nomograms that integrated all independent factors for OS and DFS in the primary cohort are shown in Figures 1(a) and 1(b), respectively. The calibration plot for the probability of survival at 1/3/5 year(s) after surgery demonstrate the optimal concordance between the nomogram prediction and actual observation (Figure 2).

*3.5. Validation of Predictive Accuracy of the Nomogram for OS and DFS.* The C-index of the nomogram for OS was 0.720 (95% CI: 0.682-0.758), and the bias-corrected C-index was 0.712. The C-index and bias-corrected C-index of the nomogram for DFS were 0.707 (95% CI: 0.670-0.744) and 0.700, respectively. For the pathological stage, the C-index and bias-corrected C-index for OS (0.672 and 0.669, respectively) and DFS (0.669 and 0.666, respectively) were significantly lower than the C-index of the nomogram ($P < 0.001$, $P < 0.001$).

The risk stratification based on the score obtained from the nomogram supported the predictive efficacy in the long-term survival of the established model (Figures 3 and 4). The patients were divided into three risk groups according to their total score for OS (low-risk group: >22 and ≤74, moderate-risk group: >74 and ≤155, and high-risk group: >155 and ≤271) and DFS (low-risk group: >22 and ≤83, moderate-risk group: >83 and ≤161, and high-risk group: >161 and ≤274), respectively.

## 4. Discussion

The present study investigated the predictive factors for long-term survival in the 503 patients who underwent resection of ESCC. The cancer characteristics were closely correlated with the long-term survival of ESCC patients. However, a large number of studies have reported that many other clinicopathological factors are also associated with the prognosis. The present study found that a history of COPD and postoperative sepsis were significantly correlated to OS and DFS in patients with ESCC. A clinical nomogram was developed which included the pathological T stage, pathological N factor, rate of positive LNs, history of COPD, and postoperative sepsis. Subsequently, a risk stratification system was constructed based on the nomogram score. These developed nomograms are more accurate than the conventional staging system for predicting prognosis in ESCC patients, and calibration plots indicated a concordance between prediction and actual observation. The C-index value for OS and DFS was 0.720 and 0.707, respectively.

A number of prior studies have demonstrated that comorbidities have an impact on the prognosis of ESCC patients [5, 6, 12, 13]. A history of COPD is one of the most common conditions, accounting for 11.5% of newly diagnosed ESCC cancer patients. Furthermore, this has an association with significantly worse prognosis [14–16]. COPD is a disease characterized by completely irreversible and usually progressive obstruction of the airways and is associated with inflammation [17]. Furthermore, in patients with ESCC, following esophageal carcinoma resection and intrathoracic gastroesophagostomy, part of the thoracic cavity is occupied by the stomach that has been pulled up. This leads to further impairment of respiratory motion and poor pulmonary function. Second, immune dysfunction plays an important role in the occurrence of COPD [17], which may facilitate the rapid development of microscopic residual disease into clinically manifested recurrence. Third, COPD was found to be a risk factor for pulmonary complications following surgery [18]. Postoperative pulmonary complications may be correlated with worse prognosis [2], although this was not found in the present study. Overall, COPD may play an important role in predicting long-term survival, and the present study revealed that this is an independent predictor of death among patients with ESCC. However, further mechanistic studies are necessary.

Table 3: Univariable and multivariable Cox analysis of prognostic factors for disease free survival in 503 patients with esophageal squamous cell carcinoma.

| | Univariable analysis | | Multivariable analysis | |
|---|---|---|---|---|
| | HR (95% CI) | *P* value | HR (95% CI) | *P* value |
| Age, year | 1.01 (0.99-1.02) | 0.17 | | |
| Sex | | | | |
| Male | Ref | | | |
| Female | 0.75 (0.54-1.02) | 0.07 | | |
| BMI, $kg/m^2$ | 0.97 (0.94-1.01) | 0.20 | | |
| Tobacco use | | | | |
| No | Ref | | | |
| Yes | 1.07 (0.84-1.36) | 0.60 | | |
| Alcohol use | | | | |
| No | Ref | | | |
| Yes | 1.12 (0.86-1.45) | 0.42 | | |
| Comorbidities | | | | |
| History of hypertension | | | | |
| No | | | | |
| Yes | 1.11 (0.87-1.41) | 0.42 | | |
| History of diabetes | | | | |
| No | Ref | | | |
| Yes | 1.26 (0.96-1.64) | 0.09 | | |
| History of COPD | | | | |
| No | Ref | | Ref | |
| Yes | 1.42 (1.02-1.97) | 0.04 | 1.65 (1.17-2.31) | 0.004 |
| History of hepatitis | | | | |
| No | Ref | | | |
| Yes | 0.96 (0.69-1.33) | | | |
| History of cardiovascular disease | | | | |
| No | Ref | | | |
| Yes | 0.91 (0.66-1.26) | | | |
| Preoperative albumin | 0.96 (0.93-0.99) | 0.04 | | |
| Preoperative platelet, $*10^9$ | 1.001 (0.999-1.003) | 0.436 | | |
| Preoperative WBC, $*10^9$ | 1.05 (0.99-1.12) | 0.08 | | |
| Preoperative NLR | 1.05 (0.99-1.11) | 0.05 | | |
| Length of tumor, cm | 1.17 (1.11-1.25) | <0.001 | | |
| Location of tumor | | | | |
| Upper | Ref | | | |
| Middle | 0.85 (0.62-1.17) | 0.33 | | |
| Lower | 0.93 (0.65-1.35) | 0.71 | | |
| Differentiation of tumor | | | | |
| Well | Ref | | | |
| Moderate | 1.62 (1.07-2.46) | 0.02 | | |
| Poor | 2.04 (1.31-3.18) | 0.002 | | |
| Pathological T stage | | | | |
| T1 | Ref | | Ref | |
| T2 | 2.92 (1.67-5.09) | <0.001 | 2.21 (1.25-3.89) | 0.006 |
| T3 | 5.23 (3.08-8.89) | <0.001 | 3.46 (2.00-5.97) | <0.001 |
| T4 | 6.42 (3.48-11.82) | <0.001 | 3.47 (1.82-6.60) | <0.001 |
| Pathological N factor | | | | |
| N0 | Ref | | Ref | |
| N1 | 2.09 (1.58-2.78) | <0.001 | 1.65 (1.30-2.49) | 0.002 |
| N2 | 3.72 (2.37-5.84) | <0.001 | 2.20 (1.27-3.79) | 0.005 |
| N3 | 5.11 (3.68-7.09) | <0.001 | 3.20 (2.07-4.93) | <0.001 |

Table 3: Continued.

| | Univariable analysis | | Multivariable analysis | |
|---|---|---|---|---|
| | HR (95% CI) | *P* value | HR (95% CI) | *P* value |
| Pathological stage | | | | |
| I | Ref | | | |
| II | 2.80 (1.63-4.81) | <0.001 | | |
| III | 7.23 (4.19-12.46) | <0.001 | | |
| IV | 7.02 (3.96-12.45) | <0.001 | | |
| Number of dissected LNs | 1.01 (0.99-1.02) | 0.28 | | |
| Number of positive LNs | 1.13 (1.10-1.17) | <0.001 | | |
| Rate of positive LNs | 9.19 (5.8-14.58) | <0.001 | 1.99 (0.90-4.36) | 0.009 |
| Postoperative complications | | | | |
| Postoperative pulmonary complications | 1.21 (0.94-1.56) | 0.14 | | |
| Postoperative anastomotic leakage | 1.44 (0.88-2.35) | 0.15 | | |
| Postoperative cardiovascular disease | 1.11 (0.84-1.47) | 0.46 | | |
| Sepsis | 1.66 (1.07-2.57) | 0.02 | 1.79 (1.16-2.79) | 0.009 |

BMI: body mass index; COPD: chronic obstructive pulmonary disease; NLR: neutrophil to lymphocyte ratio.

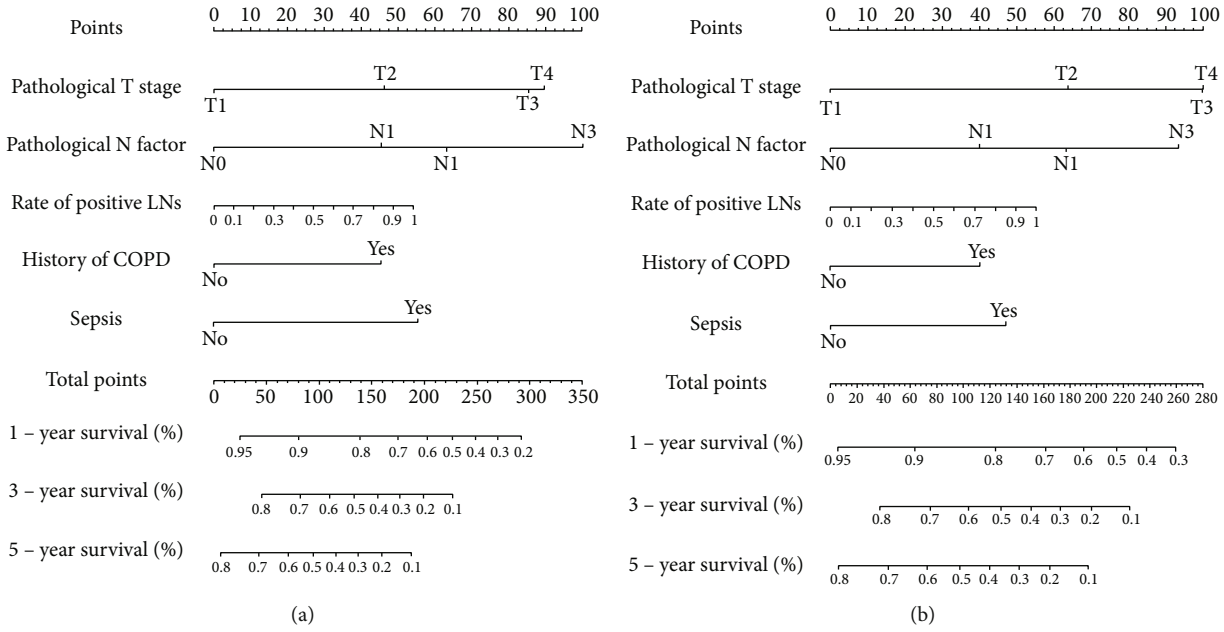



Figure 1: Survival nomogram for patients with resected esophageal squamous cell carcinoma (to use the nomogram, an individual patient's value is located on each variable axis, and a line is drawn upward to determine the number of points received for each variable value. The sum of these number is located on total point axis, and a line is drawn downward to the survival axes to determine the likelihood of 1-, 3- or 5-year survival. (a) is for overall survival; (b) is for disease free survival).

N staging is essentially based on the number of metastatic LNs, but the main source of error in the number of metastatic LNs lies in the variation of the total number of examined LNs. In the present study, the median number of examined LNs was 13 (range: 8-19), which can easily result in the stage migration phenomenon in these patients. Furthermore, the present study indicated that present AJCC staging is unable to satisfactorily distinguish between the prognosis for stage III and stage IV groups. These results are demonstrated in Figures 3(b) and 4(b). The ratio of metastatic lymph nodes is affected by the number of examined LNs. In addition, the present study found that the lymph node ratio is an independent predictor of survival for patients undergoing esophagectomy for ESCC, which is consistent with prior literatures [3, 4, 19, 20]. The lymph node ratio may compensate for the deficiencies in the AJCC nodal categories. Hence, combining the lymph node ratio and AJCC nodal categories may more accurately predict the survival, when compared to the present staging system [21].

Sepsis was the only postoperative variable associated with long-term mortality, and this finding is consistent with a previous literature [22]. For cancer patients, the occurrence of

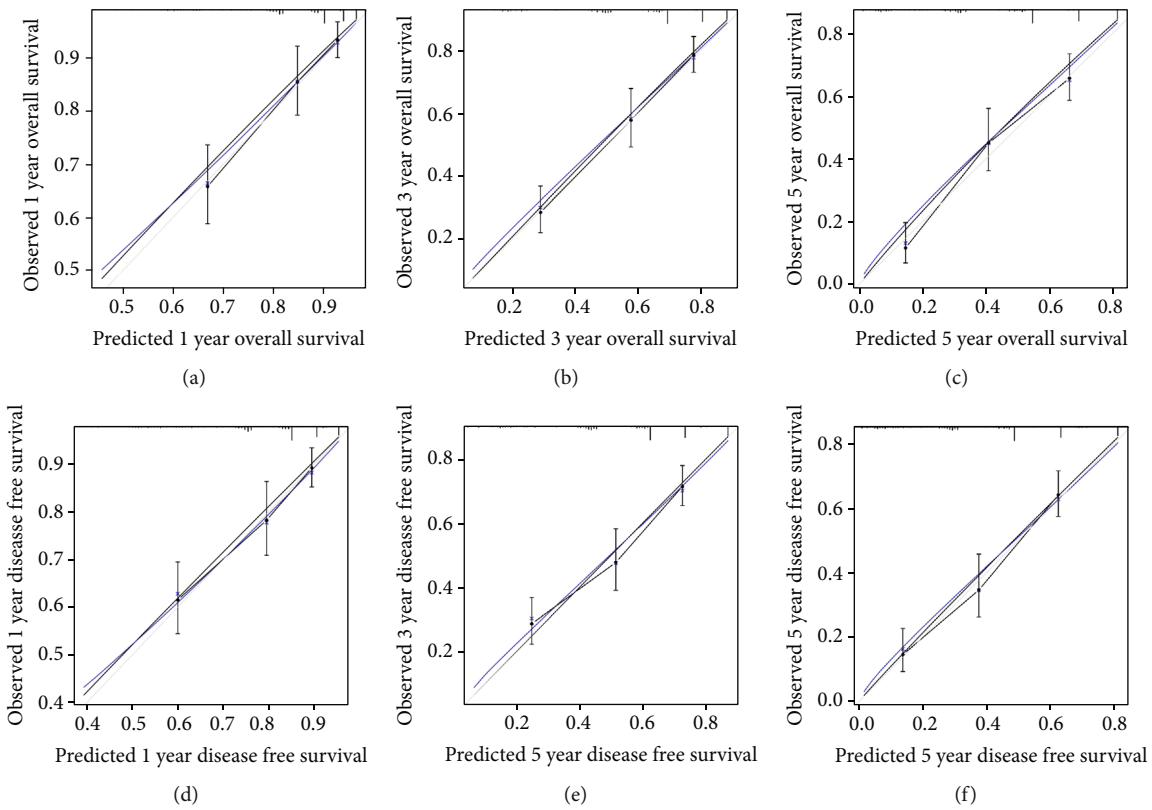



FIGURE 2: Calibration curve for predicting patient survival at (a, d) 1 year, (b, e) 3 years and (c, f) 5 years in the validation cohort. Nomogram-predicted overall survival/disease free survival (DFS) is plotted on the *x*-axis; observed overall survival/disease free survival is plotted on the *y*-axis.

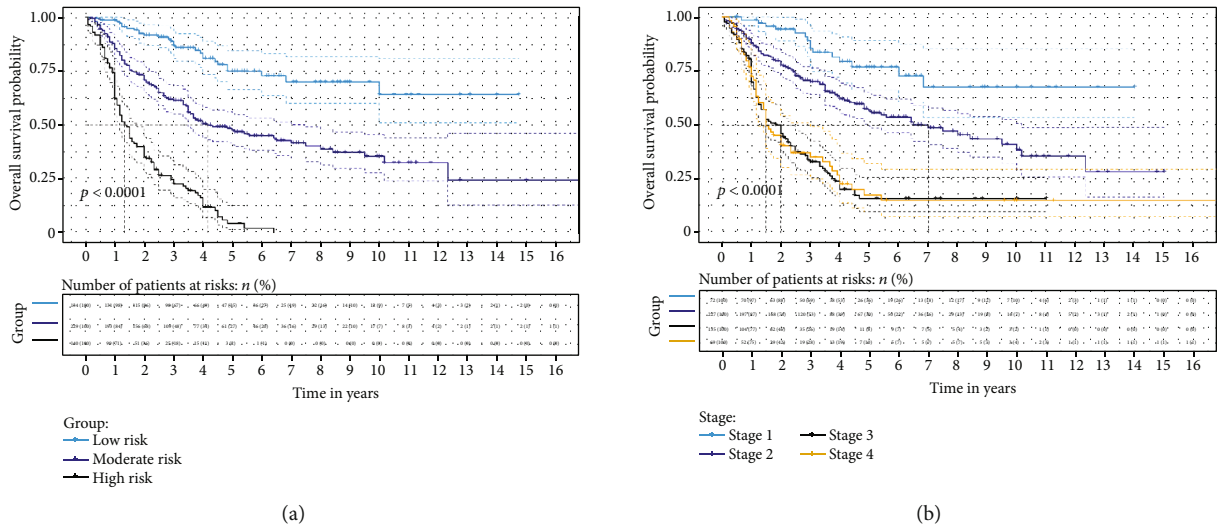
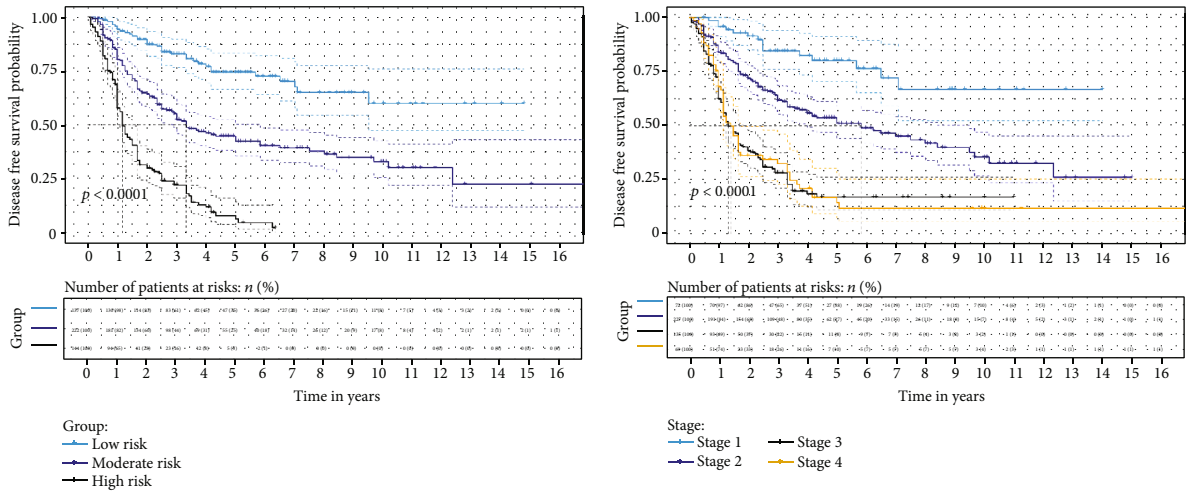



FIGURE 3: Kaplan-Meier survival curves of the primary cohort categorized by different staging systems for overall survival ((a) established model; (b) American Joint Committee on cancer (AJCC) seventh edition).

postoperative sepsis is associated with aggressive immunosuppression [23], which is potentially associated with cancer recurrence and mortality [21, 22].

The present study has some limitations. First, the present single-center study had a retrospective design. Nonetheless, the study utilized a database of more than 500 cases from a single institution that used relatively standardized surgical techniques and postoperative management, thereby avoiding some of the limitations of multicenter, population-based, or nationwide studies. Second, the present study did not include external validation. Although 200 bootstrap resamples were carried out for internal validation, there is still a risk of bias.




(a) (b)

Figure 4: Kaplan-Meier survival curves of the primary cohort categorized by different staging systems for disease free survival ((a) established model; (b) American Joint Committee on cancer (AJCC) seventh edition).

Third, patients had a median number of only 13 examined LNs. Thus, this data may not be suitable for patients with more extensive lymphadenectomy. However, previous studies have suggested that extensive lymphadenectomy do not provide any survival benefit. Furthermore, extensive lymphadenectomy introduces additional risks for complications, and may delay postoperative recovery time and reduce quality of life.

## 5. Conclusions

The present study has incorporated pathological T stage, pathological N factor, rate of positive LNs, history of COPD, and postoperative sepsis into a nomogram to predict the OS and DFS of ESCC patients. This practical system may help clinicians in both decision-making and clinical study design. The preoperative assessment of lung function in patients with COPD, and the control disease progression are needed. Furthermore, the postoperative infection of patients should be controlled. Further studies may help to extend the validation of the method, and improve the model through parameter optimization.

## Abbreviations

ESCC: Esophageal squamous cell carcinoma
COPD: Chronic obstructive pulmonary disease
OS: Overall survival
DFS: Disease-free survival
TNM: Tumor node metastasis
AJCC: American Joint Committee on Cancer
BMI: Body mass index
WBC: White blood cell
NLR: Neutrophil to lymphocyte ratio
ARDS: Acute respiratory distress syndrome
CTCAE: Common terminology criteria for adverse events
IQR: Interquartile range
AIC: Akaike's Information Criterion
ANOVA: Analysis of variance.

## Data Availability

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

## Ethical Approval

This study was approved by the ethics committees of Huashan Hospital, Fudan University, China, which waived the requirement for informed consent due to the use of anonymized retrospective data that were routinely collected during the health-screening process.

## Disclosure

The manuscript has been submitted as preprint as per the following link https://www.researchsquare.com/article/rs-37236/v1.

## Conflicts of Interest

The authors declare that they have no competing interests.

## Authors' Contributions

Zhiyong Zhao, Xiaolong Huang, and Ting Gu contributed equally to this work. Xiaolong Huang analyzed and interpreted the patient data regarding the hematological disease and the transplant. Ting Gu, Zhu Chen, and Limin Gan collected the data, and Zhiyong Zhao was a major contributor in writing the manuscript. Biao Zhu and Ning Wu designed the thoughts and methods of this research. All authors read and approved the final manuscript.

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*Research Article*

# A Novel Method of Natural Orifice Specimen Extraction Surgery (NOSES) during Laparoscopic Anterior Resection for Rectal Cancer

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We propose a modification to the reconstruction method of natural orifice specimen extraction surgery (NOSES) during laparoscopic anterior resection (LAR) for rectal cancer (RC) and evaluated its feasibility and short-term safety by comparing surgical and postoperative outcomes with those of conventional LAR. Twenty patients with RC underwent "double-purse" NOSES-LAR from October 2017 to June 2018. Data of clinicopathological characteristics, surgical and postoperative outcomes, and follow-up findings in NOSES-LAR cases were collected and retrospectively compared with those of conventional LAR to clarify the clinical benefits. The median postoperative hospital stay was lower in the double-purse NOSES group than the conventional group (6.6 vs. 7.1 days, respectively). In the conventional group, anastomotic leakage and incision site infection occurred in one patient each. In contrast, there were no complications in the double-purse group. There were no significant differences in blood loss, surgical duration, and time of the first flatus between the two groups. Additionally, "double-purse" NOSES-LAR was more economical than the conventional LAR. "Double-purse" NOSES-LAR is a safe, feasible, and minimally invasive promising procedure for LAR of RC with faster recovery, while requiring less surgical skills and lower clinical costs.

## 1. Introduction

Laparoscopic anterior resection (LAR) is widely used for rectal cancer (RC) because of the minimally invasive nature and safety of the procedure, thereby supporting its use as an alternative to open surgery [1–3]. However, current conventional laparoscopic-assisted procedures usually require additional abdominal incisions for specimen extraction and completing anastomosis, and minilaparotomy can often lead to postoperative pain, surgical site infection, incisional hernia, and poor cosmetic outcomes [4–6].

In recent years, natural orifice transluminal endoscopic surgery (NOTES) has become the focus of RC surgery. However, NOTES requires technological expertise and specialized devices, which limits its applicability in clinical practice [7, 8]. At this time, natural orifice specimen extraction surgery (NOSES) has been increasingly applied due to its advantage of a reduced risk of abdominal wounds [9–13]. There are various methods for extraction of RC specimens and digestive tract reconstruction. In combination with clinical practice, our center introduced a modified surgical procedure, called "double-purse" NOSES, for reconstruction and collection of specimens from the anus. However, the long-term efficacy of double-purse NOSES is unknown. Therefore, the aim of the present study was to evaluate the short-term efficacy of double-purse NOSES for resection of RC. This retrospective study was approved by the Ethics Committee of Huashan Hospital, Fudan University.

## 2. Materials and Methods

*2.1. Patients.* The study cohort consisted of 20 patients with RC [11 males and 9 females; median age, 63 years; age range, 40–75 years; mean body mass index (BMI), 23.5 kg/m$^2$; BMI range, 19.2–27.8 kg/m$^2$] who underwent complete LAR via

the transanal approach in the Department of General Surgery of Huashan Hospital (affiliated to Fudan University) from October 2017 to June 2018. A diagnosis of rectal adenocarcinoma was confirmed before surgery, and all procedures were performed endoscopically. Enhanced computed tomography, magnetic resonance imaging, and other auxiliary examinations were performed to ensure no invasion of the serosal layer or distant metastases, especially to the liver or lung.

The indications for LAR with double-purse NOSES were (1) a dentate line from the lower margin of the tumor > 5 cm, (2) tumor invasion depth ≤ cT3, (3) circumferential diameter of the tumor < 5 cm, and (4) body mass index < 28 kg/m$^2$. Relative contraindications were (1) tumor invasion of the serosal layer, (2) tumor diameter > 5 cm, and (3) short and thick mesentery.

According to the above indications, 20 patients who underwent traditional LAR were assigned to the control group.

Colorectal cancer in both groups was single tumor. The diameter of all patients' tumor was less than 5 cm. There were 20 cases of mass carcinoma, 15 cases of invasive carcinoma, and 5 cases of ulcerative carcinoma. There was no significant differences in age, sex ratio, BMI, tumor size, and tumor gross type between the two groups.

This retrospective study was approved by the Ethics Committee of Huashan Hospital.

*2.2. Surgical Procedures of Double-Purse NOSES-LAR.* After general surgery, the patient was placed in the lithotomy position. A curved incision was made up to the umbilicus, and a 10-mm trocar was placed. After pneumoperitoneum establishment, the abdominal cavity was explored to determine whether invasion or metastasis had occurred. Then, a 12 mm trocar was placed in the major surgical port in the right lower quadrant, and a 5 mm trocar was placed at the intersection of the level of the umbilicus and the outer edge of the right rectus abdominis. Two additional 5 mm trocars were placed symmetrically on the left side of the abdomen.

Generally, the lymphovascular trunk to the rectosigmoid colon was carefully divided and ligated. Then, the sigmoid colon and associated mesocolon were mobilized in the mediolateral direction in Toldt's fascia. Afterwards, sharp pelvic dissection with a nerve-sparing technique was performed according to the principle of total mesorectal excision. Rectal "baring" was performed at about 5 cm in the distal part of the tumor. Next, approximately 3 cm of the bowel was bared during predissection of the proximal sigmoid colon (generally 10 cm from the upper edge of the tumor). Then, the upper and lower sides of the bared area were, respectively, ligated with a blocking plier and sterilized hemp rope to prevent contamination of the surgical field, as well as tumor spread that might be caused by surgical mobilization. Afterward, an ultrasound scalpel was used to transect the bowel, and both ends were disinfected with iodine volt gauze.

After full enlargement of the anus, the distal bowel lumen containing the tumor was pulled out through the anus using oval forceps. A large amount of iodine volt gauze was used to scrub the rectal mucosa, and then the specimen was removed with purse-string forceps after the lower edge of the incision was accurately judged. At this time, the purse-string knot ("No. 1 purse") was not tied. Then, sponge forceps were used to pull the proximal sigmoid colon out of the pelvic cavity through the rectal stump under laparoscopic guidance. The detachable anvil of a circular stapler (CDH29; Ethicon Inc., Somerville, NJ, USA) was put into the proximal colon end, fixed firmly using the same purse-string instrument, and then returned back to the abdominal cavity. Afterward, the "No. 1 purse" of the rectal remnant was tightened moderately and knotted (note: the "No. 1 purse" should not be tightened too tightly so that the central hole can pass through the tip of the hemostatic forceps just enough). Then, this segment of the bowel lumen was reverted back into the abdominal cavity. After reestablishing the pneumoperitoneum, the circular stapler was inserted into the anus, and the central rod was penetrated from the "No. 1 purse" via the central hole with careful adjustment to finish the end-to-end anastomosis. The process is shown in Figure 1.

The pelvic cavity was flushed with a large amount of dilute iodine volts and normal saline, and then a pelvic drainage lumen was inserted through the trocar hole in the right lower abdomen to the pelvic floor. The trocar hole was subcutaneously injected with ropivacaine. The wound was cemented with biological adhesive without scar treatment.

*2.3. Traditional Group.* The procedure of the radical resection was the same as with the double-purse technique, but the reconstruction style differed. Briefly, the rectum was transected more than 2 cm distant from the lower margin of the tumor with a 60 mm endoscopic linear stapler (EC60A; Ethicon Endo-Surgery, Inc., Blue Ash, OH, USA). Subsequently, an incision about 5–6 cm was made to the lower abdomen, from which the specimen was resected and removed. After treatment with a purse-string instrument, the anvil was inserted into the proximal colonic ends. Then, the incision was sutured under laparoscopic vision to complete the end-to-end anastomosis.

*2.4. Postoperative Treatment.* Prophylactic use of antibiotics lasted in 48 h. The patient was offered water at 6–8 h after anesthesia, and a normal diet was offered after exhaust defecation was restored. Finally, the pelvic drainage tube was removed.

*2.5. Statistical Analysis.* All statistical analysis was carried out through a commercial statistical software package SPSS 22.0 by IBM. The Mann–Whitney $U$ tests were used to test pairwise differences between two groups for continuous variables and for ordered categorical variables. Chi-squared tests were used for categorical variables. $P$ values <0.05 were held as significant.

## 3. Results

*3.1. Patient Characteristics and Surgical Outcomes.* The baseline demographics of the two groups were comparable (Table 1). Of the 20 double-purse patients, 9 (45%) were females, and 11 (55%) were males with a median age of 63 (range, 40–75) years and mean BMI of 23.5 ± 3.0 (range, 17.9–29.8) kg/m$^2$. Of the 20 patients in the conventional

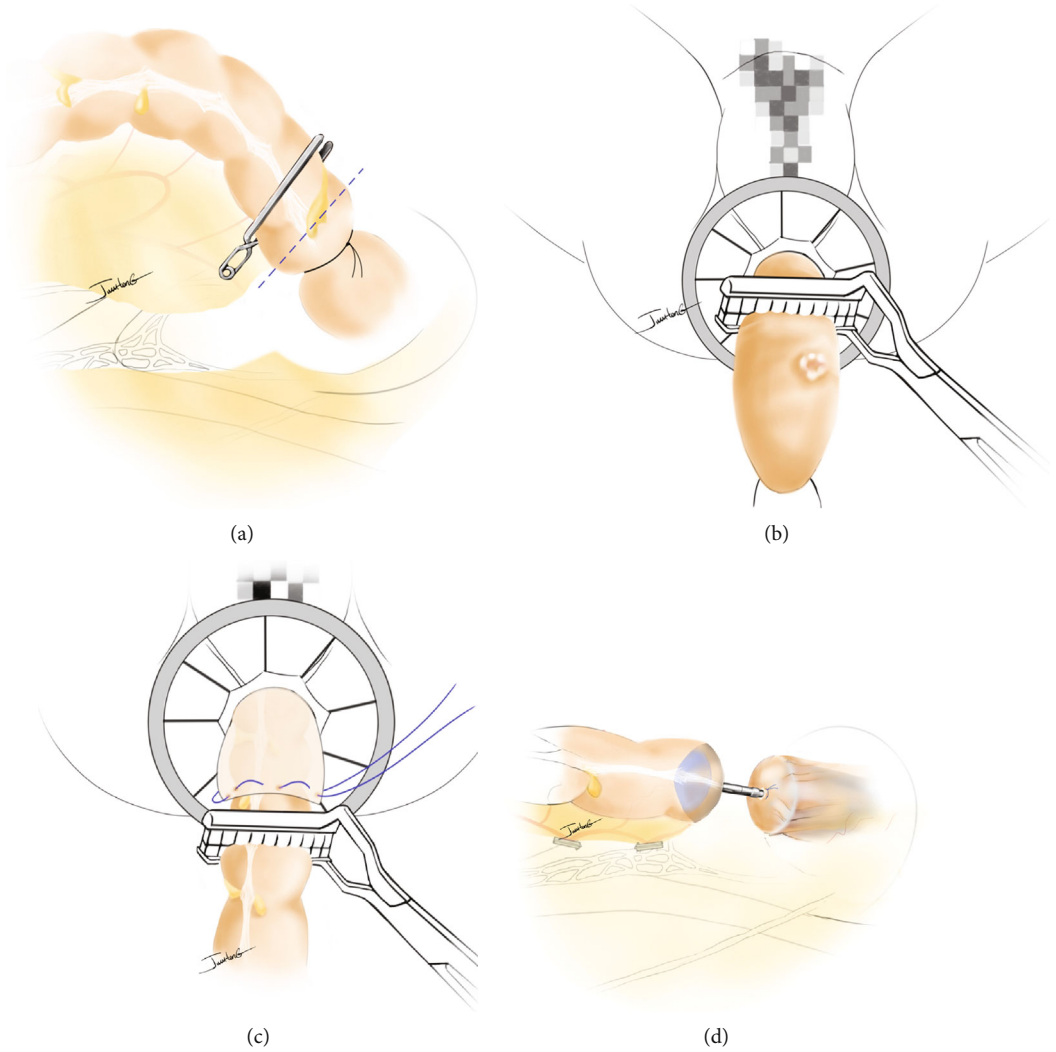

Figure 1

treatment group, 12 (60%) were males, and 8 (40%) were females with a median age of 64 (range, 38–79) years and mean BMI of 22.9 ± 2.9 (range, 16.6–30.1) kg/m$^2$. No patient had critical organ dysfunction.

There was no surgery-related death, and no prophylactic enterostomy was performed intraoperatively in either group. All patients had a negative surgical margin. There were no significant differences in mean blood loss and time to first flatus. However, four patients in the conventional group complained of pain from the incision and were treated with painkillers, while none in the double-purse group has this complaint. Meanwhile, the median postoperative hospital stay was shorter for the double-purse group than the conventional group (6.7 vs. 7.8 days, respectively), although there was no statistical significance due to the small sample size. Moreover, the hospitalization costs of our new method is less than that of the traditional method. The tumor characteristics were similar between the groups (Table 1).

*3.2. Postoperative Complications and Follow-Up.* Postoperative complications are listed in Table 2. There was no instance of postoperative abdominal or anastomotic hemorrhage in either group. However, mild anastomotic leakage was observed in one patient in the traditional surgery group, which led to a pelvic infection, and one patient developed an infection of the incision site. Both complications were solved by conservative treatment. In contrast, there were no complications in the double-purse group.

All patients postoperatively diagnosed with stage III RC received adjuvant chemotherapy with capecitabine plus oxaliplatin. At a mean follow-up duration of 9.5 ± 4.2 months after surgery, all patients survived with no instance of incisional hernia at the trocar or incisional site and no case of local recurrence. However, one patient in the conventional group was diagnosed with liver metastasis at 9 months after surgery.

## 4. Discussion

With the development of the minimally invasive concept, laparoscopic-assisted radical resection has gradually replaced laparotomy as the main surgical method for the treatment of RC. However, classic laparoscopic surgery still requires an auxiliary incision into the abdomen of 4–6 cm to complete

Table 1: Patient demographics, surgical outcomes, and tumor characteristics of both groups.

| Characteristic | Double-purse NOSES | Conventional | *P* |
|---|---|---|---|
| Patient demographics | | | |
| Age, years (range) | 63 (40-75) | 64 (38-79) | 0.547 |
| Sex ratio, male/female | 11/9 | 12/8 | 0.818 |
| Body mass index, BMI (kg/m$^2$) | 23.5 ± 3.0 | 22.9 ± 2.9 | 0.859 |
| Tumor size | 2.5 ± 0.9 | 2.3 ± 0.7 | 0.647 |
| Tumor number | Single | Single | |
| Tumor gross type | | | |
| Mass carcinoma | 10 | 10 | |
| Invasive carcinoma | 8 | 7 | |
| Ulcerative carcinoma | 2 | 3 | |
| Surgical outcomes | | | |
| Surgical duration, min | 129.7 ± 27.5 | 132.4 ± 25.8 | 0.788 |
| Blood loss, mL | 56.5 ± 24.6 | 54.2 ± 21.5 | 0.474 |
| Time to first flatus, d | 2.4 ± 0.8 | 2.7 ± 1.3 | 0.252 |
| Postoperative hospital stay, d | 6.9 ± 0.6 | 7.8 ± 3.2 | 0.158 |
| Hospitalization costs* | 4.3 ± 0.5 | 5.3 ± 0.5 | <0.05 |
| Retrieved LNs, *n* | 20.7 ± 5.9 | 22.4 ± 6.0 | 0.550 |
| Pathologic findings (*n*) b | | | |
| T1/T2/T3/T4 | 6/8/6/0 | 5/8/5/2 | 0.680 |
| N0/N1/N2/N3 | 14/5/1/0 | 12/7/1/0 | 0.645 |
| TNM stage I/II/III/IV | 8/6/6/0 | 5/7/8/0 | 0.664 |

*10 thousand yuan.

Table 2: Postoperative complications.

| Characteristic | Double-purse NOSES | Conventional |
|---|---|---|
| Intraperitoneal or digestive tract hemorrhage, *n* | 0 | 0 |
| Intraabdominal infection or abscess, *n* | 0 | 1 |
| Incision site infection, *n* | 0 | 1 |
| Anastomotic leakage, *n* | 0 | 1 |
| Anastomotic stenosis, *n* | 0 | 0 |

the removal of specimens, implantation of the anvil, and digestive tract reconstruction. An abdominal incision increases the risk of postoperative wound pain, resulting in delayed time to free movement and discharge, as well as incision-related complications, such as infection, intestinal adhesion, tumor implantation, etc. Thus, the advantages of laparoscopic minimally invasive surgery are obviously weakened [14–16].

Although still in the stage of clinical exploration, NOTES requires specific equipment and experience with the surgical technique. Therefore, surgery of nonincision specimen extraction for colorectal cancer can be considered as a transitional stage from traditional laparoscopic surgery to scar-free surgery. As a benefit of NOSES over the conventional method, specimens can be removed from a natural orifice, such as the vagina or rectum, without the need for an abdominal incision, resulting in a better aesthetic outcome, less postoperative pain, faster flatus, and earlier activity time, while lowering the risk of incision site infection and incisional hernia. However, specimen extraction via the vagina is only an option for female patients and may increase the incidence of postoperative complications due to incision of the posterior vaginal fornix. Thus, extraction of transrectal specimens is preferred. To date, various types of NOSES for LAR of RC have been reported [17–19]. Usually, the anvil is placed into the abdominal cavity through the anus and fixed in the proximal sigmoid ends under laparoscopic surveillance, while the distal rectum stump must be closed again for anastomosis. The authors consider that there are several deficiencies as follows: (1) the surgery is comparably complicated and requires the experience of a skilled team; otherwise, it is difficult for the surgeons to accurately determine whether the anvil is properly fixed, (2) the remnant rectal stump cannot be too short to apply the endo-GIA to close the ends, (3) there are still two weak horns around the anastomotic stoma after reconstruction, which are difficult to reinforce with sutures, and (4) increased surgical costs. The first three deficiencies are closely related to the occurrence of postoperative anastomotic leakage, which restricts the choices of the colorectal surgeons to a nonincisional technique.

To solve these problems, we introduced a modified NOSES method, called the "double-purse" procedure, which can be summarized in four steps, as follows: (1) two ligation

strings (or one blocking plier) are tied to the bowel after baring around the upper surgical margin of the tumor under laparoscopic vision, and the intestine lumen is transected between the strings, which not only guarantees an adequate upper margin of the incision but also complies with the tumor-free principle. (2) The diseased bowel segment is turned over and pulled from the body through the anus. Then, the specimen is removed using purse-string forceps after the lower margin is determined under direct vision. At this point, the purse string is not tightened. (3) Oval forceps are used to drag the proximal colon out from the body via the rectal stump under laparoscopic guidance, and the anvil is planted and fixed firmly using the same "purse" method. Afterward, this proximal segment is returned to the body. (4) The purse string of the valgus rectum is tightened and knotted moderately and then reversed backed into the peritoneal cavity. Under laparoscopic guidance, the pole of the stapler is pushed out from the middle of the purse string to complete an end-to-end anastomosis. As compared with the conventional method, we consider that the "double-purse" method has the following advantages: (1) the double-purse NOSES technique requires the proximal colon to be pulled out through the stump of the rectum, so the stump should not be too long, making it more suitable for tumors in the middle or even much lower rectum. (2) The procedure of placing the anvil into the proximal colonic canal for the purse string is much easier and more reliable because it is accomplished under direct vision, as compared to the conventional method, due to defects of two-dimensional vision under laparoscopy. (3) The traditional method requires the dissociation of the rectum more distally; otherwise, the endo GIA cannot be used for exact closure of the rectal stump. However, with the "double-purse" string method, it is not necessary to dissociate too many intestinal lumens, and dispensing with the closure device can also reduce costs. (4) After reconstruction of the alimentary tract by traditional methods, an area with an insufficient blood supply (weak angle) is generally found, while the weak angle is eliminated after end-to-end anastomosis by the double-purse-string method, which might reduce the incidence of postoperative anastomotic leakage.

In this study, the short-term efficacy of total laparoscopic resection of RC with NOSES was satisfactory in all 20 cases: the median surgical duration was 120 min, the median intraoperative blood loss was 35 ml, the median time to flatus was 48 h, no patient experienced severe postoperative complications, and no tumor recurrence or metastasis was observed during the follow-up period. It is considered that several important factors are essential to obtain a satisfactory curative effect and smooth operation in addition to the above procedure, which include (1) skill with the laparoscopic technique for LAR during NOSES to determine the length of the specimen, baring of the intestinal lumen, and completion of the anastomosis, which requires overcoming the anatomic dislocation of laparoscopic vision, and tacit cooperation between the operator and camera man. (2) Selection of appropriate patients (i.e., tumor distance from the dentate by 5–15 cm, no invasion of the serosa or neighboring structures (cT1–T3), tumor diameter < 5 cm, BMI < 28 kg/m$^2$, sufficient length of the sigmoid colon, and capacity to tolerate laparoscopic surgery). Patients who conform to the foregoing criteria can be considered candidates. (3) The purse string of the distal rectum stump must be tightened properly to facilitate puncture of the central rod of the circular staple. (4) Good bowel preparation and intraoperative sterile, tumor-free operations, such as ligation of the bowl before transection, iodine-volt cleaning of the rectum, and saline flushing of the pelvic cavity.

## 5. Conclusions

In conclusion, based on the principles of sterility, tumor-free surgery, and radical cure, the proposed double-purse NOSES technique for RC is more economical and might reduce the risk of postoperative complications related to the incision in some select patients. Meanwhile, the technique is safe and feasible with satisfactory short-term efficacy and is suitable for widespread application in a number of colorectal cancer treatment centers. Finally, the indications for this approach should be met, while prospective randomized controlled clinical studies are warranted to evaluate the long-term benefits.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

## Authors' Contributions

Jun Wang and Jun Hong contributed equally to this work.

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*Research Article*

# Extrapolating Prognostic Factors of Primary Curative Resection to Postresection Recurrences Hepatocellular Carcinoma Treatable by Radiofrequency Ablation

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*Objective*. Recurrence after curative resection for hepatocellular carcinoma (HCC) is a major cause of death from this disease. Factors of primary curative resection are available and potential in the prognosis of follow-up treatment. Our aim was to assess the prognostic significance of primary curative resection factors in recurrent HCC patients undergoing radiofrequency ablation therapy (RFA). *Methods*. In this retrospective study, we assessed 235 patients who underwent limited RFA of HCC recurrences (tumors ≤ 5 cm; nodules ≤ 3) after primary curative resection. Factors of primary curative resection were collected, and overall survival and recurrence-free survival were evaluated by the Kaplan-Meier method. Univariate and multivariate analyses were used to identify significant prognostic factors. *Results*. After a median follow-up of 36 months, 54 patients died, and 128 patients had hepatic recurrence. On univariate analyses, patients whose primary tumors were less differentiated ($p = 0.032$ and $p = 0.048$) and required less time to recur ($p = 0.013$ and $p = 0.001$) after curative resection displayed poorer overall survival and higher recurrence rates following RFA. On multivariate analyses, the pathologic tumor grade ($p = 0.026$ and $p = 0.038$) and recurrence-free survival after primary curative resection ($p = 0.028$ and $p < 0.001$) emerged as independent risk factors of survival and HCC recurrence. *Conclusions*. Primary tumor differentiation and time to recurrence after curative resection are viable prognostic factors of overall survival and further recurrence risk in patients undergoing RFA of recurrent HCC.

## 1. Introduction

Hepatocellular carcinoma is the fourth most common cause of cancer-related death worldwide [1]. Surgical resection represents one of the best first-line treatments for selected patients [2]. However, recurrences after curative surgery are frequent (range, 40-70%) [3, 4]. Proper follow-up for postoperative patients and recent advances in diagnostic modalities has led to an increased detection rate of recurrent tumors at an early stage. Specifically, these tumors are solitary and small, and they represent an opportunity for radical treatment.

Compared with initial treatment, more limited liver function reserve and technical difficulties in repeated hepatic resection owing to postoperative adhesion are expected in the treatment of recurrent HCC after hepatic resection [5]. Radiofrequency ablation (RFA) is regarded as an alternative curative treatment modality for small recurrent HCC instead of repeated surgical resection because of minimal invasion and damage to the liver, and some studies showed that RFA had similar outcomes as repeated surgical resection [5–7]. However, even complete tumor ablation does not ensure disease eradication. It is estimated that the cumulative 5-year recurrence rate of patients undergoing RFA is more than 70% [5–7]. To improve the long-term outcome of RFA, it is crucial to elucidate the mechanisms and risk factors associated with prognosis after RFA.

Factors of primary curative resection are available for recurrent HCC patients without additional traumatic examination, including detailed clinical and pathological information of primary tumors, which have been shown as prognostic factors related to overall survival and recurrence of primary curative resection [8–10]. However, their clinical

merits in recurrent HCC patients undergoing RFA have yet to be proven.

In the present study, we try to explore the significance of factors of primary curative resection in prognosis in recurrent HCC after treatment with RFA. Therefore, we performed a study on recurrent HCC cases after curative resection treated with RFA and investigated the relationship between factors of primary curative resection and prognosis including both overall survival and tumor recurrence after treatment.

## 2. Patients and Methods

*2.1. Ethical Approval.* All procedures involving human participants maintained standards of the Ethics Committee of Zhongshan Hospital affiliated with Fudan University, the 1964 Helsinki Declaration and its later amendments, or comparable ethical principles. The need for formal informed consent of individual participants was waived, because no patients were at risk in the retrospective analysis. Patient records were anonymized and deidentified prior to analysis.

*2.2. Patient Selection.* We conducted a retrospective analysis of prospectively collected data, contributed by 623 consecutive patients with recurrences of HCC after primary curative resection. All had undergone RFA at the Liver Cancer Institute of Zhongshan Hospital (Fudan University) between January 2010 and December 2015. In each instance, for primary tumors, the diagnosis was based on histologic assessment; for recurrent HCC, diagnosis was based on the histologic result or criteria established by the American Association for the Study of Liver Disease (AASLD). Typically, HCC is marked by intense arterial uptake of contrast agent and subsequent washout in venous-delayed phases of contrast-enhanced magnetic resonance imaging (MRI), or computed tomography (CT) [11].

Only 305 of the initial 623 study candidates met our inclusion criteria as follows: (1) no prior treatment of HCC other than curative resection; (2) time to recurrence $\geq 3$ months after curative resection; (3) Child-Pugh class A or B liver function; (4) limited recurrent disease (single nodule $\leq 5.0$ cm or $\leq 3$ nodules, the largest $\leq 3.0$ cm); and (5) no invasion of major intrahepatic vessels or extrahepatic metastasis. Another 70 patients were excluded due to incomplete ablation ($n = 5$) or loss of follow-up ($n = 65$) within 12 months. Incomplete ablation was defined as CT or MRI evidence of irregular, peripherally enhancing foci in ablation zones 4 weeks after RFA, in the absence of salvage RFA. Ultimately, 235 patients were selected for study.

*2.3. RFA Procedures.* Patients were treated either with the RITA RFA system (Starburst XL; Mountain View, CA, USA) or Cool-Tip RFA system (Covidien; Boulder, CO, US) to deliver the RF energy [12]. The procedures were performed percutaneously with real-time ultrasonic guidance, with patients pretreated with local anesthetic and intramuscular sedation. On withdrawal of the electrode needle, the tract was ablated to prevent bleeding and tumor seeding. Cardiovascular and respiratory functions were monitored, and the hyperechoic area around the electrode tip was observed by ultrasonic monitoring during the procedure. The treatment was designed to cover the whole tumor area with at least a 5 mm safety ablative margin extending into the surrounding normal hepatic parenchyma. One month after RFA, contrast-enhanced MRI or CT was performed to evaluate the effectiveness of tumor ablation. Patients with residual tumor were treated by salvage RFA.

*2.4. Follow-Up Observation after RFA Treatment.* The median follow-up period was 36 months (range, 11-82 months). Patients were followed up with an interval of 2-3 months. Serum alpha-fetoprotein (AFP) levels were measured, liver function was analyzed, and ultrasonography and CT or MRI was performed. If recurrent HCC was again detected (confirmed by typical imaging features), patients were managed accordingly, opting for repeated RFA, surgical resection, percutaneous ethanol injection, transarterial chemoembolization (TACE), or radiotherapy. Procedure-related mortality was defined as any death occurring within 30 days after RFA. Recurrence-free survival (RFS) was calculated from the date of RFA until the time at which tumor recurrence was confirmed. Patients were censored at date of death or at date of last follow-up visit if tumor recurrence was not diagnosed.

*2.5. Factors Prognostic of Primary Curative Resection Outcomes.* Factors of primary curative resection were collected including primary tumor size, tumor number, tumor differentiation, microvascular invasion or not, encapsulation invasion or not, and star lesion or not, according to the postoperative pathological report. And postoperative adjuvant TACE and time to relapse after curative resection were also included on the basis of follow-up data.

*2.6. Statistical Analyses.* Analysis was performed with SPSS 19.0 for Windows (SPSS; Chicago, IL, USA). All consecutive data were expressed as mean ± standard deviation. Differences between the two groups were analyzed using the unpaired $t$-test for continuous variables, and categorical variables were analyzed using the $x^2$ test or Fisher's exact test. The cumulative overall survival (OS) rate and RFS rate were assessed by the Kaplan-Meier method, and the difference between the two groups was evaluated with the log-rank method. Prognostic factor affecting OS and RFS was determined using univariate and multivariate analysis: significant variables obtained by univariate analysis were tested by multivariate analysis using Cox's proportional hazard model. A $p$ value of $<0.05$ was considered to be statistically significant.

## 3. Results

The median age of patients under study was 56 years (range, 21-83 years). There were 196 men and 39 women (male-to-female ratio, 5.0 : 1). Most patients (90.21%, 212/235) tested positive for hepatitis B virus (HBV) infection, rarely (0.85%, 2/235) testing positive for hepatitis C virus (HCV) infection, and none showed dual HBV/HCV positivity. Based on the Child-Pugh classification of the liver function, 95.74% (225/235) HCC patients were at class A liver function and 4.26% (10/235) at class B. After a median follow-up of 36

Table 1: Univariate analysis of the prognostic factors contributing to OS and RFS of recurrent HCC patients undergoing RFA.

| | Variables | n | OS $p$ value[1] | RFS $p$ value[1] |
|---|---|---|---|---|
| Factors of primary curative resection | Gender, male/female | 196/39 | 0.443 | 0.111 |
| | Age (>55years), yes/no | 120/115 | 0.581 | 0.769 |
| | Hepatitis, yes/no | 214/21 | 0.306 | 0.321 |
| Factors of primary curative resection | Tumor size (>3 cm), yes/no | 127/108 | 0.652 | 0.592 |
| | Tumor number, multiple/single | 51/184 | 0.729 | 0.319 |
| | Tumor differentiation (>II), yes/no | 64/171 | 0.032 | 0.048 |
| | Microvascular invasion, yes/no | 56/179 | 0.380 | 0.289 |
| | Encapsulation invasion, yes/no | 7/228 | 0.672 | 0.216 |
| | Star lesion, yes/no | 6/229 | 0.650 | 0.051 |
| | Postoperative adjuvant TACE, yes/no | 69/166 | 0.050 | 0.101 |
| | Time to recurrence after curative resection (≤9 months), yes/no | 78/157 | 0.006 | <0.001 |
| Features before RFA | TB (>17.1 $\mu$mol/l), yes/no | 30/205 | 0.129 | 0.084 |
| | ALT (>50 IU/l), yes/no | 32/203 | 0.375 | 0.718 |
| | PT (>14 s), yes/no | 14/221 | 0.066 | 0.979 |
| | Albumin (≤3.5 g/dl), yes/no | 12/223 | 0.781 | 0.361 |
| | GGT (>50 IU/l), yes/no | 95/140 | 0.002 | 0.022 |
| | AFP (>20 ng/ml), yes/no | 80/155 | 0.010 | 0.292 |
| | Tumor size (>2.0 cm), yes/no | 72/163 | 0.830 | 0.853 |
| | Tumor number, multiple/single | 36/199 | 0.622 | 0.023 |

OS: overall survival; RFS: recurrence-free survival; RFA: radiofrequency ablation; TB: total bilirubin; ALT: alanine aminotransferase; PT: prothrombin time; GGT: $\gamma$-glutamyltranspeptidase; AFP: alpha-fetoprotein; [1]log-rank test.

Table 2: Multivariate analysis of the factors associated with OS of recurrent HCC patients undergoing RFA.

| Risk factor | Hazard ratio | 95% CI | $p$ value[1] |
|---|---|---|---|
| Tumor differentiation (>II), yes/no | 1.878 | 1.077-3.275 | 0.026 |
| Time to recurrence after curative resection (≤9 months), yes/no | 1.828 | 1.066-3.135 | 0.028 |
| GGT (>50 IU/l), yes/no | 2.186 | 1.271-3.760 | 0.005 |
| AFP (>20 ng/ml), yes/no | 1.929 | 1.133-3.284 | 0.016 |

OS: overall survival; RFA: radiofrequency ablation; CI: confidence interval; GGT: $\gamma$-glutamyltranspeptidase; AFP: alpha-fetoprotein; [1]Cox proportional hazard model.

Table 3: Multivariate analysis of the factors associated with RFS of recurrent HCC patients undergoing RFA.

| Risk factor | Hazard ratio | 95% CI | $p$ value[1] |
|---|---|---|---|
| Tumor differentiation (>II), yes/no | 1.497 | 1.022-2.192 | 0.038 |
| Time to recurrence after curative resection (≤9 months), yes/no | 2.400 | 1.687-3.414 | <0.001 |
| GGT (>50 IU/l), yes/no | 1.496 | 1.050-2.133 | 0.026 |
| Tumor number, multiple/single | 1.681 | 1.075-2.628 | 0.023 |

RFS: recurrence-free survival; RFA: radiofrequency ablation; CI: confidence interval; GGT: $\gamma$-glutamyltranspeptidase; [1]Cox proportional hazard model.

months, 128 patients had developed recurrences, and 55 deaths were recorded. OS rates at 1, 3, and 5 years were 99.1%, 78.2%, and 61.2%, respectively. Corresponding RFS rates were 60.9%, 44.8%, and 35.8%, respectively.

Analysis of prognostic factors affecting OS and RFS was performed. The variables tested by univariate analysis were sex, age, hepatitis history, factors of primary curative resection, and features before RFA including total bilirubin (TB), alanine aminotransferase (ALT), prothrombin time (PT), albumin, $\gamma$-glutamyltranspeptidase (GGT), AFP, tumor size, and tumor number. The results indicated that primary tumor differentiation, time to recurrence after curative resection, GGT, and AFP before RFA were significant prognostic factors associated with OS; while primary tumor differentiation, time to recurrence after curative resection, GGT before RFA, and recurrent tumor number after curative resection

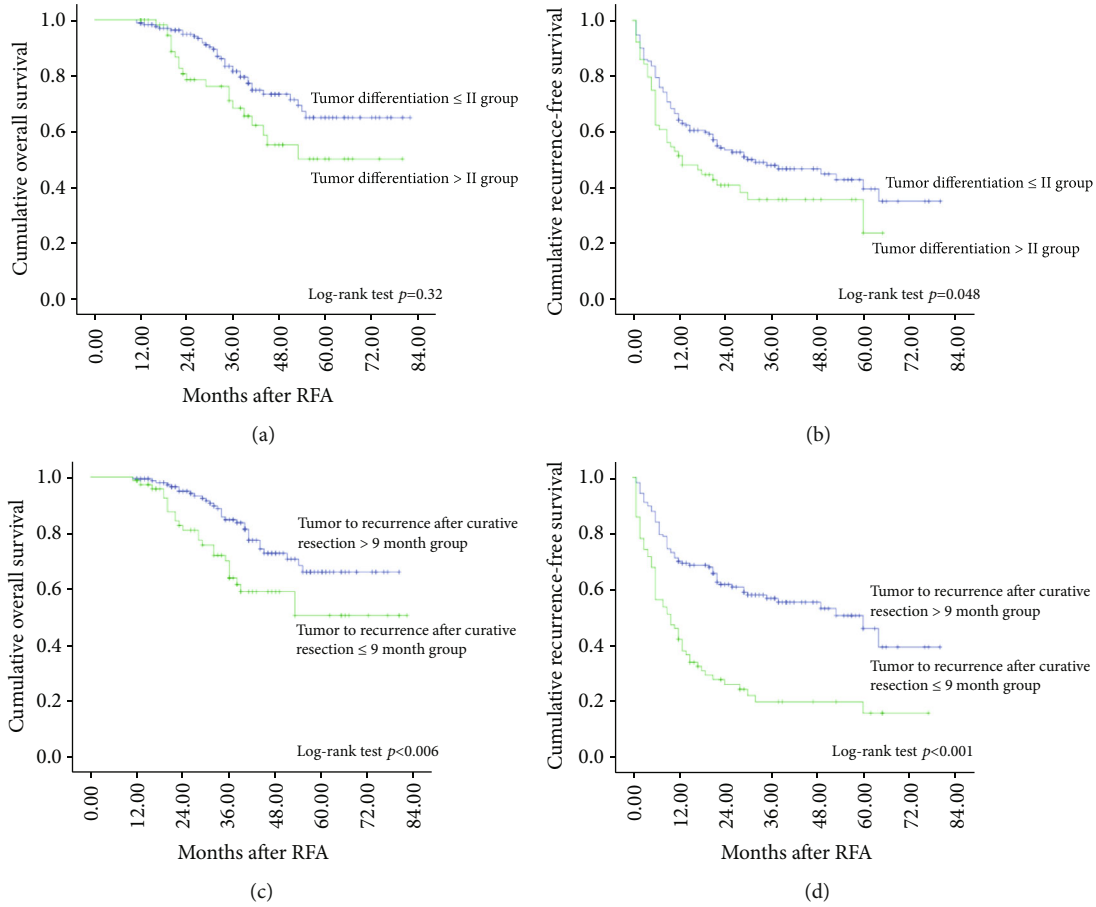


Figure 1: Worse primary tumor differentiation and short time to recurrence after curative resection bode poorly for clinical outcomes of recurrent hepatocellular carcinoma undergoing radiofrequency ablation. Univariate analyses of primary tumor differentiation demonstrated that worse tumor differentiation was associated with poorer overall survival (OS) rates (a) and poorer recurrence-free survival (RFS) rates (b) (Kaplan-Meier and log-rank tests). Prognostic performance of primary tumor differentiation was significant for both OS and RFS ($p = 0.032$ and $p = 0.048$, respectively). Univariate analyses of time to recurrence after curative resection demonstrated that short time to recurrence was associated with poorer OS rates (c) and poorer RFS rates (d) (Kaplan-Meier and log-rank tests). Prognostic performance of time to recurrence after curative resection was significant for both OS and RFS ($p = 0.006$ and $p < 0.001$, respectively).

associated with RFS (Table 1). All significant parameters and related variables were then subjected to multivariate Cox proportional hazards analysis. Primary tumor differentiation, time to recurrence after curative resection, GGT, and AFP before RFA were found to be independent risk factors of OS (Table 2), and primary tumor differentiation, time to recurrence after curative resection, GGT before RFA, and recurrent tumor number after curative resection were independent risk factors of RFS (Table 3).

Primary tumor differentiation was proven significant by univariate analysis in predicting both OS and RFS, using ≤ grade II vs > grade II tumor differentiation for dichotomous evaluation ($p = 0.032$, $p = 0.048$, respectively) (Figures 1(a) and 1(b)). In multivariate models, primary tumor differentiation emerged as independently predictive of OS (hazard ratio [HR] = 1.878, 95% confidence interval [CI]: 1.077-3.275; $p = 0.026$) and recurrence (HR = 1.497, 95% CI: 1.022–2.192; $p = 0.038$).

Time to recurrence after curative resection was proven significant by univariate analysis in predicting both OS and RFS, using >9 months vs ≤9 months to recurrence after curative resection for dichotomous evaluation ($p = 0.013$ and $p = 0.001$, respectively) (Figures 1(c) and 1(d)). In multivariate models, time to recurrence after curative resection

Table 4: Time to recurrence after curative resection predictive value of OS and RFS using different cut-off values.

| Cut-off values of time to recurrence after curative resection (months) | Log-rank analyses for OS, $p$ value[1] | Log-rank analyses for RFS, $p$ value[1] |
|---|---|---|
| 4 | 0.636 | 0.001 |
| 6 | 0.380 | <0.001 |
| 9 | 0.006 | <0.001 |
| 12 | 0.013 | 0.001 |
| 18 | 0.083 | <0.001 |
| 24 | 0.089 | 0.002 |
| 36 | 0.683 | 0.004 |

OS: overall survival; RFS: recurrence-free survival; [1]log-rank test.

Table 5: Baseline characteristics between the short time to recurrence after curative resection group and the long time to recurrence after curative resection group.

| | Variables | Short time to recurrence after curative resection ($n = 78$) | Long time to recurrence after curative resection ($n = 157$) | $p$ value |
|---|---|---|---|---|
| Factors of primary curative resection | Gender, male/female | 69/9 | 127/30 | 0.098[2] |
| | Age (years) | 53.359 ± 12.732 | 55.950 ± 11.601 | 0.120[1] |
| | Hepatitis, yes/no | 72/6 | 142/15 | 0.419[2] |
| | Cirrhosis, yes/no | 17/61 | 37/120 | 0.449[1] |
| | Tumor size (cm) | 4.172 ± 2.396 | 4.115 ± 2.687 | 0.874[1] |
| | Tumor number, multiple/single | 17/61 | 34/123 | 0.553[2] |
| | Tumor differentiation (>II), yes/no | 28/50 | 36/121 | 0.027[2] |
| | Microvascular invasion, yes/no | 25/53 | 32/125 | 0.029[2] |
| | Encapsulation invasion, yes/no | 2/76 | 5/152 | 0.574[2] |
| | Star lesion, yes/no | 2/76 | 4/153 | 0.649[2] |
| | Postoperative adjuvant TACE, yes/no | 26/52 | 43/114 | 0.214[2] |
| Features before RFA | TB (>17.1 $\mu$mol/l), yes/no | 3/75 | 27/130 | 0.452[2] |
| | ALT (>50 IU/l), yes/no | 12/66 | 20/137 | 0.578[2] |
| | PT (>14 s), yes/no | 5/73 | 9/148 | 0.836[2] |
| | Albumin (≤3.5 g/dl), yes/no | 2/76 | 10/147 | 0.212[2] |
| | GGT (>50 IU/l), yes/no | 36/42 | 59/98 | 0.207[2] |
| | AFP (>20 ng/ml), yes/no | 26/52 | 54/103 | 0.872[2] |
| | Tumor size (cm) | 1.754 ± 0.613 | 1.698 ± 0.525 | 0.470[1] |
| | Tumor number, multiple/single | 14/64 | 22/135 | 0.272[2] |

Data are expressed as the number or mean value ± standard deviation. RFA: radiofrequency ablation; TB: total bilirubin; ALT: alanine aminotransferase; PT: prothrombin time; GGT: $\gamma$-glutamyltranspeptidase; AFP: alpha-fetoprotein; [1]unpaired t test; [2]Fisher's exact test or $x^2$ test.

emerged as independently predictive of OS (HR = 1.828, 95% CI: 1.066-3.135; $p = 0.028$) and recurrence (HR = 2.400, 95% CI: 1.687-3.414; $p < 0.001$). Furthermore, $p$ values were calculated for log-rank survival analysis using different cut-offs for time to recurrence after curative resection. $p$ values reflected statistical significance when using 9 and 12 months as cut-off values in OS analysis and 4 to 36 months in recurrence-free survival analysis, suggesting good reproducibility (Table 4).

Baseline clinical characteristics of patients experiencing short and long times to recurrences after curative resection are listed in Table 5, and the distribution are shown in Figure S1. Shorter time to recurrence after curative resection was closely linked with less differentiated primary tumor and primary tumor microvascular invasion.

## 4. Discussion

RFA is a safe and effective treatment for HCC patients with solitary and small recurrent tumors of the liver after curative resection, especially for those who are not candidates for repeat resection [4, 5]. Unfortunately, recurrent HCC patients undergoing RFA, which is designed to destroy tumors locally without damage to the surrounding tissue, also have a high incidence of recurrence after completing percutaneous ablation [5–7]. Typically in HCC, most deaths are due to tumor recurrence or local progression [13]. Although factors of primary curative resection, including detailed clinical and pathological information of primary tumors, have been shown as prognostic factors related to overall survival and recurrence of primary curative resection [8–10], few studies have focused on the relationship between these factors and prognosis for recurrent HCC patients undergoing RFA. The prognostic significance of factors of primary curative resection, which are available for recurrent HCC patients without additional traumatic examination, would be very attractive for use in the clinic. In the present study, we found that worse curative resection tumor differentiation and short time to recurrence after curative resection were independently related to poorer survival and higher another recurrence incidence of patients with recurrent HCC undergoing RFA.

Worse tumor differentiation has been shown to be a poor prognostic factor related to the survival and recurrent rate in HCC patients undergoing curative resection [8–10]. In this study, our results showed that worse tumor differentiation is still predictive of poorer survival and increased risk of further recurrences, even in patients undergoing RFA of recurrent HCC. As RFA destroys tumors locally without removing tumors, pathology results of recurrent HCC undergoing RFA usually are not available. Therefore, whether the differentiation of recurrent HCC tumors is related to primary tumors has not been analyzed and needs to be further studied.

Time to recurrence after curative resection was an independent prognostic factor for outcome after recurrent tumors undergoing RFA in this study, which was similar to a previous report in recurrent HCC patients after curative resection undergoing second resection [14]. Time to recurrence was related to the origin of intrahepatic recurrence, either intrahepatic metastasis or multicentric occurrence [15, 16]. A study by Kumada in fact has shown that the incidence of intrahepatic metastasis in the 1st, 2nd, 3rd, 4th, and 5th years after resection or percutaneous ethanol injection treatment for HCC was 17.8%, 17.1%, 6.9%, 0%, and 4.4%, respectively; the incidence of multicentric occurrence in the 1st, 2nd, 3rd, 4th, and 5th year was 5.3%, 14.4%, 9.2%, 8.6%, and 13.3%, respectively, which supported the idea that intrahepatic recurrence within one postoperative year may mainly originate from intrahepatic metastasis, while it was from multicentric occurrence after three postoperative years [15]. In this study, when using 9 months or 12 months as the cut-off value of time to recurrence after curative resection, $p$ value was statistically significant in both overall survival and recurrence free survival analysis, and results suggested that short time to recurrence after curative resection was related to worse tumor differentiation and presence of microvascular invasion, indicating that poorer survival of patients with recurrent HCC undergoing RFA was mainly related to intrahepatic metastasis. Consequently, a strategy incorporating other measures to prevent such metastasis may potentially improve the survival rates of patients undergoing RFA of recurrent HCC.

In addition to factors extrapolated from primary curative resection, baseline serum GGT concentration (i.e., prior to RFA) was identified to be an independent risk factor of OS and RFS for recurrent HCC patients undergoing RFA, consistent with our previous results that the GGT level was a serum marker that may be used for prognosis in HCC treated by RFA [12]. The number of recurrent hepatic nodules present was also recognized as an independent risk factor for RFS in patients undergoing RFA of recurrent HCC. However, the tumor size was not prognostic of OS or RFS, given perhaps the limited number of patients with large tumors in this study.

In summary, the data analyzed herein indicate that primary tumor differentiation and time to recurrence after curative resection are predictive of OS and repeat postoperative recurrences of HCC in patients destined for RFA. These factors can be used as a routine assessment of such HCC patients in order to support intensive follow-up observations and to optimize management.

## Data Availability

All data are available within this manuscript or from the corresponding author upon reasonable request.

## Conflicts of Interest

The author(s) declare(s) that they have no conflicts of interest.

## Authors' Contributions

Hui Ma and Zhongchen Li contributed equally to this work.

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## Supplementary Materials

Figure S1: baseline characteristics (continuous variables) between the short time to recurrence (STR) after the curative resection group and the long time to recurrence (LTR) after the curative resection group. *(Supplementary Materials)*

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*Research Article*

# hsa_circRNA_000166 Facilitated Cell Growth and Limited Apoptosis through Targeting miR-326/LASP1 Axis in Colorectal Cancer

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Circular RNAs (circRNAs) belong to noncoding RNAs and are widely expressed in a variety of cell species, including cancers. However, the function and mechanism of circRNAs in colorectal cancer (CRC) has not been well investigated. Here, we firstly downloaded and analyzed the circRNA expression profile of CRC from the Gene Expression Omnibus (GEO) database. And we identified 181 differentially expressed circRNAs between 10 pairs of CRC and adjacent normal tissues. Interestingly, we observed that the expression of hsa_circRNA_000166 was the top increased among these circRNAs. Then, we confirmed an upregulation of hsa_circRNA_000166 in CRC tissues and cell lines and observed that higher expression of hsa_circRNA_000166 was associated with poor 5-year survival rate of patients with CRC. Next, we investigated the function of hsa_circRNA_000166 during CRC progression by knocking down its expression. Cell growth and apoptosis assay revealed that hsa_circRNA_000166 regulated the cell growth and apoptosis in CRC cell lines. Furthermore, we identified that hsa_circRNA_000166 targeted the miR-326/LASP1 pathway using bioinformatics analysis and luciferase reporter assay. Finally, suppression of miR-326 or overexpression of LASP1 could sufficiently rescue the aberrant cell growth and apoptosis in CRC cell lines. Taken together, our results indicated that downregulation of hsa_circRNA_000166 inhibited the cell growth and facilitated apoptosis during CRC development by sponging the miR-326/LASP1 pathway.

## 1. Introduction

Colorectal cancer (CRC) is one of the malignant cancers with the highest incidence and the fourth leading cause of cancer-related mortality around the world [1]. Recently, according to changes in dietary patterns and physical activity, CRC has dramatically increased in China [2]. Despite the advanced surgery technologies and medicine treatments that have been applied in CRC treatment, the survival rate of patients with CRC still remains unsatisfactory [3–7]. Hence, there is an urgent call for new breakthrough regarding the mechanisms underlying CRC progression.

Circular RNAs (circRNAs), a subgroup of noncoding RNAs, have a crucial role in regulating gene expression and function in distinct biological processes [8–12]. Different with linear RNAs, circRNAs have covalently closed continuous loops, which result in increased stability [13, 14]. Multiple evidences have demonstrated that the expression of circRNAs is aberrant in various cancers [15–19]. In general, circRNAs mainly served as upstream regulator to control the expression of microRNA (miRNA) during tumorigenesis [16, 20–22]. Previous studies have shown that circRNAs have an essential role in CRC progression by the regulation of multiple miRNAs [18, 23, 24]. However, the function of dysregulated circRNAs during the development of CRC remains to be further elucidated.

In our study, we downloaded the circRNA expression profile of CRC from the Gene Expression Omnibus (GEO)

database. After analysis, we identified 181 differentially expressed circRNAs and observed a top overexpression of hsa_circRNA_000166 among them. Subsequently, we revealed the ectopic expression of hsa_circRNA_000166 in CRC tissues and cell lines, which was associated with the 5-year survival rate of patients with CRC. Next, we knocked down the expression of hsa_circRNA_000166 using small interfering RNA (siRNA) to explore the potential roles of hsa_circRNA_000166 during CRC progression. We observed that hsa_circRNA_000166 inhibited the cell proliferation and promoted apoptosis in CRC cell lines. Moreover, we evidenced that hsa_circRNA_000166 directly regulated the miR-326/LASP1 pathway and the aberrant cell growth and apoptosis could be rescued after forced expression of miR-326 in CRC cell lines. In summary, our findings revealed that hsa_circRNA_000166 promoted the cell growth and repressed apoptosis via inducing the miR-326/LASP1 pathway during CRC tumorigenesis, which might be a promising candidate for diagnostic and therapeutic application in CRC treatment.

## 2. Materials and Methods

*2.1. Tissue Collection.* The CRC tissues and adjacent normal colon tissues were obtained from Inner Mongolia Medical University Affiliated Hospital between 2015 and 2018. Totally, 40 pairs of tissues were analyzed in the study. Patients with CRC did not experience systemic treatment of chemotherapy or radiotherapy before surgery. All of the patients had got the written informed consent. The study followed the ethics committee of Inner Mongolia Medical University Affiliated Hospital guidance. All specimens were stored at −80°C until use.

*2.2. Cell Culture.* We cultured CRC cell lines SW1116, DLD-1, HCT116, SW480, and SW620 and human normal colonic epithelial cells HCoEpiC in minimum essential medium (MEM) (Gibco, 41500034) with 1% Glutamax (Invitrogen, 35050-061), 1% Nonessential Amino Acids, 100x (Invitrogen, 11140-050), and 10% fetal bovine serum (FBS). In a humidified atmosphere containing 5% $CO_2$ at 37°C, the incubation of the cell lines mentioned above was performed. We purchased the cell lines from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China).

*2.3. Microarray Datasets.* Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/), a publicly available genomics database, is queried for all datasets. We downloaded the dataset of CRC, which was the circRNA expression profile from GEO. The selected dataset was in accordance with the following criteria. (1) They employed CRC tissue samples. (2) They took the adjacent normal tissues as control. (3) They utilized information on technology and platform.

*2.4. Quantitative Real-Time PCR Assay.* Total RNAs from tissues or cultured cells were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. For quantitative real-time PCR (qRT-PCR), the reverse transcription kit (Takara, Dalian, China) was used to reverse transcribe total RNA into cDNA according to the manufacturer's protocol, while a stem-loop RT-qPCR method was used to generate miRNAs. qRT-PCR was conducted in ABI StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, USA). U6 and GAPDH were applied as internal controls. The gene-specific primers are listed in Table 1.

*2.5. Plasmid and Transfection.* The siRNA-negative control (NC), siRNA-1, siRNA-2, siRNA-3, miR-NC, miR-326 mimics, miR-326 inhibitor (miR-326 I), hsa_circRNA_000166 wild-type (WT) plasmid, hsa_circRNA_000166 mutant (Mut) plasmid, LASP1 wild-type (WT) plasmid, mutant (Mut) plasmid, and LASP1 overexpression plasmid were constructed by GenePharma (Shanghai, China). According to the manufacturer's instructions, we transfected the plasmids into HCT116 and SW480 cells using the Lipofectamine 2000 Transfection Reagent (Invitrogen).

*2.6. Cell Counting Kit-8 Assay.* The Cell Counting Kit-8 (CCK-8) assay was used to detect cell growth of HCT116 and SW480 cells. Each group was incubated with a density of 104 cells in 96-well plates. Cells in each well were incubated which lasted for 2 h at 1, 2, and 3 days with CCK-8 reagent (Dojindo, Japan). We measured the optical density at 450 nm using an automatic microplate reader (Synergy4; BioTek).

*2.7. Colony Formation Assay.* We seeded the transfected cells into 6-well plates and cultured for 14 days and then fixed the cells with methanol and stained them with 0.5% crystal violet (Beyotime Biotechnology) for 30 min. Colonies with more than 10 cells were counted under a light microscope.

*2.8. Flow Cytometric Assay.* For apoptosis detection, the HCT116 and SW480 cells were transfected with different plasmids for 24 hours (h) before collection. Then, we used an Annexin V-FITC/PI apoptosis detection kit (Invitrogen) to label the HCT116 and SW480 cells with Annexin V and PI. Flow cytometry (FACScan; BD Biosciences) was used to detect and analyze the fluorescence (FL1) and red fluorescence (FL2).

*2.9. Target Prediction.* We obtained the sequence of hsa_circRNA_000166 from circBase (http://www.circbase.org). starBase v2.0 (http://starbase.sysu.edu.cn) and CircInteractome (https://circinteractome.nia.nih.gov) were utilized to predict the binding sites between hsa_circRNA_000166 and miRNAs.

*2.10. Dual Luciferase Reporter Assay.* We constructed pGL3-promoter driven miR-326 luciferase reporter containing the binding site for hsa_circRNA_000166. And then, we used Lipofectamine 2000 (Invitrogen) to transfect the luciferase reporter with hsa_circRNA_000166 WT with wild binding site (CCCAGAG) and hsa_circRNA_000166 Mut with mutant binding site (GGGUCCU) into the HCT116 and SW480 cells. The firefly luciferase activity was detected at

Table 1: Gene-specific primers used for qRT-PCR.

| Gene | Primer | Sequence 5′ to 3′ |
|---|---|---|
| hsa_circRNA_000166 | Forward | GCTTGGAACAGACTCACGGC |
| | Reverse | ATCTCCTGCCCAGTCTGACCT |
| miR-326 | Forward | GGCGCCCAGAUAAUGCG |
| | Reverse | CGTGCAGGGTCCGAGGTC |
| LASP1 | Forward | TGCGGCAAGATCGTGTATCC |
| | Reverse | GCAGTAGGGCTTCTTCTCGTAG |
| U6 | Forward | TGCGGGTGCTCGCTTCGCAGC |
| | Reverse | CCAGTGCAGGGTCCGAGGT |
| GAPDH | Forward | ACACCCACTCCTCCACCTTT |
| | Reverse | TTACTCCTTGGAGGCCATGT |

48 h after transfection using the Dual Luciferase Reporter Assay system (Promega).

*2.11. Western Blot Assay.* For protein isolation after transfection, cells were lysed in the RIPA buffer (Beyotime, China). The SDS-PAGE gel assay was utilized to separate the proteins, and then, we transferred the separated proteins onto nitrocellulose membranes (GE Healthcare). Primary antibodies were used to incubate the membranes overnight at 4°C, followed by washing the membranes for 5 times using phosphate-buffered saline supplemented with Tween 20 (PBST). Subsequently, the corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) were used to incubate the membranes for 2 h at room temperature. Finally, the SuperSignal West Femto kit (Pierce, Rockford, IL) was utilized to bring the bands on the membranes into visualization in the final. The primary antibodies and secondary antibody were used as follows: rabbit anti-LASP1 (1 : 2000, Abcam, ab117806), rabbit anti-GAPDH (1 : 5000, Abcam, ab181602), and goat anti-rabbit IgG H&L (HRP) (1 : 1500, Abcam, ab205718). We used GAPDH as the endogenous control in this assay.

*2.12. Statistical Analysis.* For significant difference analysis, GraphPad Prism 5.0 software was used to perform all the data. All results were analyzed using the two-tailed Student *t*-test and shown as the mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

## 3. Results

*3.1. Microarray Data Information and DEG Analysis in Colorectal Cancer.* We downloaded the circRNA expression microarray dataset GSE126094 associated with colorectal cancer from the Gene Expression Omnibus (GEO) database and normalized (Figure 1(a)). Then, we screened the dataset to obtain differentially expressed genes (DEGs) using the limma package ($|\log FC| > 1$ and FDR < 0.05). Volcano plots showed the differential expression of multiple circRNAs from the microarray dataset (Figure 1(b)). We obtained 181 DEGs from the GSE126094 dataset, including 74 upregulated circRNAs and 107 downregulated circRNAs. R-heat map software was used to draw a heat map of the top 8 upregulated circRNAs (Figure 1(c)). We found that the expression of hsa_circRNA_000166 was the highest among the upregulated circRNAs (Figure 1(c)), which suggested that hsa_circRNA_000166 might play a vital role during CRC progression.

*3.2. Upregulation of hsa_circRNA_000166 in CRC Tissues and Cell Lines.* To figure out the potential function of hsa_circRNA_000166 during CRC development, we firstly detected the expression level of hsa_circRNA_000166 in CRC tissues and adjacent normal colonic tissues. And we observed the overexpression of hsa_circRNA_000166 in CRC tissues compared with the matched normal tissues using qRT-PCR assay (Figure 2(a)). Then, we divided the patients into two groups based on higher or lower hsa_circRNA_000166 expression in CRC tissues. The Kaplan–Meier survival curve displayed that patients with higher hsa_circRNA_000166 expression had a poor 5-year survival rate than the patients with lower hsa_circRNA_000166 expression (Figure 2(b)). Moreover, we also tested the expression of hsa_circRNA_000166 in CRC cells, such as SW1116, DLD-1, HCT116, SW480, and SW620, and human normal colonic epithelial cells HCoEpiC. Consistent with that in CRC tissues, we found the transcriptional level of hsa_circRNA_000166 was significantly increased in CRC cell lines compared to normal colonic cells (Figure 2(c)). Therefore, we verified the ectopic expression of hsa_circRNA_000166 in both CRC tissues and cell lines, which related to poor 5-year survival rate of CRC patients.

Downregulation of hsa_circRNA_000166 suppressed cell growth and promoted apoptosis in CRC cells.

According to the overexpression of hsa_circRNA_000166 in CRC cells, we knocked down the transcriptional level of hsa_circRNA_000166 using small interfering RNA (siRNA) to study the role of hsa_circRNA_000166 during CRC tumorigenesis. After transfection with siRNAs, we observed that the expression of hsa_circRNA_000166 was obviously decreased in siRNA-1- and siRNA-2-treated CRC cells and no significant changes in siRNA-3-treated CRC cells compared with controls by qRT-PCR analysis

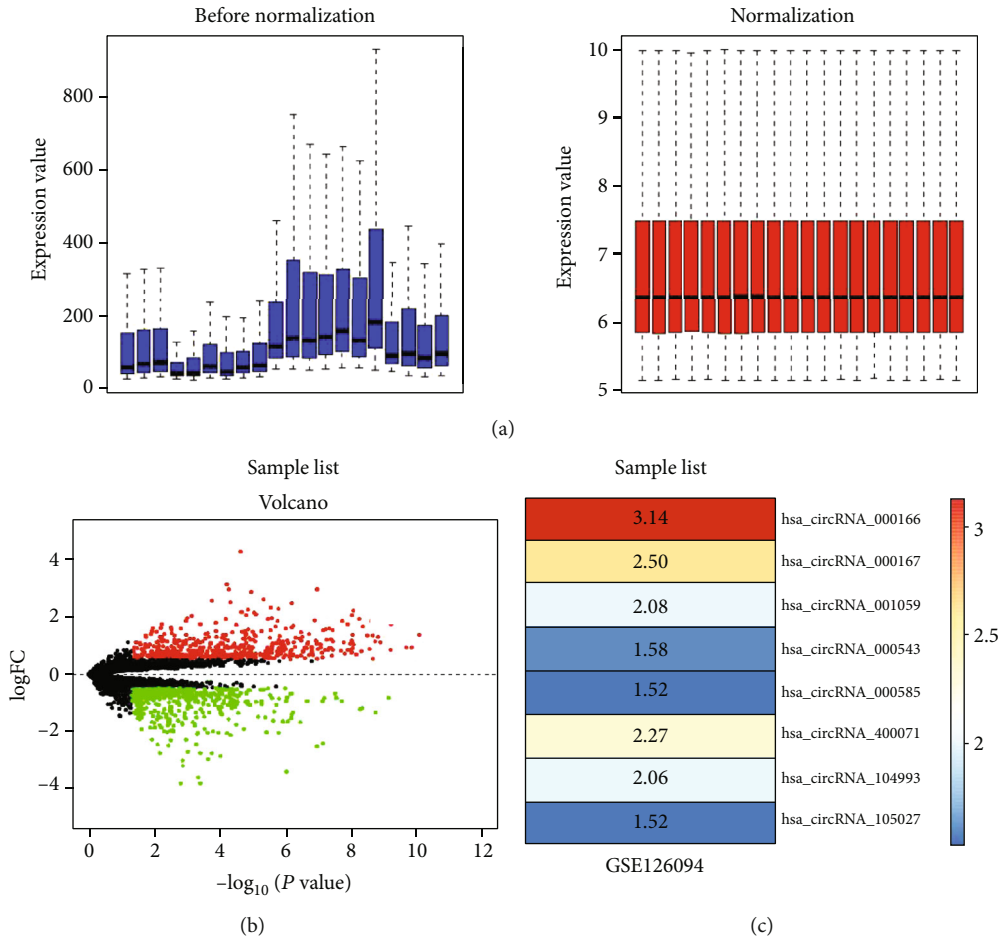



Figure 1: Microarray data information and DEG analysis in colorectal cancer. (a) The standardization of GSE126094 data. The data before normalization were displayed as the blue bar, while the normalized data were shown as the red bar. (b) The volcano plots of GSE126094 data. The red and green points, respectively, represented upregulated and downregulated genes screened on the basis of |fold change (FC)| >2.0 and a corrected $P$ value < 0.05. Genes with no significant difference were shown as the black points. (c) The abscissa was defined as GEO ID, and the ordinate was defined as the gene name. The values in the box represent the log FC values. ANOVA test was used for statistics.

(Figure 3(a)), which certified that siRNA-1 and siRNA-2 could sufficiently knock down the transcriptional level of hsa_circRNA_000166. Subsequently, to demonstrate the function of hsa_circRNA_000166 in CRC cell growth, the CCK-8 assay was performed in HCT116 and SW480 cell lines after siRNA-1 or siRNA-2 transfection. We observed that the cell proliferation of CRC was limited in siRNA-1- or siRNA-2-treated groups compared with the controls in two CRC cell lines (Figure 3(b)). Meanwhile, we also conducted the colony formation assay in HCT116 and SW480 cell lines and revealed a notable decrease of colony number in siRNA-treated groups compared to the siRNA-NC groups in the two CRC cell lines (Figure 3(c)). Furthermore, to inspect the role of hsa_circRNA_000166 in CRC apoptosis, flow cytometry was utilized to calculate the apoptotic cells in both HCT116 and SW480 cell lines, and it was found that downregulation of hsa_circRNA_000166 resulted in the dramatic elevation of apoptosis rate in siRNA-1- or siRNA-2-treated groups rather than in controls (Figure 3(d)). In brief, our findings highlighted that downregulation of hsa_circRNA_000166 could suppress cell growth and enhance apoptosis in CRC cells.

*3.3. hsa_circRNA_000166 Regulated CRC Progression by Inducing miR-326/LASP1 Axis.* To elucidate the mechanism of hsa_circRNA_000166 in controlling CRC cell proliferation and apoptosis, we predicted that miR-326 was the candidate target of hsa_circRNA_000166 using the target prediction tool. Firstly, the Venn analysis between the predicted target miRNAs of hsa_circRNA_000166 and differential expressed miRNAs in CRC cells indicated that 3 miRNAs, containing miR-326, were involved in CRC using starBase and CircInteractome (Figure 4(a)). To certify whether miR-326 was a putative downstream target of hsa_circRNA_000166, we constructed plasmids with wild binding site or mutant binding site of hsa_circRNA_000166 into pGL3 vector with luciferase reporter gene (Figure 4(b)). Also, we detected the transcriptional level of miR-326 in CRC tissues and found its downregulation in CRC tissues compared with matched normal tissues (Figure 4(c)). Similarly, the

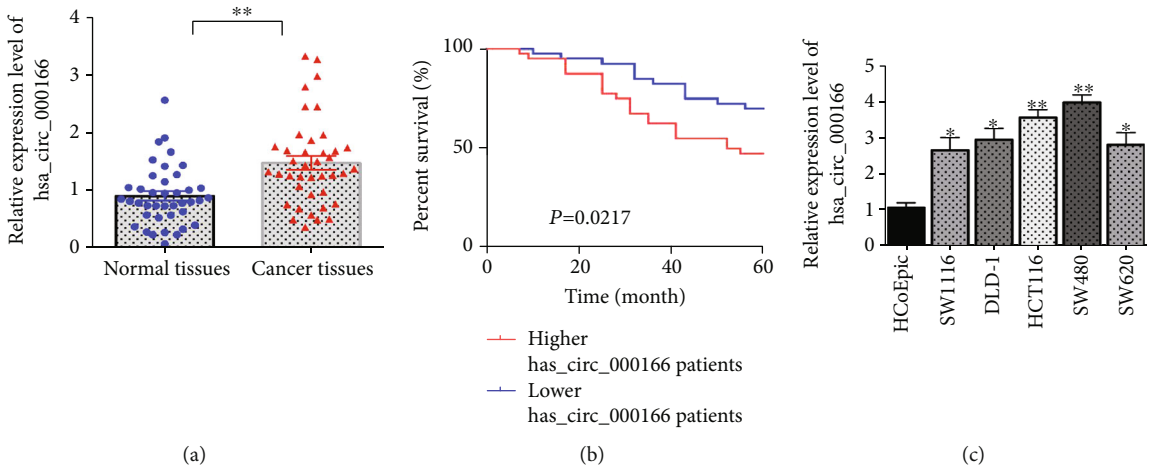



Figure 2: hsa_circRNA_000166 was upregulated in CRC tissues and cell lines. (a) qRT-PCR assay showed that the expression of hsa_circRNA_000166 was increased in CRC tissues ($n = 40$) compared with adjacent normal lung tissues ($n = 40$). (b) The Kaplan–Meier curve displayed 5-year survival rate of CRC patients with different hsa_circRNA_000166 expression levels ($n = 40$, $P = 0.0217$). (c) qRT-PCR assay showed an upregulation of hsa_circRNA_000166 in CRC cell lines compared with normal colonic cells. The Student $t$-test was used for statistics.

expression of miR-326 was decreased in CRC cell lines than in the normal cell line HCoEpiC (Figure 4(d)). As shown in Figure 3, the inhibitory effect of siRNA-1 was better than siRNA-2; we select siRNA-1 for further investigation. After transfection with siRNA-1 or siRNA-NC, we observed that the transcriptional level of miR-326 was obviously upregulated in both HCT116 and SW480 cells (Figure 4(e)). Then, miR-NC and miR-326 I were separately cotransfected with hsa_circRNA_000166 WT and hsa_circRNA_000166 Mut luciferase reporter plasmid into HCT116 cells. Luciferase assay showed the relative luciferase activity in cells cotransfected with miR-326 I, and hsa_circRNA_000166 WT luciferase reporter plasmid was significantly decreased about 50% compared with the controls, while the relative luciferase activity of cells cotransfected with miR-326 I and hsa_circRNA_000166 Mut luciferase reporter plasmid has no obvious changes compared with the controls in HCT116 cells (Figure 4(f)). Recent studies reported that miR-326 could directly control the expression of LIM and SH3 protein 1 (LASP1) to suppress cell proliferation and activate apoptosis in hepatocellular carcinoma (HCC) [25]. Thus, we measured the transcriptional and translational levels of LASP1 after transfection with siRNA-1 and found a downregulation of LASP1 in both HCT116 and SW480 cells compared with the control groups using qRT-PCR and western blot assay (Figures 4(g) and 4(h)). Moreover, the miR-NC or miR-326 mimics were separately cotransfected with LASP1 WT and LASP1 Mut luciferase reporter plasmid into HCT116 cells. Luciferase assay showed the relative luciferase activity in cells cotransfected with miR-326 mimics, and LASP1 WT luciferase reporter plasmid was significantly decreased compared with the controls, while the relative luciferase activity of cells cotransfected with miR-326 mimics and LASP1 Mut luciferase reporter plasmid has no obvious changes compared with the controls in HCT116 cells (Figure 4(i)). The outcomes strongly indicated that hsa_circRNA_000166 might participate in CRC progression through targeting the miR-326/LASP1 pathway.

*3.4. Inhibition of miR-326 or Overexpression of LASP1 Rescued the Phenotype Dominated by hsa_circRNA_000166.* To further confirm that miR-326 and LASP1 mediated the function of hsa_circRNA_000166 during CRC development, we conducted codepletion of both siRNA-1 and miR-326 I or depletion of siRNA-1 while there was overexpression of LASP1 and inspected the role of miR-326 and LASP1 in the regulation of hsa_circRNA_000166 in CRC development. CCK-8 and colony formation assay demonstrated that miR-326 downregulation or LASP1 overexpression in siRNA-1-transfected cells could restore cell growth compared with the controls in HCT116 cells (Figures 5(a) and 5(b)). Correspondingly, cells cotransfected with siRNA-1 and miR-326 I or siRNA-1 and LASP1 overexpression plasmid resulted in the significant decrease in the number of apoptotic cells compared with siRNA-1-transfected cells (Figure 5(c)). In conclusion, our studies identified hsa_circRNA_000166 was overexpressed in CRC cells and evidenced the potential function of hsa_circRNA_000166 in regulating the cell growth and apoptosis in CRC cells through directly interacting with the miR326/LASP1 axis. Therefore, hsa_circRNA_000166 might be a promising target in diagnostic and therapeutic application of CRC patient treatment.

## 4. Discussion

Colorectal cancer (CRC) is one of the solid tumors with a higher mortality among cancer-related deaths worldwide. Though advanced surgery technologies and medicine treatments have been applied in treating patients with CRC, the survival rate of patients with CRC is still poor. Therefore, there is an urgent demand for understanding the mechanisms underlying the development of CRC. In the past

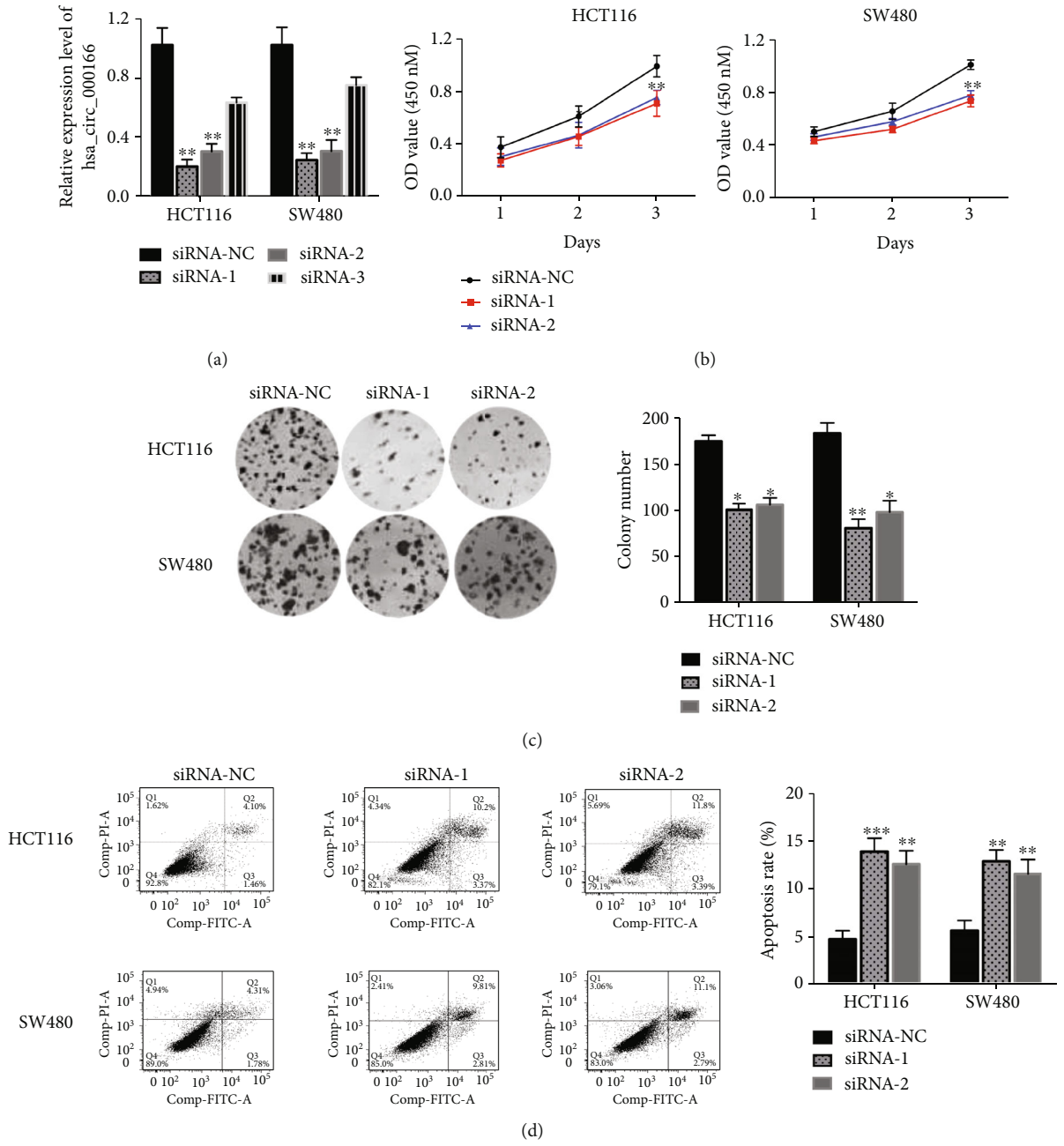



Figure 3: Decreased hsa_circRNA_000166 expression affected cell growth and apoptosis in CRC cells. (a) The transcriptional level of hsa_circRNA_000166 was measured by qRT-PCR in both HCT116 and SW480 cells after transfection with siRNAs. (b) Cell proliferation assay showed that decreased hsa_circRNA_000166 expression limited the growth of HCT116 and SW480 cells after siRNA-1 or siRNA-2 transfection compared with the controls. (c) Clone formation assay demonstrated that the number of colonies was significantly decreased after siRNA-1 or siRNA-2 transfection compared with the controls in HCT116 and SW480 cells. (d) Flow cytometric analysis displayed that the number of apoptotic cells was noticeably increased in siRNA-1- or siRNA-2-transfected groups compared with the controls. The Student *t*-test was used for statistics.

decades, circRNAs are discovered to be a subgroup of noncoding RNAs and play an essential role in regulating gene expression and function associated with cancers [15–19]. In this study, we identified that hsa_circRNA_000166 was one of the upregulated circRNAs with the highest expression level among all the upregulated circRNAs using bioinformatics analysis. Then, qRT-PCR assay was conducted to measure the expression of hsa_circRNA_000166 in CRC tissues and cell lines. We found that the transcriptional level of hsa_circRNA_000166 was notably increased, which highly correlated with poor 5-year survival rate of CRC patients. Next, we inhibited the expression of hsa_circRNA_000166 to perform further investigation. After downregulation of hsa_circRNA_000166, we observed that the cell growth and colony formation were limited and cell apoptosis was activated using corresponding assay. Similarly, Zhao and Dai have found

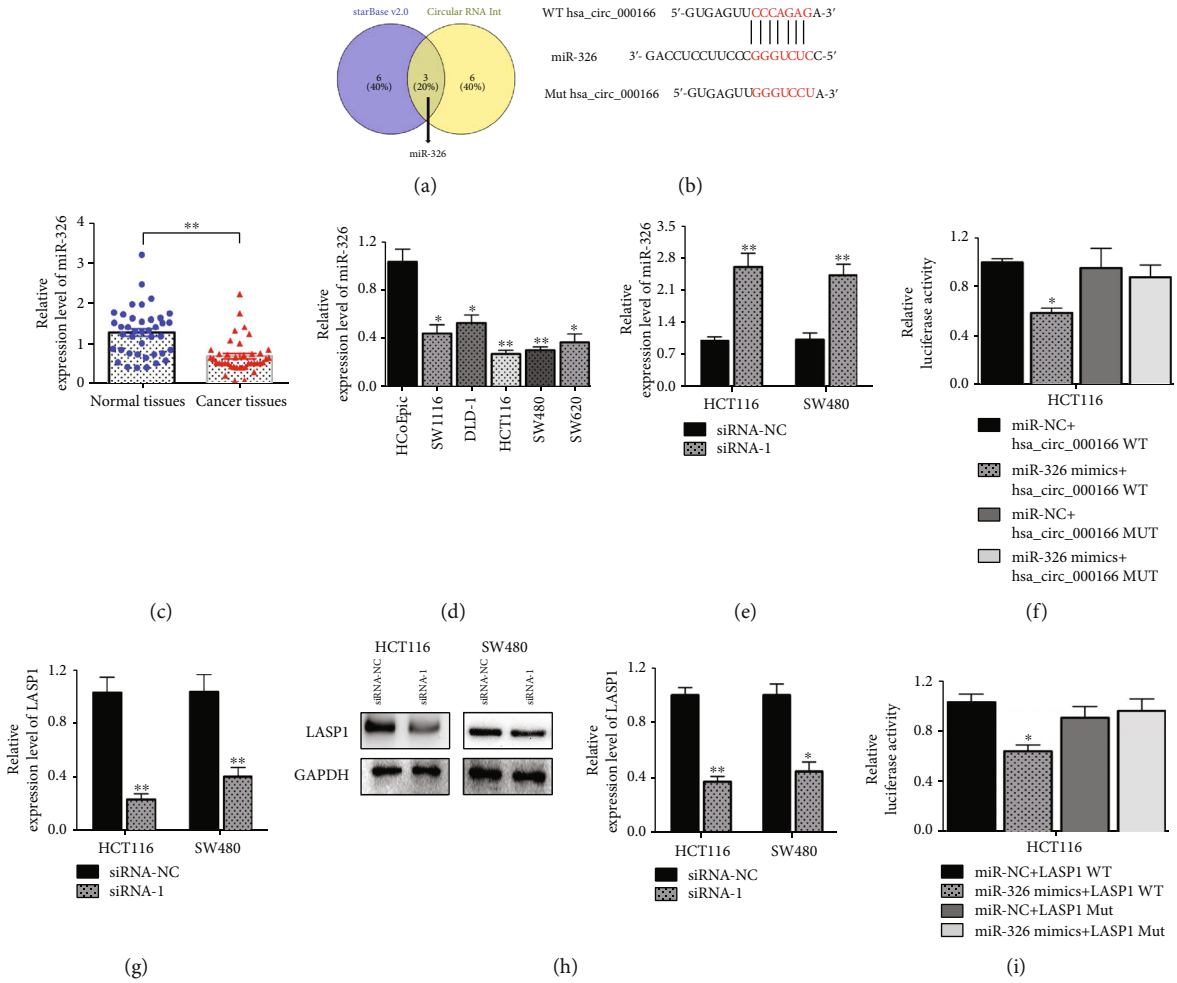
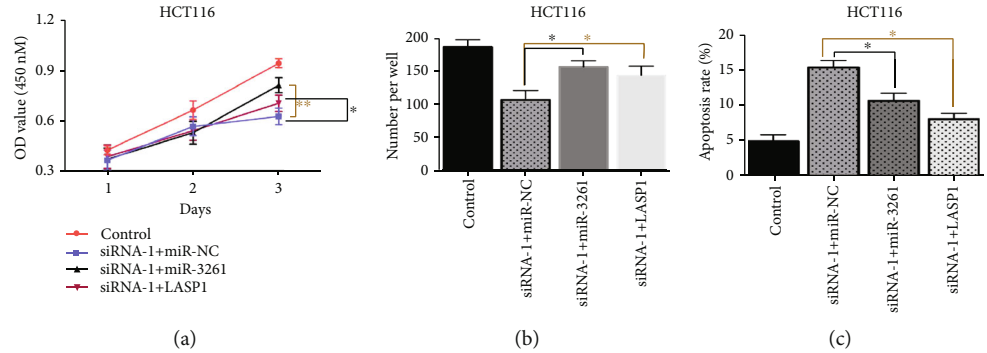



Figure 4: hsa_circRNA_000166 regulated CRC progression by targeting the miR-326/LASP1 axis. (a) The Venn analysis implied that 3 miRNAs, including miR-326, were involved in CRC. The blue represented 9 predicted miRNAs analyzed by starBase v2.0, and the yellow represented 9 predicted miRNAs analyzed by CircInteractome. (b) Binding site of hsa_circRNA_000166 in miR-326 was predicted by starBase. (c) qRT-PCR analysis showed that the transcriptional level of miR-326 was dramatically decreased in CRC tissues ($n = 40$) compared to the matched normal tissues ($n = 40$). (d) qRT-PCR analysis showed that miR-326 was notably downregulated in CRC cells compared with the normal colonic cells. (e) qRT-PCR assay displayed that miR-326 was significantly upregulated in siRNA-1-transfected groups compared with the controls in both HCT116 and SW480 cells. (f) Luciferase reporter assay indicated miR-326 dramatically repressed the WT hsa_circRNA_000166 luciferase activity but not Mut hsa_circRNA_000166 in HCT116 cells. (g, h) qRT-PCR and western blot assay showed that the transcriptional and translational levels of LASP1 were obviously downregulated in siRNA-1-transfected groups compared with the controls in both HCT116 and SW480 cells. (i) Luciferase reporter assay indicated miR-326 dramatically repressed the WT LASP1 luciferase activity but not Mut LASP1 in HCT116 cells. The Student $t$-test was used for statistics.

that hsa_circRNA_000166 could promote cell proliferation, migration, and invasion by regulating miR-330-5p/ELK1 in colon cancers previously [20]. And our results were consistent with Zhao and Dai's findings. Collectively, these data strongly demonstrated that hsa_circRNA_000166 played an important role during CRC tumorigenesis.

Generally, circRNAs are sponging miRNAs to play its function in multiple biological processes, including tumorigenesis [21–23]. Previous studies have proved that multiple miRNAs mediated the function of circRNAs in CRC progression [24, 25]. Here, combined with bioinformatics analysis, we predicted that miR-326 might be a candidate target of hsa_circRNA_000166. Subsequently, luciferase assay was performed to confirm the direct interaction between hsa_circRNA_000166 and miR-326. Also, we measured the downregulation of miR-326 in CRC tissues and cell lines compared with the matched controls. Moreover, we revealed the LASP1, a downstream effector of miR-326 [26], was downregulated after siRNA-1 transfection compared to the controls, which suggested that the miR-326/LASP1 pathway was involved in the regulation of hsa_circRNA_000166 during CRC progression. Finally, we verified that downregulation of miR-326 could compromise the phenotype in siRNA-1-treated groups. Taken together, our findings evidenced that hsa_circRNA_000166 activated the cell growth and repressed apoptosis by sponging the miR-326/LASP1 axis during CRC tumorigenesis, which might be beneficial for diagnostic and therapeutic application in CRC treatment.

In this study, we used bioinformatics analysis to screen the GSE126094 dataset in CRC and identified that hsa_




(a) (b) (c)

Figure 5: miR-326 downregulation or LASP1 upregulation rescued the phenotype dominated by hsa_circRNA_000166. (a, b) CCK-8 and colony formation assay demonstrated that cell codepletion of both siRNA-1 and miR-326 I or depletion of siRNA-1 while overexpression of LASP1 promoted cell growth compared with control groups in HCT116 cells. (c) The number of apoptotic cells in codepletion of both siRNA-1 and miR-326 I or depletion of siRNA-1 while overexpression of LASP1 groups was less than the number in control group HCT116 cells. The Student *t*-test was used for statistics.

circRNA_000166 was the top 1 among all upregulated circRNAs. We further confirmed the overexpressions of hsa_circRNA_000166 in CRC tissues and cell lines and found that 5-year survival rate of CRC patients was highly related to the expression levels of hsa_circRNA_000166. Importantly, we confirmed that the miR-326/LASP1 pathway functions downstream of hsa_circRNA_000166 for the circRNA function during CRC progression. Together, our findings manifested that hsa_circRNA_000166 had a vital role in regulating CRC tumorigenesis, which implied that hsa_circRNA_000166 had a promising value in early diagnosis and prevention of CRC.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Ethical Approval

This study was approved by the ethics board of the Inner Mongolia Medical University Affiliated Hospital.

## Conflicts of Interest

The authors declare no conflicts of interest.

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*Research Article*

# Predictive and Prognostic Factors of Synchronous Colorectal Lung-Limited Metastasis

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*Aim*. This study is aimed at investigating predictive and prognostic factors of synchronous colorectal lung-limited metastasis (SCLLM) based on The Surveillance, Epidemiology, and End Results (SEER) database. *Methods*. A multivariate logistic regression model was constructed to identify independent predictors of SCLLM. A multivariate Cox proportional hazards regression model was used to distinguish independent prognostic factors. *Results*. This study enrolled 168,007 colorectal cancer (CRC) patients without metastatic diseases and 1,298 cases with SCLLM. Eight features, involving race, tumor location, pathological grade, histological type, T stage, N stage, and tumor size as well as CEA, could be used as the independent predictors. As the nomogram shown, the T4 stage contributed the most to SCLLM, followed by the N2 stage, elevated CEA, and rectal cancer. A multivariate regression analysis discriminated 9 independent prognostic factors, including age, race, marital status, pathological grade, T stage, colectomy/proctectomy, chemotherapy, CEA, and TD. The prognostic nomogram illustrated that nonresection/NOS played as the poorest prognostic factor, followed by nonchemotherapy, ≥75-year old and T4 stage. The cumulative survival curves revealed the influence of each prognostic factor on survival after controlling the other variables. *Conclusions*. This study identified independent predictors and prognostic factors for SCLLM based on a large database of the United States. The predictors and prognostic factors can provide supporting evidence for the prevention and treatment of SCLLM.

## 1. Introduction

Colorectal cancer (CRC) ranks as the third most common malignancy in males and the second in females [1]. In spite of widespread early detection screening for CRC, approximate 25% of CRC patients are found to have distant metastases at the time of diagnosis [2, 3]. Moreover, metastasis is the main cause of high mortality among CRC patients [4].

Currently, there has been a continuous increase in the number of CRC patients diagnosed with pulmonary metastases, accounting for 32.9% of all metastatic CRCs (mCRCs) [5], after the widespread use of chest CT scans in recent years. Meanwhile, some research reported that 4-9% patients with CRC suffered from synchronous lung metastasis [6–8]. The retrospective data from China reported that lungs being the first metastatic site reached 24.5% among patients with mCRC [9]. Nevertheless, there is limited information to guide clinical practice in colorectal lung metastasis. It is a mainstream practice that the therapeutic strategy for colorectal liver metastases is applied to lung metastasis [10–12]. Undoubtedly, the treatment experience from colorectal liver metastasis is conducive to the rapid development of therapeutic strategy of colorectal lung metastasis. However, some scholars believe that there are differences involving the

Table 1: The characteristics of CRC patients associated with lung-limited metastasis.

| Characteristics | Total ($n$ = 169305) | | Without lung-limited metastasis ($n$ = 168007) | | With lung-limited metastasis ($n$ = 1298) | | $p$ value |
|---|---|---|---|---|---|---|---|
| | $n$ | % | $n$ | % | $n$ | % | |
| Gender | | | | | | | 0.899 |
| Female | 80313 | 47.44% | 79695 | 47.44% | 618 | 47.61% | |
| Male | 88992 | 52.56% | 88312 | 52.56% | 680 | 52.39% | |
| Age (years) | | | | | | | 0.072 |
| <65 | 70997 | 41.93% | 70425 | 41.92% | 572 | 44.07% | |
| 65-74 | 44114 | 26.06% | 43776 | 26.06% | 338 | 26.04% | |
| ≥75 | 54194 | 32.01% | 53806 | 32.03% | 388 | 29.89% | |
| Marital status | | | | | | | 0.001 |
| Married | 89491 | 52.86% | 88863 | 52.89% | 628 | 48.38% | |
| Unmarried/NOS | 79814 | 47.14% | 79144 | 47.11% | 670 | 51.62% | |
| Insurance | | | | | | | 0.141 |
| Yes | 160889 | 95.03% | 159667 | 95.04% | 1222 | 94.14% | |
| No/unknown | 8416 | 4.97% | 8340 | 4.96% | 76 | 5.86% | |
| Race | | | | | | | 0.010 |
| White | 133791 | 79.02% | 132814 | 79.05% | 977 | 75.27% | |
| Black | 18894 | 11.16% | 18711 | 11.14% | 183 | 14.10% | |
| Other/NOS | 16620 | 9.82% | 16482 | 9.81% | 138 | 10.63% | |
| Tumor location | | | | | | | <0.001 |
| Right colon | 72060 | 42.56% | 71738 | 42.70% | 322 | 24.81% | |
| Left colon | 45969 | 27.15% | 45677 | 27.19% | 292 | 22.50% | |
| Rectum | 49013 | 28.95% | 48345 | 28.78% | 668 | 51.46% | |
| NOS | 2263 | 1.34% | 2247 | 1.34% | 16 | 1.23% | |
| Pathological grade | | | | | | | <0.001 |
| I/II | 130151 | 76.87% | 129242 | 76.93% | 909 | 70.03% | |
| III/IV | 25628 | 15.14% | 25427 | 15.13% | 201 | 15.49% | |
| Unknown | 13526 | 7.99% | 13338 | 7.94% | 188 | 14.48% | |
| Histological type | | | | | | | 0.016 |
| Adenocarcinomas | 156108 | 92.21% | 154888 | 92.19% | 1220 | 93.99% | |
| MCC/SRCC | 13197 | 7.79% | 13119 | 7.81% | 78 | 6.01% | |
| T stage | | | | | | | <0.001 |
| Tis-2 | 65332 | 38.59% | 65117 | 38.76% | 215 | 16.56% | |
| T3 | 83185 | 49.13% | 82444 | 49.07% | 741 | 57.09% | |
| T4 | 20788 | 12.28% | 20446 | 12.17% | 342 | 26.35% | |
| N stage | | | | | | | <0.001 |
| N0 | 110089 | 65.02% | 109619 | 65.25% | 470 | 36.21% | |
| N1 | 40665 | 24.02% | 40144 | 23.89% | 521 | 40.14% | |
| N2 | 18551 | 10.96% | 18244 | 10.86% | 307 | 23.65% | |
| Colectomy/proctectomy | | | | | | | <0.001 |
| Standard resection | 121185 | 71.58% | 120545 | 71.75% | 640 | 49.31% | |
| Simplified resection | 26208 | 15.48% | 26017 | 15.49% | 191 | 14.71% | |
| Nonresection/NOS | 21912 | 12.94% | 21445 | 12.76% | 467 | 35.98% | |
| Pulmonary surgery | | | | | | | <0.001 |
| Yes | 100 | 0.06% | 0 | 0.00% | 100 | 7.70% | |
| No/unknown | 169205 | 99.94% | 168007 | 100.00% | 1198 | 92.30% | |
| Radiotherapy | | | | | | | <0.001 |
| Yes | 25351 | 14.97% | 24993 | 14.88% | 358 | 27.58% | |
| No/unknown | 143954 | 85.03% | 143014 | 85.12% | 940 | 72.42% | |

Table 1: Continued.

| Characteristics | Total ($n$ = 169305) | | Without lung-limited metastasis ($n$ = 168007) | | With lung-limited metastasis ($n$ = 1298) | | $p$ value |
|---|---|---|---|---|---|---|---|
| | $n$ | % | $n$ | % | $n$ | % | |
| Chemotherapy | | | | | | | <0.001 |
| Yes | 59540 | 35.17% | 58610 | 34.89% | 930 | 71.65% | |
| No/unknown | 109765 | 64.83% | 109397 | 65.11% | 368 | 28.35% | |
| Tumor size | | | | | | | <0.001 |
| ≤5 cm | 101949 | 60.22% | 101357 | 60.33% | 592 | 45.61% | |
| 5-10 cm | 41599 | 24.57% | 41177 | 24.51% | 422 | 32.51% | |
| >10 cm | 4149 | 2.45% | 4092 | 2.44% | 57 | 4.39% | |
| NOS | 21608 | 12.76% | 21381 | 12.73% | 227 | 17.49% | |
| CEA | | | | | | | <0.001 |
| Normal | 59541 | 35.17% | 59262 | 35.27% | 279 | 21.49% | |
| Elevated | 35452 | 20.94% | 34835 | 20.73% | 617 | 47.53% | |
| NOS | 74312 | 43.89% | 73910 | 43.99% | 402 | 30.97% | |
| TD | | | | | | | <0.001 |
| Negative | 133508 | 78.86% | 132910 | 79.11% | 598 | 46.07% | |
| Positive | 13672 | 8.08% | 13448 | 8.00% | 224 | 17.26% | |
| NOS | 22125 | 13.07% | 21649 | 12.89% | 476 | 36.67% | |
| PNI | | | | | | | <0.001 |
| Negative | 132991 | 78.55% | 132292 | 78.74% | 699 | 53.85% | |
| Positive | 13079 | 7.73% | 12863 | 7.66% | 216 | 16.64% | |
| NOS | 23235 | 13.72% | 22852 | 13.60% | 383 | 29.51% | |
| Median survival (months) | 30 (13-53) | | 30 (13-53) | | 18 (8-33) | | <0.001 |

MCC: mucinous cell carcinoma; SRCC: signet ring cell carcinoma; CEA: carcinoembryonic antigen; TD: tumor deposits; PNI: perineural invasion; NOS: not otherwise specified.

metastatic pattern between the colorectal liver and lung metastasis [13, 14]. Thus, it is important to further investigate the risk factors of colorectal lung metastasis. In addition, in order to exclude the interference from other metastatic sites, this study focused on synchronous colorectal lung-limited metastasis (SCLLM), which was defined as colorectal cancer with lung-limited metastases at the time of diagnosis.

SCLLM is considered less frequent due to the different metastatic route. The routine metastatic process of CRC involves discrete steps (CRC cancer cells initially migrate to the liver via the portal system, followed by the lungs and finally other locations) [15, 16], while the spread of metastatic CRC to the lungs, either in isolation or as the first of several distant sites, may be attributable to venous drainage which bypasses the portal system and instead enters systemic circulation [17]. Nevertheless, the frequency of synchronous lung metastasis increased significantly by a nearly 3-folds in the past decades [15].

Due to the rareness of SCLLM, a large public database is needed to explore this issue. The Surveillance, Epidemiology, and End Results (SEER) database is a kind of population-based cancer registration system of the USA taking 34.6% Americans into account, which can provide some necessary clinical data and be used to be an excellent database to explore issues regarding various cancers.

Therefore, this study is aimed at investigating predictive and prognostic factors of SCLLM based on SEER database.

## 2. Materials and Methods

*2.1. Patients.* This retrospective analysis used data from the SEER-linked database. The SEER program of the National Cancer Institute is an authoritative source of information on cancer incidence and survival in the United States (U.S.) with updated annually. SEER currently collects and publishes cancer incidence and survival data from population-based cancer registries covering approximately 34.6% of the U.S. population [18]. Data from SEER was used to identify patients with CRC diagnosed between 2010 and 2016, and 230,301 patients were diagnosed with colorectal adenocarcinoma (ICD-O-3: 8140, 8141, 8143, 8144, 8145, 8147, 8201, 8210, 8211, 8213, 8220, 8221, 8230, 8253, 8255, 8260, 8261, 8262, 8263, 8280, 8440, 8441, 8460, 8470, 9471, 8481, and 8490) between these years in total. Exclusion criteria: (1) without positive histology ($n$ = 1,591); (2) autopsy/death certificate only cases and survival months = 0 ($n$ = 12,460); (3) M1b, M1NOS, and metastases to other organs ($n$ = 36,818); (4) incomplete information regarding stage T and stage N ($n$ = 10,127). The final study sample contained 169,305 CRC patients, including 1,298 SCLLM patients.

For each patient, the following data was acquired: age at diagnosis, married status, insurance, gender, race, grade, histological type, T stage, N stage, regional nodes examined (RNE), CEA, surgery for primary tumor, surgery for hepatic metastasis, tumor deposits (TD), perineural invasion (PNI),

Table 2: Univariable and multivariable logistic regression model analyses.

| Characteristics | Univariable analysis | | | | Multivariable analysis | | | |
|---|---|---|---|---|---|---|---|---|
| | OR | 95% CI lower | 95% CI upper | *p* value | OR | 95% CI lower | 95% CI upper | *p* value |
| Gender | | | | 0.899 | | | | |
| Female | | Reference | | | | NA | | |
| Male | 0.993 | 0.890 | 1.108 | 0.899 | | | | |
| Age (years) | | | | 0.197 | | | | |
| <65 | | Reference | | | | NA | | |
| 65-74 | 0.951 | 0.831 | 1.088 | 0.462 | | | | |
| ≥75 | 0.888 | 0.780 | 1.010 | 0.072 | | | | |
| Marital status | | | | 0.001 | | | | |
| Married | | Reference | | | | Reference | | |
| Unmarried/NOS | 1.198 | 1.074 | 1.336 | 0.001 | 1.112 | 0.995 | 1.243 | 0.062 |
| Insurance | | | | 0.142 | | | | |
| Yes | | Reference | | | | NA | | |
| No/unknown | 1.191 | 0.943 | 1.503 | 0.142 | | | | |
| Race | | | | 0.001 | | | | 0.021 |
| White | | Reference | | | | Reference | | |
| Black | 1.330 | 1.135 | 1.558 | <0.001 | 1.256 | 1.068 | 1.476 | 0.006 |
| Other/NOS | 1.138 | 0.952 | 1.361 | 0.156 | 1.004 | .838 | 1.203 | 0.968 |
| Tumor location | | | | <0.001 | | | | <0.001 |
| Right colon | | Reference | | | | Reference | | |
| Left colon | 1.424 | 1.215 | 1.669 | <0.001 | 1.430 | 1.217 | 1.680 | <0.001 |
| Rectum | 3.078 | 2.694 | 3.518 | <0.001 | 2.633 | 2.287 | 3.031 | <0.001 |
| NOS | 1.586 | 0.959 | 2.625 | 0.073 | 1.193 | 0.719 | 1.980 | 0.495 |
| Pathological grade | | | | <0.001 | | | | <0.001 |
| I/II | | Reference | | | | Reference | | |
| III/IV | 1.124 | 0.964 | 1.310 | 0.135 | 0.871 | 0.743 | 1.023 | 0.092 |
| Unknown | 2.004 | 1.711 | 2.347 | <0.001 | 1.900 | 1.603 | 2.251 | <0.001 |
| Histological type | | | | 0.016 | | | | <0.001 |
| Adenocarcinomas | | Reference | | | | Reference | | |
| MCC/SRCC | 0.755 | 0.600 | 0.950 | 0.016 | 0.623 | 0.492 | 0.787 | <0.001 |
| T stage | | | | <0.001 | | | | <0.001 |
| Tis-2 | | Reference | | | | Reference | | |
| T3 | 2.722 | 2.338 | 3.170 | <0.001 | 1.953 | 1.644 | 2.319 | <0.001 |
| T4 | 5.066 | 4.269 | 6.013 | <0.001 | 3.143 | 2.579 | 3.831 | <0.001 |
| N stage | | | | <0.001 | | | | <0.001 |
| N0 | | Reference | | | | Reference | | |
| N1 | 3.027 | 2.671 | 3.431 | <0.001 | 2.142 | 1.873 | 2.450 | <0.001 |
| N2 | 3.925 | 3.396 | 4.536 | <0.001 | 2.797 | 2.388 | 3.277 | <0.001 |
| Tumor size | | | | <0.001 | | | | <0.001 |
| ≤5 cm | | Reference | | | | Reference | | |
| 5-10 cm | 1.755 | 1.548 | 1.989 | <0.001 | 1.229 | 1.079 | 1.400 | 0.002 |
| >10 cm | 2.385 | 1.814 | 3.135 | <0.001 | 1.518 | 1.144 | 2.015 | 0.004 |
| NOS | 1.818 | 1.559 | 2.120 | <0.001 | 1.784 | 1.511 | 2.107 | <0.001 |
| CEA | | | | <0.001 | | | | <0.001 |
| Normal | | Reference | | | | Reference | | |
| Elevated | 3.762 | 3.264 | 4.336 | <0.001 | 2.679 | 2.317 | 3.098 | <0.001 |
| NOS | 1.155 | 0.991 | 1.346 | 0.065 | 1.194 | 1.023 | 1.394 | 0.025 |

MCC: mucinous cell carcinoma; SRCC: signet ring cell carcinoma; CEA: carcinoembryonic antigen; NOS: not otherwise specified; NA: unavailable.

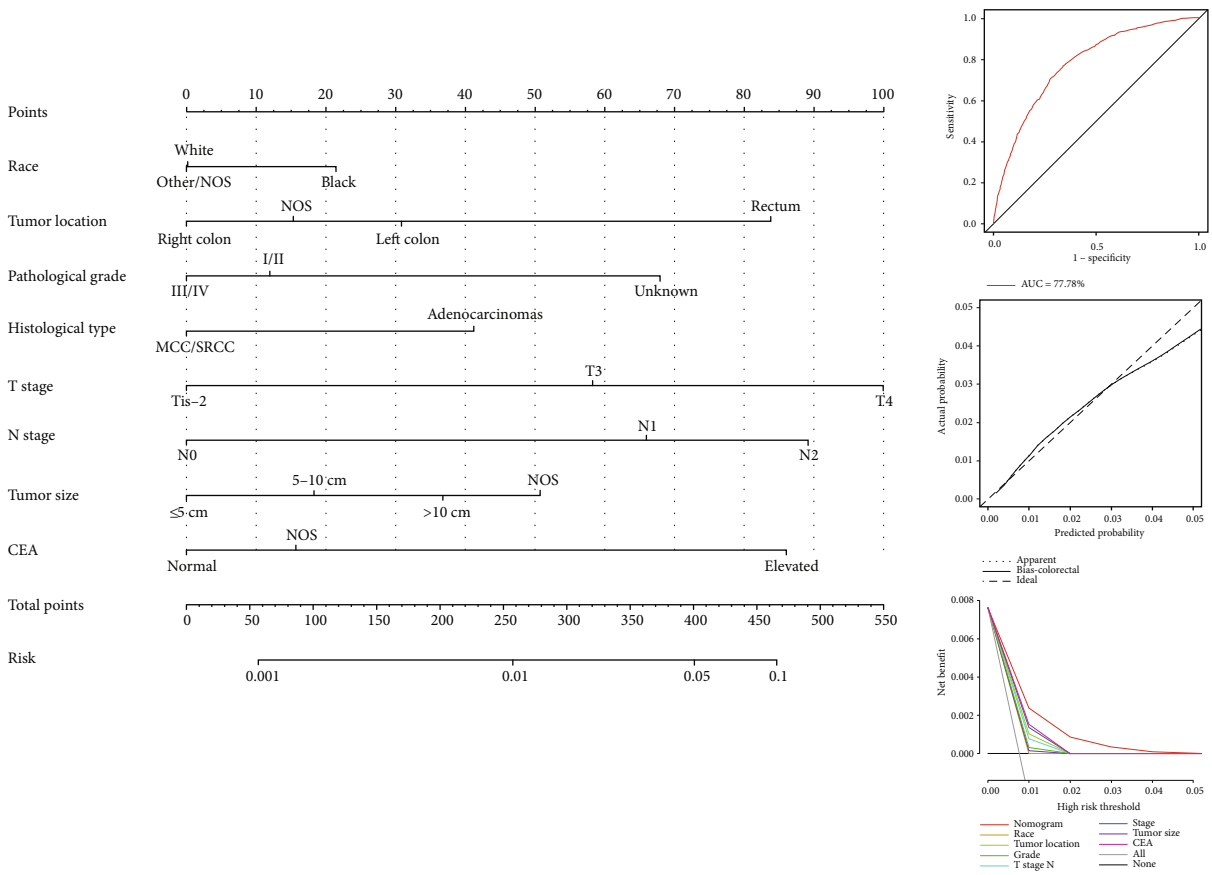


Figure 1: The weight of each independent predictor of SCLLM.

radiotherapy, and chemotherapy. We defined colectomy/-proctectomy with RNE ≥ 12 as standard colectomy/proctectomy and colectomy/proctectomy with RNE < 12/NOS as simplified colectomy/proctectomy.

*2.2. Statistical Analysis.* Intergroup comparisons were analyzed using Pearson's chi-square test and Mann-Whitney *U* test depending on the nature of the data. A multivariate logistic regression model was constructed, including all independent variables that showed statistical significance on univariate analysis, to identify independent predictors of SCLLM. Meanwhile, a multivariate Cox proportional hazards regression model was used to distinguish independent prognostic factors. Univariate analysis of variables with significant differences was included in the Cox regression model for multivariate analysis. Cumulative survival function was also calculated by the multivariate Cox analysis for comparing the effect of each independent prognostic factor. Statistical analyses were performed using IBM SPSS statistics trial ver. 25.0 (IBM, Armonk, NY, USA). All reported *p* values lower than 0.05 were considered significant.

## 3. Results

*3.1. Patient Characteristics.* This study enrolled 168,007 CRC patients without metastatic diseases and 1,298 cases with SCLLM. The entire cohort was predominantly elderly (≥65, 58.07%) and white people (75.27%). The rectum was the main site occurring lung-limited metastases in CRC. Besides, SCLLM was related to marital status, race, pathological grade, and histological type. Meanwhile, there were significant differences regarding the depth of tumor invasion and regional lymph node status between the two cohorts. Moreover, a lower rate of surgery but a significantly higher rate of chemotherapy and radiotherapy can be observed in the patients with SCLLM. Furthermore, SCLLM patients suffered a larger tumor size and a higher positive ratio of CEA, TD, and PNI, as well as a shorter median survival (Table 1).

*3.2. Predictive Factors of Synchronous Colorectal Lung-Limited Metastasis.* This section of the study excluded therapeutic variables and postoperative variables, including colectomy, pulmonary surgery, radiotherapy, chemotherapy, TD, and PNI. All variables with *p* values less than 0.05 in the univariate logistic regression model were brought into the multivariate regression analysis, which displayed that 8 features, involving race, tumor location, pathological grade, histological type, T stage, N stage, and tumor size as well as CEA, could be used as the independent predictors (Table 2). Furthermore, a nomogram was constructed to clearly show the weight of each independent predictor. As the nomogram shown, the T4 stage contributed the most to SCLLM, followed by the N2 stage, elevated CEA, and rectal cancer (Figure 1). Various methods, including ROC curves, calibration curves and decision curve analysis (DCA), were utilized to evaluate the discriminating superiority of the

Table 3: Univariable and multivariable Cox regression model.

| Characteristics | Univariable analysis | | | | Multivariable analysis | | | |
|---|---|---|---|---|---|---|---|---|
| | OR | 95% CI lower | 95% CI upper | *p* value | OR | 95% CI lower | 95% CI upper | *p* value |
| Gender | | | | 0.609 | | | | |
| Female | | Reference | | | | NA | | |
| Male | 1.039 | 0.898 | 1.203 | 0.609 | | | | |
| Age (years) | | | | <0.001 | | | | <0.001 |
| <65 | | Reference | | | | Reference | | |
| 65-74 | 1.318 | 1.089 | 1.594 | 0.004 | 1.278 | 1.050 | 1.557 | 0.014 |
| ≥75 | 2.531 | 2.136 | 3.000 | <0.001 | 2.014 | 1.663 | 2.440 | <0.001 |
| Marital status | | | | <0.001 | | | | 0.003 |
| Married | | Reference | | | | Reference | | |
| Unmarried/NOS | 1.427 | 1.231 | 1.654 | <0.001 | 1.263 | 1.082 | 1.475 | 0.003 |
| Insurance | | | | | | | | |
| Yes | | Reference | | | | NA | | |
| No/unknown | 1.126 | 0.830 | 1.527 | 0.447 | | | | |
| Race | | | | 0.040 | | | | 0.035 |
| White | | Reference | | | | Reference | | |
| Black | 0.866 | 0.700 | 1.071 | 0.185 | 0.950 | 0.760 | 1.188 | 0.653 |
| Other/NOS | 0.730 | 0.558 | 0.954 | 0.021 | 0.695 | 0.528 | 0.916 | 0.010 |
| Tumor location | | | | 0.008 | | | | 0.465 |
| Right colon | | Reference | | | | Reference | | |
| Left colon | 0.742 | 0.600 | 0.916 | 0.006 | 0.930 | 0.746 | 1.158 | 0.515 |
| Rectum | 0.788 | 0.663 | 0.936 | 0.007 | 0.840 | 0.677 | 1.043 | 0.114 |
| NOS | 1.246 | 0.696 | 2.232 | 0.459 | 0.988 | 0.538 | 1.812 | 0.968 |
| Pathological grade | | | | <0.001 | | | | <0.001 |
| I/II | | Reference | | | | Reference | | |
| III/IV | 1.426 | 1.172 | 1.734 | <0.001 | 1.526 | 1.241 | 1.878 | <0.001 |
| Unknown | 1.475 | 1.204 | 1.807 | <0.001 | 1.011 | 0.808 | 1.266 | 0.920 |
| Histological type | | | | 0.214 | | | | |
| Adenocarcinomas | | Reference | | | | NA | | |
| MCC/SRCC | 1.204 | 0.898 | 1.614 | 0.214 | | | | |
| T stage | | | | <0.001 | | | | <0.001 |
| Tis-2 | | Reference | | | | Reference | | |
| T3 | 0.746 | 0.612 | 0.909 | 0.004 | 1.268 | 1.000 | 1.607 | 0.050 |
| T4 | 1.172 | 0.943 | 1.456 | 0.154 | 1.962 | 1.511 | 2.548 | <0.001 |
| N stage | | | | 0.036 | | | | 0.169 |
| N0 | | Reference | | | | Reference | | |
| N1 | 0.804 | 0.681 | 0.949 | 0.010 | 0.958 | 0.796 | 1.154 | 0.653 |
| N2 | 0.901 | 0.743 | 1.092 | 0.287 | 1.168 | 0.925 | 1.476 | 0.193 |
| Colectomy/proctectomy | | | | <0.001 | | | | <0.001 |
| Standard resection | | Reference | | | | Reference | | |
| Simplified resection | 1.294 | 1.041 | 1.608 | 0.020 | 1.434 | 1.138 | 1.805 | 0.002 |
| Nonresection/NOS | 1.914 | 1.631 | 2.246 | <0.001 | 2.895 | 2.078 | 4.034 | <0.001 |
| Pulmonary surgery | | | | <0.001 | | | | 0.246 |
| Yes | | Reference | | | | Reference | | |
| No/unknown | 2.061 | 1.512 | 2.808 | <0.001 | 1.208 | 0.878 | 1.663 | 0.246 |
| Radiotherapy | | | | 0.003 | | | | 0.124 |
| Yes | | Reference | | | | Reference | | |
| No/unknown | 1.289 | 1.090 | 1.523 | 0.003 | 1.172 | .957 | 1.436 | 0.124 |

Table 3: Continued.

| Characteristics | Univariable analysis | | | | Multivariable analysis | | | |
|---|---|---|---|---|---|---|---|---|
| | OR | 95% CI lower | 95% CI upper | *p* value | OR | 95% CI lower | 95% CI upper | *p* value |
| Chemotherapy | | | | <0.001 | | | | <0.001 |
| Yes | | Reference | | | | Reference | | |
| No/unknown | 2.694 | 2.314 | 3.137 | <0.001 | 2.179 | 1.830 | 2.594 | <0.001 |
| Tumor size | | | | <0.001 | | | | 0.220 |
| ≤5 cm | | Reference | | | | Reference | | |
| 5-10 cm | 1.144 | 0.966 | 1.355 | 0.119 | 1.069 | 0.898 | 1.272 | 0.454 |
| >10 cm | 2.040 | 1.466 | 2.838 | <0.001 | 1.436 | 1.016 | 2.030 | 0.040 |
| NOS | 1.453 | 1.186 | 1.780 | <0.001 | 1.104 | 0.877 | 1.390 | 0.401 |
| CEA | | | | 0.004 | | | | 0.006 |
| Normal | | Reference | | | | Reference | | |
| Elevated | 1.376 | 1.129 | 1.675 | 0.002 | 1.381 | 1.128 | 1.692 | 0.002 |
| NOS | 1.362 | 1.106 | 1.676 | 0.004 | 1.182 | .952 | 1.468 | 0.131 |
| TD | | | | <0.001 | | | | 0.001 |
| Negative | | Reference | | | | Reference | | |
| Positive | 1.493 | 1.216 | 1.832 | <0.001 | 1.494 | 1.194 | 1.868 | <0.001 |
| NOS | 1.807 | 1.535 | 2.128 | <0.001 | .908 | .673 | 1.224 | 0.525 |
| PNI | | | | <0.001 | | | | 0.404 |
| Negative | | Reference | | | | Reference | | |
| Positive | 1.188 | 0.967 | 1.459 | 0.101 | 1.162 | 0.923 | 1.462 | 0.201 |
| NOS | 1.524 | 1.291 | 1.798 | <0.001 | 1.060 | 0.867 | 1.297 | 0.569 |

MCC: mucinous cell carcinoma; SRCC: signet ring cell carcinoma; CEA: carcinoembryonic antigen; TD: tumor deposits; PNI: perineural invasion; NOS: not otherwise specified; NA: unavailable.

nomogram. The area under the curve (AUC) values of ROC were 77.78%. The calibration curves illustrated agreement between model prediction and actual observations. The DCA demonstrated net benefits of the nomogram and each prognostic factor.

*3.3. Prognostic Factors of Synchronous Colorectal Lung-Limited Metastasis.* The qualified variables, that identified by a univariate Cox regression model, were further analyzed by a multivariate regression analysis, which discriminated 9 independent prognostic factors, including age, race, marital status, pathological grade, T stage, colectomy/proctectomy, chemotherapy, CEA, and TD (Table 3). In order to visually demonstrate the impact of each prognostic factor on survival, the cumulative survival curves and nomogram were utilized in accordance with the result of the multivariate Cox regression model. The prognostic nomogram illustrated that nonresection/NOS played as the poorest prognostic factor, followed by nonchemotherapy, ≥75-year-old and T4 stage (Figure 2). Meanwhile, the AUC values of ROC were 79.67%, 79.67%, and 76.97% regarding nomograms predicting 1-, 2-, and 3-year OS. The calibration curves demonstrated optimal agreement between model prediction and actual observations for 1-, 2-, and 3-year OS. The DCA indicated net benefits of the nomogram and each prognostic factor. Moreover, the cumulative survival curves revealed the influence of each prognostic factor on survival after controlling the other variables (Figure 3).

## 4. Discussion

To the best of our knowledge, this analysis was the first to look into the predictive and prognostic factors regarding OS for CRC with synchronous lung-limited metastasis. Colorectal oncologists have mainly focused on CRC with liver metastasis. Nevertheless, there is limited research on CRC with lung metastasis. The treatment of SCLLM commonly learns from the clinical experiences and strategies of treatment of colorectal hepatic metastasis [19]. In order to further improve treatment, it is essential to identify the specialized predictive and prognostic factors of SCLLM. CRC patients with high risk factors of lung metastasis should receive the particular treatments against prognostic factors and increase the frequency of follow-up.

Previous studies reported that the pattern of colorectal lung metastasis was the direct invasion of cancer cells into the systemic circulation through the veins [13], which was different from the method of colorectal liver metastasis, that was thought to result from the lymphatic drainage of the colon and rectum [14]. It may be the reason why the T stage can be used as both predictor and prognostic factor but the N stage can only play as a predictor of SCLLM. Moreover, numerous researches reported that TD was associated with reductions in survival [20, 21]. In fact, most of TD were thought to arise from lymphovascular invasion [22] and significantly related to T staging [22, 23]. Therefore, TD may be a manifestation of the ability and depth of tumor invasion affecting the survival of SCLLM patients.

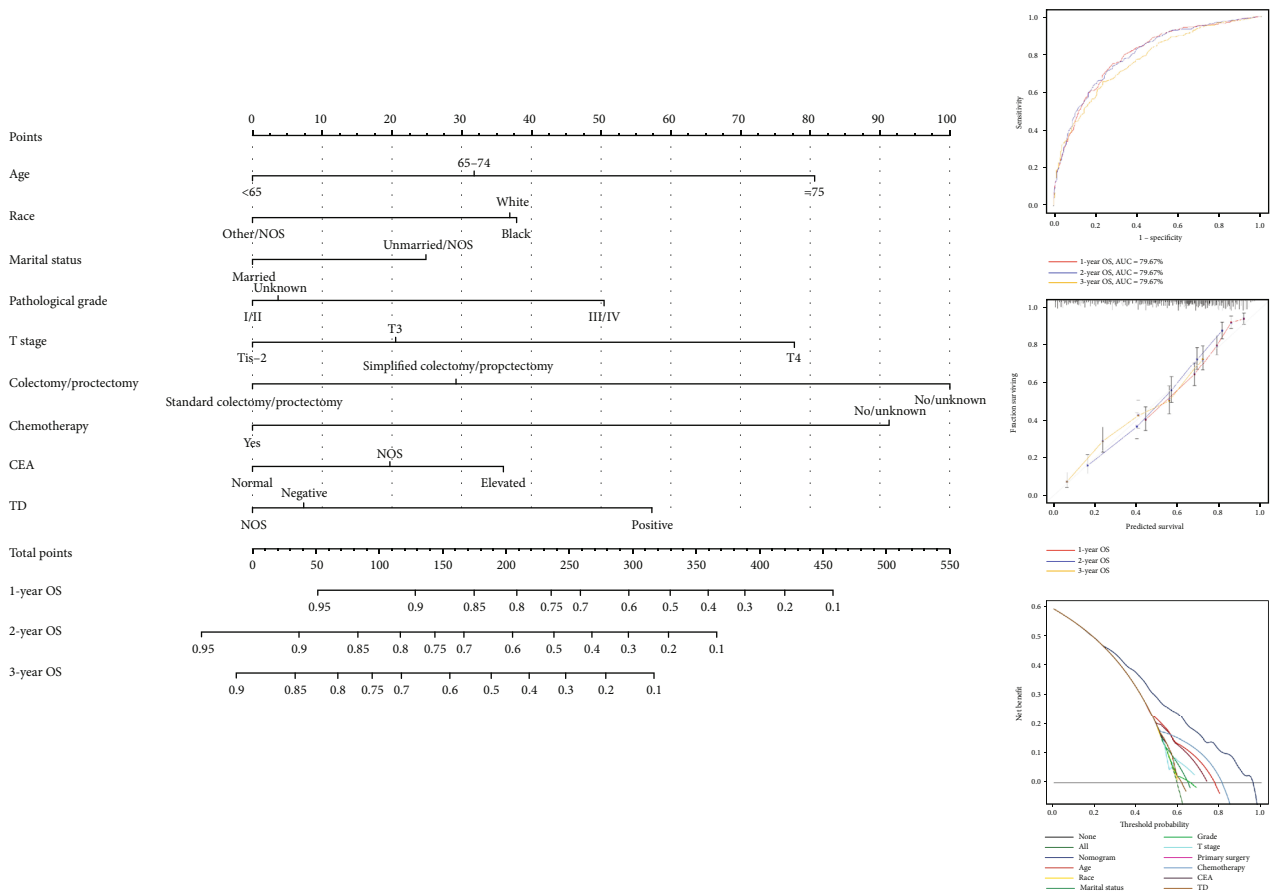


Figure 2: The impact of each prognostic factor on survival for patients with SCLLM.

RNE were considered as the priority for the assessment of the quality of surgery, which was mentioned in previous study [24], especially for the lack of the data concerning total mesorectal excision (TME) and complete mesocolic excision (CME) in the SEER database. The prognostic nomogram and survival curve manifested that standard colectomy/proctectomy with RNE ≥ 12 owned the clearest survival benefit comparing with noncolectomy and simplified resection. It is a consensus that high-quality colectomy/proctectomy means sufficient circumferential resection margin (CRM), which can be used as a specific therapeutic indicator against the depth of tumor invasion. Considering the critical role of T staging in patients with SCLLM, eligible TME/CME may be the most effective way to treat and prevent colorectal lung metastasis.

It is feasible to remove the primary tumor and liver metastasis in a simultaneous or staged approach for patients present with synchronous colorectal liver metastasis [25, 26]. Although existing some controversy concerning the order of resection of the liver metastasis and the primary tumor [19], none of synchronous, sequential liver-first, or bowel-first surgery appeared inferior to the others [25, 26]. Can the experience from colorectal liver metastasis be completely applied to SCLLM? The result of this study confirmed that independent pulmonary surgery, as a nonindependent prognostic factor in Cox regression analysis, did not improve the survival for SCLLM patients. Therefore, we believe that the approach of lung resection before resection of the primary tumor may be unreasonable for patients with SCLLM. Besides, more studies are needed to confirm whether the pulmonary surgery following by the colectomy/proctectomy cutting off the source of cancer cells and chemotherapy eliminating micrometastases can provide a survival benefit. In addition, CRC patients with metastatic diseases should receive radiation therapy cautiously [19]. This study believed that radiotherapy cannot improve survival for SCLLM patients as a whole. Nevertheless, it is meaningful to identify CRC patients who are sensitive to radiotherapy, as some other studies did [27, 28].

A growing body of data has shown that the location of the primary tumor can be both prognostic and predictive of response to EGFR inhibitors in metastatic colorectal cancer [29–31]. This study demonstrated inconsistent risk of lung-limited metastasis among right colon, left colon, and rectum. Several studies also proposed that rectal cancer is prone to metastasize to the lungs [15, 32]. Interestingly, there was no correlation between the primary site and the prognosis of patients with SCLLM. The mainstream opinions presently considered that targeted chemotherapy drugs, like cetuximab and panitumumab, improve survival for left-side colon patients but confer little benefit to right-side colon patients with metastatic diseases [29–31]. Does the consistent prognostic coefficient mean that the existing targeted drugs may not significantly prolong survival in all patients with SCLLM,

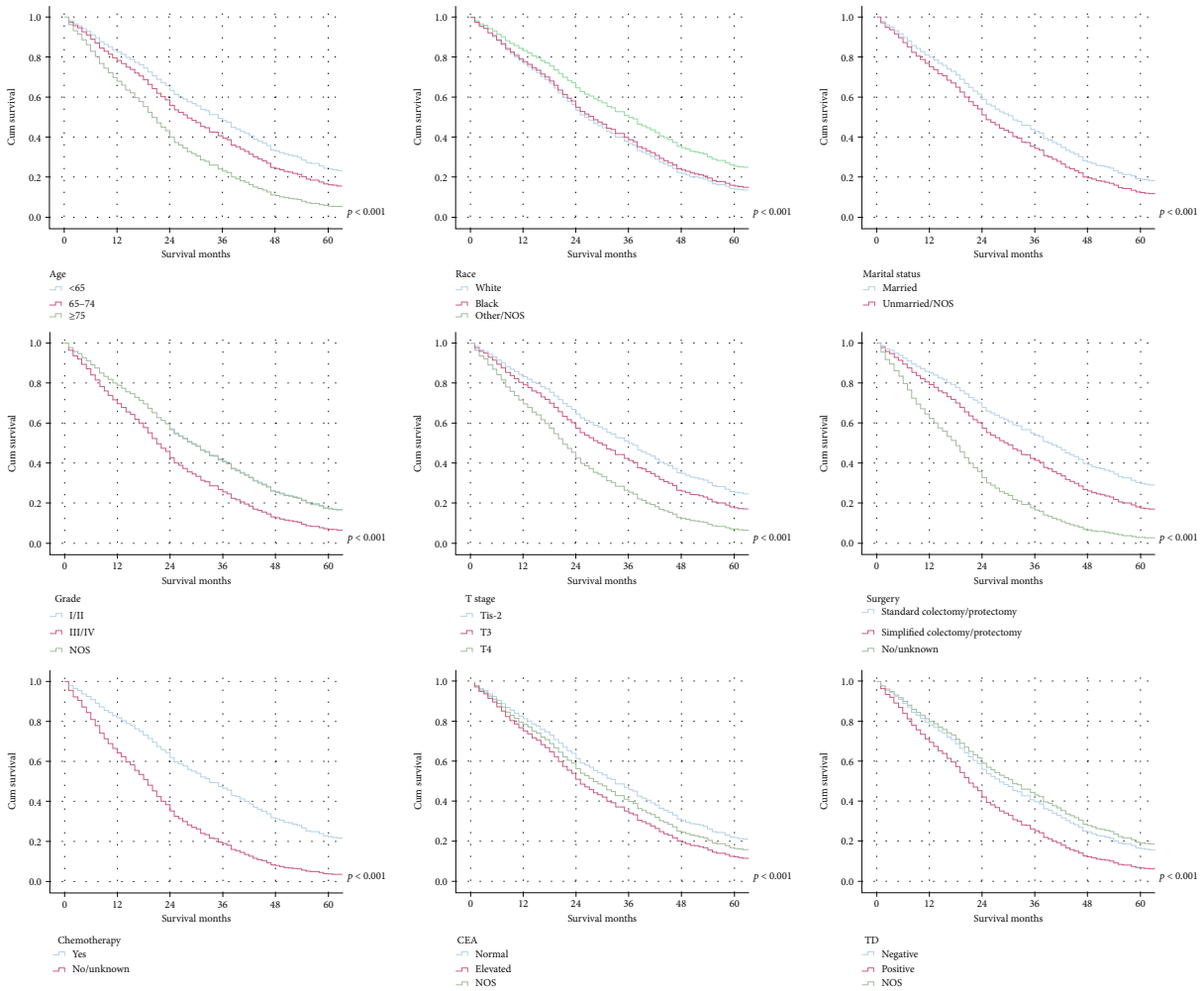


Figure 3: The cumulative survival curves revealed the influence of each prognostic factor on survival after controlling the other variables.

including left colon and rectal cancer? It is uncertain and requires prospective research to verify.

A recent study involved the prognostic factors regarding cancer-specific survival for CRC with synchronous lung-limited metastasis [33]. However, study only focusing on cancer-specific survival inevitably misses some cases, such as those being not first tumor. Meanwhile, it is more reasonable to choose OS as the research endpoint since SCLLM, as a systemic disease, is able to affect the whole-body function. Limitations of this study include the following: (1) the use of retrospective data; (2) detailed treatment information for included patients were not recorded in the SEER cohort, and we could not investigate specific options, including chemotherapy regimen and specific surgical method, in the survival of SCLLM patients; and (3) the lack of some important genetic indicators, such as KRAS, NRAS, and BRAF. Future studies can focus on the molecular mechanisms of CRC with lung-limited metastasis.

## 5. Conclusion

This study identified independent predictors and prognostic factors for SCLLM based on a large database of the United States. The predictors and prognostic factors can provide supporting evidence for the prevention and treatment of SCLLM.

## Data Availability

These data were derived from the Surveillance, Epidemiology, and End Results (SEER) database (https://seer.cancer.gov/) and identified using the SEER*Stat software (Version 8.3.5) (https://seer.cancer.gov/seerstat/).

## Consent

Patients' informed consent was waived because of the retrospective nature of the study design.

## Conflicts of Interest

The authors declare that they have no competing interests and consent for publication.

## Authors' Contributions

Yuqiang Li, Zhongyi Zhou, and Da Liu contributed equally to this article as co-first author. Fengbo Tan and Wenxue Liu contributed equally to this article as co-corresponding authors.

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