A positive feedback loop consisting of C12orf59/NF-κB/CDH11 promotes gastric cancer invasion and metastasis

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Abstract

Background: Metastasis remains the main cause of cancer-related death for gastric cancer (GC) patients, but the mechanisms are poorly understood. Using The Cancer Genome Atlas (TCGA) database and bioinformatics analyses, we identified C12orf59 might act as a potential oncogenic protein in GC.

Methods: We investigate the expression pattern and clinical significance of C12orf59 in two independent cohorts of GC samples. In the training cohort, we used the X-tile program software to generate the optimal cutoff value for C12orf59 expression in order to classify patients accurately according to clinical outcome. In the validation cohort, this derived cutoff score was applied to examine the association of C12orf59 expression with survival outcome. A series of in vivo and in vitro assays were then performed to investigate the function of C12orf59 in GC.

Results: C12orf59 was significantly upregulated, and associated with poor survival outcome in two cohorts of GC samples. Gain- and loss-of-function studies demonstrated C12orf59 promotes GC cell invasive and metastatic capacity both in vitro and in vivo, and induces epithelial–mesenchymal transition and angiogenesis. Mechanically, C12orf59 exerts oncogenic functions by up-regulating CDH11 expression via NF-κB signaling. Interestingly, CDH11 could in turn promote NF-κB bind to C12orf59’s promoter and form a positive feedback loop to sustain the metastatic ability of GC cells. Additionally, downregulation of miR-654-5p is another important mechanism for C12orf59 overexpression in GC.

Conclusion: Our finding suggested the newly identified C12orf59/NF-κB/CDH11 feedback loop may represent a new strategy for GC treatment.

Keywords: Gastric cancer, C12orf59, NF-κB, CDH11, Metastasis
Cancer Genome Atlas (TCGA) database. We found that, Chromosome 12 open reading frame 59 (C12orf59), localized on Chromosome 12p13.2, was significantly up-regulated in cancerous tissues of GC samples (Additional file 1: Figure S1a). C12orf59, a newly identified gene in kidney, was predicted to encode transmembrane proteins and associated with the RNA-binding protein HuR [8, 9]. Currently, the expression pattern and potential role of C12orf59 in human cancer types is largely unknown. Only one study reported that decreased C12orf59 expression was correlated with poor prognosis and von Hippel-Lindau (VHL) mutation in human Renal cell carcinoma (RCC) [10]. The significant aberration of C12orf59 in GC promoted us to further explore its potential oncogenic role in GC pathogenesis, although the function of C12orf59 in this disease is largely unknown.

In this study, we found that overexpression of C12orf59 correlated with poor survival prognosis of GC patients, and promoted GC cell invasion and metastasis both in vitro and in vivo. Mechanically, C12orf59 induces GC cell epithelial–mesenchymal transition (EMT) and angiogenesis by up-regulating CDH11 gene expression via NF-κB signaling. More importantly, CDH11 could in turn promote NF-κB bind to C12orf59’s promoter and form a positive feedback loop to sustain the metastatic ability of GC cells.

Methods
Patients and specimen collection
Two independent cohorts of 302 formalin-fixed paraffin-embedded (FFPE) tumor tissues and adjacent normal tissues (ANTS) of GC samples were included in present study. The training cohort was collected from 170 GC patients who underwent surgical resection from Sun Yat-Sen University Cancer Center (SYSUCC), between January 2010 and December 2011. In parallel, we obtained another validation cohort that consisted of 132 GC samples from the First Affiliated Hospital of Sun Yat-sen University, between January 2007 and May 2009. The patients enrolled were diagnosed with stage I-III GC during surgery resection, and did not receive any treatment before or after operation. The clinicopathologic characteristics of the patients in each cohort are summarized in Table 1.

In addition, eight fresh pairs of GC tissues and matched ANTs, were frozen and stored in liquid nitrogen until further use. A total of 20 pairs of primary and metastatic sites were collected.

Table 1 Association of C12orf59 expression with patient’s clinicopathological features in GC.

| Variables         | Case | Training cohort |          | Validation cohort |          | P-value |
|-------------------|------|----------------|----------|-------------------|----------|---------|
|                   |      | Low expression | High expression | Case | Low expression | High expression | |
| Age (years)       |      |                |          |                   |          |
| ≤60               | 100  | 65 (50.9%)     | 35 (49.1%) | 78                | 51 (54.5%) | 27 (45.5%) | 0.119 |
| >60               | 70   | 37 (58.1%)     | 33 (41.9%) | 0.112             | 54        | 28 (35.3%) | 26 (46.5%) | 0.53  |
| Gender            |      |                |          |                   |          |
| Male              | 109  | 64 (50.0%)     | 45 (50.0%) | 88                | 51 (50.0%) | 37 (50.0%) | 0.53  |
| Female            | 61   | 38 (63.3%)     | 23 (36.7%) | 0.648             | 44        | 28 (63.3%) | 16 (36.7%) | 0.92  |
| Tumor grade       |      |                |          |                   |          |
| G1                | 56   | 37 (61.4%)     | 17 (57.8%) | 38                | 23 (65.8%) | 15 (34.2%) | 0.288 |
| G2+3              | 114  | 63 (59.3%)     | 51 (40.7%) | 0.027             | 94        | 56 (46.7%) | 38 (53.3%) | 0.079 |
| Tumor size (cm)   |      |                |          |                   |          |
| ≤5                | 91   | 59 (51.0%)     | 32 (49.0%) | 97                | 62 (53.1%) | 35 (46.9%) | 0.017 |
| >5                | 79   | 43 (57.1%)     | 36 (42.9%) | 0.016             | 35        | 17 (55.1%) | 18 (44.9%) | 0.756 |
| T status          |      |                |          |                   |          |
| T1/2              | 37   | 25 (54.2%)     | 12 (45.8%) | 44                | 31 (50.0%) | 13 (50.0%) | 0.876 |
| T3                | 133  | 77 (54.1%)     | 56 (45.9%) | 0.288             | 88        | 48 (55.4%) | 40 (44.6%) | 0.079 |
| N status          |      |                |          |                   |          |
| N0                | 58   | 43 (34.5%)     | 15 (65.5%) | 61                | 43 (72.4%) | 18 (27.6%) | 0.001*|
| N1/2              | 112  | 59 (62.3%)     | 53 (37.7%) | 0.007*            | 71        | 36 (46.4%) | 35 (53.6%) | 0.021*|
| Clinical stage    |      |                |          |                   |          |
| I + II            | 69   | 50 (37.8%)     | 19 (62.2%) | 70                | 49 (67.6%) | 21 (32.4%) | 0.011*|
| III               | 101  | 52 (63.9%)     | 49 (36.1%) | 0.006*            | 62        | 30 (45.9%) | 32 (54.1%) | 0.011*|

a Mean age. b Mean tumor size. *Statistically significant difference
Fig. 1 (See legend on next page.)
matched metastatic GC specimens were collected from archived paraffin-embedded tissues.

All the patients were followed up on regular basis and the Overall survival (OS) time was calculated from the date of surgery to the date of the death or when censored at the latest date if patients were still alive. Samples were obtained after given informed consent in accordance with the approval by institutional ethical review board.

Cell culture
Five gastric cancer cell lines (AGS, GC-823, HGC-27, MNK-28, MNK-45), and one immortalized human gastric epithelial mucosa cell line (GES-1) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were grown in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen).

RNA isolation and quantitative real-time PCR
Total RNA was isolated using (Invitrogen, Calsbad, CA) from cultured cells and fresh tissues following the manufacturer’s instructions. Real-time PCR was performed on ABI7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) system using SYBR Green SuperMix (Roche, Basel, Switzerland). Expression data were normalized to the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6.

Cell proliferation, wound-healing, migration and invasion assays
The CCK-8 assay was performed to test cell proliferation. Wound healing assays and transwell assays were performed to detect cell migration and invasion [11, 12]. The details were described in our previous study.

Chromatin immunoprecipitation (ChIP), Western blotting analyses and immunofluorescence analyses
ChIP analysis, Western blotting analyses and immunofluorescence analyses were conducted according to the method described previously [11, 12]. The following antibodies were used in this study: C12orf59 (Novus), CDH11 (abcam), GAPDH (Cell Signaling Technology), E-cadherin (abcam), α-catenin (abcam), Vimentin (abcam), Fibronectin (abcam), NF-κB p65 (Cell Signaling Technology), p84 (Cell Signaling Technology), β-actin (R&D system), CXCR4 (abcam), FLT4 (Abanova), PTEN (Cell Signaling Technology) and TNFSF10(R&D system).

Vector construction, transfection and retroviral infection
Plasmids encoding the human 1κBα mutant (1κBα-mut) were gifts from Professor Song Libing (SYSUCC, Guangzhou, China). The following reagents were purchased from the GeneCopoeia Company (Guangzhou, China): CDH11 expression vector and its control vector; C12orf59 expression vector and its control vector; short hairpin RNA (shRNA) directed against C12orf59 and scrambled control; short interfering RNA (siRNA) specifically against p65 and scrambled control. Transfection was formed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells transfected with empty vector were used as controls. The vectors were packaged into 293FT cells using the ViraPower Mix (GeneCopoeia, Guangzhou, China). After 48 h’s culture, the lentiviral particles within the
Table 2: Univariate and Multivariate analysis of C12orf59 expression and various clinicopathological parameters in training and validation cohort patients with GC

| Variables                  | Training cohort | Validation cohort |
|----------------------------|-----------------|-------------------|
|                            | Case HR 95% CI  | P-value           | Case HR 95% CI  | P-value           |
| Univariate analysis        |                 |                   |                 |                   |
| Age ≤ 60<sup>a</sup>       | 100 1.0  | 78 1.0 |                   |                   |
| Age >60                    | 70 1.751 1.059–2.895 0.029 | 54 1.195 0.620–2.301 0.596 | 88 1.0 | 54 1.195 0.620–2.301 0.596 |
| Gender                     |                 |                   |                 |                   |
| Female                     | 109 1.0 | 88 1.0 | 44 1.316 0.673–2.574 0.422 | 88 1.0 | 54 1.195 0.620–2.301 0.596 |
| Male                       | 61 0.992 0.588–1.672 0.975 | 44 1.316 0.673–2.574 0.422 | 88 1.0 | 54 1.195 0.620–2.301 0.596 |
| WHO grade                  |                 |                   |                 |                   |
| G1/2                       | 56 1.0 | 38 1.0 |                   |                   |
| G3                         | 114 1.265 0.723–2.214 0.411 | 94 1.411 0.836–4.364 0.125 | 94 1.411 0.836–4.364 0.125 | 94 1.411 0.836–4.364 0.125 |
| Tumor size(cm)             |                 |                   |                 |                   |
| ≤ 5<sup>b</sup>           | 91 1.0 | 92 1.0 |                   |                   |
| >5                         | 79 1.758 1.060–2.916 0.029* | 54 1.195 0.620–2.301 0.596 | 92 1.0 | 92 1.0 | 92 1.0 |
| T status                   |                 |                   |                 |                   |
| T1/2                       | 37 1.0 | 44 1.0 |                   |                   |
| T3/4                       | 133 7.234 2.264–23.119 0.001* | 88 2.304 1.008–5.264 0.048* | 44 1.0 | 44 1.0 | 44 1.0 |
| N status                   |                 |                   |                 |                   |
| N0                         | 58 1 | 61 1 |                   |                   |
| N1                         | 112 3.722 1.833–7.568 0.01 | 71 3.823 1.738–8.409 0.001* | 61 1 | 61 1 | 61 1 |
| Clinical stage             |                 |                   |                 |                   |
| I + II                     | 69 1 | 70 1 |                   |                   |
| III                        | 101 4.677 2.399–9.227 < 0.001 | 62 4.308 2.022–9.180 < 0.001* | 70 1 | 70 1 | 70 1 |
| C12orf59 expression        |                 |                   |                 |                   |
| Low expression             | 102 1.0 | 79 1.0 |                   |                   |
| High expression            | 68 13.497 6.790–26.832 < 0.001* | 53 3.326 1.680–6.582 0.001* | 79 1.0 | 79 1.0 | 79 1.0 |
| Multivariate analysis      |                 |                   |                 |                   |
| Age ≤ 60<sup>a</sup>       | 100 1.0 | 78 1.0 |                   |                   |
| Age >60                    | 70 1.751 1.059–2.895 0.029 | 54 1.195 0.620–2.301 0.596 | 88 1.0 | 54 1.195 0.620–2.301 0.596 |
| T status                   |                 |                   |                 |                   |
| T1/2                       | 37 1.0 | 44 1.0 |                   |                   |
| T3/4                       | 133 7.234 2.264–23.119 0.001* | 88 2.304 1.008–5.264 0.048* | 44 1.0 | 44 1.0 | 44 1.0 |
| N status                   |                 |                   |                 |                   |
| N0                         | 58 1 | 61 1 |                   |                   |
| N1                         | 112 3.722 1.833–7.568 0.01 | 71 3.823 1.738–8.409 0.001* | 61 1 | 61 1 | 61 1 |
| Tumor size(cm)             |                 |                   |                 |                   |
| ≤ 5<sup>b</sup>           | 91 1.0 | 97 1.0 |                   |                   |
| >5                         | 79 1.758 1.060–2.916 0.029* | 54 1.195 0.620–2.301 0.596 | 97 1.0 | 97 1.0 | 97 1.0 |
| C12orf59 expression        |                 |                   |                 |                   |
| Low expression             | 102 1.0 | 79 1.0 |                   |                   |
| High expression            | 68 13.497 6.790–26.832 < 0.001* | 53 3.326 1.680–6.582 0.001* | 79 1.0 | 79 1.0 | 79 1.0 |

<sup>a</sup> Mean age. <sup>b</sup> Mean tumor size. Cox propotional hazard regression model, enter; HR Hazard ratio, CI Confidence interval; *Statistically significant difference
Fig. 2 (See legend on next page.)
supernatant were harvested, filtered by centrifugation at 500 g for 10 min, and then transfected into GC cells.

**MiR-654-5p mimic, antagonim-654-5p, and transient transfection**

The 3'-UTRs of C12orf59 were amplified and cloned downstream to the luciferase gene in a modified pGL3 control vector. MiR-654-5p mimic, antagonim-654-5p and their corresponding control oligonucleotides (Ribobio, GuangZhou, China) were transfected into GC cells cultured in six-well plates using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions.

**Human umbilical vein endothelial cells (HUVEC) tube-formation assay**

HUVECs were plated in a 24-well plate (5 × 10^3 per well) coated with Matrigel (BD Pharmingen, San Jose, CA, USA), and cultured at 37 °C in 5% CO2 for 24 h in the absence or presence of GC cell culture medium. The number of branch points of individual polygons of the capillary network was quantified in five low-power fields. Transwell assay was applied to examine the migration of HUVECs.

**Immunohistochemistry (IHC) staining assays and selecting the optimal cutoff value**

The Dako Real EnVision FLEX (K5007, Dako) was used in IHC staining to visualize protein expression with primary antibody: C12orf59 (Novus) or CDH11 (abcam). Two independent, experienced pathologists evaluated and performed the IHC scoring as follows: The intensity of staining was graded: 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. Tumor cells in five visual fields were randomly selected and scored on the basis of the percentage of positively stained cells (0–100%). The final IHC score was determined by multiplying the intensity score with the percentage of positive cells (range from 0 to 3).

To achieve the optimal cutoff value for C12orf59 IHC staining, we performed the X-tile plots analysis (Yale University School of Medicine, New Haven, CT, USA) [13]. At first, we used the X-tile program software to obtain the optimal C12orf59 IHC cutoff score to accurately classify patients according to clinical outcome in the training cohort. In the validation cohort, the cutoff score derived from X-tile analysis was investigated to test the association of C12orf59 expression with patients’ overall survival. X-tile data were presented in a right triangular grid where each point represents a different cut point. The intensity of the color of each cutoff point represents the strength of the association. The X-tile program can automatically select the optimal data cut point according to the highest chi-square value (minimum P-value) defined by Kaplan–Meier survival analysis and log-rank test [14]. X-tile plots were performed with X-tile software version 3.6.1 (Yale University School of Medicine, New Haven, CT, USA).

**Cell fractionation assay**

The cell fraction assay was carried out with the PARIS Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s instructions and p84 was used as positive control for nucleus and β-actin was used as a positive control for cytoplasm.

**Experimental in vivo metastasis model**

Six 4-week-old male BALB/c nude mice in each experimental group were injected with MKN-45/shC12orf59 or MKN-45/shcontrol, and HGC-27/C12orf59 or HGC-27/vector control cells, respectively. Briefly, 2 × 10^5 cells were injected intravenously through tail vein into each mouse in a laminar flow cabinet. Six weeks later, mice were killed and examined. All the procedures were carried out in accordance with the guidelines of the Laboratory Animal Ethics Committee of Sun Yat-Sen University.

**Luciferase reporter assay**

The wild type C12orf59 and CDH11 promoter and a promoter with mutated NF-kB-binding sites were constructed from Gene Copoeia company (Guangzhou, China), and the Cignal Finder 10-Pathway Reporter Array plates were purchased from QIAGEN company (Dusseldorf, GER). The reporter plasmids for detecting the transcriptional activity of NF-kB were generated as described previously.
Fig. 3 (See legend on next page.)
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[11]. The firefly luciferase construct was cotransfected with a control Renilla luciferase vector. Luciferase and renilla signals were measured 48 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega Corporation), according to the manufacturer’s protocol.

The cancer genome atlas (TCGA) data analysis
The Stomach Adenocarcinoma (STAD) patient clinical and RNA Sequencing data were downloaded from the Broad GDAC Firehose database (Broad Institute TCGA Genome Data Analysis Center (2016)).
C12orf59 expression levels of STADs and normal tissues were compared by paired \( t \)-test, the two patient cohorts were compared using the Kaplan-Meier method, and the Cox proportional hazards model was used to compute the hazard ratio. \( P \) value of \(< 0.05\) was considered statistically significant. For statistical assessments and plotting, R software version 3.4.3 (R Core Team (2017)) was used.

Results
C12orf59 expression is increased and associated with poor outcome in GC
Consistent with the TCGA data analysis (Additional file 1: Figure S1a), we detected that the mRNA and protein level of C12orf59 was significantly up-regulated in 8 fresh GC tumor samples, compared with paired normal tissues. We also found that C12orf59 expression was higher in five GC cells than in GES-1 (Fig. 1a).

To investigate the prognostic significance of in GC samples, we performed IHC staining for in two independent cohorts of GC samples. C12orf59 protein was stained in the cytoplasm, and significantly higher expressed in tumor tissues than in adjacent normal mucosal tissues (Fig. 1b). In the training cohort, the cut-off point (IHC score: 1.7) for dividing tumors into low-and high-expression of C12orf59 was determined using X-tile software. The optimal cut point determined by the training cohort was then applied to the validation cohort, which also identified high statistical significance again (\( P < 0.001\); Fig. 1c). According to this cut point, high C12orf59 expression was observed in 102 of 170 (60.0%) GC samples in the training cohort (\( P < 0.001\)), and 79 of 132 (59.8%) GC samples in the validation cohort. This prognostic value for C12orf59 expression levels was also confirmed using a large cohort of 415 GC patients retrieved from the TCGA database (\( P = 0.017\), hazard ratio (HR) = 1.59, 95% confidence interval (CI): (1.09–2.34), Additional file 1: Figure S1b).

In addition, high expression of C12orf59 was positively associated with N status (\( P = 0.007\); \( P = 0.012\), respectively) and overall clinical stage (\( P = 0.006\); \( P = 0.011\), respectively) both in training and validation cohorts of GC cases (Table 1), implying a potential role of C12orf59 in promoting GC metastasis. To investigate the role of C12orf59 in GC metastasis, C12orf59 expression was compared using IHC assay in 20 pairs of primary and metastatic GCs specimens. Overall, 18 pairs of GCs (90%) showed higher levels of C12orf59 expression in metastatic lesions, compared with the corresponding primary tumor samples (Fig. 1d).

Multivariate analysis showed that high expression of C12orf59 was an independent risk factor for adverse overall survival (OS) in both training (HR: 13.012; 95% CI: 6.402–26.446; \( P < 0.001\); Table 2) and validation cohorts (HR: 2.656, 95% CI: 1.311–5.380, \( P = 0.007\); Table 2). To further confirm the prognostic value of C12orf59 expression in GC, receiver operating characteristic (ROC) curves were plotted to test patient survival status. According to the ROC curve analysis, C12orf59 was found to be a promising predictor for survival status both in training (area under the curve (AUC) = 0.840; \( P < 0.001\) and validation cohort (AUC = 0.663; \( P = 0.004\); Fig. 1e).
Fig. 4 (See legend on next page.)
C12orf59 promotes GC invasion and metastasis

To explore the oncogenic role of C12orf59 in GC, we suppressed C12orf59 expression in MKN-45 and AGS cell lines that have high levels of C12orf59, and over-expressed C12orf59 expression in HGC-27 cell lines that have low level of C12orf59 (Fig. 2a). We found that C12orf59 knockdown did not influence cell proliferation (Additional file 2: Figure S2). However, suppression of C12orf59 significantly inhibited the ability of MKN-45 and AGS cells to fill the wound gap (Fig. 2b) and migrate through Transwell membranes (Fig. 2d). Also, C12orf59 inhibition largely reduced GC cell invasive ability, as measured by Boyden chamber assays (Fig. 2d). On the other side, enforced overexpression of C12orf59 in HGC-27 cells substantially increased cell migration and invasive capacity (Fig. 2c and e).

To further validate the effect of C12orf59 on tumor metastasis, in vivo metastasis assay was performed in nude mice: MKN-45/shC12orf59 or MKN-45/shControl, and HGC-27/C12orf59 or HGC-27/shControl cells were injected into nude mice via tail vein respectively. In accordance with previous results, the mice injected with MKN-45/shC12orf59 cells formed fewer nodes per lung compared with the shcontrol group (6.5 ± 2.4 versus 2.7 ± 1.2, P < 0.001), while the mice injected with HGC-27/C12orf59 cells formed more nodes per lung than HGC-27/shControl (2.2 ± 1.7 versus 7.0 ± 2.8, P < 0.001), thus we also explored whether C12orf59 is likely to be involved in GC angiogenesis using a murine tapetal vessel endothelial cell (HUVEC) model. We observed that overexpressing C12orf59 potently enhanced, while silencing C12orf59 strongly inhibited, the ability of GC cells to induce angiogenesis and migration of HUVECs (Fig. 3c and d). In addition, the microvesSEL density (MVD) (a, indicated by anti-CD31 staining) was significantly stronger in GC samples with C12orf59 high expression than GC samples with C12orf59 low expression in the training cohort of 170 samples (Additional file 3: Figure S3).

C12orf59 promotes GC cells’ migration and invasion abilities by enhancing CDH11 expression

To further determine the potential downstream targets regulated by C12orf59 in promoting GC cells invasion and/or metastasis, mRNA expression profiles of MKN-45/shC12orf59 cells were compared with that of control MKN-45/shControl cells using a Human Tumor Metastasis RT2 Profiler PCR Array containing 84 cell metastasis-related genes. As shown in Fig. 4a, three down-regulated genes (that is, CDH11, CXC4R4 and FLT4) and two upregulated genes (that is, PTEN and TNFSF10), showed a more than twofold change in mRNA levels in MKN-45/shC12orf59 cells compared with that in MKN-45/shControl cells (Additional file 4: Table S1). Subsequently, we validated that two targets CDH11 and CXC4R4 were up-regulated in protein level by western blot assay in cell after C12orf59 knockdown, but the other genes did not exhibit differential protein expression before and after depletion of C12orf59 (Fig. 4b).

Next, we try to explore whether CDH11 is involved in C12orf59-induced GC cell invasiveness and EMT. Wound-healing and Transwell assays showed that after CDH11 was introduced, the compromised migrative and
Fig. 5 (See legend on next page.)
invasive capacities of C12orf59-silenced MKN-45 cells were all largely restored (Fig. 4d and e). And meanwhile, we observed the decreased expression of epithelial markers (E-cadherin and α-catenin) and increased expression of mesenchymal markers (fibronectin and vimentin) after overexpression of CDH11 in C12orf59-silenced cells (Fig. 4f). IF staining of E-cadherin and vimentin also confirmed this result (Fig. 4g). In addition, the tube formation and migration of HUVECs were also largely strengthened when CDH11 was restored in C12orf59-silenced cells (Fig. 4h and i). The in vivo metastasis ability assay also showed that after re-introduction CDH11 in MKN-45/shC12orf59 cells, the in vivo metastasis ability was largely enhanced (Additional file 5: Figure S4).

C12orf59 promotes CDH11 expression via NF-κB dependent transcription

To identify the potential downstream pathway modulated by C12orf59, we performed Luciferase experiments using the Signal Finder 10 Pathway Reporter Arrays and Dual-Glo Luciferase Assay System. Among the ten different pathways, transcriptional activity of NF-κB pathway was compromised, predominantly when C12orf59 was overexpressed (Fig. 5a). This phenomenon promoted us to examine the influence of C12orf59 on NF-κB in GC cells. We further confirmed that, the stimulatory effect of C12orf59 on NF-κB activation was dramatically inhibited upon κBα-mut (κBα-dominant-negative mutant), which is widely known to inhibit the NF-κB pathway transfection (Fig. 5a). Moreover, subcellular fraction assays showed overexpressing C12orf59 led to stronger nuclear localization of NF-κB p65 (Fig. 5b). Collectively, these results suggest that C12orf59 plays an important role in regulation of NF-κB signaling in gastric cancer.

As NF-κB could activate transcription of specific genes and was involved in C12orf59-mediated GC pathogenesis, we thus try to investigate if C12orf59 upregulated CDH11 expression is mediated by NF-κB in GC cells. We analyzed CDH11’s promoter sequence using the PROMO algorithm and identified one putative NF-κB p65-binding site inside the putative CDH11 promoter region (Fig. 5c). As anticipated, the chromatin immunoprecipitation results showed that the enrichment of NF-κB p65 on the promoter of CDH11 was substantially enhanced in HGC-27/C12orf59 cell line. On the other hand, after knockdown of p65 by siRNA in HGC-27 cells, the enrichment of NF-κB on the CDH11 promoter was substantially reduced (Fig. 5d). Next, we cloned the putative CDH11 promoter sequence into a pGL4-basic vector and transfected into GC cell. The dual luciferase reporter assay showed that overexpression of C12orf59 dramatically enhanced the transcription activity of firefly luciferase. However, when the binding sequence was deleted, the firefly luciferase expression dropped significantly (Fig. 5e). Further functional studies showed that C12orf59-mediated enhanced CDH11 expression, migrative/invasive capacity, EMT and angiogenesis ability of HGC-27/C12orf59 cells were all prevented, when NF-κB p65 was inhibited (Fig. 5f, g, h, i, j, k and l).

Bidirectional regulation between C12orf59 and CDH11 via NF-κB in GC

To identify the upregulation mechanism of C12orf59 in GC, we performed In silico analysis, and detected that C12orf59 promoter contained putative NF-κB p65 binding sites (Fig. 6a). Additional study detected that overexpression of CDH11 in GC could enhance NF-κB-induced luciferase activity, and promote nuclear localization of NF-κB p65, suggesting CDH11 is a positive regulator of NF-κB signaling (Additional file 6: Figure S5). Thus, we tried to explore whether CDH11 could in turn up-regulate C12orf59 expression via NF-κB. The chromatin immunoprecipitation results showed that after overexpression of CDH11, more p65 proteins were recruited to the binding sites in C12orf59 promoter. When NF-κB p65 was inhibited by siRNA, the enrichment of p65 protein on C12orf59 promoter was significantly decreased (Fig. 6b). The dual-luciferase
Fig. 6 (See legend on next page.)
reporter assay revealed that ectopic expression of CDH11 activated the transcription of firefly luciferase that was driven by the wide-type C12orf59 promoter. When the binding sequence was deleted, firefly luciferase expression dropped dramatically (Fig. 6c). Furthermore, we observed that overexpression of CDH11 increased C12orf59 mRNA and protein levels in GC cells. On the other hand, C12orf59 levels were decreased when NF-κB p65 was inhibited (Fig. 6d). Thus, CDH11 could in turn enhance C12orf59 expression via NF-κB, and thus form a positive feedback loop, which sustained the metastatic and aggressive phenotype of human GC cells (Fig. 6e).

C12orf59 is negatively regulated by miR-654-5p

It is known that post-translational regulation such as microRNA (miRNA) might play critical roles in protein regulation, thus we investigated the potential miRNA regulators of C12orf59 in GC. We performed bioinformatics analyses (TARGETSCAN) and overlapped the predicted
miRNA regulators with the downregulated miRNAs from miRNA expression profiles of GC [18]. The result showed that miR-654-5p was singled out as a potential regulator of C12orf59 (Fig. 7a). Our qRT-PCR analysis showed that miR-654-5p was indeed downregulated in GC tissues and cell lines examined (Fig. 7b). In addition, overexpression of miR-654-5p decreased the luciferase activity of C12orf59 3′-UTR (Fig. 7c). Meanwhile, the C12orf59 mRNA and protein levels were all substantially reduced after overexpression of miR-654-5p in GC cells (Fig. 7d and e). However, miR-654-5p-mut failed to exert none of the above effects. These data suggested miR-654-5p directly suppress C12orf59 expression and decreased miR-654-5p might contribute to C12orf59 overexpression in GC at least partially. At last, we examined investigate the phenotypes of miR-654-5p in GC cells. After overexpression of miR-654-5p in GC cell, we detected that the invasion and metastatic ability was largely compromised (Additional file 7: Figure S6).

Discussion

In the present study, we identified the putative oncogene, C12orf59, is frequently overexpressed in GC cell lines and tissues. More importantly, high C12orf59 expression was associated with malignant clinicopathological characteristics and poor survival outcome in two independent cohorts of GC samples. Thus, the examination of C12orf59 expression by IHC could be used as an additional tool in distinguishing GC patients at high risk of metastasis and/or progression, which might provide some useful information for clinicians in optimizing individual therapy management for GC disease. As a newly identified gene, the expression pattern and clinical feature of C12orf59 in human cancers is largely unknown. In contrast to our finding, Xie reported the loss of C12orf59 expression is a common feature in Renal cell carcinoma that is correlated with increased aggressive tumor behavior and predicts poor survival outcomes of patients, suggesting a potential tumor-suppressive role of C12orf59 in this disease. [10]. These results, collectively, suggested that the biologic/clinical significance of C12orf59 expression in different human cancers may be tissue-specific. Clearly, further investigations are substantially needed to identify the expression dynamics of C12orf59 and its clinicopathological/prognostic significance in other human cancer types.

Based on the clinical finding, we carried out a series of in vitro and in vivo experiments to explore the potential mechanisms. Our results show that enforced knockdown or ectopic overexpression of C12orf59 in GC cells substantially repressed or promoted the capacities of invasion, migration, and EMT, respectively. In an experimental in vivo metastasis model, we further noticed that the tail injection of ectopic overexpression of C12orf59 in GC cells led to a significant increase in the number of lung metastatic lesions. These data supported strongly the view that C12orf59 has a crucial oncogenic role in the promotion of GC cells invasive and/or metastatic process, and C12orf59 could provide an attractive potential target for the development of novel therapeutic interventions in GC.

To gain a further insight into the downstream molecular events involving C12orf59 and GC invasion and/or metastasis, we used a Human Tumor Metastasis RT2 Profiler PCR Array containing 84 cell metastasis-related genes was used to compare mRNA expression profiles between MKN-45/shControl and MKN-45/shC12orf59 cells. We found and validated that CDH11 was the downstream target of C12orf59 in GC cells. To date, the gene functions of CDH11 in human cancers are complicated and it exhibited distant role in different tumor types. The CDH11 functioned as a metastase in certain human cancers, such as breast, pancreatic, colorectal and prostate cancers [19–23]. In glioma, osteosarcoma and cervical cancer, however, it was reported to exert tumor-suppressor functions [24–27]. Up to date, however, the expression pattern and potential biological function of CDH11 in human GC have not been elucidated. Our finding, for the first time, reported that C12orf59 enhances the binding of NF-κB to the promoter of CDH11, thereby promoting the aggressive phenotype of GC cells. Moreover, our study also found that CDH11 overexpression could in turn enhance C12orf59 expression via NF-κB. Thus, the bidirectional regulation of C12orf59 and CDH11 forms a positive feedback to promote the progression of GC cells. Previous studies have shown that positive feedback regulatory network could form a self-sustained mode which is autonomous to the original stimuli [28–30]. Thus, we speculate that, once induced by NF-κB, the C12orf59/NF-κB/CDH11 feedback loop allows GC cells to become more autonomous, which could endow GC cells with more aggressive and invasive phenotype (Fig. 6c).

Conclusion

In summary, our current report describes, for the first time, the important oncogenic role of C12orf59 in GC metastasis. Functional and mechanistic investigations showed that C12orf59 promoted GC metastasis and invasion by up-regulating CDH11 through NF-κB. Interestingly, up-regulated CDH11 could in turn enhance C12orf59 expression via NF-κB, which form a positive feedback loop, and thus sustained the metastatic and aggressive nature of human GC cells. Therefore, targeting C12orf59/NF-κB/CDH11 loop may represent a new therapeutic strategy to improve treatment and survival of GC patients.
Additional files

**Additional file 1:** Figure S1. TCGA data analysis of C12orf59 expression pattern and prognostic value in GC tissues. a. Expression of C12orf59 mRNA level in 68 paired GC and adjacent normal tissues from TCGA database. The mean level of C12orf59 expression in the GC tissues was significantly higher than in normal tissues (P < 0.001, paired t-test). b. High expression of C12orf59 in GC is correlated with poor overall survival in the TCGA gastric cancer data set. Kaplan-Meier analysis of overall survival (OS) of patients with GC for whom OS information was available in TCGA the Stomach Adenocarcinoma (STAD) data set. C12orf59-low and C12orf59-high are defined by the median value of C12orf59 expression. (DOCX 149 kb)

**Additional file 2:** Figure S2. Suppression or overexpression of C12orf59 did not alter the GC cellular growth rate. (DOCX 155 kb)

**Additional file 3:** Figure S3. The microvesSEL density (MVD), as indicated by CD31 staining was noticeably stronger in GC samples with C12orf59 high expression compared with GC samples with C12orf59 low expression in the training cohort of 170 samples. *P < 0.05. (DOCX 188 kb)

**Additional file 4:** Table S1. List of genes differentially expressed in MKN-45 cells after C12orf59 knockdown using a Human Tumor Metastasis Real-time PCR Array. (DOCX 24 kb)

**Additional file 5:** Figure S4. Restoration of CDH11 in GC cells enhanced the in vivo metastasis ability. Representative hematoxylin and eosin (H&E) staining depicting metastatic tumors in the lung of nude mice that originated from MKN-45/shC12orf59/CDH11 and MKN-45/shC12orf59/control cells injected via the tail vein (left). Number of metastatic nodules formed in the lung of mice 6 weeks after tail vein injection of MKN-45/shC12orf59/CDH11 or MKN-45/shC12orf59/control cells. Six mice per group; **P < 0.01. (DOCX 156 kb)

**Additional file 6:** Figure S5. CDH11 activates NF-κB signaling. a. Luciferase reporter assay showed NF-κB signaling pathway is activated after CDH11 was overexpressed. b. Western blotting showed increased expression level of nuclear NF-κB p65 after overexpression of CDH11 in GC cells. (DOCX 120 kb)

**Additional file 7:** Figure S6. miR-654-5p inhibited GC cell invasion and metastatic ability in vitro. a. Overexpression of miR-654-5p dramatically reduced the migratory ability of GC cells in wound healing assay. b. Overexpression of miR-654-5p resulted in a decrease in the migratory and invasive abilities of GC cells as determined by Transwell analysis. *P < 0.05. (DOCX 141 kb)

Abbreviations

AUC: Area under the curve; C12orf59: Chromosome 12 open reading frame 59; ChIP: Chromatin immunoprecipitation; CI: Confidence interval; EMT: Epithelial-mesenchymal transition; GC: Gastric cancer; HR: Hazard ratio; OS: Overall survival; ROC: Receiver operating characteristic; TCGA: The Cancer Genome Atlas

Acknowledgements

Not applicable.

Funding

The study was supported by the National Science Foundation of China (Nos. 81472514, 81772539, and 81772359), Pearl River S&T Nova Program of Guangzhou (Nos. 201806010005) and National Key R&D Program of China (Nos. 2017YFC0913000, 2016YFC1302305).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

DX and RHX conceived and designed the experiments. JXZ, SHF, DLC, and KQ contributed to the sample collection and interpretation of the data. YG, YH, ZSZ, HWW, and YM performed the statistical analysis. DX, SY and RHX revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The research protocol was reviewed and approved by the Ethical Committee and Institutional Review Board of the Sun Ya-Sen University. No informed consent (written or verbal) was obtained for use of retrospective tissue samples from the patients within this study, most of whom were deceased, since this was not deemed necessary by the Ethics Committee, who waived the need for consent. All samples were anonymous.

Consent for publication

All data during the current study is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Received: 18 November 2018 Accepted: 19 February 2019

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