METTL3-Mediated DLGAP1 Antisense RNA 2 Promotes Gastric Cancer Tumorigenesis Through Facilitating C-Myc-Dependent Warburg Effect

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Research Article

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Abstract

Background

The critical roles of N⁶-methyladenosine (m⁶A) modification have been demonstrated by more and more evidence. However, the cross-talking of m⁶A and long non-coding RNAs (lncRNAs) in gastric cancer (GC) tumorigenesis is still unclear. Here, our work focused on the functions and molecular mechanism of m⁶A-modified lncRNA DLGAP1 antisense RNA 2 (DLGAP1-AS2) in GC.

Methods

LncRNA expression profile data was derived from GEO. M⁶A profile was screened using Methylated RNA immunoprecipitation sequencing (MeRIP-Seq). The metabolism assays were conducted using quantitative analysis of glucose, lactate, ATP and extracellular acidification rate (ECAR). The m⁶A level of specific RNA was identified using MeRIP-qPCR. The molecular interaction was detected using RIP assay.

Results

Microarray analysis found that lncRNA DLGAP1-AS2 up-regulated in GC cells. Clinical data showed that DLGAP1-AS2 high-expression was correlated with advanced pathological stage and poor prognosis. Functionally, DLGAP1-AS2 promoted the Warburg effect (aerobic glycolysis) and knockdown of DLGAP1-AS2 suppressed the tumor growth of GC cells. Mechanistically, m⁶A methyltransferase METTL3 enhanced the stability of DLGAP1-AS2 via m⁶A site binding. Moreover, DLGAP1-AS2 interacted with YTHDF1 to enhance the stability of c-Myc mRNA through DLGAP1-AS2/m⁶A/YTHDF1/c-Myc mRNA.

Conclusions

In conclusion, our work indicates the functions of m⁶A-modified DLGAP1-AS2 in the GC aerobic glycolysis, disclosing a potential m⁶A-dependent manner for GC treatment.

Introduction

Gastric cancer (GC) is a worldwide digestive system malignant tumor with a significant high mortality and morbidity[1, 2]. In spite of the achievement on the GC therapy, including chemotherapy, radiotherapy and surgery, the overall survival rate or survival time of GC sufferers remains unimproved[3]. The reason for the lethal trait of GC is metastases and recurrence, however, the molecular mechanisms for GC progression is still poorly understood[4]. Therefore, it is significantly critical to explore the underlying mechanism of GC development, covering prognostic biomarkers, diagnostic and therapeutic targets.
Long non-coding RNAs (lncRNAs) are novel noncoding RNAs longer than 200 nucleotides length without protein-coding potential[5, 6]. Over the years of research, growing number of lncRNAs are identified in the GC occurrence[7, 8]. For example, lncRNA MAGI2-AS3 promotes the tumor progression through sponging miR-141/200a to overexpress ZEB1 level, highlighting an ideal biomarker for GC potential therapeutic target[9]. SNHG11 post-transcriptionally promotes oncogenic autophagy progression through aggravating ATG12 and CTNNB1 through miR-483-3p and miR-1276[10]. In summary, these findings suggest that lncRNA may function as an essential regulator in GC progression.

N^6^-methyladenosine (m^6^A) is the most prevalent internal modification connected with eukaryotic mRNAs fate, including mRNA metabolism, splicing, export and stability[11–13]. Besides, in various tumor physiological events, m^6^A participates in these tumor progression related progresses[14]. For example, major m^6^A methyltransferase METTL3 expression elevated in GC tissue and was predictive of poor prognosis. The stability of ZMYM1 mRNA was enhanced by through METTL3 relying on reader protein HuR[15]. Wilms' tumour 1-associated protein (WTAP) highly expressed in GC cells and tissue, which indicates a poor prognosis for GC patients and correlated with a cell-related immune response[16]. Thus, these evidences support that m^6^A modification significantly modulates the GC carcinogenesis.

Herein, our work focused on the cross-talking of m^6^A and lncRNAs in gastric GC tumorigenesis to uncover the functions and molecular mechanism of m^6^A-modified lncRNA DLGAP1 antisense RNA 2 (DLGAP1-AS2). We found that lncRNA DLGAP1-AS2 was highly expressed in GC, which was correlated with poor prognosis and advanced clinical stage. Moreover, m^6^A methyltransferase METTL3 enhanced the stability of DLGAP1-AS2 and then DLGAP1-AS2 interacted with YTHDF1 to enhance the stability of c-Myc mRNA in m^6^A-dependent manner. Overall, these findings provided a potential m^6^A-dependent therapeutic strategy for GC treatment.

**Materials And Methods**

**Patient tissues specimens collection**

A total of 50 GC tissue samples in the cohort, which were formalin-fixed and paraffin-embedded, were recruited between January 2016 and December 2018 from patients who had underwent operation in Fujian Provincial Hospital. All samples were staged according to the criteria of the 7th Edition of the AJCC Cancer Staging Manual (Stomach, 2010)[17]. This study was approved by the Fujian Provincial Hospital Ethics Review Committee. Informed consent was written by each patient before the study.

**Cell culture**

GC cell lines (AGS, SNU-216, MKN45, AGS) and the immortalized human gastric epithelial cell line (GES-1) were purchased from the Cell Bank of the Chinese Academy of Sciences. The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and antibiotics at 37 °C under 5% CO_2_.

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Transfection vector construction

For the stable silencing of DLGAP1-AS2, lentivirus vector pLKD-U6-shRNA-EF1a-LUC-F2A-Puro-DLGAP1-AS2 (Obio Technology, Shanghai, China) or control vectors were packaged and transfected into AGS cells. Cell were grown in 1 μg/ml puromycin (Invitrogen) to select the stable transfected cells. Moreover, for the overexpression of DLGAP1-AS2, the cDNA of DLGAP1-AS2 was PCR-amplified ad then sub-cloned into pcDNA 3.1 vector (Invitrogen, Carlsbad, Calif, USA). The efficiency of silencing or overexpression was subsequently quantified by qRT-PCR. The siRNAs and their controls (si-NC) were provided by GenePharma (Shanghai, China) and transfected using Lipofectamine 2000 (Invitrogen) when 70 % confluent growth.

Quantitative real-time PCR (qRT-PCR)

Total RNA from GC tissues or cell lines was extracted by TRIzol Reagent (Invitrogen, CA, USA). For the concentration and purity of RNA, RNA was detected with ultraviolet spectrophotometer using 260 nm and 280 nm. The real-time PCR was performed using SYBR Premix Ex Taq (Takara) on the 7900 Real-time PCR System. Reverse transcription was performed using PrimerScript RT Reagent Kit (TaKaRa, Kyoto, Japan). Fast SYBR green master mix (Life Technologies) applied to perform qRT-PCR. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. The β-actin acted as the internal mRNA control. The primer sequences were displayed in Table S1.

Western blot assay

The procedure details were conducted according to previous study. Proteins from transfected GC cells were extracted using RIPA buffer (Beyotime, China) and authenticated using BCA Kit (Pierce, Rockford, IL, USA). Protein was resolved using the SDS-PAGE Electrophoresis System and then transferred to PVDF membranes (Millipore, USA). Specific primary antibodies (anti-METTL3, 1:1000, ab195352, anti-c-MYC, 1:1000, ab32072) were incubated at 4 °C overnight. β-actin was the internal reference.

Methylated RNA immunoprecipitation (MeRIP) Sequencing and qPCR

All the specific manipulations were performed according to the protocol of Magna MeRIP™ m⁶A Kit (Merck Millipore). In brief, total RNAs were isolated from AGS cells and chemically fragmented into 100-300 nt. Fragmented fragments were incubated with m⁶A antibody for immunoprecipitation. The MeRIP was performed according to the manufacturer's instructions. Samples were sequenced with the HiSeq PE150 platform. The enrichment of m⁶A containing mRNA was sent for quantitative RT-PCR. The RT-qPCR primers were listed in Table S1.

Quantitative analysis of glucose, lactate and ATP

The glucose uptake was quantificationally detected using Glucose Uptake Assay Kit (Fluorometric, Abcam, ab136956). The lactate production was quantificationally detected using Lactate Colorimetric/Fluorometric Assay Kit (BioVision, K607–100). The ATP generation was quantificationally detected using ATP Assay Kit (Beyotime, S0026).
Measurement of extracellular acidification rate (ECAR)

ECAR was analyzed using Seahorse XFe96 analyzer (Seahorse Bioscience, Agilent) as previously described. Briefly, knockdown and overexpression of DLGAP1-AS2 transfected cells (1×10^4 cells/well) seeded into 96-well XF cell culture microplate. Medium (pH 7.4) was sequentially added with 10 mM glucose, 1 mM glutamine, 50 mM 2-DG and 1 µM oligomycin. ECAR was shown in mP H/min and measured using an XF96 analyzer.

Actinomycin D assay

AGS cells were seeded in 6-well plates (2×10^5 cells/well). 24 hours later, cells were exposed to Actinomycin D (Act D, 2 µg/ml, Sigma) and harvested at indicated time point. The RNA remaining level was analyzed using qRT-PCR and normalized to mock group (0 hour).

RNA immunoprecipitation (RIP) assay

RIP assay was performed using EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) according to manufacturer’s protocol. Specific antibodies (anti-YTHDF1, Abcam) was conjugated with magnetic beads and then dissolved in RIP buffer. Approximate 90% confluence, cells were lysed using complete RIP lysis buffer (100 µl) containing protease inhibitor and RNase Inhibitor (Millipore). Mouse anti-IgG antibody (Cell Signaling Technology, USA) acted as negative control. Then, total RNA was retrieved and the relative expression was detected by qRT-PCR analysis.

Subcellular localization

Probes were obtained from Genepharma (Shanghai, China). Fluorescence in situ hybridization (FISH) was performed using fluorescent in situ hybridization kit according to the manufacturer’s protocols (Genepharma). Cy3-labeled probe for DLGAP1-AS2 and FAM-labeled probe for c-Myc, YTHDF1 were used for localization of DLGAP1-AS2, c-Myc and YTHDF1 in GC cells. Nuclei was stained with 4,6-diamidino-2-phenylindole (DAPI) and images were obtained using confocal microscope (Olympus).

Animal models in vivo

Approximately 2×10^6 GC cells (AGS), transfected with sh-DLGAP1-AS2 or controls, were suspended in 100 µl PBS and then injected into flank of male BALB/c nude mice (5-week old). In the following observation, tumor size was recorded by vernier caliper. At the indicated time, mice were anesthetized and sacrificed for tumors neoplasm acquisition. All animal assays were performed in the light of institutional Fujian Provincial Hospital Animal Ethics Committee guidelines.

Statistical analysis

The data was expressed as mean ± S.D (standard deviation). All results were conducted using SPSS Statistics software (Armonk, NY, USA) or GraphPad prism 7.0 (La Jolla, CA, USA). Difference within
groups was calculated using One-way ANOVA test, non-parametric Mann-Whitney test and student's t-test. Overall survival rate was calculated using Kaplan-Meier analysis and log-rank test. *P ≤ 0.05 or **P ≤ 0.01 was considered statistically significant.

Results

DLGAP1-AS2 was an up-regulated IncRNA in GC

Using the microarray data (GSE147167), we found that numerous IncRNAs were dysregulated in the GC tissue samples (Figure 1A). Moreover, we found that a potential up-regulated IncRNA (DLGAP1-AS2) might be correlated with the GC carcinogenesis. In GC cell lines, expression of DLGAP1-AS2 over-expressed as comparing to the normal cell lines (Figure 1B). In GC samples, DLGAP1-AS2 levels significantly over-expressed as comparing to the normal adjacent tissue (Figure 1C, Table 1). Kaplan-Meier analysis and log-rank test found that GC tissue samples with high DLGAP1-AS2 levels displayed a lower survival rate as the control cohort (Figure 1D). Therefore, these data revealed an up-regulated IncRNA DLGAP1-AS2 in GC.

MeRIP-Seq revealed the m^6^A modification of DLGAP1-AS2

MeRIP-Seq in GC cells revealed the m^6^A peaks density, including 5'-UTR, coding region (CDS) and 3'-UTR (Figure 2A, 2C). The significant m^6^A motif for the m^6^A peaks was GGAC (Figure 2B). MeRIP-Seq demonstrated that there was a remarkable m^6^A modification site in the 3'-UTR of DLGAP1-AS2 (Figure 2D). In the 3'-UTR sequences of DLGAP1-AS2, there was a remarkable m^6^A site (AGAC) (Figure 2E). In the exploration which m^6^A enzyme catalyzes the m^6^A modification on DLGAP1-AS2, we found that m^6^A reader YTHDF1 was positively correlated to the level of DLGAP1-AS2 (Figure 2F). Taken together, these MeRIP-Seq data indicated the m^6^A modification of DLGAP1-AS2.

METTL3 enhanced the stability of DLGAP1-AS2 in m^6^A-dependent manner

In the MeRIP-Seq, we noticed a m^6^A modification of DLGAP1-AS2, thus, DLGAP1-AS2 might be modulated in m^6^A-dependent manner. To investigate the possibility which m^6^A enzyme catalyzes the m^6^A modification on DLGAP1-AS2, we found that m^6^A methyltransferase METTL3 was positively correlated to the level of DLGAP1-AS2 (Figure 3A). In GC cells, METTL3 overexpression was transfected (Figure 3B). After METTL3 overexpression, the m^6^A modification levels were found to be up-regulated (Figure 3C, 3D). RNA stability analysis found that the METTL3 overexpression accelerated the RNA stability of DLGAP1-AS2 in MKN45 cells and AGS cells (Figure 3E, 3F). In summary, these findings revealed that m^6^A-modified DLGAP1-AS2 demonstrated higher stability mediated by METTL3.

DLGAP1-AS2 promoted the aerobic glycolysis of GC
In the functional experiments, enhanced expression or silencing of DLGAP1-AS2 was constructed in MKN45 or AGS cells (Figure 4A). Subsequently, the biological functions of DLGAP1-AS2 on GC aerobic glycolysis were investigated. Glucose uptake analysis illustrate that enforced DLGAP1-AS2 expression promoted the glucose consumption in MKN45 cells, and knockdown of DLGAP1-AS2 repressed the glucose consumption in AGS cells (Figure 4B). Lactate production analysis unveiled that enforced DLGAP1-AS2 expression accelerated the lactate production in MKN45 cells, and knockdown of DLGAP1-AS2 inhibited the lactate production in AGS cells (Figure 4C). ATP quantitative analysis illustrated that enforced DLGAP1-AS2 expression facilitated the ATP production in MKN45 cells, and knockdown of DLGAP1-AS2 restrained the ATP production in AGS cells (Figure 4D). ECAR analysis for GC glycolytic ability illustrated that enforced DLGAP1-AS2 expression promoted the extracellular acidification accumulation (Figure 4E), and knockdown of DLGAP1-AS2 repressed the acidification (Figure 4F).

Moreover, the in vivo animal experiments indicated that knockdown of DLGAP1-AS2 inhibited the tumor neoplasm growth of GC cells (Figure 4G). In conclusion, these findings demonstrated that DLGAP1-AS2 promoted the aerobic glycolysis of GC.

**DLGAP1-AS2 targeted c-Myc via YTHDF1-dependent manner**

On the basis of finding that DLGAP1-AS2 regulated the aerobic glycolysis, we assumed that DLGAP1-AS2 might regulate certain key enzyme of glycolysis. Firstly, the localization analysis using RNA-FISH found that the distribution of DLGAP1-AS2 was in cytoplasm, which was in accordance with the c-Myc and YTHDF1 (Figure 5A). The coincident cytoplasmic distribution of DLGAP1-AS2, YTHDF1 and c-Myc indicated the potential interaction within them. MeRIP-Seq presented that there was a m\(^6\)A site in the c-Myc mRNA, suggesting a potential regulation for DLGAP1-AS2 via m\(^6\)A-dependent manner (Figure 5B). In the GEPIA database, c-Myc was positively correlated with DLGAP1-AS2 and YTHDF1, indicating that c-Myc might act a target of DLGAP1-AS2 (Figure 5C). Besides, the c-Myc levels were highly expressed in the stomach neoplasm (STAD, Stomach adenocarcinoma) (Figure 5D). In summary, these data illustrated that c-Myc acted as a target of DLGAP1-AS2.

**DLGAP1-AS2 promoted c-Myc mRNA stability through m\(^6\)A-YTHDF1-dependent manner**

The MeRIP-Seq demonstrated that there was a m\(^6\)A modification site in the 3′-UTR of c-Myc mRNA, which was in accord with the CAGAC motif (Figure 6A). MeRIP-qPCR suggested us that the m\(^6\)A enrichment on c-Myc mRNA was higher in the GC cells (MKN45, AGS) (Figure 6B). Moreover, the RNA immunoprecipitation (RIP) unveiled that enhanced DLGAP1-AS2 expression promoted the c-Myc mRNA level precipitated by the anti-YTHDF1 antibody (Figure 6B, left). Besides, knockdown of DLGAP1-AS2 inhibited the c-Myc mRNA level (Figure 6B, right). RT-qPCR found that enhanced DLGAP1-AS2 expression amplified the c-Myc mRNA level (Figure 6D, up). Moreover, the knockdown of DLGAP1-AS2 decreased the c-Myc mRNA level (Figure 6D, down). RNA stability analysis found that DLGAP1-AS2 overexpression promoted the c-Myc mRNA remaining level, and the knockdown of DLGAP1-AS2 repressed the c-Myc mRNA remaining (Figure 6E). In summary, these findings illustrated that DLGAP1-AS2 promoted c-Myc mRNA stability through m\(^6\)A-YTHDF1-dependent manner (Figure 7).
Discussion

As one of the most prominent characteristics of malignant tumors, aerobic glycolysis (also known as Warburg effect) is a complex process affected by genetic and epigenetic modifications[18, 19]. For the regulation of tumorigenesis, (Warburg effect) aerobic glycolysis exerts critical roles on the progress of tumor cell energy metabolism, providing the main energy supplement. In the current study, we demonstrated that m6A-modified DLGAP1-AS2 expression was increased in GC tissues and cell lines. Furthermore, a high DLGAP1-AS2 expression implied a poor prognosis for GC.

Here, we found that DLGAP1-AS2 expression was significantly up-regulated in the GC and moreover promoted the glucose uptake, lactate production and ATP generation. Our results reflect this possibility that DLGAP1-AS2 may act as an energy metabolism-related oncogene in GC. Accumulating evidences have illustrated that IncRNA plays critical roles in cancer aerobic glycolysis. For instance, the over-expressed LINC00242 promotes the GC proliferation and aerobic glycolysis in vitro and relieves the tumorigenesis in vivo through directly targeting G6PD[20]. LINC01391 overexpression hampers the proliferation, invasion and aerobic glycolysis in vitro and tumor growth in vivo of GC, while LINC01391 knockdown shows the opposite results[21]. Overexpressed DLX6-AS1 in GC significantly promotes cell viability and colony formation, and then regulates aerobic glycolysis by targeting phosphoinositide-dependent protein kinase 1 (PDK1)[22]. These results consistently emphasized the regulation of IncRNAs for GC aerobic glycolysis.

To further validate the biological function of IncRNA on GC, we utilized the MeRIP-Seq and found that there was a potential m6A site in the 3'-UTR of DLGAP1-AS2, suggesting the m6A modification for DLGAP1-AS2. The specific m6A modification on DLGAP1-AS2 could significantly modulate its fate. In our research, we found that the m6A methyltransferase METTL3 could specifically bind with the m6A site on the DLGAP1-AS2. Besides, METTL3 could assist the stability increasing for DLGAP1-AS2. DLGAP1-AS2, on the other hand, connected with its downstream targets through m6A binding. Mechanistically, we found that m6A-modified DLGAP1-AS2 functions through a METTL3-involved m6A mechanism. DLGAP1-AS2 integrated with c-Myc mRNA via the m6A-mediated combination, constructing a DLGAP1-AS2/m6A/YTHDF1/c-Myc mRNA axis.

On this basis of m6A-DLGAP1-AS2, we realize the importance that m6A-modified IncRNA provides an important regulatory pattern for human cancer. For instance, IncRNA LINRIS is upregulated in colorectal cancer, and LINRIS knockdown attenuates the degradation of IGF2BP2 and especially MYC-mediated glycolysis[23]. For another example, m6A is highly enriched on the IncRNA THOR transcripts, including GAACA, GGACU and UGACU motifs. Besides, m6A-modified IncRNA THOR regulates the cancer cells’ proliferation via an m6A-reader-dependent manner[24]. In colorectal cancer, m6A-induced IncRNA RP11 triggers the progression and metastasis via post-translational Zeb1 upregulation through RP11/hnRNPA2B1/mRNA complex[25]. These results consistently demonstrated the m6A-IncRNA interaction regulation for human cancer.
Conclusion

In summary, our findings in this study revealed that DLGAP1-AS2 promoted the tumorigenesis of GC through m\(^6\)A/YTHDF1/c-Myc mRNA axis, thus triggering c-Myc mRNA stability and accelerating the aerobic glycolysis of GC cells. These m\(^6\)A-dependent RNA-protein interactions could maintain the oncogenic characteristic of IncRNA DLGAP1-AS2. Based on the findings, DLGAP1-AS2 may serve as potential target in the GC treatment.

Declarations

Ethics approval and consent to participate

This study was approved by the Fujian Provincial Hospital Ethics Review Committee. Informed consent was written by each patient before the study.

Consent for publication

All authors agree with the publication.

Availability of data and materials

Not applicable.

Competing interests

All authors declare no conflicts of interest

Funding

Not applicable.

Authors’ contributions

Changshun Yang and Yu Zhang performed the assays and were responsible for the writing. Xuefei Cheng was responsible for the statistic. Weihua Li was responsible for the funding and design.

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References

12. Z. Ma, J. Ji, N6-methyladenosine (m6A) RNA modification in cancer stem cells, Stem cells (Dayton, Ohio), (2020).
15. B. Yue, C. Song, L. Yang, R. Cui, X. Cheng, Z. Zhang, G. Zhao, METTL3-mediated N6-methyladenosine modification is critical for epithelial-mesenchymal transition and metastasis of gastric cancer, Molecular cancer, 18 (2019) 142.

Tables

Table 1. DLGAP1-AS2 expression and GC patients' clinicopathological characteristic.
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Well: Well-differentiated adenocarcinoma; Moderate, moderately differentiated adenocarcinoma; Poor, poorly differentiated adenocarcinoma; TNM, tumor-node-metastasis. *P<0.05 represents statistical differences.

**Figures**

**Figure 1**

<sup>** strong>DLGAP1-AS2 was an up-regulated lncRNA in GC.</sup> (A) Based on the lncRNA microarray data (GSE147167), heatmap demonstrated the numerous de-regulated lncRNAs in the GC tissue as comparing to normal tissue. (B) In GC and normal cell lines cell lines, expression of DLGAP1-AS2 was detected using RT-qPCR. (C) In GC samples, DLGAP1-AS2 level was detected using RT-qPCR as comparing to the normal adjacent tissue. (D) Survival rate analysis showed the lower survival rate of GC patients with high DLGAP1-AS2 levels, while the higher survival rate of GC patients with low DLGAP1-AS2 levels. Data were expressed as mean ± SD. **p &lt; 0.01, *p &lt; 0.05.</sup>
Figure 2

MeRIP-Seq revealed the m<sup>6</sup>A modification of DLGAP1-AS2. (A, C) MeRIP-Seq revealed the m<sup>6</sup>A peaks density in GC cells, including 5'-UTR, coding region (CDS) and 3'-UTR. (B) The significant m<sup>6</sup>A motif for the m<sup>6</sup>A peaks was GGAC. (D) MeRIP-Seq showed that there was a remarkable m<sup>6</sup>A modification site in the 3'-UTR of DLGAP1-AS2. (E) Genomic analysis displayed the sequences of DLGAP1-AS2 3'-UTR containing m<sup>6</sup>A site (AGAC). (F) The motif of METTL3 targeting DLGAP1-AS2 m<sup>6</sup>A site.

Figure 3

METTL3 enhanced the stability of DLGAP1-AS2 in m<sup>6</sup>A-dependent manner. (A) GEPIA dataset revealed the positive correlation within METTL3 and DLGAP1-AS2 in STAD (Stomach adenocarcinoma, http://gepia.cancer-pku.cn/). (B) The METTL3 protein was detected in GC cells upon METTL3 overexpression transfection. (C, D) The m<sup>6</sup>A modification levels were detected using m<sup>6</sup>A RNA Methylation Quantification Kit (Colorimetric). (E, F) RNA stability analysis revealed the RNA remaining of DLGAP1-AS2 in MKN45 cells and AGS cells treated by Act D (actinomycin D, 1 μg/m). LncRNA DLGAP1-AS2 was determined by qRT-PCR assay. *p < 0.05.

Figure 4

DLGAP1-AS2 promoted the aerobic glycolysis of GC. (A) Detection of DLGAP1-AS2 levels by qRT-PCR in GC cell lines (MKN45, AGS) respectively transfected with DLGAP1-AS2 overexpression plasmids or lentivirus shRNA. (B) Glucose uptake quantity was quantificationally detected using glucose uptake kit in MKN45 and AGS cells. (C) Lactate production was quantificationally detected using lactate analysis kit. (D) ATP quantitative analysis was performed in MKN45 or AGS cells. (E, F) The glycolysis levels of MKN45 and AGS was detected using a seahorse analyzer. (G) In vivo animal experiments were performed to test the tumor neoplasm growth of GC cells (AGS) transfected with DLGAP1-AS2 silencing (sh-DLGAP1-AS2). The data were presented as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01.

Figure 5

DLGAP1-AS2 targeted c-Myc via YTHDF1-dependent manner. (A) Co-localization analysis using fluorescence in situ hybridization (FISH) displayed the distribution of DLGAP1-AS2, YTHDF1 and c-Myc in AGS cells. (B) IGV viewer showed the m<sup>6</sup>A site in the c-Myc mRNA
based on MeRIP-Seq data. (C) GEPIA database demonstrated the positive correlation within c-Myc and DLGAP1-AS2, or c-Myc with YTHDF1 (p<0.001). (B) TCGA database illustrated the high-expression of c-Myc in STAD (Stomach adenocarcinoma, www.tcga.org/).

**Figure 6**

(A) An m<sup>6</sup>A modification site in the 3'-UTR of c-Myc mRNA was discovered by MeRIP-Seq. The appropriate motif is CAGAC motif. (B) MeRIP-qPCR detected the m<sup>6</sup>A enrichment on c-Myc mRNA in GC cells (MKN45, AGS) using m<sup>6</sup>A antibody and IgG control. (C) RNA immunoprecipitation (RIP) assays unveiled the c-Myc mRNA level precipitated by the anti-YTHDF1 antibody in in GC cells (MKN45, AGS) transfected with DLGAP1-AS2 overexpression (DLGAP1-AS2) and knockdown (sh-DLGAP1-AS2). (D) RT-qPCR detected the c-Myc mRNA level in GC cells (MKN45, AGS) transfected with DLGAP1-AS2 overexpression and knockdown. (E) The levels of c-Myc expression in DLGAP1-AS2-overexpressing (DLGAP1-AS2), DLGAP1-AS2 knockdown (sh-DLGAP1-AS2) and their corresponding control were detected by qRT-PCR. GC cells (MKN45, AGS) transfected GC cells treated with actinomycin D (2 µg/mL). **P <0.01 vs vector control.

**Figure 7**

METTL3/DLGAP1-AS2/YTHDF1/m<sup>6</sup>A/c-Myc axis promotes the aerobic glycolysis (Warburg effect) of GC.

**Supplementary Files**

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- SupplementTableS1.docx