URG11 promotes gastric cancer growth and invasion by activation of β-catenin signalling pathway

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Abstract

Upregulated gene 11 (URG11), a new gene upregulated by Hepatitis B Virus X protein (HBx), was previously shown to activate β-catenin and promote hepatocellular growth and tumourigenesis. Although the oncogenic role of URG11 in the development of hepatocellular carcinoma has been well documented, its relevance to other human malignancies and the underlying molecular mechanisms remain largely unknown. Here we reported a novel function of URG11 to promote gastric cancer growth and metastasis. URG11 was found to be highly expressed in gastric cancer tissues compared with adjacent non-tumourous ones by immunohistochemical staining and western blot. Knockdown of URG11 expression by small interfering RNA (siRNA) effectively attenuated the proliferation, anchorage-independent growth, invasiveness and metastatic potential of gastric cancer cells. URG11 inhibition led to decreased expression of β-catenin and its nuclear accumulation in gastric cancer cells and extensive costaining between URG11 and β-catenin was observed in gastric cancer tissues. Transient transfection assays with the β-catenin promoter showed that it was inhibited by URG11-specific small inhibitory RNA. Moreover, suppression of endogenous URG11 expression results in decreased activation of β-catenin/TCF and its downstream effector genes, cyclinD1 and membrane type 1 matrix metalloproteinase (MT1-MMP), which are known to be involved in cell proliferation and invasion, respectively. Taken together, our data suggest that URG11 contributes to gastric cancer growth and metastasis at least partially through activation of β-catenin signalling pathway. These findings also propose a promising target for gene therapy in gastric cancer.

Keywords: URG11 ¦ proliferation ¦ invasion ¦ gastric cancer ¦ β-catenin

Introduction

Upregulated gene 11 (URG11) was identified as one of the genes upregulated in HBxAg positive HepG2 cells compared to HBxAg negative ones by PCR select cDNA subtraction [1]. Located at chromosome 11q11, URG11 encodes a 70 kD protein which consists of five Chordin-like cysteine-rich (CR) repeats (also referred to as von Willebrand factor type C (VWC) modules) and a single C-type lectin domain. These domains are of major importance in mediating cell adhesion, migration, cell/matrix interaction and immune response [2–6]. URG11 was shown to be a natural effector of HBxAg in promoting hepatocellular growth and tumourigenesis [1]. Further studies demonstrated that URG11 transcriptionally activated β-catenin promoter in Hep3B cells, resulting in the accumulation of β-catenin, and promoted β-catenin-dependent growth in serum-free medium [7]. These findings suggest that URG11 may be an oncogene operating in hepatocarcinogenesis and a regulatory element in the β-catenin signalling pathway.

β-catenin is a major structural component of cell–cell adherens junctions that are critical for establishment and maintenance of epithelial layers. In addition, it also serves as the central downstream effector of Wnt signalling that plays a key role in the regulation of growth and development [8, 9]. In the absence of Wnt signalling, the cytoplasmic level of β-catenin is kept low through

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interaction with Axin – adenomatous polyposis coli (APC) – glycogen synthase kinase (GSK) 3β complex that can phosphorylate β-catenin and target it to ubiquitin-mediated proteasomal degradation [10]. However, failure of this degradation in cells may occur as a result of gene mutation in APC, β-catenin, axin/conductin, or dysregulation of Wnt ligands [11, 12]. Consequently, the accumulated β-catenin in cytoplasm is translocated to the nucleus, where it binds to the transcription factor T-cell factor (Tcf)/lymphoid enhancer factor (Lef) and thereby activates gene transcription [13]. Numerous genes relevant for tumour formation and progression have been identified as targets of β-catenin/Tcf [14]. Thus, it is not surprising that activation of β-catenin signalling has been shown to participate in carcinogenesis of many human cancers as well as tumour invasion and metastasis. Specifically for gastric cancer, accumulating evidence has revealed the importance of β-catenin pathway activation in gastric cancer development and progression. Frequent mutations in APC have been observed in gastric adenocarcinomas, signet-ring cell carcinomas and intestinal-type gastric cancer [15, 16]. Activating β-catenin mutations have also been found in both diffuse- and intestinal-type gastric cancers [16, 17] and β-catenin nuclear localization was reported to occur in approximately one-third of gastric adenocarcinomas [17]. In addition, β-catenin mutations and nuclear accumulation are observed during late progression stage of rat stomach adenocarcinoma development [18]. More recently, Lowy et al. [19] reported that introduction of mutant β-catenin by adenoviral infection enhanced invasiveness and proliferation of infected gastric cancer cells.

As URG11 has been shown to activate β-catenin in hepatocellular carcinoma cells, it is of great interest to find out whether URG11 is implicated in gastric cancer development and progression by activating β-catenin signalling pathway. Here, we reported for the first time that URG11 was highly expressed in gastric cancer tissues compared with adjacent nontumorous tissues, and that URG11 expression was correlated with differentiation grade, TNM stage, as well as lymph node metastasis of gastric cancer. We also revealed that URG11 promoted proliferation and invasiveness of gastric cancer cells at least partially through activation of β-catenin/TCF pathway and subsequent up-regulation of cyclinD1 and membrane type 1 matrix metalloproteinase (MT1-MMP).

Materials and methods

Patients and tissue samples

For immunohistochemistry (IHC) analysis of URG11, archived formalin-fixed, paraffin-embedded samples were obtained from 100 gastric cancer patients (71 men and 29 women) who underwent curative gastrectomy between January 2004 and August 2007 at the Department of General Surgery in Xian hospital (X’an, China). Patients’ ages ranged from 36 to 75 years with a mean age of 56.4 years. None of the patients had received preoperative radiation therapy or chemotherapy. Histologically, all cases were gastric adenocarcinoma. There were 35 out of 100 patients with regional lymph node metastasis and 65 without metastasis. Histopathological features of the specimens were assessed according to the WHO classification [20] and cancer staging criteria set by the International Union Against Cancer (Union International Contre le Cancer [UICC]) [21]. For Western blotting, eight paired surgical specimens of gastric adenocarcinoma and normal gastric mucosa were obtained from patients who received curative gastrectomy at the Department of General Surgery in our hospital. Fresh specimens were immediately frozen in liquid nitrogen after surgical removal and stored at −80°C until the analysis. The study was approved by the Hospital’s Protection of Human Subjects Committee and informed consent was obtained from all patients.

Immunohistochemistry

Sections from 3–5 μm of formalin-fixed paraffin-embedded specimens were made. Slides were deparaffinized in xylene and rehydrated serially with alcohol and water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min., followed by heating in a microwave oven for epitope retrieval. The sections were then blocked in 10% normal goat serum and 0.3% Triton X-100 in phosphate buffer saline (PBS) for 1 hr and incubated overnight at 4°C with mouse anti-human URG11 monoclonal antibody 4C10 (diluted 1:1000; prepared and characterized by our laboratory [22]) or anti-β-catenin antibody (diluted 1:5000 Sigma Chemical Co., St. Louis, MO, USA). The slides were washed in PBS three times for 5 min. each. The tissues were incubated in biotin-labelled rabbit anti-mouse (1:2000, Santa Cruz) for 30 min., rinsed with PBS, and incubated with avidin–biotin–peroxidase complex for 1 hr. The signal was detected using 3,3-diaminobenzidine as the chromogen. Negative control slides using normal mouse immunoglobulin G (Vector Labs, Burlingame, CA, USA) as the primary antibody were included in all assays. The ratio of positive cells per specimen was evaluated quantitatively and scored as follows: 0 = staining of <1%; 1 = staining of 2% to 25%; 2 = staining of 26% to 50%; 3 = staining of 51% to 75%; and 4 = staining of >75% of the cells examined. Intensity was graded as follows: 0 = no signal; 1 = weak; 2 = moderate; and 3 = strong. A total score of 0 to 12 was finally calculated and graded as negative (−; score: 0–1), weak (+; score: 2–4), moderate (++; score: 5–8), and strong (+++; score: 9–12) according to staining intensity in the majority of gastric epithelium cells [23].

Cell culture

Human SV40-transformed immortal gastric epithelial cell (GES-1) and gastric cancer cell lines SGC7901, AGS, MKN45, MKN28, as described previously [24], were maintained at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco RL, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Protein preparation and Western blot

Fresh tissue blocks (0.1 mg) or harvested cells (2 × 10⁶) were put into 1.5-ml Eppendorf tubes and homogenized with 400 μl lysis buffer (50 mmol/l Tris-HCl, pH 8.0, 150 mmol/l NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate 0.02% sodium azide, 100 μg/ml PMSF, 1 μg/ml aprotinin). The tissue samples were then homogenized by ultrasonic vibration, heated in 95°C for 5 min. Cell lysates were centrifuged at 4°C for 5 min. at 10,000 rpm, and the protein-containing supernatant was
Table 1 PCR primers and reaction parameters

| Gene       | Primers                                      | PCR conditions               | Products size |
|------------|----------------------------------------------|------------------------------|---------------|
| URG11      | 5'-TGATACTAAGGGACGCCGCTGAC-3'               | (94°C/30 sec., 60°C/30 sec.,| 325 bp        |
|            | 5'-GCATCTCAGTGGACACAAAGC-3'                | 72°C/35 sec.) × 28 cycles    |               |
| β-catenin  | 5'-ATTGGTGGATGATGGCATGG-3'                  | (94°C/30 sec., 62°C/30 sec.,| 217 bp        |
|            | 5'-TTTTCTTGAAGCAGTTAATGA-3'                | 72°C/30 sec.) × 26 cycles    |               |
| cyclinD1   | 5'-CTGGAGCCCGTGAAAGAAGC-3'                 | (94°C/30 sec., 55°C/30 sec.,| 434 bp        |
|            | 5'-CTGGAGGAGGAAGGCTGAGG-3'                 | 72°C/40 sec.) × 24 cycles    |               |
| MT1-MMP    | 5'-CCAGAAGCTGAAGGATAGAAGCGG-3'             | (94°C/30 sec., 60°C/30 sec.,| 414 bp        |
| β-actin    | 5'-ATGATAGCCTGGAGGATTCTGA-3'               | (94°C/30 sec., 56°C/30 sec.,| 580 bp        |
|            | 5'-GCTGCGGTGAGATTTCTGA-3'                 | 72°C/45 sec.) × 18 cycles    |               |

removed to fresh tubes for experimental purposes. Nuclear proteins were extracted from the cell lines by Schreiber's method [25]. Briefly, the cells were collected and resuspended in 200 μl of hypotonic lysis buffer A (10 mmol/l N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid [HEPES]-KOH, pH 7.9, 1.5 mmol/l MgCl2, 10 mmol/l KCl, 0.5 mmol/l DTT, 0.2 mmol/l phenylmethylsulfonyl fluoride [PMSF]) and put on ice for 10 min. After centrifugation at 10,000 rpm at 4°C for 3 min., the nuclear protein was prepared by washing the pellet with 20 μl of hypertonic extraction buffer B (20 mmol/l HEPES-KOH, pH 7.9, 25% glycerol, 420 mmol/l NaCl, 1.5 mmol/l MgCl2, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 0.2 mmol/l PMSF) and letting it stand on ice for 20 min. with intermittent vortexing. The supernatant was collected after centrifugation at 10,000 rpm at 4°C for 5 min. Protein concentration was measured using the Bio-Rad protein assay kit (Hercules, CA, USA).

For western blot, total or nuclear proteins (60 μg) were electrophoresed on 10% SDS polyacrylamide gels and then transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). Following blocking with 10% fat-free milk in TBS (20 mmol/l Tris, 0.15 mol/l NaCl [pH 7.0], 0.1% Tween 20), the membranes were incubated with a primary antibody: anti-URG11 (rabbit) 1:1500, anti-β-catenin (Sigma Chemical Co.) diluted 1:2500 and anti-cyclinD1 (Cell Signalling Technology) diluted 1:1000, anti-MT1-MMP, anti-α-PA, anti-MMP2, anti-MMP7 and anti-MMP9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200. After repeated washing, the membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:2000. Following incubation, the membranes were washed with distilled water, stained with 0.1% Ponceau S, and destained in 10% methanol and 1% acetic acid. The membranes (Millipore, Bedford, MA, USA) were exposed to X-ray film (Kodak, Rochester, NY, USA). Relative protein levels were calculated by densitometry scanning (software: Bio Image IQ, BioImage, Ann Arbor, MI, USA). Relative protein levels were calculated by referring them to the amount of β-actin protein.

**RT-PCR**

Cells (2.0 × 10^6) were harvested and placed into 1ml Trizol Reagent (Invitrogen, Carlsbad, CA, USA) for 30 sec., followed by extraction according to the protocol provided by the manufacturer. The quality of RNA was checked by electrophoresis on a denaturing agarose gel in 3-[Nmorpholine]-propanesulfonic acid buffer, and the concentration of total RNA was determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. Total RNA (1 μg) was reversely transcribed into complementary DNA (cDNA) using the First-Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Appropriate cycles were chosen to ensure the termination of PCR amplification before reaching a stable stage in each reaction. The PCR primers and reaction parameters used for URG11 and β-catenin signalling pathway genes amplification are listed in Table 1. PCR products were loaded onto a 1.5% agarose gel and electrophoretically separated. The gel was then visualized under ultraviolet light following ethidium bromide staining. Gene expression was presented by the relative yield of the PCR product from target sequence to that from β-actin gene.

**Plasmid construction and transfection**

pSilencer3.1 (Ambion, Austin, TX, USA) was used for construction of human URG11 small interfering RNA (siRNA) vectors URG11si1 and URG11si2 according to the manufacturer’s protocol. Two pairs of oligonucleotides (U1, U2) were annealed and then subcloned into the BamHI/HindIII restriction site of pSilencer3.1, respectively. For oligo-1, S: 5’-GATCGCGAGCGAGCTGATTGAGATAACTCAAGATTTGCCAGAATCGGTGATGTGTTTTTTGAAA-3’, AS: 5’-AGCTTTTTCAAAAACAAAAGCGGATTGCTGATCAACTCTCAGTGTTGAGTTGACAGCAATCCGCGCTTGTGTTGTGGAAA-3’. pGL3-β-catenin reporter vector containing a fragment −298 to +139 of the β-catenin promoter was constructed as described [7]. All the resulting plasmids were verified by direct DNA sequencing. Synthetic siRNAs for β-catenin and nonspecific control pools were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Cell transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Briefly, cells were plated and grown to 70–90% confluence without antibiotics and then transfected with 1 μg plasmids or 200 pmol siRNA. For transient transfection, cells were harvested for further experiments after 48 hrs of transfection. For stable transfection, G418 (MKN28 600 μg/ml, SGC7901 400 μg/ml) was added into cells after 48 hrs of transfection.
Mixed clones were screened and expanded for an additional 6 weeks. Gastric cancer cell line MKN28 stably transfected with URG11si1, URG11si2 and pSilencer3.1 were designated as MKN28-sir1, MKN28-sir2 and MKN28-cont, respectively. And SGC7901 cell line stably transfected with URG11si1, URG11si2 and pSilencer3.1 were designated as SGC7901-sir1, SGC7901-sir2 and SGC7901-cont, respectively.

Monolayer growth rate

The monolayer culture growth rate was determined as described previously [26]. Briefly, cells were plated in 96-well plates (1 × 10^4 cells/well in a final volume of 200 μl) and grown under normal conditions. After 1, 2, 3, 4, 5 and 6 days of cell culture, 20 μl of 3-(4,5)-dimethylthiazol-2-yl)-5,5-diphenyltetrazolium bromide (MTT, 5 mg/ml; Sigma) was added into each well and incubated for 4 hrs at 37°C. The supernatant was then aspirated, and 150 μl of dimethyl sulfoxide (DMSO) was added to dissolve the crystals by agitation for 10 min. at room temperature. Absorbance values were determined by an ELISA reader (Bio-Rad Laboratories, Richmond, CA, USA) at a wavelength of 490 nm. Growth curves from SGC7901-cont and MKN28-cont cells were generated in parallel for comparison.

Soft agar clonogenic assay

Anchorage-independent growth was determined by soft agar clonogenic assay described previously [27]. Briefly, cells were detached and plated in 0.3% agarose with a 0.5% agarose underlay (1 × 10^5 cells/well in six-well plates). Cells were incubated at 37°C in 5% CO₂ and the number of foci (> 100 μm) was counted after 17 days.

Cell cycle analysis

The flow cytometry assay was performed by propidium iodide staining. Subconfluent cells were washed with ice-cold PBS, suspended in 0.5 ml of 70% ethanol, and then kept at 4°C for 30 min. The suspension was filtered through 50 μm nylon mesh, and the DNA content of stained nuclei was analyzed by a flow cytometer (EPICS XL, Coulter, Miami, FL, USA). The cell cycle was analyzed using Multicycle-DNA Cell Cycle Analyzed Software (FACScan, Becton Dickinson, San Jose, CA, USA).

Invasion assay

Cell invasion assays were performed as described by others [28] using Transwells (8-μm pore size, Corning Costar Corp., Cambridge, MA, USA). Matrigel (Becton Dickinson) was diluted to a concentration of 2 mg/ml, and 50 μl of this solution was placed on the lower surface of a polycarbonate filter and air-dried. After being rinsed with PBS, the filters were placed into wells and 700 μl of DMEM containing 10% bovine serum was added into the lower compartment. Freshly trypsinized and washed cells were suspended at 2 × 10^5 per ml in DMEM containing 1% bovine serum and were added to the upper chamber. After incubation for 24 hrs at 37°C, cells on the upper surface of the filter were removed with the cotton swab; cells that had invaded into the bottom surface of the filter were fixed with methanol and stained with hematoxylin. The invasive ability was determined by counting the penetrating cells under a microscope at 200 magnification on 10 random fields in each well.

Tail vein metastatic assay

All procedures for animal experimentation were performed in accordance with the Institutional Animal Care and Use Committee guidelines. Cells were trypsinized and washed three times with PBS, and single cell suspensions were prepared. Cells (2 × 10^5) in 0.1 ml of PBS were injected into the lateral tail vein of 8-week-old female BALB/c nude mice (five for each group). The mice were then monitored for overall health and total body weight. At the end of 4 weeks, all mice were sacrificed and the liver tissues were dissected out and preserved in buffered formalin. The number of visible tumours on the liver surface was counted with naked eyes before the liver tissues were made serial sections and stained with H&E.

Dual luciferase reporter assay

SGC7901 cells were plated at a density of 1.5 × 10^5 per 35 mm dish for 24 h before transfection. The plasmid pGL3-β-catenin (0.2 μg) was always transfected and pRL-TK vector (Promega, Madison, WI, USA, 0.04 μg) was used as an internal control. Cotransfection experiments were performed with 0.2, 0.4 and 0.8 μg of URG11si1 plasmids. After cultivation for 48 hrs, the transfected cells were harvested, lysed and subjected to the luciferase assay. Luciferase activity was measured as chemiluminescence in a luminometer (Perkin-Elmer, Norwalk, CT, USA) using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer’s protocol.

The TCF reporter plasmid kit, which contained the TOPflash, FOPflash, and TK vectors, was purchased from Upstate Biotechnology (Lake Placid, NY, USA). SGC7901 cells were cotransfected transiently with 0.8 μg of URG11si1 (or pSilencer3.1), 0.2 μg of TOPflash (or FOPflash) and 0.04 μg of TK vectors using Lipofectamine 2000. Twenty-four hours later, cells were collected and analyzed using the dual luciferase reporter assay system. The ratio between firefly luciferase activity (TOPflash or FOPflash) and renilla luciferase activity (TK vector) was used to estimate changes in β-catenin-mediated transcription.

Statistical analysis

Each experiment was repeated at least three times. Bands from Western blot or RT-PCR were quantified by Quantity One software (Bio-Rad). Relative protein or mRNA levels were calculated by referring them to the amount of β-actin. Numerical data are presented as mean ± SD. The difference between means was analyzed with analysis of variance (ANOVA) and then a post-hoc test. The Spearman correlation test was used to determine the correlation between URG11 and β-catenin expression. All statistical analyses were performed using SPSS11.0 software (Chicago, IL, USA). Differences were considered significant when P < 0.05.

Results

URG11 is overexpressed in gastric cancer tissues

Histological reevaluation was performed on each of the tissue blocks before further studies. Totally, 53 normal gastric mucosae,
41 premalignant lesions (26 atrophic gastritis, 15 intestinal metaplasia) and 100 primary tumour tissues from all 100 gastric adenocarcinoma patients were examined for IHC analysis. In addition, tissues of lymph node metastases are available from 25 out of 35 gastric cancers with regional lymph node metastasis and were also studied for URG11 immunostaining. It was found that URG11 was predominantly located in the cytoplasm of gastric cancer cells (Fig. 1A), which was consistent with our previous results [22]. In primary tumour tissues, 69% (69 of 100 patients) had positive staining for URG11, which was significantly higher than 39.0% (16 of 41 patients) in premalignant lesions (P < 0.01; Fig. 1A b and c) and 20.8% (11 of 53 patients) in normal gastric mucosae (P < 0.001; Fig. 1A a). Further analysis of the clinicopathological features of these gastric cancer specimens showed that the average expression of URG11 in moderately (G2, with average staining score of 4.87 ± 1.94) or poorly differentiated tumour tissues (G3, with average staining score of 5.96 ± 2.19) was significantly higher than that in well-differentiated ones (G1, with average staining score of 2.97 ± 1.56) (P < 0.001, respectively), indicating a correlation between URG11 expression and the differentiation grade of gastric cancer. URG11 overexpression was also significantly correlated with advanced TNM stage (P < 0.001).
URG11 enhances invasive and in vivo metastatic abilities of gastric cancer cells

SGC7901 and MKN28 cells were demonstrated to have invasive ability to penetrate Matrigel and metastatic ability to metastasize mainly to liver instead of lung and other organs [29]. We then evaluated the specific role of the endogenous URG11 in the invasiveness and metastasis of gastric cancer cells. As shown in Fig. 3A, URG11 siRNA transfection produced a marked inhibition of invasion in SGC7901 and MKN28 cells through Matrigel on transwell assay, with the average inhibiting rate of 41.7% and 47.5%, respectively (P < 0.01). Tail vein metastatic assay in nude mice was performed to examine the in vivo metastatic potential of SGC7901-siR1 and MKN28-siR1 cells. Compared with control cells transfected with empty vector, intravenous inoculation of SGC7901-siR1 and MKN28-siR1 cells led to significantly less visible tumours in liver surface (Fig. 3B and C P < 0.05 and P < 0.01, respectively). These data suggested that URG11 had a potential to promote metastasis of gastric cancer.

URG11 upregulates expression of β-catenin in vitro and in vivo

URG11 has been demonstrated to activate β-catenin in hepatocellular carcinoma cells, we examined if this applies in gastric cancer cells. First, we measured the total and nuclear protein levels of β-catenin in SGC7901-siR1, MKN28-siR1 and control cells. Western blotting showed that the total and nuclear protein of β-catenin was markedly downregulated in URG11si1-transfected SGC7901 and MKN28 cells compared with control cells (Fig. 4A upper panel). Furthermore, RT-PCR showed that URG11 siRNA reduced the mRNA level of β-catenin in SGC7901 and MKN28 cells (Fig. 4A lower panel). Next, we investigated whether β-catenin was transcriptionally activated by URG11 using dual luciferase reporter assay. SGC7901 cells were transiently cotransfected with pGL3-β-catenin reporter vector plus URG11si1 or pSilencer3.1 plasmid. It was shown that cotransfection with different contents of URG11si1 led to a 2.4- to 3.2-fold decrease in relative luciferase activity compared to pSilencer3.1 transfection (Fig. 4B). These

URG11 promotes gastric cancer cell proliferation and anchorage-independent growth

Because URG11 expression was stronger in gastric cancerous tissues than noncancerous tissues, we addressed its effect on gastric cancer cell growth. SGC7901 and MKN28 cells that had strongest endogenous expression of URG11 (as shown by Western blot) were selected for the experiments. We first generated genetically stable SGC7901 and MKN28 clones transfected with URG11-specific siRNA vectors (URG11s1 and URG11s2) or control plasmid as described in Materials and methods. The expression of URG11 in stably transfected cells was determined by Western blot. URG11s1 could downregulate the expression of URG11 in SGC7901 and MKN28 cells effectively, whereas the effect of URG11s2 on URG11 expression was modest (Fig. 2A). Thus, the cells stably transfected with URG11-s1 were chosen for further cellular assay. When the growth curves of these cell lines were compared in a medium containing 10% FCS (Fig. 2B), the curves for SGC7901-siR1 and MKN28-siR1 were significantly lower than control cells (pSilencer3.1-transfected cells and parental cells). Anchorage-independent growth is one of the important characteristics of in vitro tumour growth. Therefore, we examined whether URG11 knockdown would cause an inhibition of cell growth in soft agar. As shown in Fig. 2C, URG11-s1-transfected SGC7901 and MKN28 cells yielded significantly lower numbers of colonies compared to empty vector-transfected and parental cells (P < 0.01). Hence, inhibition of URG11 could attenuate gastric cancer cell growth.

To further investigate the mechanisms by which URG11 promotes cell growth, the effect of URG11-si1 on the cell cycle of SGC7901 and MKN28 cells was evaluated by flow cytometry. As seen in Fig. 2D, the percentages of SGC7901 and SGC7901-cont cells in S-phase were 47.95% and 47.80%, whereas 32.92% of SGC7901-siR1 cells were in S-phase (P < 0.01). Similarly, 46.41% of MKN28 and 48.18% of MKN28-cont cells were in S-phase compared 30.61% of MKN28-siR1 cells (P < 0.01). There was no alteration in G2-phase in URG11s1-transfected cells compared with control cells. Together, these data suggested that URG11 might promote the growth of gastric cancer cells, at least in part, through accelerating G1 to S phase transition in the cell cycle.
Table 2 Correlation of URG11 immunohistochemistry with clinicopathologic parameters

|                  | Cases tested | URG11 Immunostaining | Average score | P-value |
|------------------|--------------|-----------------------|---------------|---------|
|                  |              | – | + | ++ | +++ |               |               |
| Normal gastric mucosa | 53 | 42 | 5 | 4 | 2 | 1.59 ± 1.20 |       |
| Premalignant lesions | 41 | 25 | 7 | 5 | 4 | 2.63 ± 1.50 | *       |
| Atrophic gastritis | 26 | 17 | 4 | 3 | 2 | 2.35 ± 1.36 |       |
| Intestinal metaplasia | 15 | 8 | 3 | 2 | 2 | 3.03 ± 1.54 |       |
| Gastric cancer | 100 | 31 | 23 | 25 | 21 | 4.68 ± 2.09 | **#       |
| Gender | | | | | | |
| Male | | | | | | |
| Female | 29 | 10 | 6 | 7 | 6 | 4.54 ± 1.86 |       |
| Age | | | | | | |
| >60 | | | | | | |
| ≤60 | 59 | 19 | 15 | 13 | 12 | 4.49 ± 1.99 |       |
| Tumour size | | | | | | |
| >5.5cm | | | | | | |
| ≥5.5cm | 57 | 17 | 14 | 15 | 11 | 4.52 ± 2.02 |       |
| Depth of invasion | | | | | | |
| T1 | | | | | | |
| T2 | 22 | 7 | 5 | 5 | 5 | 4.60 ± 1.79 |       |
| T3 | 43 | 12 | 11 | 12 | 8 | 4.75 ± 1.99 |       |
| T4 | 19 | 6 | 4 | 4 | 5 | 4.92 ± 1.73 |       |
| Histopathologic grade | | | | | | |
| G1 | 32 | 16 | 8 | 5 | 3 | 2.97 ± 1.56 |       |
| G2 | 31 | 9 | 7 | 8 | 7 | 4.87 ± 1.94 |       |
| G3 | 37 | 6 | 8 | 12 | 11 | 5.96 ± 2.19 |       |
| TNM stage | | | | | | |
| I + II | 48 | 21 | 14 | 8 | 5 | 3.27 ± 1.70 |       |
| III + IV | 52 | 10 | 9 | 17 | 16 | 5.97 ± 2.26 |       |
| Lymph node metastasis | | | | | | |
| Absent | 65 | 26 | 17 | 13 | 9 | 3.74 ± 1.84 |       |
| Present | 35 | 5 | 6 | 12 | 12 | 6.41 ± 2.24 |       |
| Lymph node metastases | 25 | 4 | 5 | 7 | 9 | 6.28 ± 2.08 |       |

Abbreviation: NS, not significant.
URG11 staining was graded as negative (–; score: 0–1), weak (+; score: 2–4), moderate (++; score: 5–8), and strong (+++; score: 9–12).

* P < 0.01 in comparison with normal gastric mucosa group; ** P < 0.001 in comparison with normal gastric mucosa group; # P < 0.001 in comparison with premalignant lesion group.

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results indicated that URG11 may upregulate β-catenin by activating its promoter.

To test whether the expression of β-catenin is upregulated in vivo by URG11, tumourous and adjacent nontumorous gastric samples from 100 patients were stained for URG11, and consecutive slides were stained for β-catenin. As shown in Fig. 4C, strong membranous labelling, weak cytoplasmic staining and rare nuclear location are the typical distribution pattern of β-catenin in noncancerous mucosa, whereas reduced and even diminished membranous labelling of β-catenin were observed in 87% (87/100) of cancer tissues. β-catenin cytoplasm staining was observed in 82 (82%) of cancer tissues, and costaining with URG11 was observed in 65 (65%) of cancer tissues (P < 0.01). Nuclear β-catenin was observed in 30 (43.5%) of URG11-positive tumours, but only in 5 (16.1%) of URG11-negative tumours (P < 0.01), implying that URG11 expression is associated with the activation of β-catenin in gastric cancer (Table 3).

We then investigated the correlation of upregulated URG11 and altered β-catenin distribution in gastric cancer specimens with lymph node metastasis. The data showed that among 35 metastasis...
cases, URG11 expression was detected in 30 cases (85.7%) and cytoplasmic β-catenin accumulation in 32 cases (91.4%), in which 20 cases (57.1%) with distinct nuclear translocalization. Again, URG11 expression correlated with β-catenin cytoplasmic accumulation in 26 cases (P < 0.01) and nuclear translocalization in 18 cases (P < 0.05). Although in specimens without lymph node metastasis, URG11 expression was detected in 39 cases (60.0%) and nuclear translocalization of β-catenin only in 15 cases (23.1%), suggesting that activated β-catenin is associated with URG11-induced lymph node metastasis.

**URG11 activates β-catenin/TCF pathway and increases the expression of its downstream target genes in gastric cancer cells**

It is well known that β-catenin interacts with the LEF/TCF family of transcription factors upon activation of Wnt signalling pathway. To determine whether β-catenin had a role in gene regulation during URG11-induced gastric cancer cell proliferation and invasion, SGC7901 cells were transiently transfected with the TOPflash vector that contained wild type TCF/LEF binding sites before a luciferase reporter gene. Changes in β-catenin-mediated transcription were examined after URG11-si1 transfection. Decrease in luciferase activity was observed, probably reflecting a steady decrease in the nuclear accumulation of β-catenin (Fig. 5A). In contrast, luciferase activity remained at the background level in cells transfected with the FOPflash vector, which contained mutated TCF/LEF binding sites.

Elevated β-catenin level and accumulation of β-catenin in the nucleus could lead to activation of β-catenin/TCF pathway and its downstream target genes, such as cyclinD1, c-myc, MT-MMP1, urokinase plasminogen activator (uPA) and matrix metalloproteinase-7 (MMP-7), which have been linked to cancer growth, invasion and metastasis [30]. Therefore, we sought to determine whether these β-catenin target genes could be upregulated by URG11. As shown in Fig. 5B, the protein levels of cyclinD1 and MT1-MMP were markedly down-regulated by URG11 siRNA.
transfection in SGC7901 and MKN28 cells, whereas the uPA and MMP-7 protein levels were not altered. Furthermore, RT-PCR showed that URG11 siRNA reduced the mRNA level of cyclinD1 and MT1-MMP in SGC7901 and MKN28 cell (Fig. 5C). We also showed that URG11 had no effect on the expression level of MMP2 and MMP9 proteins that also play important roles in tumour metastasis.

/H9252-catenin signalling pathway is involved in URG11-induced growth and invasion of gastric cancer cells

To investigate whether /H9252-catenin was required for the promoting effects of URG11 on growth and invasion of gastric cancer cells, SGC7901-siR1 and SGC7901-cont cells were transiently transfected with /H9252-catenin-specific or irrelevant siRNA, and the effects on cell proliferation and invasion were determined as before. As shown in Fig. 6A, /H9252-catenin siRNA inhibited the proliferation of SGC7901-siR1 and SGC7901-cont cells. The inhibiting rate of /H9252-catenin siRNA on proliferation of SGC7901-cont cells were 26.4% (day 4) and 33.9% (day 5), respectively, higher than 14.7% (day 4) and 22.7% (day 5) of SGC7901-siR1 cells. Similarly, /H9252-catenin siRNA also caused an inhibition of the invasive ability of SGC7901-siR1 and SGC7901-cont cells. The inhibiting rate of /H9252-catenin siRNA on invasive ability of SGC7901-cont cells was 37.3%, higher than 27.1% of SGC7901-siR1 cells (Fig. 6B). These data suggested that the ability of URG11 to stimulate gastric cancer cell growth and invasion is partially dependent on the upregulated expression of /H9252-catenin.

Discussion

Gastric cancer is the second leading cause of cancer death worldwide. Although some progress has been made, the molecular pathogenesis of gastric cancer remains poorly understood, impeding the development of effective targeted therapeutic strategies [31]. Thus, searching for the molecular regulators of carcinogenesis and/or progression has been a major goal of gastric cancer research. Here, we present the first evidence that URG11 may promote gastric cancer growth and metastasis through activation of /H9252-catenin and subsequent up-regulation of cyclinD1 and MT1-MMP in gastric cancer cells.

Lian et al. [7] first identified URG11 as a natural effector of HBx and could promote the development of hepatocellular carcinoma (HCC). The authors also showed that URG11 expression was

Table 3 Correlation of clinicopathologic parameters with URG11 expression and /H9252-catenin distribution

| Cases tested          | Membrane | β-catenin immunostaining | Cytoplasm | Nucleus |
|-----------------------|----------|--------------------------|-----------|---------|
|                       | − + +/+ + | − + +/+ + + | − + +/+ + + | − + +/+ + + |
| Normal gastric mucosa | 53       | 0 4 49                   | 25 19 9   | 50 3    |
| Premalignant lesions  | 41       | 0 7 34                   | 9 16 16   | 36 5    |
| Gastric cancer        | 100      | 45 23 32                 | +/− 36 46 | +/− 65 35 +/− 26 15 20 45 |
| Lymph node metastasis |          | 0.041 0.073 0.002        |           |
| Absent                | 65       | 22 15 28                 | 15 23 27  | 50 15 |
| Present               | 35       | 23 8 4                   | 3 13 19  | 15 20 |
| TNM stage             |          | 0.064 0.043 0.016        |           |
| I + II                | 48       | 17 9 22                  | 13 16 19  | 37 11  |
| III + IV              | 52       | 28 14 10                 | 5 20 27  | 28 24 |
| URG11                 |          | 0.212 0.002 0.008        |           |
| Positive              | 69       | 33 19 17                 | 4 25 40  | 39 30 |
| Negative              | 31       | 12 4 15                  | 14 11 6  | 26 5   |

β-catenin staining was graded as negative (−; score: 0–1), weak (+; score: 2–4), moderate (++; score: 5–8), and strong (+++; score: 9–12). *P < 0.001 in comparison with normal gastric mucosa group; #P < 0.001 in comparison with premalignant lesion group; ##P < 0.01 in comparison with premalignant lesion group.
upregulated in a variety of tumours other than HCC compared to peritumour and normal, uninfected tissues, suggesting that URG11 may play a role in the pathogenesis of many human cancers, in addition to HCC. However, considering the small number of samples in each tumour type, chance or potential association cannot be ruled out. Our IHC analysis based on 100 gastric cancer cases showed that whereas only 11 out of 53 (20.8%) normal gastric mucosa had positive staining for URG11, URG11 expression was detected in 16 of 41 (39.0%) premalignant lesions, in which gastric cancer is more likely to occur than in its normal counterpart. And in gastric cancer tissues, the positive rate for URG11 staining was up to 69%. These findings suggested that URG11 might be involved in the early stage of gastric cancer carcinogenesis. Interestingly, URG11 was found to be highly expressed in metastatic gastric cancers compared to nonmetastatic ones, whereas no difference in URG11 expression was found between the primary and metastatic sites of lymph node in metastatic gastric cancer, suggesting that the alteration of URG11 expression may be one of the early determinant events of metastasis. In addition, we also assessed the expression pattern of URG11 in varied human cancers besides gastric cancer by IHC using a monoclonal antibody specific for URG11 [22]. In agreement with previous report, we found that in colon cancer, lung cancer, esophageal carcinoma and breast cancer, the expression of URG11 protein was consistently higher in tumour tissues than in adjacent nontumorous tissues (data not shown). All these data suggest that URG11 may play a critical role in the pathogenesis of a broad range of human cancers, including gastric cancer.

To further investigate the pathobiological role of URG11 in the tumour biology of gastric cancer, we introduced the URG11-specific siRNA constructs into SGC7901 and MKN28 cells and assessed the effects on cell malignant phenotype. It was shown that suppression of URG11 with URG11-siRNA inhibited the cell proliferation and anchorage-independent growth in soft agar, in accordance with that observed in HepG2 cells. Furthermore, we found that URG11 inhibition also led to reduced invasive and in vivo metastatic abilities of SGC7901 and MKN28 cells, which corroborated the results of IHC showing higher URG11 expression in metastatic gastric cancers. Thus, URG11 may play a fundamental role in facilitating the aggressive biological behaviour of gastric cancer cells.

In line with a previous report [7], we observed a close relationship between expression level of URG11 and β-catenin in gastric cancer cells and tissues. Further, URG11 was demonstrated to transcriptionally activate β-catenin promoter, resulting in the elevated expression and nuclear accumulation of β-catenin. Of note,
this finding reveals a new mechanism underlying β-catenin activation in gastric cancer cells, which are mostly caused by inactivating mutations in APC, β-catenin, axin/conductin or dysregulation of Wnt ligands. Considering the well-defined role of β-catenin signalling in regulating cancer cell proliferation and metastasis, we assumed that the promoting effects of URG11 on proliferative and invasive abilities of gastric cancer cells may at least partially be mediated by its ability to activate β-catenin. In support of this postulation, we demonstrated that β-catenin-specific but not irrelevant siRNA could inhibit the proliferation and invasion of gastric cancer cells induced by URG11. A number of genes important for tumour growth and invasion have been identified as targets of the β-catenin pathway. Thus, it is intriguing to determine which transcriptional targets might be responsible for the effects after β-catenin signalling is activated by URG11.

CyclinD1 is a major regulator of the progression of cells into the proliferative stage of the cell cycle [32]. Gene amplification and abnormal expression of cyclinD1 have been described in several human cancers [33–35]. In addition, cyclinD1 has been previously identified as a target of the β-catenin pathway [36]. Our experiment data suggested that URG11 might promote gastric cancer cell proliferation through accelerating G1 to S phase transition in the cell cycle. Moreover, we showed that cyclinD1 expression could be downregulated by URG11 siRNA. These findings suggest that URG11 may use β-catenin-cyclinD1 pathway to promote gastric cancer growth.

MT1-MMP is the first discovered member of the membrane-type MMPs (MT-MMPs) family and is frequently expressed in a variety of human tumours, including gastric cancer [37–39]. It was identified as an activator of proMMP-2 and also acts as a processing enzyme for CD44 and collagen I [40–42]. When overexpressed, MT1-MMP strongly promotes cellular invasion and lymph node metastasis [43, 44]. It has been reported that specific down-regulation of MT1-MMP expression was sufficient to cause significant inhibition of the migration and invasion of tumour cells [45]. Moreover, competitive disruption of MT1-MMP function by dominant negative mutants could prevent the metastatic spread of gastric cancer cells into the peritoneal cavity in vivo [46]. Our present work revealed that knockdown of URG11 led to decreased expression of MT1-MMP. We also demonstrated that the expression of MMP-7 and uPA, both of which are previously described β-catenin target genes, were not altered by URG11. Lowy et al. [19] reported that MT3-MMP was upregulated by β-catenin activation and is critical to β-catenin-mediated invasive phenotype in gastric cancer cells. Instead, they did not found MT1-MMP, as well as MMP-7 and MMP-26 to be upregulated in the gastric cancer cell lines tested. The discrepancy in these results may reflect the complexity of β-catenin activation and cell specificity of β-catenin target genes.

In conclusion, our findings reveal URG11 as an oncogenic factor that is critical for maintenance of such malignant cell phenotypes as anchorage-independent growth, invasion and metastasis. Evidence strongly indicates that URG11-induced cell growth and invasion are mediated, at least in part, by trans-activation of β-catenin, and subsequent up-regulation of cyclinD1 and MT1-MMP.

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