RP11-323N12.5 promotes the malignancy and immunosuppression of human gastric cancer by increasing YAP1 transcription

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Abstract
Background YAP1 is a core protein of the Hippo signaling pathway and is associated with malignancy and immunosuppression. In the present study, we discovered a novel lncRNA, RP11-323N12.5, with tumor promotion and immunosuppression activities through enhancing transcription of YAP1.

Methods RP11-323N12.5 was identified using GEPIA. Its expression levels and their relationship with clinical features were investigated using clinical samples. The regulation of YAP1 transcription by RP11-323N12.5 was investigated in both GC and T cells, the tumor and immunosuppression promotion roles of RP11-323N12.5 were explored in vitro and in vivo.

Results RP11-323N12.5 was the most up-regulated lncRNA in human GC, based on data from the TCGA database. Its transcription was significantly positively correlated with YAP1 transcription, YAP1 downstream gene expression which contribute to tumor growth and immunosuppression. RP11-323N12.5 promoted YAP1 transcription by binding to c-MYC in the YAP1 promoter region. Meanwhile, transcription of RP11-323N12.5 was also regulated by YAP1/TAZ/TEADs activation in GC cells. RP11-323N12.5 had tumor- and immunosuppression-promoting effects by enhancing YAP1 downstream genes in GC cells. Excessive RP11-323N12.5 was also observed in tumor-infiltrating leukocytes (TILs), which may be exosome-derived and also be related to enhanced Treg differentiation as a result YAP1 up-regulation. Moreover, RP11-323N12.5 promoted tumor growth and immunosuppression via YAP1 up-regulation in vivo.

Conclusions RP11-323N12.5 was the most up-regulated lncRNA in human GC and it promoted YAP1 transcription by binding to c-MYC within the YAP1 promoter in both GC and T cells. RP11-323N12.5 is an ideal therapeutic target in human GC due to its tumor-promoting and immunosuppression characteristics.

Keywords RP11-323N12.5 · YAP1 · Hippo signaling · Immunosuppression · Gastric cancer

Abbreviations
GC Gastric cancer
lncRNA Long non-coding RNA
TILs Tumor infiltrating leukocytes
TCGA The Cancer Genome Atlas Program
GTEx The Genotype-Tissue Expression
STAD Stomach adenocarcinoma

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Background

Long noncoding RNAs (lncRNAs) have gained widespread attention by biological and medical researchers recently. However, decades ago, lncRNAs were termed “junk DNA” due to its transposons, pseudogenes, and simple repeats, which make up approximately 70% of the human genome [1, 2]. With the evolution of high-throughput RNA sequencing and genetic modification techniques, more functional lncRNAs have been investigated in various human diseases, especially in malignancies, such as lung cancer, gastric cancer, liver cancer, colorectal cancer, and leukemia [3–6]. The functional mechanisms of lncRNAs are varied and include chromatin remodeling and chromatin interactions, as well as acting as enhancers, promoters, or introns of other genes; pseudogenes; or natural antisense transcripts [7, 8]. In addition, lncRNAs may also host one or more small RNAs within their transcriptional units. Besides their function within tumor cells, tumor-derived lncRNAs also modulate the tumor microenvironment via exosomes, switching tumor immunity from anti-cancer to pro-cancer by targeting signaling pathways in different immunocytes [9–12].

Hippo signaling is one of the tumor- and tumor immunosuppression-related signaling pathways that was first discovered in Drosophila melanogaster through genetic mosaic screens [13, 14]. The Hippo signaling pathway consists of the NDR family protein kinase, Warts (Wts); the WW domain–containing protein, Salvador (Sav); the Ste20-like protein kinase, Hippo (Hpo); and the adaptor protein Mob as tumor suppressor (Mats) [13, 14]. Dysregulation of Hippo pathway components has been demonstrated in many cancers [15]. Besides its promotion of cell proliferation, dysregulation of Hippo signaling has also been shown to regulate many normal biological and disease processes, including survival, metabolism, stemness, angiogenesis, invasion, and immunosuppression [15, 16]. In addition to classical stimuli of Hippo signaling, many studies have reported its regulation by lncRNAs [17, 18]. Therefore, an investigation of the diagnostic value and mechanisms of lncRNAs that target Hippo signaling is warranted.

Materials and methods

TCGA database analysis and bioinformatics analysis

We used GEPIA, an online TCGA and GTEx analysis tool, to identify differentially expressed genes in the stomach adenocarcinoma (STAD) subset. TRANSFAC (www.gene-regulation.com) and PROM (https://alggen.lsi.upc.es/) were used to predict the potential binding sites in the promoter regions of YAP1 or RP11-323N12.5 (1000 bp upstream flanking sequence from the transcription start site).

Patients

This hospital-based case–control study included 67 GC patients. All subjects were recruited from the First People’s Hospital of Kunshan, affiliated with Jiangsu University, between January 2017 and December 2018. All patients underwent surgery for primary GC and subjects with a previous history of cancer or chemotherapy were excluded. This study was approved by the Ethics Review Board of the First People’s Hospital of Kunshan (KSL-2017017) and all patients provided written informed consent.

Cell lines and reagents

Human GC cell lines, including MKN45 and MGC-803, were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and maintained at 37°C in a humidified atmosphere containing 5% CO2. Lysophosphatidic acid (LPA) was purchased from ENZO (BML-LP100-0025; Farmingdale, NY, USA) and an shRNA targeting YAP was purchased from Santa Cruz Biotechnology (sc-38637-V; Dallas, TX, USA). Positive clones were obtained using a puromycin screen.

Real-time PCR

Total RNA was isolated from GC tissues and cell lines using TRIzol reagent (Invitrogen, 15596026). Gene expression was assessed using a SYBR green-based real-time PCR method (AceQ Universal SYBR qPCR Master Mix, Vazyme,
Nanjing, China, Q511-02). The primers used in this study are listed in Supplementary Table 1.

**Multicolor immunohistochemistry, immunofluorescence, and in situ hybridization**

For multicolor staining, we processed slides strictly according to the instructions of the Opal 4-Anti-Rabbit Automation IHC kit (NEL830001KT; AKOYA Biosciences, Marlborough, MA, USA). Slides were incubated at 4 °C overnight with primary anti-YAP1 (#4912; Cell Signaling Technology, Danvers, MA, USA), anti-Survivin (ab76424; Abcam, Cambridge, UK) anti-human CD4 (ab181724, Abcam), anti-mouse CD4 (ab183685, Abcam), anti-COX2 (ab15191, Abcam), anti-human Foxp3 (ab20034, Abcam), and anti-mouse Foxp3 antibodies (ab75763, Abcam) and then photographed using a digital microscope camera (Nikon, Tokyo, Japan).

In situ hybridization (ISH) was performed using a Boster ISH kit (Boster, Wuhan, China). Cells were fixed and permeabilized using methanol and protease to allow biotin-labeled probes to access the cells. Cells were treated with 30% H2O2 in ddH2O, at a ratio of 1:10, for 5 min, after which pepsase, diluted in 3% citric acid, was applied for 20 s. The second fixation was performed using 1% paraformaldehyde/0.1 M PBS. Next, the cells were incubated with pre-hybridization solution at 40 °C for 2 h and then with IncRNA target probes at 30 °C overnight, followed by two washes with 2× saline sodium citrate. After blocking, a biotin-labeled anti-digoxin antibody was added and cells were incubated for 60 min. The following sequence was used for the RP11-323N12.5 probe: ctctcgctgcccgcgccgc.

**Western blotting**

For western blotting, proteins were extracted from cultured cells using RIPA buffer containing phenylmethanesulfonyl fluoride (Beyotime, Nantong, China). An equal amount of protein per sample (100 μg) was separated by 7.5%/12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The following primary polyclonal antibodies were used: anti-YAP1 (#4912, Cell Signaling Technology), anti-phospho(S127)-YAP1 (#13008, Cell Signaling Technology), anti-Survivin (ab76424, Abcam), anti-MMP9 (ab219372, Abcam), and anti-COX2 (ab15191, Abcam). HRP-conjugated anti-rabbit secondary antibodies were used (Abcam). The blots were developed using ECL reagent (WBKLS0500; Merck Millipore, Burlington, MA, USA). An equal amount of protein loading in each lane was confirmed using an anti-β-Actin antibody (ab8226, Abcam). ImageJ software was used to quantify the integrated density of the protein bands.

**Luciferase gene reporter assay**

The promoter region of the YAP1 gene containing wild-type (WT) or mutant (MU) potential target sites for c-MYC, as well as the promoter region of the RP11-323N12.5 gene containing wild-type or mutant binding sites for TEAD1, were designed and synthesized by Genescript, Co. (Nanjing, China) and inserted into the pGL4 Vector (Promega, Madison, WI, USA). For luciferase assays, MKN45, MGC803, and Jurkat cells were transfected with pGL4-YAP1 WT pro or pGL4-YAP1 c-MYC MU pro, pGL4-RP11-323N12.5 WT pro or pGL4-RP11-323N12.5 TEAD1 MU pro, or control vector, using Lipofectamine 2000 (11668019, ThermoFisher Scientific, Waltham, MA, USA). LPA-treated cells were harvested 48 h after transfection and a luciferase assay was performed using a Dual-Luciferase® Reporter Assay System (E1910, Promega, Madison WI), according to the manufacturer’s protocol.

**Chromatin isolation by RNA purification assay (ChIRP)**

Chromatin isolation by RNA purification (ChIRP) assays were performed using an EZ- Magna ChIRP RNA Interactome Kit (17-10495, Millipore). The sequence of the RP11-323N12.5 probe was GGGCGTTGGAGGCTGC. Isolated RNA was used for qRT-PCR analysis to quantify the enrichment of chromatin. The qRT-PCR primer sequences used in this study were as follows: YAP1 promoter: 5'-AATGAGTTTCACTGAGGAGAT-3' (forward) and 5'-CAAAAACAAGCAATGTAGGTTT-3' (reverse).

**CCK8 cell proliferation, cell colony formation, migration, and invasion assays**

A CCK-8 assay kit (CK04-11, Dojindo, Kumamoto, Japan) was used to detect cell proliferation, according to the manufacturer’s protocol. Cells were seeded into 96-well plates (500 cells/well in 100 μL of medium) and 10 μL of CCK-8 solution was added to each well at the same time each day for each time-point. After incubation for 2 h at 37 °C, the absorbance (450 nm) of each well was measured.

For cell colony formation assays, GC cells were plated in 6-well plates, at a density of 500 cells/well, and incubated for 2 weeks. Proliferating colonies were fixed with 75% alcohol and stained with crystal violet. Colonies containing ≥ 50 cells were counted under a microscope. All procedures were performed in triplicate.

For migration and invasion assays, cells were placed into the upper chamber of a transwell plate (8-μm pore size; Milipore), which was coated with (invasion assay) or without (migration assay) a Matrigel mix (BD Biosciences, San Jose, CA, USA), according to the manufacturer’s protocol. After
incubation for 24 h, the cells located on the upper membrane of the transwell chambers were removed with cotton wool, while cells that had migrated or invaded through the membrane were fixed with methanol for 10 min and stained with 0.1% crystal violet for 30 min at 37 °C. Cells in three randomly selected microscopic fields were counted for each group. Experiments were independently repeated three times.

ELISA

The secretion of PGE2 and CXCL15 into the supernatant of MKN45 and MGC-803 cells, RP11-323N12.5-overexpressing cells, and RP11-323N12.5 + YAP1 shRNA-transfected cells was measured using ELISA kits (ab133021 for PGE2, ab234567 for CXCL15; Abcam).

Treg cell differentiation and flow cytometric analysis

Various genetically modified Jurkat cells were obtained for the in vitro stimulation of Treg cell polarization. For the in vitro generation of Treg cells, Jurkat cells were stimulated with recombinant human TGF-β (1 ng/mL; Peprotech, Rocky Hill, NJ, USA) for 7 days. Dead cells were excluded based on staining with a live/dead fixable dye. Thereafter, cells were fixed, permeabilized with IntraPrep reagent (BD Pharmingen), and then stained with fluorochrome-conjugated antibodies against CD4 (20 μL per test, 555346), CD25 (20 μL per test, 555432), or Foxp3 (20 μL per test, 560082, BD Pharmingen). Data were acquired on a BD FACSVersa™ flow cytometer (BD Pharmingen).

Tumor-infiltrating leukocyte isolation

Fresh tissue–infiltrating lymphocytes were obtained as described previously [20]. Briefly, GC tissue specimens were cut into small pieces and digested in RPMI 1640. Dissociated cells were filtered through a 75 μm cell strainer and separated by Ficoll centriﬁgation. The mononuclear cells were then washed and resuspended in RPMI 1640, supplemented with 10% FBS (Gibco, Waltham, MA, USA). T cells were puriﬁed using anti-CD3 magnetic Dynabeads (Invitrogen Life Technologies, CA, USA) according to manufacturer’s instruction.

Subcutaneous tumorigenesis assay

For subcutaneous tumorigenesis assays, tumor cells were injected subcutaneously in mice (2 × 10^6 cells/100 μl per flank containing 1 × 10^6 GC tumor cells and 1 × 10^6 peripheral mono-nuclear blood cells obtained from non-GC healthy volunteers) [20]. Four weeks after injection, the mice were euthanized. The tumor volume was calculated using the formula (width^2 × length)/2. The tumor tissues were then harvested for further analysis.

Statistical analysis

Data are presented as mean ± SD. Chi square tests and a two-tailed Student’s t test analysis of variances were used to evaluate statistical differences in demographic and clinical characteristics. All in vitro expression experiments were repeated at least three times, each with triplicate samples. Pearson’s correlation analysis was used to analyze the relationship between associated factors. Statistical analyses were performed using STATA 9.2 (StataCorp, College Station, TX, USA) and presented using Prism software (GraphPad, San Diego, CA, USA). In all cases, P < 0.05 was considered significant.

Results

**RP11-323N12.5 is the most overexpressed IncRNA in human gastric cancer, based on the TCGA database**

Based on the newly developed interactive web server, GEPIA [19], we found 3746 significantly up-regulated genes in stomach adenocarcinomas (STADs) compared to their paired normal tissues (n = 36) using an ANOVA to analyze TCGA data. The chromosomal distribution of the top 104 differentially expressed genes is presented in Fig. 1a. The top 25 up-regulated genes are listed in Fig. 1b. **RP11-323N12.5** was found to be the most significantly up-regulated lncRNA in human GC tissues (Gene ID ENSG00000267601.1), with a median expression of 27.970 in tumors and 0.000 in normal tissues, with an adjusted P value 1.36e-67 (Fig. 1b). **RP11-323N12.5** is an anti-sense non-coding RNA located on chr17:78855478–78855844, with a transcript of 367 bp.
RP11-323N12.5 was over-expressed in human GC tissues and was associated with the dysregulation of Hippo signaling. a Transcription of RP11-323N12.5 in paired human GC and adjacent normal stomach tissues (\(n=67\)). b RP11-323N12.5 expression at different stages of human GC. c Transcription of YAP1 in paired human GC and adjacent normal stomach tissues (\(n=67\)). d Linear correlation analysis of RP11-323N12.5 and YAP1 in human GC. e–e Transcription of BIRC5, AXL, and PTSG2 in paired human GC and adjacent normal stomach tissues (\(n=67\)). f–h Linear correlation analysis of RP11-323N12.5 and BIRC5, AXL, or PTSG2 in human GC. i Representative figures of immunofluorescence (IF) staining of YAP1 and Survivin in human GC tissues with high and low levels of RP11-323N12.5 expression. j, k Integrated optical intensity (IOD) analysis of YAP1 and Survivin protein expression in human GC tissues with high (\(n=33\)) and low (\(n=34\)) levels of RP11-323N12.5 expression. l, m Linear correlation analysis of RP11-323N12.5 and YAP1 or Survivin in human GC with high (\(n=33\)) and low (\(n=34\)) levels of RP11-323N12.5 expression. Each experiment was performed in triplicate. Data are presented as the mean ± SEM and were analyzed by Student’s \(t\) test (\(P<0.01\)).

Moreover, using a one-way ANOVA to analyze samples from the GTEx database, we found that RP11-323N12.5 was also overexpressed in human GC tissues compared to normal tissues (GC \(n=408\) vs. normal \(n=211\), Fig. 1c). We found that RP11-323N12.5 expression levels were significantly higher in advanced GC (TMN Stage I–II vs. Stage III, IV; Fig. 1d). Survival analysis showed that high RP11-323N12.5 expression was significantly correlated with disease-free survival (\(P=0.047\)), but not with overall survival of post-surgery GC patients (Fig. 1e).

Through pathway enrichment analysis between RP11-323N12.5-high and RP11-323N12.5-low GC samples (\(n=408\)), we found that RP11-323N12.5 expression was significantly related to Hippo signaling (data not shown). Furthermore, we confirmed this correlation by investigating the correlation between RP11-323N12.5 and components of the Hippo signaling pathway and its downstream genes. These results indicated that RP11-323N12.5 expression levels were significantly positively correlated with YAP1, AXL, and TGFBR1 expression levels (Fig. 1f–h). Since, the inactivation of Hippo signaling is significantly associated with a suppressive tumor microenvironment (TME) in human solid tumors, we also investigated the correlation between RP11-323N12.5 expression and the expression of genes representing different types of T helper cells. We found that RP11-323N12.5 was significantly correlated with FOXP3 and TGFBR1 expression (Fig. 1i, j), which implied that RP11-323N12.5 was associated with Treg cells, but not Th1, Th2, or Th17 cells in the TME of human GC (Fig. 1k–m).

RP11-323N12.5 was associated with YAP1 expression in human GC

To confirm the overexpression of RP11-323N12.5 in human GC, we collected 67 paired human GC and para-tumor tissues. We found that RP11-323N12.5 was overexpressed in GC tissues compared to paired normal tissue (Fig. 2a). Moreover, RP11-323N12.5 expression levels were also significantly higher in advanced stage GC (stage III–IV) compared to early stage GC (stage I–II, Fig. 2b). The transcription levels of YAP1 were also determined by real-time PCR in 67 paired GC and normal tissues. Similar to previously published results [19], YAP1 was found to be overexpressed in GC tissues and its expression levels were positively correlated with RP11-323N12.5 expression levels (\(P=0.0004, r=0.6275\)). Moreover, we found that the downstream genes of Hippo signaling, BIRC5, AXL, and PTSG2 were overexpressed in GC tissues (Fig. 2c, d, e) and their expression levels were also positively correlated with RP11-323N12.5 expression levels in human GC tissues (BIRC5 vs. RP11-323N12.5: \(r=0.7515, P<0.0001\); AXL vs. RP11-323N12.5: \(r=0.3885, P=0.0012\); and PTSG2 vs. RP11-323N12.5: \(r=0.5000, P=0.0009\); Fig. 2f, g, h).

We also detected the protein expression of YAP1 and the downstream, protein, Survivin, in human GC tissues by immunofluorescence staining, in two groups according to RP11-323N12.5 expression (RP11-323N12.5-high, \(n=33\) and RP11-323N12.5-low, \(n=34\)). We found that YAP1 and Survivin levels were higher in the RP11-323N12.5-high group than in the RP11-323N12.5-low group (Fig. 2i, j, k). YAP1 and Survivin protein levels were also positively correlated with RP11-323N12.5 expression levels (RP11-323N12.5 vs. YAP1: \(P=0.0001, r=0.4535\); RP11-323N12.5 vs. Survivin: \(P<0.0001, r=0.5079\)). Based on these data, we proposed that RP11-323N12.5 may be associated with YAP1 transcription and downstream gene activation.

RP11-323N12.5 and YAP1 promoted the transcription of each other by promoter binding in GC cells

Since clinical studies have indicated that RP11-323N12.5 expression is positively correlated with YAP1 transcription, we further investigated the detailed regulatory mechanisms of RP11-323N12.5 and YAP1. Firstly, we overexpressed and knocked down RP11-323N12.5 expression in the human GC cell lines, MKN45 and MCG803, respectively. We found that the changes in YAP1 transcription were synchronized to RP11-323N12.5 expression (Fig. 3a). We also investigated
RP11-323N12.5 was overexpressed in GC and its transcription was positively correlated with Hippo inactivation, based on our clinical investigation and data from the TCGA database, we next investigated whether YAP1 activation could promote RP11-323N12.5 transcription. Firstly, LAP-induced YAP1 activation was shown to significantly increase RP11-323N12.5 transcription in both MKN45 and MCG 803 cells, as determined by both real-time PCR and ISH analyses (Fig. 3i, j). When we queried the promoter region of RP11-323N12.5, we found that a TEAS1 binding site existed within this region (Fig. 3k). Therefore, we performed a luciferase gene reporter assay and found that LAP did not increase RP11-323N12.5 promoter activity when the TEAD1 binding site was mutated (Fig. 3l). These results indicated that RP11-323N12.5 and YAP1 can promote each other’s expression in human GC.

RP11-323N12.5 promoted GC cell proliferation and invasion, as well as immunosuppression, by increasing YAP1 expression

Since RP11-323N12.5 and YAP1 can promote each other’s expression in human GC, we investigated the mechanisms of this phenomenon in vitro. Three types of cell lines were constructed including WT cells; cells overexpressing RP11-323N12.5 (RP11-323N12.5); and cells overexpressing RP11-323N12.5, with YAP1 knocked down (RP11-323N12.5 + YAP1 shRNA). Cell proliferation was monitored using CCK8 assay and colony formation assays. We found that RP11-323N12.5 promoted the proliferation of both MKN45 and MCG803 cells; however, this effect was mainly dependent on YAP1 expression (Fig. 4a, b). RP11-323N12.5 markedly increased the migration and invasion ability of both GC cell lines. When YAP1 was knocked down by a specific shRNA, cell migration and invasion decreased to levels significantly less than those observed in WT cells (Fig. 4c). These data implied that RP11-323N12.5 is a YAP1-dependent oncogene. These oncogenic effects may be carried out by up-regulating Survivin, MMP9, and AXL, which were is a YAP1-dependent oncogene. These oncogenic effects may be carried out by up-regulating Survivin, MMP9, and AXL, which were up-regulated by RP11-323N12.5 overexpression and down-regulated by knocking down YAP1 (Fig. 4d, e).

Since previous studies have shown that inactivation of Hippo signaling is significantly related to immunosuppression [21, 22] and data from TCGA also implied that RP11-323N12.5 was significantly correlated with Treg cell-related immunosuppression, we analyzed genes downstream of YAP1 and immunosuppression-related genes, including PSTG2, CSF1, CXCL15, and CSF3. We found that RP11-323N12.5 significantly promoted the expression of these genes in a YAP1-dependent manner (Fig. 4d, e).
**RP11-323N12.5 was related to Treg cell-and MDSC-related immunosuppression in the human GC tumor microenvironment**

Based on in vitro studies, we found that *RP11-323N12.5* may be related to Treg cell differentiation via cytokine secretion. We also investigated the relationship between *RP11-323N12.5* expression and Treg cell distribution by multicolor immunohistochemistry (IHC). We found that in tissues with high levels of *RP11-323N12.5* expression, the tested genes are downstream of YAP1 activation. The protein downstreams of YAP1 activation were measured by western blotting in GC-803 and MKN45 cells with different treatments. Each experiment was performed in triplicate. Data are presented as the mean ± SEM and were analyzed by Student's *t* test (**P*< 0.01).

The image shows graphs and tables illustrating the effects of *RP11-323N12.5* on cell proliferation, migration, and invasion. The graphs depict the percentage of Treg cells and MDSCs in GC tissues with high and low levels of *RP11-323N12.5* expression.
expression (Figs. 5f, g, S1a, b). Furthermore, we found that serum PGE2 and CXCL15 levels were markedly higher in GC patients with high levels of RP11-323N12.5-expression (Fig. 5h, i).

We next investigated PGE2 and CXCL15 secretion by human GC cell lines after various treatments. We found that RP11-323N12.5 promote the secretion of these proteins in a YAP1-dependent manner (Fig. 5j, k). We also co-cultured human T cells, isolated from six non-GC individuals, with MKN45 cells, after various treatments, for 7 days. We found that Treg cell differentiation was significantly increased by co-culture with RP11-323N12.5-overexpressing MKN45 cells, but this effect was inhibited by YAP1 depletion in MKN45 cells (Fig. 5l).

**RP11-323N12.5 promoted Treg cell differentiation by enhancing YAP1 transcription in T cells**

Since we observed that RP11-323N12.5 was related to Treg cell-induced immunosuppression by tumor-derived secretion, we next investigated whether endogenous RP11-323N12.5 could promote Treg cell differentiation. Firstly, we investigated YAP1 protein expression in CD4+ T cells using multicolor IHC. We found that T cell expression levels of YAP1 were higher in GC tissues expressing high levels of RP11-323N12.5 compared to those expressing low levels of RP11-323N12.5. Moreover, RP11-323N12.5 levels were also significantly positively correlated with the expression levels of these genes (Fig. 6c). These results indicated that endogenous RP11-323N12.5 in T cells was not only related to tumor cell RP11-323N12.5 expression, but also related to Treg cell differentiation. TILs were isolated from human GC tissues and RP11-323N12.5 levels were found to be significantly higher in GC tissues expressing high levels of RP11-323N12.5 than in those with low levels of RP11-323N12.5 expression. Treg cell-related genes, including YAP1, FOXP3, TGFB, and IL10 were all expressed at significantly higher levels in T cells isolated from tissues expressing high levels of RP11-323N12.5 compared to those expressing low levels of RP11-323N12.5. We wondered that RP11-323N12.5 whether can promote YAP1 transcription in T cells.

We used Jurkat cells, a human T cell line, to further investigate this mechanism. Firstly, we found that overexpression of RP11-323N12.5 in Jurkat cells enhanced Treg cell differentiation and this effect was YAP1 activation dependent (Fig. 6f). Both mRNA and protein expression of YAP1 were up-regulated by RP11-323N12.5 (Fig. S2a, b). Next, we used a ChIRP assay to show that RP11-323N12.5 promoted YAP1 transcription by binding to c-MYC, specifically in the promoter region of the YAP1 gene (Fig. 6g). Similar results were seen in luciferase gene reporter assays, indicating that YAP1 promoter activity can be significantly increased by RP11-323N12.5 in a c-MYC binding-dependent manner, since promoter activity was fully restored once the c-MYC binding site was mutated (Fig. 6h). However, using both luciferase gene reporter assays and real-time PCR, we found that YAP1 activation had no effect on RP11-323N12.5 transcription in Jurkat cells (Fig. S2c, d). Next, we measured RP11-323N12.5 levels in both GC patient serum samples and in cell culture supernatants. We found that RP11-323N12.5 can be detected in the exosomes and its quantity varied according to RP11-323N12.5 expression levels within tumor cells (Fig. 6i, j, k). Furthermore, we co-cultured RP11-323N12.5 shRNA-transfected Jurkat cells with human GC cells showing different levels of RP11-323N12.5 expression. The result indicated that RP11-323N12.5 can transfer into Jurkat cells by exosome delivery (Figs. 6j, k, S2e). Therefore, we concluded that the high levels of RP11-323N12.5 expression in T cells were exosome derived.

**RP11-323N12.5 promoted tumor growth and immunosuppression via YAP1 up-regulation in vivo**

To investigate the pro-cancer and pro-immunosuppressive effects of RP11-323N12.5 in vivo, we performed a subcutaneous tumorigenesis assay using wild-type MKN45 cells; MKN45 cells overexpressing RP11-323N12.5; and MKN45 cells overexpressing RP11-323N12.5, with YAP1 knockdown. Simultaneously, the human mono-nuclear peripheral blood cells mainly containing T cells and monocytes were implanted subcutaneously with the tumor cells. RP11-323N12.5 significantly promoted tumor growth, but this effect was completely inhibited by YAP1 knockdown in vivo (Fig. 7a). The TME derived from different tumor cells was also investigated by multicolor IHC staining of YAP1, Foxp3, and CD4. We found that YAP1 was up-regulated by RP11-323N12.5 in vivo and the number of infiltrating Treg cells increased in tumors derived from RP11-323N12.5-overexpressing MKN45 cells. However, the number of infiltrating Treg cells decreased significantly when YAP1 was knocked down in MKN45-derived tumor tissue (Fig. 7b–d). Furthermore, TILs were isolated from the tumors and the numbers of both Treg cells and MDSCs were found to be increased in RP11-323N12.5-overexpressing tumors. However, their numbers decreased markedly when YAP1 expression was silenced in RP11-323N12.5-overexpressing tumor tissues (Fig. 7e, f).
RP11-323N12.5 High

b) COX2

RP11-323N12.5 Low

c) Treg

d) $R = 0.45$, $P = 0.0001$

e) $R = 0.59$, $P = 0.0001$

f) TILs Treg

g) MDSCs

h) PGE2

i) CXCL15

j) PGE2

k) CXCL15

l) CD25

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RP11-323N12.5 was involved in Treg cell-related immunosuppression in the human GC tumor microenvironment. Representative figures of multicolor staining of CD4, COX2, and FOXP3 in human GC tissues with high and low levels of RP11-323N12.5 expression. b, c Integrated optical density (IOD) values of COX2 and CD4+FOXP3+ cell percentage (CD4+FOXP3+/CD4) in human GC tissues with high \( n=33 \) and low \( n=34 \) levels of RP11-323N12.5 expression. d, e Linear correlation analysis of RP11-323N12.5 and CD4+FOXP3+ percentage (log2) or COX2 IOD values (log2) in human GC. f, g Tumor-infiltrating leukocytes were isolated from human GC tissues with high \( n=33 \) and low \( n=34 \) RP11-323N12.5 expression levels and the percentages of Treg cells (CD4+CD25+FOXP3+) and MDSCs (CD11b+CD33+) were determined by flow cytometry. h, i The concentrations of PGE2 and Cxcl15 secreted from MKN45 and MGC-803 cells after different treatments were determined by ELISA. i The human T cell isolated from six non-GC persons was co-cultured with MKN45 cells treated with TGF-β (1 ng/mL) for 7 days and the percentage of Treg cells (CD4+CD25+FOXP3+) was determined by flow cytometry. Each experiment was performed in triplicate. Data are presented as the mean ± SEM and were analyzed by Student’s \( t \) test (\( **P<0.01 \)).

In summary, in the present study, we used data from the TCGA database to identify RP11-323N12.5 as the most differentially expressed IncRNA between human GC and normal tissue. RP11-323N12.5 was shown to promote the transcriptional activation of YAP1 in GC cells and further promote immunosuppression by Treg cell differentiation and MDSCs infiltration via a YAP1 activation dependent manner (Fig. 8).

Discussion

In the present study, we found that a novel IncRNA, RP11-323N12.5, contributed to the overexpression of YAP1 in human GC. Although it is a controversial notion that YAP overexpression alone is related to the development of GC, several studies have identified YAP1 as an oncogene in the stomach. In normal and proliferating normal gastric epithelium, low or moderate levels of YAP1 expression are detected, whereas significantly elevated YAP1 levels are consistently observed in both primary and metastatic GC [23, 24]. YAP1 expression is strongly positively correlated with lymphatic metastasis and shortened overall survival time, indicating that YAP may be used as an independent prognostic marker for GC [25–27]. Moreover, knockdown of YAP markedly suppresses cell proliferation, colony formation, and metastasis in GC cell lines, both in vitro and in vivo [28–30]. Besides the overexpression of YAP1, dysregulation of other genes within the Hippo signaling pathway have been reported more frequently. These include NF2, MST1/2, LAT51/2, and RASSF1A, which can increase the transcriptional activity of YAP1/TAZ/TEADs in human GC. Hippo signaling is also associated with immunosuppression [21]. On one hand, the activation of YAP1 signaling in tumor cells induces immunosuppression by up-regulating various cytokines, chemokines, and other molecules associated with the recruitment of M2 macrophages, Tregs, and MDSCs and by inhibition the infiltration and activity of NK cells and effector T-cells. On the other hand, YAP1 overexpression in immune cells facilitates immunosuppressive cell differentiation and proliferation [21]. For example, YAP1 induces the differentiation of naïve T-cells to Treg cells by up-regulating TGFBR2 [31]. In stromal cells, YAP1 promotes the transcription of myofibroblast marker genes, such as α-SMA, CYR61, and CTGF to activate cancer-associated fibroblasts (CAFs) in breast cancer. Activated YAP1 then recruits CAFs, MDSCs, and Tregs, leading to the impairment of CD8+ T-cells [32]. Our data confirmed this mechanism. We found that RP11-323N12.5 not only promoted the malignancy of human GC, but also promoted immunosuppression by upregulating YAP1 activation-related cytokines. We found that once YAP1 levels decreased in GC cells, the RP11-323N12.5-induced immunosuppressive TME was totally reversed and the percentage of Treg cells and MDSCs decreased markedly. For decades, the “on” state of the Hippo pathway has been regarded as a tumor suppressor pathway, due to its inhibition of proliferation and promotion of apoptosis [15, 16, 28, 30]. Dysregulation of the Hippo pathway is associated with a broad spectrum of cancers, mainly due to the core protein, YAP-1 [15]. YAP1 has been identified as an oncogene, associated with many different types of cancer [15]. Zhang et al. showed that YAP expression is markedly elevated in clinical hepatocellular carcinoma (HCC) samples and that YAP is a key driver gene in hepatitis B virus X protein (HBx)-induced hepatocarcinogenesis in a CREB-dependent manner. YAP may serve as a novel target in HBV-associated HCC therapy [33]. Moreover, there are several clinical studies showing that YAP protein is associated with the progression and prognosis of human GC. For example, Zhang et al. first reported that YAP is strongly expressed...
RP11-323N12.5 promotes the malignancy and immunosuppression of human gastric cancer by...

in gastric adenocarcinomas and that YAP knockdown may inhibit gastric cancer cell proliferation and metastasis [34]. Chol et al. showed that YAP and TAZ are oncogenic initi-
tors and drivers in human GC, with MYC as the downstream effector [35].

Besides the protein-coding genes that regulate Hippo signaling, recently, a greater number of non-coding RNAs have been reported to modulate Hippo signaling. MAYA, a lncRNA, has been reported to mediate the methylation and upstream kinase activity of MST1/2 in Hippo signaling, which consequently, leads to the failure of LATS1 phosphorylation and YAP1 target gene activation in bone metastasis [36]. A recent study also identified a lncRNA, BCAR4, as a direct transcriptional target of YAP, involved in YAP-dependent glycolysis. Mechanistically, the long-term activation of YAP up-regulates the transcription of BCAR4, which, in turn, promotes the transcription of two glycolysis-related enzymes, HK2 and PFKFB3, through Hedgehog effector GLI2/p300 complex-mediated histone acetylation, marked by H3K27ac [37]. Similar to the lncRNAs, MAYA and BCAR4, RP11-323N12.5 is also a Hippo signaling-associated lncRNA. However, RP11-323N12.5 functions differently, in that it is an enhancer of YAP1 transcription by binding to c-MYC, which is a transcriptional factor that regulates YAP1 [38].

Several studies have shown that lncRNAs can act as enhancers for certain genes by binding to DNA or protein. The lncRNA, MAGI2-AS3, prevents the development of HCC by recruiting KDM1A and promoting H3K4me2 demethylation of the RACGAP1 gene promoter [39]. Chen et al. identified an exosome-derived lncRNA, termed lymph node metastasis-associated transcript 2 (LNMAT2), which stimulates human lymphatic endothelial cell (HLEC) tube formation and migration in vitro and enhances tumor lymphangiogenesis and LN metastasis in vivo. LNMAT2 was shown to be present at high levels in bladder cancer-derived exosomes by directly interacting with heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1). Subsequently, exosomal LNMAT2 is internalized by HLECs and epigenetically up-regulates prospero homeobox 1 (PROX1) expression by the recruitment of hnRNPA2B1 and by increasing H3K4 trimethylation levels in the PROX1 promoter, ultimately resulting in lymphangiogenesis and lymphatic metastasis [40].

Conclusion

In the present study, we identified the lncRNA, RP11-323N12.5, as the most significantly up-regulated lncRNA in human GC, based on data from the TCGA database. Mechanistically, RP11-323N12.5 promoted YAP1 transcription by binding to c-MYC in its promoter region. RP11-323N12.5 also promoted malignancy and immunosuppression in a YAP1 activation-dependent manner. Moreover, RP11-323N12.5 expression can be increased by YAP1/TAZ/TEADs. Therefore, RP11-323N12.5 is a novel lncRNA contributing to the increased YAP1 expression seen in human GC.
Fig. 7 RP11-323N12.5 promoted tumor growth and immunosuppression in vivo. a Subcutaneous tumorigenesis assay using MKN45 cells with different levels of gene expression, as indicated (n = 6 per group, left panel). Comparison of tumor volume in all three groups (right panel). b Multicolor staining of YAP1, CD4, and Foxp3 in tumor tissues. c, d Comparison of YAP1 expression and CD4+ Foxp3+ cells within tumor tissues of all three groups. e, f Comparison of Treg cells and MSDCs within tumor-infiltrating lymphocytes isolated from tumor tissues of all three groups. Each experiment was performed in triplicate. Data are presented as the mean ± SEM and were analyzed by Student’s t test (* P < 0.01)

Fig. 8 Graphic summary of RP11-323N12.5 promoted tumor growth and immunosuppression in human gastric cancer

Author contributions Conception and design: JRQ and YYL. Collection and assembly of data: JJW, FH, SX, and YXS data analysis and interpretation: JJW and FH. Contribution of reagents, materials, and analysis tools wrote the paper: JJW, FH and QHZ. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declared no competing interests.

Ethical approval Animal experiments were approved and performed in accordance with the institutional guidelines for animal care of animal ethics committee of the First People’s Hospital of Kunshan (KSL-2017017). All institutional and national guidelines for the care and use of laboratory animals were followed.

Human and animal rights All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions.

Informed consent Informed consent to be included in the study, or the equivalent, was obtained from all patients.

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