N6-methyladenosine (m6A) RNA modification of G protein-coupled receptor 133 increases proliferation of lung adenocarcinoma

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Abstract
Lung adenocarcinoma (LUAD) accounts for almost 40% of lung cancers, leading to...
significant associated morbidity and mortality rates. However, the mechanism of LUAD tumorigenesis remains far from clear. Here, we scanned downregulated genes involved in LUAD sourced from Cancer Genome Atlas and Gene Expression Omnibus data and focused on G protein-coupled receptor 133 (GPR133). We offer compelling evidence that GPR133 was expressed at low levels in the setting of LUAD and higher expression was positively related with a better prognosis among LUAD patients. Functionally, GPR133 inhibited cell proliferation and tumor growth in vitro and in vivo. Regarding the mechanism, flow cytometry assays and western blot assays showed that GPR133 enhanced p21 and decreased cyclin B1 expression, thus triggered LUAD cells at G2/M-phase arrest. Consistent with this, we evaluated the expression levels of cell-cycle biomarkers and found that Bioinformatics analysis combined with N6-methyladenosine (m6A; methylation at the N6 position in adenosine) RNA immunoprecipitation-qPCR (MeRIP-qPCR) assay indicated that GPR133 expression was downregulated by this modification. Moreover, we observed that methyltransferase-like 3 was impaired in LUAD and that it is able to significantly increase levels of GPR133 by enhancing its RNA stability. In conclusion, we found that GPR133 expression was downregulated in LUAD via m6A modification. Increasing GPR133 levels could suppress LUAD cell proliferation and tumor growth.

Keywords: G protein–coupled receptor 133, proliferation, cell cycle, lung adenocarcinoma

1. Introduction

Lung adenocarcinoma (LUAD), which develops along the outer edges of the lungs within glandular cells in the small airways, accounts for approximately 40% of all lung cancer cases and constitutes a major cause of cancer mortality worldwide[1]. Recent efforts have focused on identifying biomarkers for the diagnosis and treatment of LUAD. Several genes, such as epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and reactive oxygen species (ROS), were found to exhibit genomic mutations in LUAD[2], while other research found that molecularly targeted therapies directed at receptor tyrosine kinases were clinically successful. Studies are also currently being conducted on other targeted therapies directed against alterations in the KRAS, ERBB2, BRAF, MET, RET, NTRK1, and NTRK2 genes[3]. Encouragingly, immune checkpoint inhibitors targeting programmed cell death 1 receptor/programmed death-ligand 1
(PD-1/PD-L1) mediated immunosuppression, showing efficacy in up to 30% of LUAD patients[4]. However, many LUAD patients have no common genetic mutation or acquire resistance to EGFR tyrosine kinase inhibitors, even experiencing no response to immune checkpoint inhibition therapy. Thus, it is urgent to advance the understanding of the regulatory mechanisms involved in the development and progression of LUAD. Also, more sensitive novel biomarkers need to be identified for early diagnosis and therapeutic purposes.

The adhesion family forms a large branch of the pharmacologically important superfamily of G protein–coupled receptors (GPCRs), which play important roles in receptor recognition, signal transduction, the cell cycle, and cell differentiation[5]. GPR133, also known as ADGRD1, is an orphan adhesion GPCR member. GPR133 contains a large N-terminal extracellular domain, with a signal peptide and a pentraxin/concanavalin A domain [6]. Research suggests that the height and length of the R–R interval in the adult cardiac electrical cycle are related with the single-nucleotide polymorphisms of GPR133[7,8]. Notably, GPR133 correlates with altered bone mineral density in mouse knockouts, suggesting that it is a causal genetic driver of such disease in humans[9]. GPR133 expression increased as a function of World Health Organization grade and peaks in glioblastoma[10]. However, the role of GPR133 in LUAD remains unknown.

Here, we conducted a bioinformatics analysis of downregulated genes in LUAD using several databases, focusing in particular on GPR133. Our investigation demonstrated that GPR133 expression was decreased in LUAD and positively related with better outcomes among patients with LUAD. Moreover, increased GPR133 expression may significantly suppress the proliferation of LUAD cells in vitro and in vivo.

2. Materials and Methods

2.1 Bioinformatics analysis

The messenger RNA (mRNA) expression profiles and outcomes of LUAD patients were obtained from The Cancer Genome Atlas (TCGA) data portal (https://tcga-data.nci.nih.gov/tcga/) and Gene Expression Omnibus (GEO) datasets (https://www.ncbi.nlm.nih.gov/). The downregulated genes with fold changes of two or more and \( p < 0.05 \) were chosen during subsequent analysis. We merged the above downregulated genes for intersection and focused on the GPR133 gene. Meanwhile, the R software (R Foundation for Statistical Computing, Vienna,
Austria) was used to match \textit{GPR133} expression levels with the outcomes of LUAD patients via TCGA data. Similarly, we analyzed \textit{GPR133} expression levels and the prognosis of LUAD patients using GEO datasets. Moreover, gene set enrichment analysis (GSEA) was implemented via GSEA version 2.2.2 (http://www.broadinstitute.org/gsea) to investigate the biological characteristics of \textit{GPR133}. The genes correlated with \textit{GPR133} were downloaded from the cBioPortal for Cancer Genomics (https://www.cbioportal.org/) and placed into the DAVID Bioinformatics Resources database (https://david.ncifcrf.gov/) to predict their function by Gene Ontologies (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. The N^6\textsuperscript{-}methyladenosine (m^6A) sites on the \textit{GPR133} transcript were analyzed using the SRAMP online software program (http://www.cuilab.cn/sramp). The expression pattern of methyltransferase-like 3 (\textit{METTL3}) and the relationship between \textit{METTL3} and \textit{GPR133} was estimated using Gene Expression Profiling Interactive Analysis (GEPIA) data (http://gepia.cancer-pku.cn/).

\section*{2.2 Cells and clinical samples}

The BEAS-2B human bronchial epithelial cell line and several kinds of LUAD cells (A549, H1299, H1650, H1975, and PC9) were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco Laboratories, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco Laboratories), incubated at 37°C with 5% CO\textsubscript{2} in a humidified incubator. A total of 28 cases of LUAD tissue samples and matched adjacent normal tissues were collected from patients in the People’s Hospital of Wuzhou between December 2011 and March 2019. There were 14 cases male and 14 cases female patients. The average age is 53.78 ± 4.67 years old. Another cohort of normal tissues (n = 17, 5 female and 11 male, the average age is 39.41 ± 10.27 years old) and LUAD tissues (n = 63, 25 female and 38 male, the average age is 64.39 ± 9.17 years old) with prognosis information was compiled from the biological resource specimen bank of the People’s Hospital of Wuzhou. The written informed consent has been obtained from each patients for use of their tissue samples in research. This study was approved by the ethics committee of the People’s Hospital of Wuzhou.

\section*{2.3 Transfection, qRT-PCR and western blot assays}

For transfection assay, GPR133 restored expression plasmid, METTL3 overexpression plasmid and vector plasmid were obtained from GeneCoopia biotechnology. Lipofectamine 3000
Reagent were got from Thermo Fisher Scientific, USA. Cells were seeded into 6-well plate overnight. For a well per 6-well plate, 3 μg plasmid were incubated with 5 μL P3000 in 250μL Opti-MEM. 5 μL Lipofectamine 3000 were mixture with 250μL Opti-MEM. Then these two mixture were mixed together and incubated for 20 mins at room temperature. At last, the mixture were added into one plate with 1.5 mL complete culture. 48 h later, the cells were harvested for other assays.

For qRT-PCR assay, cells were treated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNAs were extracted and reversely transcribed into complementary DNA. qRT-PCR assays were performed to measure the expression levels of target genes. All the primers used are listed in Table 1.

For western blot assay, total protein was obtained from cells with lysis buffer and protease inhibitor. BCA methods (Thermo Fisher Scientific, Waltham, MA, USA) were used to determine protein concentrations. Proteins were loaded in gel channels to begin sodium dodecyl sulfate–polyacrylamide gel electrophoresis; transferred into polyvinylidene fluoride or polyvinylidene difluoride membranes; and incubated with 5% bovine serum albumin, primary antibodies, and horseradish peroxidase as a secondary antibody, respectively. All the primary antibodies were listed by a manner as name, catalog name, dilution rate: GAPDH (CST, 5174, 1:1000), GPR133 (Affinity, DF4947, 1:500), p21 (CST, 2947, 1:500), Cyclin B1 (CST,12231, 1:500), Cyclin D1 (CST, 55506, 1:500), CDK4 (CST, 12790, 1:600), Cdc2 (CST, 9116, 1:500), METTL3 (CST, 86132, 1:600). Finally, an ECL chemiluminescence kit (Millipore, Burlington, MA, USA) was used to evaluate protein levels in the samples.

2.4 MeRIP-qPCR assay

The m6A sites of GPR133 were validated using the EZ-Magna RNA immunoprecipitation kit (Millipore, Burlington, MA, USA) according to the users’ instructions. Cells were seeded into a 10-cm cell culture dish for overnight. Briefly, cells were collected and lysed by RNA immunoprecipitation lysis buffer for 20 minutes, then centrifuged at 13,000 rpm for 10 minutes. The supernatant was incubated with m6A antibody and normal rabbit immunoglobulin G overnight, respectively. RNAs were extracted from magnetic beads and we analyzed the level of GPR133 in above groups by qRT-PCR assay.

2.5 Immunohistochemistry (IHC)
Tissues were deparaffinized in xylene, rehydrated with graded alcohol, and boiled in 0.01M citrate buffer (pH: 6.0). Subsequently, the tissues were treated with 0.3% hydrogen peroxide, followed by normal goat serum.

All sections were incubated with the primary antibodies (GPR133, 1:100) overnight and followed by the biotinylated second antibody, streptavidin alkaline phosphatase each for 10 minutes. Then, the sections were then counterstained with hematoxylin, dehydrated, and mounted. All immunohistochemical staining was evaluated and scored by at least two independent pathologists. The scores were set into 4 groups: negative (0-3), weak positive (3-6), middle positive (6-9) and strong positive (9-12).

2.6 **Cell viability assay**

Cells were seeded in 96-well plates at the concentration of 1500 cells per well. MTS was added into wells at 0, 1, 2, 3, 4, and 5 days, respectively, for 3 hours. The absolute absorbance value at 490 nm was measured using the Varioskan LUX system (Thermo Fisher Scientific).

2.7 **Colony-formation assay and soft agar colony-formation assay**

For colony-formation experiments, the cells were seeded into six-well plates at a density of 750 cells per well and cultured. After 14 days, cells were fixed with 4% formaldehyde for 15 minutes and stained with 0.1% crystal violet for 10 minutes. Finally, the colonies were photographed and any colonies larger than 1 mm (> 50 cells/clone) were counted.

For soft agar colony-formation assay, 1500 cells were fully mixed with complete RPMI 1640 medium and 0.75% agarose, then quickly placed on the complete RPMI 1640 medium curing layer with 1.5% agarose. Next, 0.5 mL of RPMI 1640 was added every 5 days to each well to feed cells. After 3 weeks, colonies larger than 50 μm were photographed.

2.8 **RNA stability assay**

A549 cells with decreasing METTL3, H1299 cells with decreasing METTL3 concentrations, and control-group cells were seeded into six-well plates, respectively. All cells were treated with 5 μg/mL of actinomycin D and total RNA samples were obtained at 0, 1, 2, and 3 hours. The mRNA expression level of GPR133 was estimated by qRT-PCR assay.

2.9 **Flow cytometry**
A cell-cycle staining kit was obtained from MultiSciences Biotech (Hangzhou, China) and cell cycles were analyzed by flow cytometry assay using this kit according to the manufacturer’s instructions. Cells were seeded in a six-well plate and starvation treatment was deployed overnight. Then, cells were harvested and washed with cold phosphate-buffered saline solution and were incubated with DNA staining solution and permeabilization solution for 30 minutes at room temperature. Finally, cell samples were determined by using FACS (Becton, Dickinson & Co., Franklin Lakes, NJ, USA).

2.10 Animal experiments

All animal experiments were approved by the ethics committee of the People’s Hospital of Wuzhou. Four-week-old immunodeficient mice were purchased from Guangdong Animal Center (Guangzhou, China). Animals were randomly divided into groups (n=4). Each group had 2 males and 2 females. A total of $5 \times 10^5$ cells were subcutaneously injected into the nude mice (n = 4 per group). Tumor growth was analyzed by measuring the tumor length (L) and width (W) and calculating the volume (V) using the formula $V = LW^2/2$. The tumor tissues were then embedded in paraffin and analyzed.

2.11 Statistical analysis

For all statistical tests, a two-tailed $p$-value of less than 0.05 was considered to be statistically significant. The Student’s $t$-test and chi-squared test were performed to compare a single gene’s expression levels between two groups. Overall survival curves were estimated by Kaplan–Meier analysis.

3. Results

3.1 GPR133 was downregulated and associated with better prognosis in LUAD

To explore the tumorigenesis and development of LUAD, we searched for downregulated genes in LUAD among TCGA and GEO datasets and located a total of 319 genes downregulated in LUAD among TCGA, GSE43767, and GSE43458 datasets (Fig. 1A). Notably, the expression level of GPR133 was much lower in 497 samples of LUAD tissue than that in 54 samples of normal tissue. GPR133 expression was also decreased in 80 samples of LUAD tissue as compared with that in 30 samples of normal tissue in GSE43458; similar results were obtained in GSE43767 (Fig. 1B). Consistently, we found that GPR133 expression was more significantly suppressed in
28 samples of LUAD tissue than in paired adjacent tissues (Fig. 1C). Further, we estimated GPR133 expression in LUAD cells, where not only the mRNA but also the protein expression level of GPR133 was much lower in LUAD cells than in normal BEAS-2B cells (Fig. 1D). Next, we selected A549 and H1299 cells given their low expression of GPR133. To investigate the clinical implications of GPR133 in LUAD, we conducted Kaplan–Meier analysis and found that patients with high GPR133 expression levels had a better overall survival (OS) and disease-free survival (DFS) (Fig. 1E). In GSE31210, we performed the chi-squared test and found that the expression of GPR133 was related with the tumor stage of LUAD but not with age, sex, or smoking history (Table 2). Collectively, these results suggested that GPR133 expression is decreased in LUAD and may be a potential biomarker of a better prognosis in LUAD patients.

3.2 Restored expression of GPR133 inhibited LUAD proliferation

To discover the effects of GPR133 on tumor progression, we firstly enhanced GPR133 expression in A549 and H1299 cells (Fig. 2A). Interestingly, the cell viability of both A549 and H1299 cells were sharply decreased by accelerating GPR133 in these cells (Fig. 2B). Consistent with this observation, colony-formation and soft agar colony-formation assays also demonstrated that LUAD cells with restored GPR133 expression formed significantly smaller and fewer colonies than the control group (Figs. 2C and 2D). These data indicate that GPR133 inhibited the proliferation ability of LUAD cells.

3.3 GPR133 triggered cell-cycle arrest in LUAD cells

GSEA analysis was carried out to discover the possible mechanism through which GPR133 is involved in the proliferation of LUAD. The results revealed that GPR133 expression was correlated with the cell cycle in LUAD (Fig. 3A). As shown in Fig. 3B, GO annotations suggested that GPR133 co-expressed genes that were mainly involved in the cell cycle. Thus, we estimated the mRNA and protein expression levels of several biomarkers for the cell cycle. It was noted that GPR133 expression reduced cyclin B1 and enhanced p21 but had no effect on cyclin D1, CDK4, or Cdc2 (Fig. 3C). Additionally, a flow cytometry assay was performed to measure the effect of GPR133 expression on the cell cycle of LUAD cells and revealed that overexpressing GPR133 triggered G2/M-phase arrest in LUAD cells (Fig. 3D). Collectively, these results suggest that GPR133 plays a role in G2/M-phase arrest in LUAD cells.

3.4 GPR133 expression is enhanced by METTL3-mediated m^6A modification in LUAD
To discover the mechanism of GPR133 downregulation in LUAD, the m\textsuperscript{6}A sites on the \textit{GPR133} transcript were analyzed using SRAMP and it was predicted that several m\textsuperscript{6}A sites exist on the \textit{GPR133} transcript (Fig. 4A). We next performed MeRIP-qPCR assays to test this hypothesis and the results revealed a substantial increase in the m\textsuperscript{6}A level in A549 and H1299 cells (Fig. 4B). Moreover, we examined the correlation between the expression levels of \textit{GPR133} and m\textsuperscript{6}A writers in TCGA. As shown in Fig. 4C, \textit{METTL3} expression was impaired in 483 samples of LUAD tissue and 347 samples of normal tissue (p < 0.01). Although TCGA and GTEx datasets of LUAD showed weak positive correlation of METTL3 and GPR133 (R=0.092), further cell line results strengthened it. Notably, qRT-PCR and western blot assays revealed that the expression levels of METTL3 in A549 and H1299 cells were much lower than those in BEAS-2B cells (Fig. 4D). On the basis of METTL3 expression, we transfected METTL3 overexpression plasmid into LUAD cells to restore an appropriate level of METTL3 expression (Fig. 4E). Actually, accelerating METTL3 may enhance not only the mRNA but also the protein expression levels of GPR133 (Fig. 4F). To verify whether METTL3 promoted the stability of \textit{GPR133} mRNA, we used actinomycin D to observe the mRNA level of \textit{GPR133}. Upon increasing the actinomycin D treatment time, the decay of \textit{GPR133} mRNA was reduced in A549 and H1299 cells with overexpressed METTL3 as compared with in the respective control groups (Fig. 4G). Given these results, we presumed that METTL3 modulates \textit{GPR133} in an m\textsuperscript{6}A-dependent manner.

\textbf{3.5 GPR133 inhibited LUAD tumor growth in vivo}

Briefly, we estimated GPR133 expression patterns in LUAD by IHC assay. The results revealed that GPR133 expression was downregulated in 63 samples of LUAD tissue and in 17 samples of normal tissue (Fig. 5A). Meanwhile, Kaplan–Meier analysis demonstrated that LUAD patients with high GPR133 expression levels had a better outcome (Fig. 5A). To further investigate the effects of GPR133 on tumorigenicity \textit{in vivo}, A549 cells with increasing GPR133 expression were subcutaneously injected into nude mice and the subsequent tumor growth was assessed; ultimately, the results showed that the overexpression of GPR133 could markedly suppress tumor growth \textit{in vivo} (Fig. 5B). As compared with in the control group, restored GPR133 expression may significantly suppress both tumor weight and tumor growth (Fig. 5B). All these data support that GPR133 is a tumor inhibitor in LUAD.

\textbf{4. Discussion}
GPR133, a member of the GPCR superfamily, regulates cell adhesion and cell metabolism. In the current study, we provided the first evidence that GPR133 expression is upregulated in LUAD by METTL3 in an m^6^A-dependent manner. However, METTL3 was impaired in LUAD. Bioinformatics analysis combined with experiments suggested that GPR133 expression is positively related with a better prognosis among patients with LUAD. Functionally, GPR133 suppressed the proliferation of LUAD cells both in vivo and in vitro. Importantly, we showed that GPR133 triggered G2/M-phase arrest in LUAD cells. Hence, targeting GPR133 might represent a novel strategy to treat LUAD.

Recently, N^6^-methyladenosine (m^6^A) has been discovered to be a reversible RNA methylation factor. This dynamic RNA methylation factor is enriched around stop codons, in 3′ untranslated regions, and within internal long exons[11]. The act of m^6^A modification affects fundamental aspects of mRNA metabolism, results in posttranscriptional dysregulation of gene expression relating to cell differentiation, cell homeostasis, the cellular response to stress, and cancer[12]. In this modification system, m^6^A “writers” are composed of core catalytic components (METTL3/methyltransferase-like 14) to install m^6^A modification[13]. Alkylation repair homolog protein 5 (ALKBH5) and fat-mass and obesity-associated protein (FTO), which are termed as m^6^A erasers, focus on removing m^6^A modification[14,15]. The function of m^6^A is executed by m^6^A “readers” that bind to m^6^A directly (YTH domain-containing proteins, eIF3 and IGF2BPs) or indirectly (HNRNPA2B1)[16-18].

METTL3 has been recognized as an essential factor in conditions including diabetes, cancers and cardiovascular disease[19,20]. The METTL3-mediated m^6^A modification on AFF4 could promote its expression. In addition, AFF4 is bound to the promoter of MYC. As such, METTL3/AFF4/MYC axis contributes to bladder cancer tumorigenesis[21]. METTL3 targeted the 3′ untranslated region of HK2 mRNA. Moreover, METTL3 recruited YTHDF1 to enhance HK2 stability, thereby promoting the Warburg effect of cervical cancer[22]. Consistent with the aforementioned study, we found that METTL3 was expressed at low levels in LUAD. METTL3 was positively correlated with GPR133 via the GEPIA databank. Moreover, the overexpression of METTL3 may significantly enhance the mRNA stability of GPR133.

To discover the function of GPR133 expression in LUAD, we performed cell-viability and colony-formation assays, where the results showed that increasing the GPR133 expression sharply
suppressed the proliferation of LUAD cells. Further investigation determined that GPR133 inhibited tumor growth in animal experiments of LUAD. However, it was reported that GPR133 was selectively expressed in hypoxic regions of GBM, while GPR133 knockdown abrogated tumor initiation[10]. Several genes, including GPR133, were found to be up-regulated in gastrointestinal stromal tumors, but their functions remain unknown[23].

To discover the potential regulatory mechanism of GPR133 in LUAD, GO and KEGG analyses were performed based on GPR133-related genes. The results indicated that the cell cycle was the most likely possible regulatory mechanism of GPR133 in LUAD. The cell cycle is a key event of cells and targeting the cell cycle may be an important approach in cancer therapy[24]. It is well known that cell-cycle machinery is controlled by cyclin-dependent kinase (CDK), cyclins, and CDK-inhibitory proteins[25]. Hence, we quantified the mRNA and protein expression levels of cell cycle biomarkers in LUAD cells with increasing GPR133 expression. Our results indicated that GPR133 inhibited cyclin B1 and enhanced p21 expression in LUAD cells. Notably, cyclin B1 and p21 are famous G2/M-phase biomarkers. Next, we performed a flow cytometry assay to discern that GPR133 significantly induced G2/M-phase arrest in LUAD cells. Consistently, PP9 (a natural steroidal saponin) was reported to effectively induce G2/M-phase arrest by upregulating p21 and suppressing cdc25C, cyclin B1, and cdc2[26]. Evidently, avasimibe dose-dependently inhibited the proliferation of U251 and U87 human glioblastoma cells. Further research revealed that avasimibe suppressed the expression of CDK2, cyclin E1, CDK4, cyclin D, CDK1, cyclin B1, Aurora A, and PLK1, while inducing the expression of p53, p21, p27, and GADD45A[27].

Our encouraging data presented herein lay the foundation for further research of GPR133 in LUAD as a therapeutic target. Indeed, we are currently conducting experiments geared toward further target validation as well as toward developing GPR133 inhibitors via small biomolecules.

Author contributions
GW and DZ performed most of the experiments and analyzed results. JX and ZL did the bioinformatics analysis. XL and SZ analyzed the data. ZZ designed the research, GW wrote the paper, ZZ and XL revised the paper.
Conflict of Interest
The authors declare that they have no conflict of interest.

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Data Availability
All supporting data are included in the manuscript.

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Figure legends

**Fig. 1 The expression pattern of GPR133 in LUAD.** (A) Gene-expression information was downloaded from TCGA and GEO databases, and a Venn diagram was used to identify differentially expressed genes. (B) The expression pattern of GPR133 in LUAD was displayed in TCGA and GEO databases. vs. normal, **** p < 0.0001. (C) Total RNAs were obtained from 28 samples of LUAD tissue and matched adjacent normal tissues and qRT-PCR assays were implemented to detect GPR133 expression in these tissues. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. (D) The mRNA and protein expression levels of GPR133 in LUAD cells were measured by qRT-PCR and western blot assays, respectively. Data were reported as mean ± SD for three independent
experiments, statistical analysis were performed via the Student’s t-test. vs. BEAS-2B, **p < 0.01. (E) Kaplan–Meier analysis was conducted according to GPR133 expression in LUAD patients from TCGA database and GSE31210 dataset.

Fig. 2 GPR133 suppressed cell proliferation in LUAD. GPR133 overexpression plasmid was transfected into A549 and H1299 cells, respectively. (A) qRT-PCR assays and Western blot assays were carried out to verify GPR133 in the mentioned cells. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. vs. Vec, **p < 0.01. (B) The above cells were seeded into 96 wells and MTS was added into each well at 0, 1, 2, 3, 4, and 5 days. The cell viability of each well was measured at A490 nm. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. vs. Vec, *p < 0.05, **p < 0.01. Soft agar colony-formation assay (C) and colony-formation assay (D) were performed using the above cells. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. vs. Vec, **p < 0.01. Scale bar: 100 μm.

Fig. 3 Effect of GPR133 on cell-cycle distribution in LUAD cells. (A) GSEA assays were performed to explore the mechanism of GPR133 function in LUAD cells. (B) GPR133 co-expressed genes were analyzed by GO and KEGG using the clusterProfiler software package on the R platform. (C) In LUAD cells with accelerating GPR133 and control groups, the expression levels of cell cycle biomarkers were evaluated by qRT-PCR and western blot assays. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. vs. Vec, *p < 0.05, **p < 0.01. (D) Flow-cytometry assays were conducted to detect the cell cycle in these cells.

Fig. 4 Identification of METTL3 targeting GPR133 in LUAD cells. (A) SRAMP presumed that the m6A was abundant in GPR133 transcripts. (B) The cells were isolated and RNA immunoprecipitation assay was performed. m6A antibody was used to trigger target RNAs, while immunoglobulin G was used as a negative control. The expression of GPR133 was determined in this experiment by qRT-PCR assay. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. vs. IgG, **p < 0.01. (C) The expression pattern of METTL3 in LUAD was exhibited. The relationship between METTL3 and GPR133 was analyzed by Pearson’s correlation coefficient using the GEPIA databank. vs.
normal, *p < 0.05. (D) The expression levels of METTL3 in BEAS-2B, A549, and H1299 cells were detected by qRT-PCR and western blot assays. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. BEAS-2B, **p < 0.01. (E) METTL3 overexpression plasmid was transfected into LUAD cells and the mRNA and protein expression profiles of METTL3 were assessed by qRT-PCR and western blot assays. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. vs. Vec, **p < 0.01. (F) In LUAD cells with increasing METTL3 expression, the mRNA and protein expression levels of GPR133 were evaluated by qRT-PCR and Western blot assays. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. vs. Vec, **p < 0.01. (G) The decay rate of GPR133’s mRNA at the indicated times after actinomycin D (5 µg/mL) treatment in A549 and H1299 cells with restored METTL3 expression was measured. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. vs. Vec, **p < 0.01.

**Fig. 5 GPR133 inhibited tumor growth in vivo.** (A) Immunohistochemistry assays were implemented to determine GPR133 expression in normal tissues and LUAD tissues. Data were reported as mean ± SD for three independent experiments, statistical analysis were performed via the Student’s t-test. vs. normal, **p < 0.01. The relationship between GPR133 expression and the prognosis of LUAD patients was established by Kaplan–Meier analysis. Scale bar: 100 µ m. (B) A total of 5 × 10^5 A549 cells with overexpressed GPR133 and the control group were subcutaneously injected into nude mice (n = 4 per group), respectively. The tumor size was detected every two days; tumor weights were also measured and the tumor growth curve was drawn. Data were reported as mean ± SD, statistical analysis were performed via the Student’s t-test. vs. Vec, *p < 0.05, **p < 0.01.
Table 1. Primers in this study.

| Name         | sequence (5’-3’)                      |
|--------------|---------------------------------------|
| GPR133-F     | AAAGTCCCGGAGTGATACGTGA                |
| GPR133-R     | TTGGTGAGATTCAAGGCTGTC                 |
| GAPDH-F      | ACAACTTTGGATCTGGAAGGG                 |
| GAPDH-R      | GCCATCACGCCACAGTTTC                   |
| Cyclin D1-F  | GCTGCGAAGTGGAACCATC                   |
| Cyclin D1-R  | CCTCCTTTCTGCACACATTTGAA              |
| Cyclin B1-F  | AATAAGGCGAAGATCAACATGTC              |
| Cyclin B1-R  | TTTGTACCAAATGTCACCACAGAG             |
| CDK4-F       | ATGGCTACCTCTCAGAAGG                   |
| CDK4-R       | CATTGGGGACTTCACACTCT                  |
| p21-F        | TGTCCGTACAGAACCACCCATGC              |
| p21-R        | AAAGTCAAGTCCATGCCTTTGCT              |
| Cdc2-F       | GGATGTGCTTTAGCAGGATTTGCC             |
| Cdc2-R       | CATGTACTGACAGGAGGGATAG               |
| IGF2BP3-F    | ACGAAATATCCCGCCTCATTTAC              |
| IGF2BP3-R    | GCAGTTCCGGAGTGCTGTTCA                |
Table 2. The correlationship between GPR133 expression and the clinical parameters of LUAD in GSE31210

|               | Case (n) | High  | Low  | P value |
|---------------|----------|-------|------|---------|
|               | case(n)  | Rate(%) | case(n) | Rate(%) |    |
| Age           |          |        |       |         |
| ≥60           | 140      | 49     | 35.00 | 91      | 65.00 | 0.297 |
| <60           | 106      | 44     | 41.51 | 62      | 58.49 |
| Gender        |          |        |       |         |
| Male          | 116      | 39     | 33.62 | 77      | 66.38 | 0.201 |
| Female        | 130      | 54     | 41.54 | 76      | 58.46 |
| Smoking       |          |        |       |         |
| Yes           | 123      | 43     | 34.96 | 80      | 65.04 | 0.357 |
| No            | 123      | 50     | 40.65 | 73      | 59.35 |
| Tumor Stage   |          |        |       |         |
| I             | 169      | 68     | 40.24 | 101     | 59.76 |
| II            | 58       | 6      | 10.34 | 52      | 89.66 | 0.000 |
Figure 4

A. Prediction Score Distribution along the Query Sequence

B. IgG and anti-mA

C. Scatter plot showing Log2 METTL3 TPM vs. Log2 ADGC1 TPM, with p<0.05 and R=0.092

D. METTL3 Relative Expression

E. METTL3 and GPR133 Relative Expression

F. Relative mRNA Level of GPR133

G. Relative mRNA Level of GPR133 for different treatments
