Effect of Linc-00475 Regulation of p53 on Glycolysis and Survival in Gastric Cancer

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Research Article

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Abstract

Background: Abnormal glucose metabolism leading to tumor proliferation and metastasis is an important area of interest in cancer treatment. The purpose of this study was to investigate the effects of clinical expression of glucose metabolism-related genes Linc-00475 and p53 on glycolysis and survival.

Methods: A key differential gene Linc-00475 was screened using a metabolic database, and its downstream gene, p53, was predicted. A total of 107 gastric cancer tissue samples from patients diagnosed at our center between 2011 and 2013 were selected. The expression levels of Linc-00475 and p53 were detected via in situ hybridization or immunohistochemistry. Chi-square test was used to analyze the relationship between Linc-00475 and p53 expression and clinicopathological factors. Kaplan-Meier method and log rank test were used to analyze patients' overall survival. To determine the effect of Linc-00475 on glycolysis, qRT-PCR and western blot were utilized to evaluate the regulatory relationship between Linc-00475 and p53.

Results: High expression of Linc-00475 ($P < 0.001$) and low expression of p53 ($P < 0.01$) were associated with poor prognosis. There was a negative correlation between the expression of Linc-00475 and p53 in gastric cancer (Pearson's coefficient test, $r = -0.405$; $P < 0.001$). The co-expression of high-level Linc-00475 and low-level p53 can thus be used as an independent prognostic factor ($P = 0.001$). Linc-00475 was also shown to regulate aerobic glycolysis. Western blot and qRT-PCR demonstrated that Linc-00475 regulates the expression of p53.

Conclusion: The co-expression of Linc-00475 and p53 can be used as a reference index for evaluating the prognosis of gastric cancer. Linc-00475 regulates p53, thereby affecting glycolysis.

Background

Gastric cancer remains the sixth most common cancer and the third leading cause of cancer death in the world [1]. Even though surgery and adjuvant chemotherapy prolong patient survival, the five-year survival rate is still low at only ~32% [2]. Early detection and treatment are the key to prevention. Therefore, finding specific early markers and targets is of great significance for the diagnosis and treatment of gastric cancer.

Normal cells metabolize glucose through the oxidative mitochondrial phosphorylation pathway, while tumor cells supply energy via aerobic glycolysis even under the condition of sufficient oxygen, which is known as the Warburg effect or aerobic glycolysis. More and more evidence show that tumor progression mainly depends on aerobic glycolysis [3]. As a transcription unit with a length of more than 200 nt, LincRNA can interact with DNA, mRNA, and protein, regulate cell metabolism, proliferation, transfer, and drug resistance at both the transcription and translation levels, and participate in different regulatory mechanisms according to its subcellular localization. Furthermore, it plays a crucial regulatory role in many important biological processes and disease progression [4, 5]. Linc-00475 can promote tumor progression in gliomas and is associated with poor prognosis [6–7].

As a tumor suppressor gene, p53 plays an important regulatory role in signal transduction pathways, such as cell cycle regulation, DNA repair, oxidative stress, autophagy, aging, and apoptosis. The p53-mediated signal transduction pathway is closely related to its post-translational modification. The abnormal expression of tumor suppressor gene p53 is one of the factors affecting tumor aerobic glycolysis rate [8–12].

Studies have found that p53 contains an enhanced region that regulates Linc-00475, but Linc-00475, in turn, also regulates p53 and affects its expression [13]. Currently, there is no research on Linc-00475 and p53 in gastric cancer. In the present study, the expression of Linc-00475 and p53 in gastric cancer and adjacent normal tissue was detected using bioinformatics analysis combined with immunohistochemistry. The correlation between these two proteins and clinicopathological characteristics of gastric cancer, as well as clinical significance of their co-expression, were investigated. This regulatory relationship may provide a reference for clinical treatment of gastric cancer.

Materials And Methods

Study patients

By analyzing the sequencing results from the GEO gastric cancer public database (GSE96856), the metabolism-related long-chain noncoding RNA (Linc-00475) was screened to predict its downstream gene (p53). A total of 107 tissue specimens were selected for radical gastric cancer surgery in the Department of Gastrointestinal Nutrition and Hernia Surgery, The Second Hospital of Jilin University between June 2014 and June 2015. Among the 107 patients, 52 were aged <60 years and 55 were aged ≥60 years. There were 27 males and 80 females. The cohort included 42 cases with a tumor diameter of <5 cm and 65 cases with a tumor diameter of ≥5 cm. The degree of differentiation was high/moderate in 30 cases and low in 77 cases. Tumors were located in the upper region in 16 cases and middle and lower in 91 cases. A total of 83 cases were of Borrmann types I, II, and III, and 24 cases were of Borrmann type IV. The TNM stage was identified based on the Eighth Edition of AJCC, where 22 cases were in stage T1–2 and 85 cases were in stage T3–4. There were 32 cases of N0 and 75 cases of N1–3. All patients were single-focus. The patients did not receive radiotherapy and chemotherapy before the operation, and their postoperative follow-up data were complete. The study protocol was approved by the hospital ethics committee, and all patients signed the informed consent form.

Immunohistochemical standing and in situ hybridization (ISH)

Paraffin specimens were kept in the pathology department of our hospital. Tissue specimens were cut into 4 µm-thick sections via continuous sectioning. After dewaxing and hydration, tissue sections were soaked in a 3% H₂O₂ solution at room temperature for 10 min and then repaired in a sodium citrate antigen repair solution (pH 6.0) at 121°C for 2 min. The slices were then incubated with a primary antibody for p53 (1:1000, Abcam, Cambridge, MA, USA) at 4°C overnight, followed by incubation with a secondary antibody at 37°C for 30 min, DAB staining, hematoxylin re-staining for 1 min, and 1% hydrochloric acid alcohol differentiation. After washing with tap water and blue returning for 10 min, the

samples were dehydrated in gradient solutions of ethanol, treated with xylene, and air dried to create transparent tissue sections. They were then sealed and prepared for microscopic observation.

ISH, dewaxing, and hydration methods were the same as described above. The sections were treated with protease, diluted with citric acid for 30 min, and fixed with 1% paraformaldehyde for 10 min at room temperature. A total of 20 μ L of pre-hybridization solution were added to each slice for 2–4 h of hybridization, followed by 20 μ L of Linc-00475 probe hybridization solution, and kept constant at 37°C overnight. Blocking solution was added at room temperature for 1 h on the next day. Then, biotinylated mouse anti-human digoxin antibody was added dropwise and incubated at room temperature for 60 min, followed by dropwise addition of the streptavidin-biotin complex and incubation at room temperature for 30 min, and finally biotinylated peroxidase and incubation at room temperature for 30 min. The samples were then stained with DAB, counterstained with hematoxylin, dehydrated in gradient ethanol, treated with xylene, air dried, sealed, and prepared for microscopic observation.

Immunoreactivity scoring

Staining intensity grading: 0 points for colorlessness, 1 point for yellow, 2 points for brown and 3 points for dark brown; ratio of positive cells: <5% for 0 points, 5%-25% for 1 point, 25%-50% for 2 points, 50%-75% for 3 points, and >75% is 4 points. The staining intensity score is multiplied by the score of the proportion of positive cells as the final score; ≥ 6 is considered as positive expression, and ≤ 4 is considered as negative expression [14].

Cell culture and virus transfection

GSE-1, MKN-28, BGC-823, AGS, MKN-45 were cultured in DMEM and F-12 medium (Gibco, Thermo Fisher, China) containing 10% fetal bovine serum (Gibco, Thermo Fisher, China). After digestion, centrifugation, and resuspension, the cells are counted. Add 1×10^5 cells to each well, and wait until the cells are fused 50%-70% for use the next day; add the calculated virus to the 12-well plate, Change the medium after 14 hours. After the cells are fused to about 80%, they will be transferred to the culture dish and continue to be cultured. Puromycin is added for selection until stable. The transfection efficiency is verified by Western blot and qRT-PCR, and subsequent experiments are performed.

qRT-PCR

The qRT-PCR analysis of Linc-00475 expression in 38 fresh gastric cancer pathological tissue samples and cell lines obtained between May and August 2020 at our center was performed as described previously [14]. The primer sequences for qRT-PCR are as follows. GAPDH mRNA was used as the internal reference gene. Data assessment was determined by the $-\Delta\Delta C_t$ method.

Linc-00475 forward: 5'-GAGGGTTCACAAACGAGCCT-3'

Linc-00475 reverse: 5'-CCCAGGCTCCTAACCATGTC -3'

Extracellular acidification rate (ECAR)

Cells were divided into blank and experimental groups and inoculated into 96-micro-well plates for ECAR testing. A total of 200 μ L of hydration solution were added to each well in addition to glutamine detection and stock solutions from the glycolysis kit prior to testing.

Oxygen consumption rate (OCR)

OCR testing methods were the same as those for ECAR. The glycolysis pressure kit (Abcam, Britain) was configured using stock compounds, which were successively added into the hole and tested on a computer.

Lactic acid, glucose, and ATP level detection

Cells in the blank and experimental groups were inoculated into six-well plates at a density of 2×10^5 cells per well. After reaching 90% confluence, the cells were collected and centrifuged at room temperature and 500 rpm for 5 min. Culture medium supernatant was then collected into an Eppendorf tube and analyzed according to the instructions for the glucose, lactic acid, and ATP testing kit (BioVision, USA) to determine the optical density (OD) value of each group using a microplate reader. Glucose consumption and lactic acid level of the supernatant in each group were calculated according to the formula provided in the manufacturer instructions, such that the higher the OD value, the higher the ATP content.

Statistical analysis

SPSS 22.0 and GraphPad Prism 7.0 software were used for statistical analysis. The measurement data were compared between the two groups via a non-paired t-test. Chi-square test was used to analyze the relationship between Linc-00475 and p53 expression and clinicopathological factors. Pearson test was used to analyze the correlation between Linc-00475 and p53 expression. Kaplan-Meier method was used for survival analysis, while log rank test was utilized to calculate survival differences between different groups. Univariate and multivariate Cox risk ratio models were fitted to screen out independent prognostic factors. $P < 0.05$ indicated statistically significant differences.

Results

Relationship between Linc-00475 and p53 expression in gastric cancer and clinicopathological factors

The GEO Gastric Cancer Metabolism Database (GSE96856) was used to screen the highly expressed Linc-00475 as the research target (Figure 1). A literature review allowed to identify the key gene p53 related to downstream glucose metabolism. Clinical significance of Linc-00475 and p53 expression in gastric cancer was studied via immunohistochemical experiments.

ISH and immunohistochemical staining results (Figure 2A, B) showed that Linc-00475 and p53 proteins were mainly expressed in the cytoplasm and nucleus. There was a negative correlation between Linc-00475 and p53 expression in gastric cancer (Pearson test, $r = -0.405$; $P < 0.001$, Figure 2C).

The high Linc-00475 expression was significantly related to tumor location, Bowman type, and p53 expression ($P < 0.05$; Table 1), while low p53 expression was related to gender.

Table 1
Relationship between the Linc-00475 and p53 expression and clinical parameter

Variables	Linc-00475 Expression		Pvalue	p53 Expression		Pvalue
	High(n=72)	Low(n=35)		High(n=37)	Low(n=70)	
Age			0.218			0.412
≤60	32	20		20	32	
≥60	40	15		17	38	
Sex						
Male	17	10	0.579	16	11	0.002
Female	55	25		21	59	
Size						
<5(cm)	32	10	0.115	14	28	0.828
≥5(cm)	40	25		23	42	
Differentiation						
Well/ Moderate	23	7	0.197	7	23	0.127
Poor	49	28		30	47	
Tumor location						
Upper	7	9	0.030	8	8	0.160
Middle/ Lower	65	26		29	62	
Borrmann type						
Ⅰ Ⅱ Ⅲ	60	23	0.040	32	51	0.108
Ⅳ	12	12		5	19	
Invasive depth						
T1-T2	14	8	0.682	8	14	0.844
T3-T4	58	27		29	56	
lymph node metastasis						
N0	21	11	0.811	9	23	0.359
N1-N3	51	24		28	47	
p53 Expression						

Variables	Linc-00475 Expression		Pvalue	p53 Expression	Pvalue
Low	53	17	0.011		
High	19	18			

Relationship Between Linc-00475 And P53 Expression And Prognosis

In 107 gastric cancer patients, the prognosis for individuals with a high expression of Linc-00475 was worse than that for patients with a low expression (5 years OS 59.21% vs. 17.85%, $P < 0.001$). High p53 expression was associated with a better prognosis than low expression (20.38% vs. 52.04%, $P < 0.01$; Figure 2C, D).

In univariate analysis, Borrmann type IV (hazard ratio (HR) = 1.912, 95% confidence interval (CI): 1.088–3.359, $P = 0.024$), T3–4 stage (HR = 2.713, 95% CI: 1.227–5.998, $P = 0.014$), N + stage (HR = 2.269, 95% CI: 1.207–4.265, $P = 0.011$), high expression of Linc-00475 (HR=2.955, 95% CI: 1.601–5.455, $P = 0.001$), high expression of p53 (HR = 0.417, 95% CI: 0.231–0.751, $P = 0.001$), and expression of Linc-00475 combined with p53 (HR = 3.929, 95% CI: 2.208–6.994, $P < 0.001$) were associated with overall survival (OS). Univariate significant factors were included in the Cox multivariate analysis, which further confirmed that Borrmann type, T stage, N stage, and expression of Linc-00475 combined with p53 were independent prognostic factors for OS (HR = 1.816, 95% CI: 1.032–3.356, $P = 0.039$; HR = 2.771, 95% CI: 1.236–6.213, $P = 0.013$; HR = 2.159, 95% CI: 1.128–4.131, $P = 0.020$; HR = 4.537, 95% CI: 2.499–8.238, $P = 0.001$, respectively; Table 2).

Table 2
The univariate and multivariate analyses of factors associated with overall survival

Variable	univariate Cox regression			multivariate Cox regression		
	HR	95%CI	P-value	HR	95%CI	P-value
Age (≥ 60)	1.045	0.634-1.725	0.862			
Sex(male)	1.660	0.862-3.197	0.130			
Size(≥ 5 cm)	0.671	0.408-1.106	0.118			
Differentiation(Poor)	0.801	0.470-1.136	0.413			
Tumor location(Middle low)	1.328	0.604-2.920	0.480			
Borrmann type (Ⅱ)	1.912	1.088-3.359	0.024	1.816	1.032-3.356	0.039
Invasive depth T3-T4	2.713	1.227-5.998	0.014	2.771	1.236-6.213	0.013
lymph node metastasis (+)	2.269	1.207-4.265	0.011	2.159	1.128-4.131	0.020
High Linc-00475 expression	2.955	1.601-5.455	0.001			
High p53 expression	0.417	0.231-0.751	0.004			
Linc-00475+/ p53-	3.929	2.208-6.994	≤ 0.001	4.537	2.499-8.238	0.001

High expression of Linc-00475 and low expression of p53 were both associated with poor prognosis. The prognosis of patients with a high expression of Linc-00475 and low expression of p53 was worse (HR = 3.03; 95% CI: 1.783–5.149; $P = 0.001$; Figure 2F). These results further demonstrate that Linc-00475 combined with p53 may be a more promising prognostic indicator.

Linc-00475 Expression In Tissues And Cell Lines

Analysis results for the RNA expression levels of 38 fresh frozen cancer (T) and adjacent (N) tissues showed that Linc-00475 mRNA expression in cancer tissues was higher than that in adjacent tissues (Figure 3A). The qRT-PCR was used to detect the expression of gastric cancer cell lines AGS, BGC-823, MKN-45, MKN-28, and GES-1. It showed that Linc-00475 expression was higher in AGS and MKN-45 cells (Figure 3B), which were selected for subsequent experiments (Figure 3C, D).

Effect of Linc-00475 expression on ECAR level in gastric cancer

ECAR was determined in stably silenced cell lines overexpressing Linc-00475. The results showed that the ECAR level decreased after Linc-00475 silencing, suggesting that the glycolysis rate decreased (Figure 4). The ECAR level increased after Linc-00475 overexpression, showing that the glycolysis rate increased.

Effect of Linc-00475 expression on OCR level in gastric cancer

OCR was detected in stably silenced cell lines overexpressing Linc-00475. The results showed that the level of OCR increased after silencing Linc-00475, suggesting that mitochondrial energy metabolism increased and glycolysis rate decreased (Figure 5). The level of OCR decreased after Linc-00475 overexpression, suggesting that mitochondrial energy metabolism decreased and glycolysis rate increased.

Effects of Linc-00475 expression on glucose uptake and aerobic glycolytic metabolites (lactic acid and ATP) in gastric cancer

Glucose uptake and metabolites were detected in stably silenced cell lines overexpressing Linc-00475. The results showed that glucose uptake decreased and lactate and ATP production decreased after Linc-00475 silencing, suggesting that the rate of glycolysis decreased (Figure 6). Linc-00475 overexpression increased glucose uptake and lactate and ATP production, showing an increase in glycolysis rate.

Linc-00475 Regulates P53 Expression

The p53 expression increased after silencing the expression of Linc-00475, and decreased after Linc-00475 overexpression (Figure 7).

Discussion

Metabolic abnormalities are closely related to tumor progression. Studies have shown that regulating the aerobic glycolysis level has a positive significance in inhibiting gastric cancer progression [15–19]. Therefore, exploring the molecular mechanism between aerobic glycolysis and malignant progression of gastric cancer will be key in improving gastric cancer prevention and treatment.

Database analysis was used to screen Linc-00475, which is related to metabolism. Linc-00475 also affects cancer progression via glycolysis. Jin et al. have found that Linc-MSC-AS1 promotes the proliferation and glycolysis of gastric cancer cells by regulating the expression of PFKFB3 [20]. The study by Du et al. has found that Linc-MIR210HG promotes aerobic glycolysis by regulating the expression of HIF-1 α , which leads to the progression of triple-negative breast cancer [21]. ISH analysis in the present study determined that Linc-00475 is highly expressed in gastric cancer. The high expression of Linc-00475 is related to tumor location, Bowman classification, and p53 expression. This shows that Linc-00475 may play an oncogene role in gastric cancer. Yu et al. [6] have found that Linc-00475 promotes the progression of glioma via the miR-141-3p/YAP1 axis. In glioma under hypoxia, the Linc-00475-microRNA-449b-5p-AGAP2 axis promotes the disease progression [7]. The present study also demonstrated that patients with a high Linc-00475 expression had a poor prognosis (HR = 2.955, 95% CI: 1.601–5.455, P = 0.001). Linc-00475 is highly expressed in gastric cancer tissues and cell lines and may be an oncogene that leads to a poor prognosis. It is known that lincRNA is closely related to aerobic glycolysis. However, whether Linc-00475 has an effect on glycolysis has not been reported in the literature. Functional experiments showed that the low expression of Linc-00475 can reduce the production of lactic acid and

ATP by reducing sugar consumption in order to reduce the glycolysis rate. The low expression of Linc-00475 can reduce ECAR and its glycolytic ability, while an increase in OCR means that the low expression of Linc-00475 promotes the metabolism of mitochondrial energy. Linc-00475 promotes the progression of gastric cancer by affecting glycolysis, which then affects patient prognosis. Its specific mechanism may also be related to its downstream genes.

The abnormal expression of the tumor suppressor gene p53 is one of the factors that affects the rate of aerobic glycolysis in tumors. The two downstream transcription targets of p53 are TP53-induced glycolysis and apoptosis regulator (TIGAR) and cytochrome oxidase 2 (synthesis of cytochrome oxidase 2, SCO2) [22, 23]. The TIGAR sequence is similar to that of the phosphofructokinase (PFK), which can hydrolyze fructose 2,6-diphosphate, thereby inhibiting aerobic glycolysis. In addition, p53 can activate SCO2, improve mitochondrial respiration efficiency, and then inhibit the efficiency of aerobic glycolysis [24]. It can also inhibit the functions of GLUT1, GLUT3, and GLUT4, reduce the ability of glucose uptake, and thereby inhibit aerobic glycolysis [25]. Finally, p53 can inhibit the activity of G6PD and regulate other factors (PTEN, AKT, HIF, and PUMA) to indirectly modulate aerobic glycolysis [26–29]. In summary, tumor suppressor gene p53 can reduce the efficiency of glycolysis, thereby affecting tumor progression.

As a transcription factor, p53 was found to contain a new binding enhancer (Linc-00475). It was also confirmed that the function of p53 was maintained by Linc-00475. At the same time, with a decrease in Linc-00475 level, its binding to p53 also decreased [13]. LincRNA can also be used as a transcription regulator. LincMALAT1 can regulate the expression of p53 and can also be regulated by p53 [30]. In addition, Linc-00475 can regulate the expression of p53, and there is a positive correlation between them. This shows that Linc-00475 affects the prognosis of gastric cancer patients via glycolysis, which may be realized by p53. Linc-00475 and p53 expression alone is not an independent prognostic factor, while expression of Linc-00475 combined with p53 is an independent prognostic factor. The study conclusions suggest that co-expression of Linc-00475 and p53 may be more suitable as a marker of gastric cancer than one alone. Whether the dual drugs targeting Linc-00475 and p53 can be used as a new strategy for gastric cancer treatment will become the focus of future investigations.

The present study has several limitations. First, this was a retrospective study, which may be subject to selection bias. Second, the specific mechanism of Linc-00475 regulating p53 needs to be identified in further research. The present study revealed that co-expression of Linc-00475 and p53 may be a prognostic marker for gastric cancer.

Conclusion

In summary, high Linc-00475 expression is associated with poor prognosis, which becomes worse when accompanied by low expression of p53. Linc-00475 may affect glycolysis by regulating p53, which leads to poor patient prognosis.

Declarations

Authors' contributions

XDW designed the research. STZ collected the data, analyzed the data. CZ analyzed the data and wrote the manuscript. All the authors approved the final manuscript.

Acknowledgements

None.

Conflict of Interest

The authors report no competing interest in this study.

Availability of data and materials

Please contact author for data requests

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was granted approval by the ethics committee of The Second Hospital of Jilin University and carried out in compliance with ethical principles of the Declaration of Helsinki. The written informed consents of included patients were obtained prior to surgery.

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Figures

Figure 1

GEO public database (GSE96856). (A) Differential genes expression analysis; (B) Heat map

Figure 2

IHC staining for Linc-00475 and p53. (A) Expression of Linc-00475; (B) Expression of p53; (C) correlation analysis of the expression level between Linc-00475 and p53; (D) OS in Linc-00475 expression; (E) OS in p53 expression; (F) OS for Linc-00475 and p53 co-expression

Figure 3

Expression of Linc-00475 in gastric cancer. (A) Linc-00475 expression level in tissues; (B) Linc-00475 expression level in cell lines; (C) Silencing of Linc-00475; (D) Overexpression of Linc-00475

Figure 4

Effects of silencing and overexpression of Linc-00475 on ECAR level in gastric cancer cells: (A) Changes of ECAR after silencing expression of Linc-00475 in Gastric Cancer Cell Line AGS; (B) Changes of ECAR after silent expression of Linc-00475 in Gastric Cancer Cell Line MKN-45; (C) Changes of ECAR after overexpression of Linc-00475 in gastric cancer cell line AGS; (D) Changes of ECAR after overexpression of Linc-00475 in gastric cancer cell line MKN-45

Figure 5

Effects of silencing and overexpression of Linc-00475 on OCR level in gastric cancer cells: (A) Changes of OCR after silencing expression of Linc-00475 in Gastric Cancer Cell Line AGS; (B) Changes of OCR after silent expression of Linc-00475 in Gastric Cancer Cell Line MKN-45; (C) Changes of OCR after overexpression of Linc-00475 in gastric cancer cell line AGS; (D) Changes of OCR after overexpression of Linc-00475 in gastric cancer cell line MKN-45

Figure 6

Effects of silencing and overexpression of Linc-00475 on glucose uptake and aerobic glycolytic metabolites in gastric cancer cells: (A) Changes of glucose uptake after silencing and overexpression of Linc-00475 in gastric cancer cell line; (B) Changes of lactate production after silencing and overexpression of Linc-00475 in gastric cancer cell line; (C) Changes of ATP level after silencing and overexpression of Linc-00475 in gastric cancer cell line

Figure 7

Linc-00475 regulates the key downstream gene p53: (A) Expression of p53 after silencing Linc-00475 in gastric cancer cell lines; (B) Expression of p53 after overexpression Linc-00475 in gastric cancer cell lines