

## Characterization of long non-coding RNAs and MEF2C-AS1 identified as a novel biomarker in diffuse gastric cancer



Tianhang Luo<sup>\*,1</sup>, Jiangman Zhao<sup>†,1</sup>, Zhengmao Lu<sup>\*</sup>, Jianwei Bi<sup>\*</sup>, Tao Pang<sup>\*</sup>, Hangtian Cui<sup>\*</sup>, Biao Yang<sup>\*</sup>, Wushuang Li<sup>†</sup>, Yu Wang<sup>†</sup>, Shouxin Wu<sup>†</sup> and Xuchao Xue<sup>\*</sup>

<sup>\*</sup>Department of General Surgery, Changhai Hospital, The Second Military Medical University, Shanghai, 200433, China; <sup>†</sup>Zhangjiang Center for Translational Medicine, Shanghai, Biotecon Diagnostics Co. Ltd, Shanghai 201204, China

### Abstract

Previous studies proved that long noncoding RNAs (lncRNAs) play important role in human cancer. However, the knowledge of genome scale expression of lncRNAs and their potential biological function in gastric cancer is still lacking. Next generation RNA sequencing (RNA-seq) was performed on tumor tissues and matched adjacent normal tissues of six diffuse gastric cancer (DGC) patients. Then we performed a comprehensive analysis on lncRNAs and mRNA. Fifty-eight lncRNAs were upregulated and 54 lncRNAs were downregulated in diffuse gastric cancer tissue compared with adjacent tissue. The numbers of up- and downregulated mRNAs were 306 and 161, respectively. In addition, we inferred the function of lncRNAs by construction of a co-expression network for deregulated mRNAs and lncRNAs. Co-expressed genes of MEF2C-AS1 and FENDRR were enriched to RAS and TGF-beta signaling pathway. MEF2C-AS1 and FENDRR expression were re-evaluated by Real-time Quantitative PCR in 42 DGC patients' tumor and normal tissues, and other 46 DGC patients' and 21 healthy controls' plasma. Validation data showed MEF2C-AS1 and FENDRR were significantly downregulated in tumor tissues compared with normal tissues. And decreased FENDRR are associated with aggressive tumor characteristics including more advanced stage ( $P = .030$ ), poor differentiation ( $P = .043$ ) and lymphatic metastasis ( $P = .001$ ). The expression level MEF2C-AS1 was significantly lower in DGC patients' plasma than that in healthy controls' plasma. In gastric cancer cell lines, knock-down of MEF2C-AS1 or FENDRR reduced the protein levels of *FAT3*, *NTN1* and *LYVE1* (the co-expressed genes), which were related with gastric cancer cell proliferation and invasion by previous studies. In addition, knock-down of MEF2C-AS1 or FENDRR promoted aggressive tumor behaviors in in-vitro assays. In this study, we provide a valuable resource of lncRNAs which might play important roles in the function of oncogenes or tumor suppressors affecting the development and progression of diffuse gastric cancer.

*Translational Oncology* (2018) 11, 1080–1089

### Introduction

Gastric cancer is an important health problem, being the fourth most common cancer and the second leading cause of cancer death worldwide, and it is especially prevalent in Asia [1,2]. According to the Lauren classification, gastric carcinomas are separated into two main histological types, diffuse and intestinal, in addition to the mixed and indeterminate types. [3]. Diffuse carcinomas are poorly differentiated and are composed of solitary or poorly cohesive tumor cells in the absence of gland formation [4].

Address all correspondence to: Xuchao Xue, Department of General Surgery, Changhai Hospital, 168 Changhai Road, Yangpu District, Shanghai 200433, China. or Shouxin Wu, Zhangjiang Center for Translational Medicine, Shanghai Biotecon Diagnostics Co. Ltd, 180 Zhongheng Road, Shanghai 201204, China. E-mail: [xuexch@163.com](mailto:xuexch@163.com)

<sup>1</sup>Tianhang Luo and Jiangman Zhao contributed equally to this work.

Received 11 March 2018; Revised 12 June 2018; Accepted 15 June 2018

© 2018 The Authors. Published by Elsevier Inc. on behalf of Neoplasia Press, Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1936-5233/18

<https://doi.org/10.1016/j.tranon.2018.06.007>

Long non-coding RNAs (lncRNAs) are a large class of non-protein-coding transcripts that are with more than 200 nucleotide in length. Over the past few years, lots of lncRNAs have been proved to play important roles in a large number of biological processes such as chromatin remodeling, transcription, post-transcriptional processing and intracellular trafficking [5,6]. The development of high throughput deep sequencing technology provided the possibility of a nearly comprehensive view of lncRNAs profile in cancer [7,8]. lncRNAs are aberrantly expressed in many types of cancers. A series of lncRNAs have been identified and confirmed as oncogenes or tumor suppressors [6]. Some lncRNAs, such as HOTAIR [9], H19 [10], GAPLINC [11], ANRIL [12], MALAT1 [13] are reported to be oncogenic molecules in gastric cancer. Other lncRNAs act as tumor suppressors, including GAS [14], MEG3 [15], and LEIGC [16]. Taken together, lncRNAs play a multifaceted role in gastric cancer carcinogenesis and might be novel biomarkers for gastric cancer diagnosis and prognosis, as well as provide effective therapeutic targets for gastric cancer treatment. However, the clinical value of lncRNA is still largely unknown in gastric cancer, especially in diffuse gastric cancer. Recent advance in RNA sequencing (RNA-seq) allowed researcher to comprehensively annotate and characterize lncRNA transcripts.

To better understand the roles of lncRNAs in DGC development and progression, in this study, we described a comprehensive analysis of lncRNAs in DGC tissues and respective adjacent normal tissue by RNA-seq (rRNA depleted). We found a group of lncRNAs were aberrantly expressed in six diffuse gastric cancer tissues. Then, we performed co-expression network analysis to study the function of lncRNAs. Among these, we evaluated these two lncRNAs (MEF2C-AS1 and FENDRR) expression level in tumor tissues and adjacent normal tissues from 42 DGC patients. We evaluated the associations of MEF2C-AS1 and FENDRR levels with clinicopathological characteristics. In addition, expression levels in plasma of MEF2C-AS1 and FENDRR were measured in other 46 DGC patients and 21 healthy controls. Finally, we performed knock-down of MEF2C-AS1 and FENDRR to evaluate their effects on potential target genes and aggressive tumor behaviors in in-vitro assays.

## Material and Methods

### Patients and Samples

Fresh primary DGC tumor tissues and matched normal adjacent tissues were collected from 48 pathologically confirmed DGC patients in Changhai Hospital (Shanghai, People's Republic of China) in 2016. None of them had received therapeutic procedures, for instance chemotherapy or radiotherapy. RNA-seq was performed on six DGC patients. Clinicopathological features of 6 diffuse-type gastric cancer patients for RNA-seq were shown in Table 1. Forty-two cases were recruited for validation by Real Time PCR. And Table 2

Table 1. Clinicopathological features of 6 diffuse-type gastric cancer patients for RNA-seq

Patients	Sex	age	TNM stage	Histological types	Differentiation	Location of stomach	Tumor size (cm)
DGC1	male	48	pT3N0M0	DGC	Poor	Body	2.5*2
DGC2	male	57	pT3N2M0	DGC	Poor	Antrum	3.5*2*2
DGC3	male	64	pT4aN2M0	DGC	Poor	Antrum	6*4*1.5
DGC4	male	45	pT1bN0M0	DGC	Poor	Antrum	3*2*0.6
DGC5	male	50	pT3N0M0	DGC	Poor	Cardia	3*2.5*0.5
DGC6	male	76	pT3N1M0	DGC	Poor	Body	9*5.5*2.5

Table 2. Correlation between MEF2C-AS1, FENDRR expression and different clinicopathological features in patients with diffuse-type gastric cancer

Characteristics	Total	MEF2C-AS1 expression		P value	FENDRR expression		P value
		Up	Down		Up	Down	
Age	Median 62.5 (range: 31–81)						
>60	23	7	16	0.661	8	15	0.495
≤60	19	7	12		4	15	
Gender							
Male	35	12	23	1	9	26	0.387
Female	7	2	5		3	4	
Stage							
I-II	14	6	8	0.335	7	7	0.030*
III-IV	28	8	20		5	23	
Differentiation							
Poor	21	6	15	0.513	3	18	0.043*
Moderate-well	21	8	13		9	12	
Lymphatic metastasis							
Positive	32	10	22	0.707	5	27	0.001***
Negative	10	4	6		7	3	
Location							
Cardia of stomach	16	5	11	0.894	2	14	0.313
Body of stomach	13	5	8		4	9	
Antrum of stomach	13	4	9		6	7	
Tumor size							
<5 cm	21	7	14	1	8	13	0.306
≥5 cm	21	7	14		4	17	
Invasion depth							
T1, T2	7	4	3	0.197	4	3	0.088
T3, T4	35	10	25		8	27	

Footnotes: \*P < .05, \*\*P < .01, \*\*\*P < .001.

showed clinicopathological features of 42 DGC patients. Samples were frozen immediately in liquid nitrogen and stored at -80 °C until analysis. 5 mL of morning fasting venous blood were collected from other 46 DGC patients and 21 healthy controls by EDTA anticoagulant tube, and plasma were separated immediately which were subjected to 800 × g centrifugation for 5 min and 13,000 × g centrifugation for 10 min at 4 °C, then stored at -80 °C.

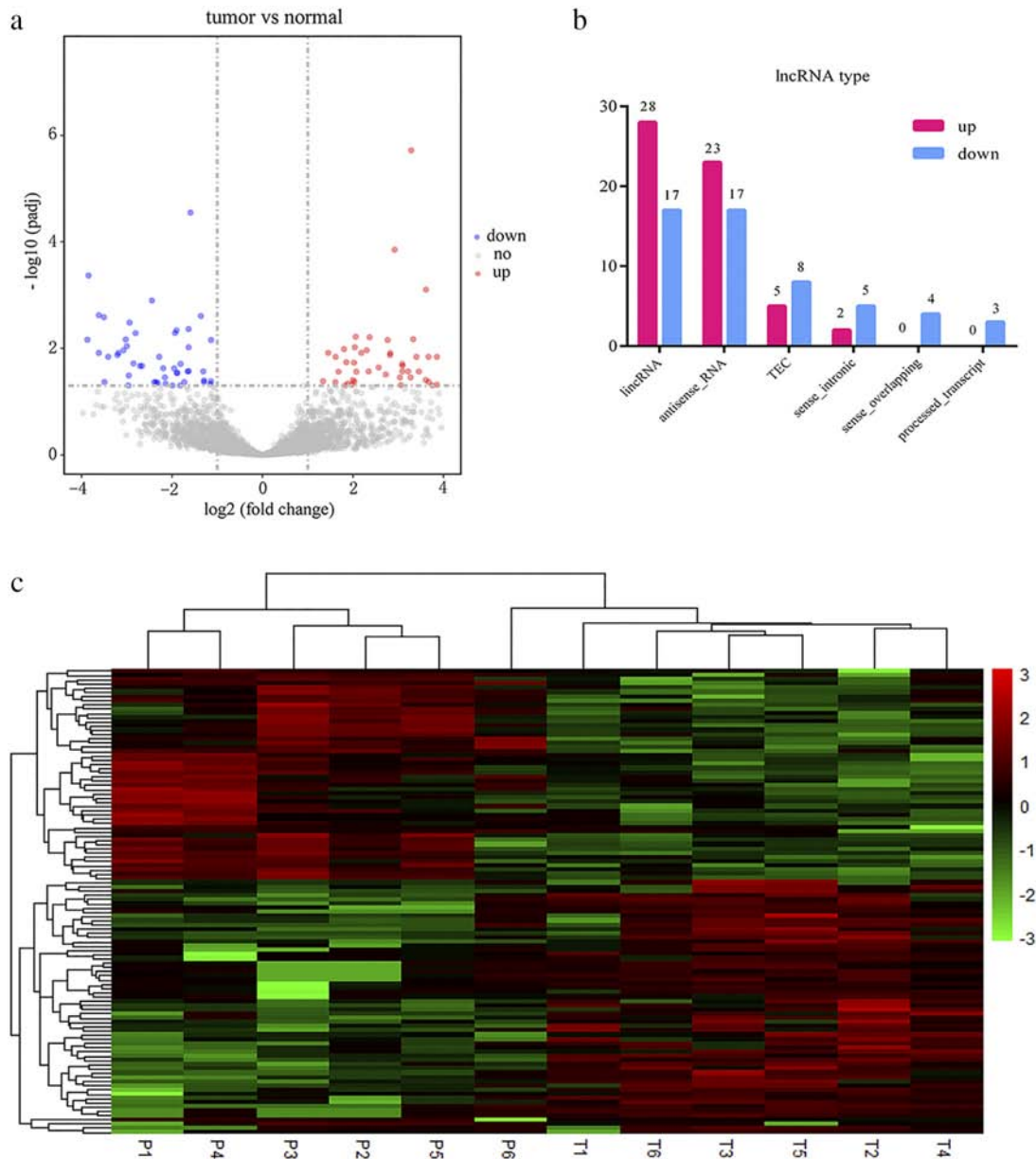
All procedures followed were in accordance with the ethical standards of Clinical Research Ethics Committee of Changhai Hospital (Approval number: CHEC2015–105) and with the Helsinki Declaration of 1964 and later versions. Informed consent was obtained from all individuals included in the study (Chinese Clinical Trial Registry, Registration number: ChiCTR-CDC-15007379).

### RNA-Seq

Total RNA from 6 paired DGC and adjacent normal tissue samples was isolated and quality controlled. The preparation of whole transcriptome libraries and deep sequencing were performed by Novogene Bioinformatics Technology Cooperation (Shanghai, People's Republic of China). Ribosomal RNA was removed and strand-specific sequencing libraries were generated following the manufacturer's instructions. RNA-Seq was performed on Illumina HiSeq 2000 platform (San Diego, CA, USA), and 100 bp paired-end reads were generated according to the Illumina's protocol.

### Bioinformatics Analysis

The clean reads after filtering low quality data were mapped to the human reference genome (Ensembl *Homo sapiens* GRCh37/hg19) using the HISAT2 program. Then transcripts were spliced and merged by StringTie. Then lncRNAs and its genome location were annotated referring to GENCODE lncRNAs.



**Figure 1.** Differentially expressed lncRNAs in diffuse gastric cancer. a Volcano plot of the differential expression analysis of lncRNAs. b Bar graph showing the number of differentially expressed lncRNAs in each category. c Heatmap of unsupervised hierarchical clustering of differentially expressed lncRNAs in all samples.

For each transcription region, a FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated to quantify its expression abundance and variations, using StringTie software. RNAs with false discovery rate (FDR) below 0.05 and absolute fold change  $\geq 2$  were considered differentially expressed by DESeq2 software between two different groups (gastric cancer vs. adjacent normal tissue).

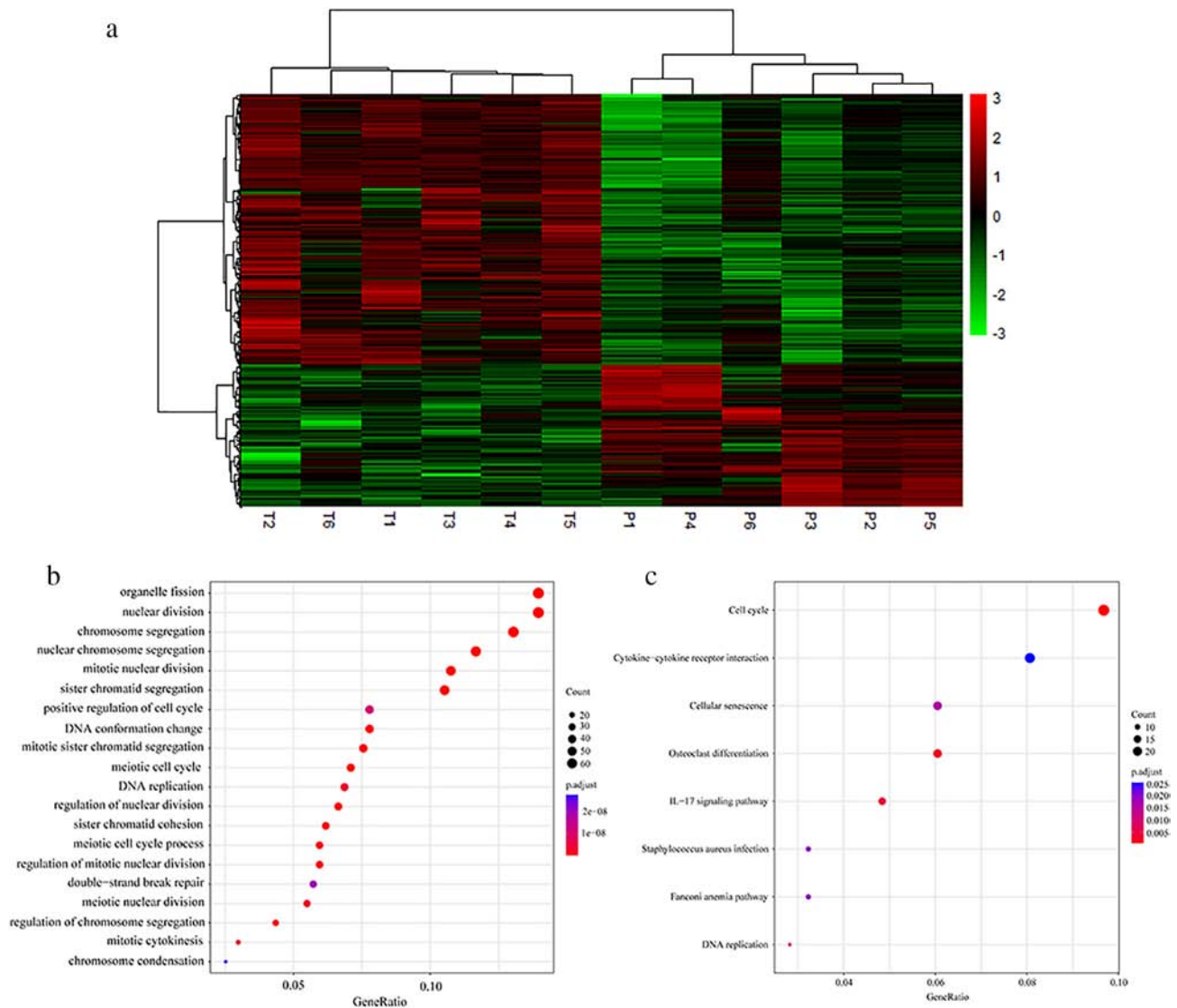
We performed gene ontology (GO) and KEGG enrichment analysis to investigate the biological significance of those differentially expressed genes by ClusterProfiler software. The 0.05 cut-off of the adjust p value was used. We used gene ontology system, which covers three domains: biological processes, molecular functions and cellular components, to annotate the genes.

We calculated Pearson correlation coefficient of expression levels between lncRNAs and protein coding RNAs. All deregulated lncRNAs and protein coding genes were considered. Then a co-

expression network of lncRNAs and protein coding RNAs was visualized using Cytoscap software, with the Pearson correlation above 0.9. We also performed Gene Ontology and KEGG pathway enrichment on the protein-coding genes of co-expression network.

#### Validation of lncRNAs by RT-qPCR

Total RNA was extracted from tissues by TRIzol reagent (Thermo Fisher Scientific), and total RNA from plasma was extracted by Bioteke Fast RNA Blood Kit (Bioteke Corporation, Beijing, China). RNA was reverse transcribed into cDNA with PrimeScrip™ RT Master Mix Kit (Takara). Real-time PCR was performed with SYBR Premix ExTaq II Kit (Takara). The qRT-PCR assays and data collection were performed on ABI 7500. Data were normalized to GAPDH levels (forward: 5'-CTCTGACTTCAACAGCGACACC-3', reverse: 5'-CTGTTGCTGTAGCCAAATTGGTT-3'). The PCR



**Figure 2.** Differentially expressed mRNA in diffuse gastric cancer. a Heatmap visualization of the patterns of change for differentially expressed genes in all samples. Pathway analysis of deregulated genes in DGC by GO (b) and KEGG (c) enrichment.

primers for MEF2C-AS1 were 5'-ACTTGTTGCCTACTATCA TACCTG-3' (forward) and 5'-ATAGCCATACAATAAGTTG CTCT-3' (reverse). The PCR primers for FENDRR were 5'-AGTAC GGAACCTTTATTACCTGCT-3' (forward) and 5'-ACCAGGATA TAATCAAACACGAGA-3' (reverse). Relative expression was calculated using the comparative cycle threshold (CT)( $2^{-\Delta\Delta C_t}$ ) method with GAPDH as the endogenous control to normalize the data.

### Cell Cultures

Gastric cancer cell lines AGS and SNU-1 from ATCC were maintained in Dulbecco's Modified Essential Medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were cultured in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C.

### Knock-Down

The small interference RNA (siRNA) against human MEF2C-AS1 (NR\_104031.1) or FENDRR (NR\_033925.1) was synthesized by GenePharma (Shanghai, China). The sequences were as follows. MEF2C-

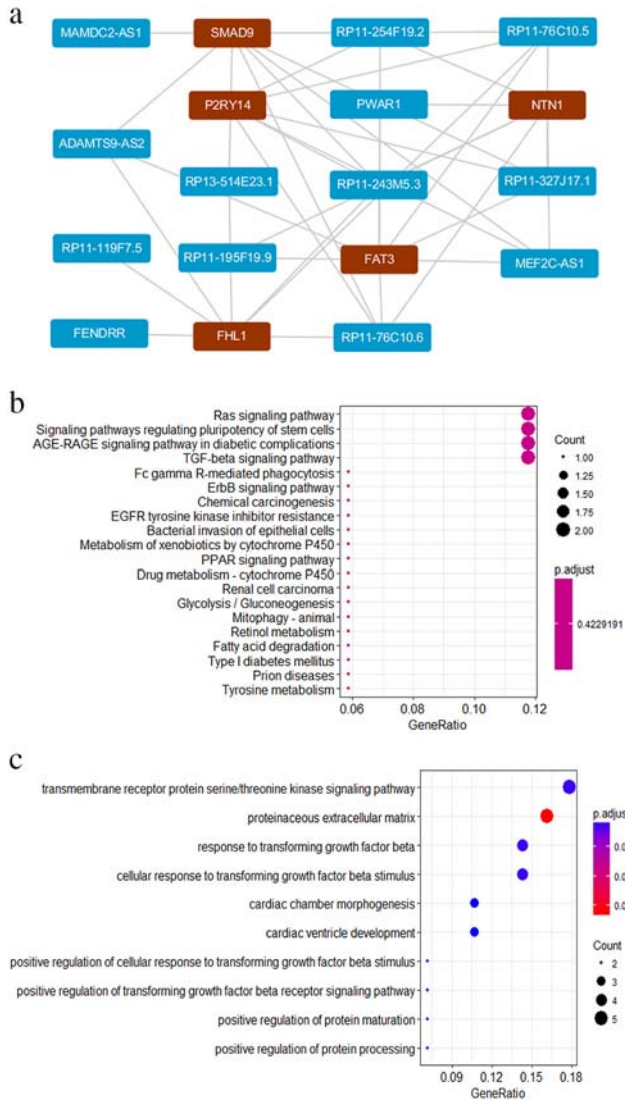
AS1 siRNA: AGCTCTCCTTCAAACCTGCCTTATCT. MEF2C-AS1 scramble: AGCTCCTAACTGTCATTCCTACTTCT. FENDRR siRNA: GAGCCGGGATTATCCTACCCTTTGA. FENDRR scramble: GAGAGGGTATTATCCTCCCTCCTGA. The siRNAs were transfected at the final concentration of 20 nM for 48 hours using Lipofectamine 2000 (Invitrogen).

### Western Blot

Proteins were extracted from cell lines using RIPA lysis buffer. Protein samples (50 µg) were resolved by SDS-PAGE and probed with FAT3, NTN1 and LYVE1 antibodies from Abcam (ab121896), Abcam (ab126729), Abcam (ab14917), respectively.

### Colony Formation

Cells lines transfected with MEF2C-AS1 or FENDRR siRNA were plated in duplicate in a six-well plate. After incubation at 37 °C for 14 days, the colonies were stained with Crystal Violet solution in methanol for 15 min.



**Figure 3.** Co-expressed genes with MEF2C-AS1 and FENDRR. a Sub-network showing important genes/lncRNAs co-expressed with MEF2C-AS1 and FENDRR (Pearson correlation above 0.95). Pathway analysis of co-expressed genes (Pearson correlation above 0.9) with MEF2C-AS1 and FENDRR by KEGG enrichment (b) and GO (c).

**MTT Assay**

Cell lines transfected with MEF2C-AS1 or FENDRR siRNA were seeded into a 96-well plate in triplicate at the concentration of  $4 \times 10^3$  cells per well. The cell growth was measured by MTT assay at day 1, 3, 5 and 7, respectively. Cells were incubated with 5 mg/ml MTT for 4 h, and subsequently solubilized in DMSO. The absorbance at 570 nm was then measured using an ELISA reader.

**Transwell Migration Assay**

Cell lines transfected with MEF2C-AS1 or FENDRR siRNA were trypsinized and resuspended as single-cell suspension. A total of  $1 \times 10^5$  cells in 0.2 mL serum-free DMEM were seeded in 8-um pore chambers inserted in a transwell apparatus (Corning). Then, 600  $\mu$ l DMEM with 10% FBS was added to the lower chamber. After incubation for 24 h at 37 °C, the cells on the top surface of the insert were removed and the cells that migrated to the bottom surface of the insert were fixed in 100% methanol and stained with 0.5% crystal violet.

**Statistical Analysis**

Statistical analyses were performed using SPSS 19.0 software (IBM, NY, USA). Differences in distributions between clinical-pathological and lncRNA expression level were assessed with Chi-square or the Fisher's exact test, as appropriate. Differences in lncRNA expression level between categorical variables groups were compared by Mann-Whitney U or Kruskal-Wallis H. Graphical plots were generated using GraphPad Prism 6.0 software (La Jolla, CA, USA), and R project. P value less than .05 was considered statistically significant. Receiver operating characteristic (ROC) curve was used to analyze the sensitivity and specificity of lncRNA expression level for distinguishing gastric cancer patients from healthy controls, with IBM SPSS statistics 19.

**Results**

**Sequencing Data and Reads Mapping**

To systematically identify lncRNAs related to DGC tumorigenesis, a total of 1.3 billion reads for 12 samples from six DGC patients were sequenced using total RNAs-seq (rRNA depleted). 668 million reads were from gastric cancer tissues, and 637 million reads were from the matched normal tissues. The normalized gene expression level was gauged by fragments per kilobase of exon per million fragments mapped (FPKM).

**Recurrently Deregulated lncRNAs in Tumors**

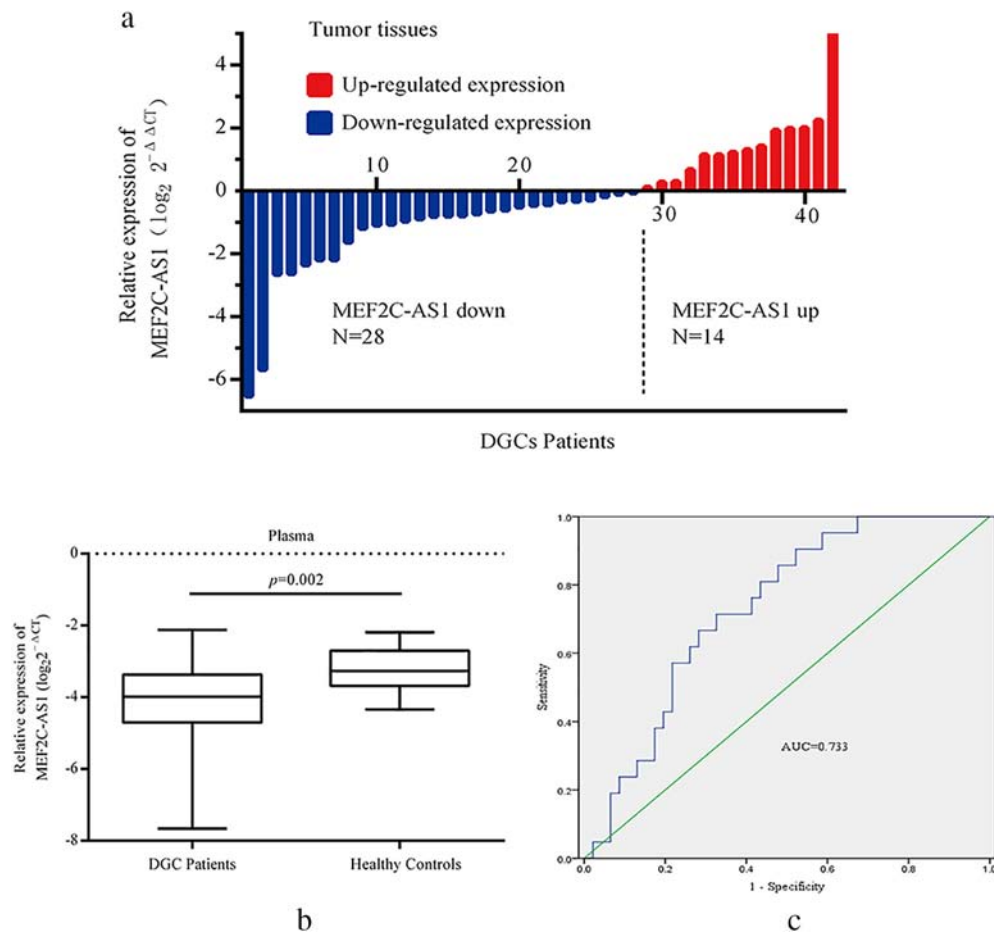
15,906 lncRNAs annotated by GENCODE were detected in our samples. And we characterized genomic location, expression abundance, transcript length for annotated lncRNAs. Firstly, different expressed lncRNAs were analyzed in six paired DGC and adjacent normal tissues. The different expressed lncRNAs were filtered by the criteria of fold change  $\geq 2$  and FDR  $< 0.05$  between cancer and adjacent normal tissues.

We identified 112 lncRNAs that were differentially expressed in tumor tissues as compared to normal gastric tissues. Of 112 deregulated lncRNAs, 58 lncRNAs were found to be upregulated and 54 to be downregulated. (Figure 1a, Supplementary Table S1). The most significant upregulated lncRNAs were AC011288.2, RP11-115D19.1 and AC006000.5. And the most significant downregulated lncRNAs were RP11-554A11.9, RP11-526P6.1 and LINC00982. Unsupervised hierarchical cluster analysis revealed two separate clusters between gastric cancer and normal tissues (Figure 1c). Then we analyzed the differentially expressed lncRNAs based on their categorizations, which were showed in Figure 1b.

**Recurrently Deregulated mRNA in Tumors**

19,753 expressed genes were detected by requiring that the FPKM value was greater than one. With the threshold of fold change  $\geq 2$  and FDR  $< 0.05$ , we totally detected 467 significantly differentially expressed genes between cancer and normal samples. The numbers of up- and downregulated genes were 306 and 161, respectively (Figure 2a, Supplementary Table S2).

The upregulated gene with the most significant expression difference between normal and cancer samples is carcinoembryonic antigen related cell adhesion molecule 6 (CEACAM6), with a 117 fold higher expression in cancer samples. This gene has been reported to be a prognostic biomarker and potential therapeutic target for gastric carcinoma [17]. And the most significant downregulated gene in cancer samples is PGA3, which encoding pepsinogen 3. PGA3 was rarely reported in gastric cancer field.



**Figure 4.** Validation results of MEF2C-AS1. a MEF2C-AS1 relative expression in 42 DGC tissues compared with adjacent normal tissues by RT-PCR. b MEF2C-AS1 expression level was significantly lower in plasma of 46 DGC patients than that of 21 healthy controls. c ROC curve of MEF2C-AS1 expression in plasma between 46 DGC patients and 21 healthy controls.

#### Functional Enrichment Analysis of Deregulated Genes

To better understand the biological function of these differentially expressed genes, GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis were performed. We found that all differentially expressed genes were categorized into 333 functional categories by GO enrichment (Supplementary Table S3). Figure 2b showed the top 20 functional categories according to gene ratio and adjust p value. The over-represented GO categories include “organelle fission”, “nuclear division” and “chromosome segregation”. All deregulated genes were categorized into 8 pathways by KEGG enrichments (Figure 2c, Supplementary Table S4). The most significantly pathways were “cell cycle”, “cytokine-cytokine receptor interaction”.

#### Co-expression Network of lncRNAs and mRNAs

To predict the potential functional and regulatory mechanisms of lncRNAs with respect to the molecular etiology of DGC, we constructed a co-expression network of protein-coding genes and lncRNAs. The co-expression network consisted of 108 deregulated lncRNAs and 356 deregulated mRNAs (Supplementary Table S5).

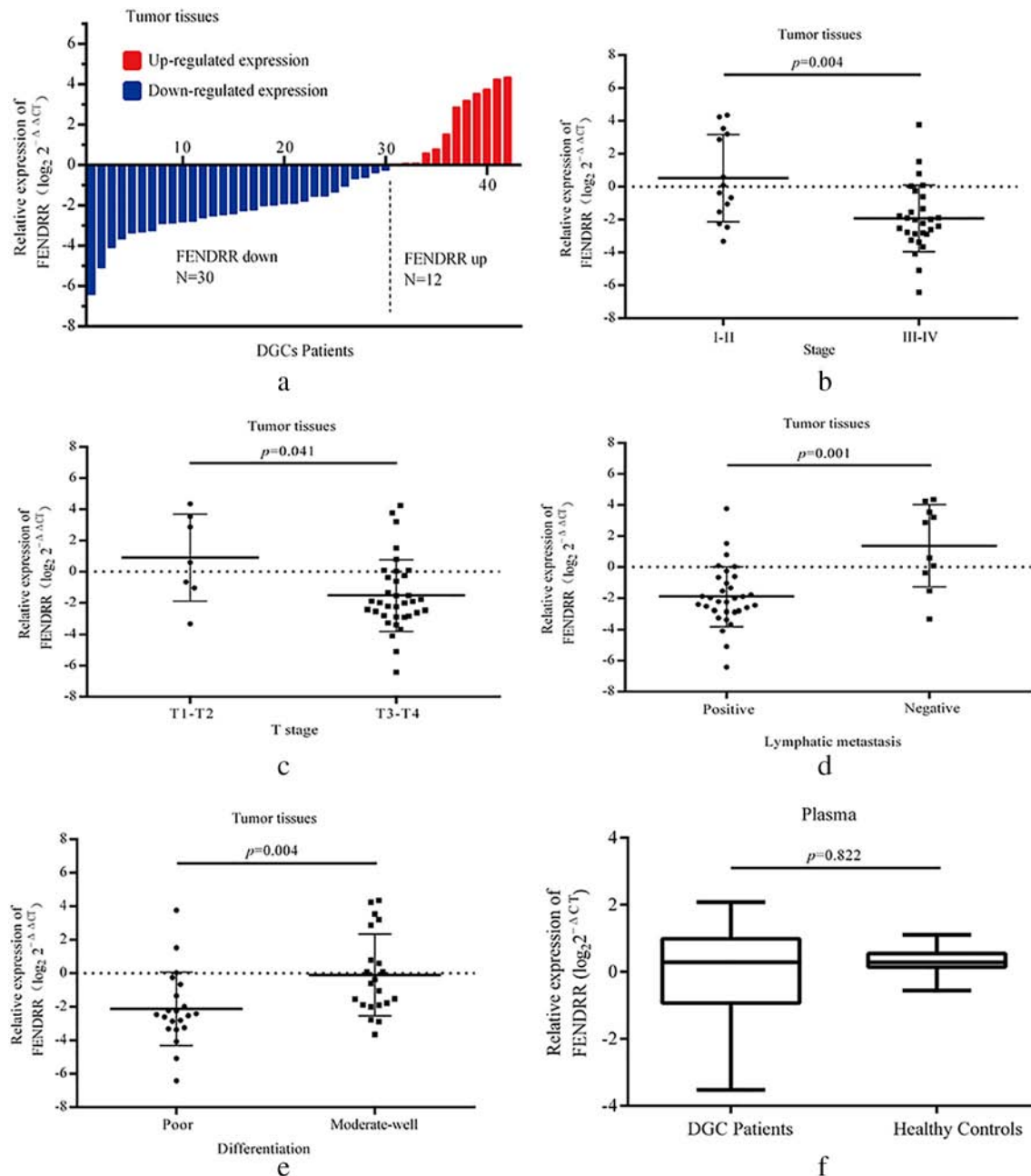
Among these, FENDRR and MEF2C-AS1 expression level were correlated with 27 and 17 protein-coding genes' expression, respectively, with Pearson correlation above 0.9. KEGG pathway enrichment analysis suggest that the protein-coding genes which were co-expressed with FENDRR and MEF2C-AS1 are mostly associated

with RAS and TGF-beta signaling pathway (Figure 3b), which have been shown to play essential roles in diverse processes, including cell proliferation, differentiation, motility and adhesion [18]. Furthermore, some gastric cancer related genes was found in FENDRR and MEF2C-AS1 co-expression protein-coding genes. For example, the decrease expression of four and a half LIM domains 1 (*FHL1*) was reported to be related to the incidence, progression, invasiveness, and metastatic potential of gastric cancer [19,20]. And *FHL1* was co-expressed with 17 lncRNAs. Another interesting gene is *NTN1*. Previous study reported netrin-1 promotes gastric cancer cell proliferation and invasion via the receptor neogenin through PI3K/AKT signaling pathway [21]. And 16 lncRNAs were co-expressed with *NTN1*. Figure 3a showed the sub-network of MEF2C-AS1, FENDRR and their co-expressed protein-coding genes with Pearson correlation above 0.95.

Considering their diverse functions in carcinogenesis, two lncRNAs (MEF2C-AS1, FENDRR) from downregulated subgroup were selected to be further studied.

#### MEF2C-AS1 Expression in Tumor Tissues and Plasma of DGC Patients

MEF2C antisense RNA 1 (MEF2C-AS1) gene is located at 5q14.3, and consists of 16 exons. MEF2C-AS1 expression levels were investigated in 42 paired gastric cancer tissues and adjacent normal tissues by qPCR. MEF2C-AS1 expression was significantly lower in



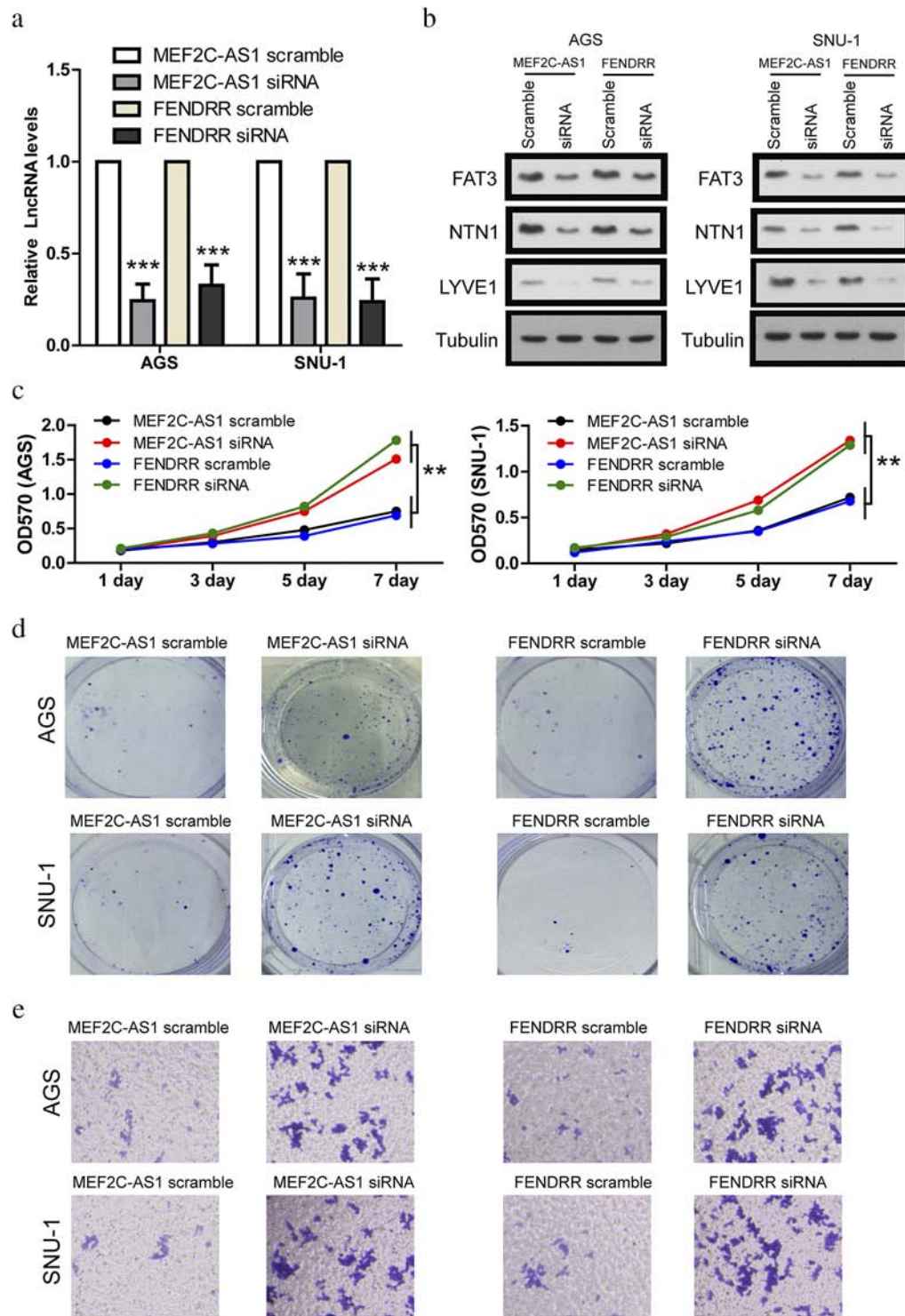
**Figure 5.** Validation results of FENDRR. a FENDRR relative expression in 42 DGC tissues compared with adjacent normal tissues by RT-PCR. FENDRR expression levels were significantly lower in III-IV stage (b), T3-T4 stage (c), positive lymphatic metastasis (d), poor differentiation (e) patients. f FENDRR expression patterns in plasma of 46 DGC patients and 21 healthy controls.

tumor tissues than that in adjacent normal tissues ( $P < .05$ ). Among 42 DGC patients, MEF2C-AS1 of 28 patients' tumors were down-regulated (Figure 4a). We analyzed the associations of MEF2C-AS1 expression levels with clinicopathological features in DGC patients' tumors. But no significant correlation was found (Table 2).

We further detected MEF2C-AS1 expression levels in plasma of 46 DGC patients and 21 healthy controls. Also, MEF2C-AS1 expression in DGC patients' plasma was significantly lower than that in healthy controls' plasma ( $P = .002$ ) (Figure 4b). ROC curve was performed for examining the expression difference of MEF2C-AS1 in DGC patients' and healthy controls' plasma. The Area Under Curve (AUC) was 0.733 (Figure 4c), which showed well sensitivity and specificity of plasma MEF2C-AS1 to distinguish DGC patients from healthy controls.

#### *FENDRR Expression in Tumor Tissues and Plasma of DGC Patients*

FOXF1 adjacent non-coding developmental regulatory RNA (FENDRR) gene is located at 16q24.1, and consists of seven exons. FENDRR expression was significantly lower in tumor tissues than that in adjacent normal tissues ( $P < .05$ ). Among 42 DGC patients, FENDRR of 30 patients' tumors were down-regulated (Figure 5a). Decreased FENDRR expression was significantly associated with more advanced stage ( $P = .030$ ), poor differentiation ( $P = .043$ ) and lymphatic metastasis ( $P = .001$ ) (Table 2). And the expression levels of III-IV stage ( $P = .004$ ), T3-T4 stage (0.041), lymphatic metastasis positive (0.001), poor differentiation (0.004) patients' tumor tissues were significantly lower than that of I-II stage, T1-T2 stage, lymphatic metastasis negative, and moderate-well differentiation



**Figure 6.** MEF2C-AS1 and FENDRR modulate target gene expression and in-vitro tumor behaviors. (a) Validation of MEF2C-AS1 and FENDRR knock-down by RT-PCR. (b) Protein levels of indicated genes measured by western blot after knock-down of MEF2C-AS1 or FENDRR. (c) Cell proliferation measured by MTT assay after knock-down of MEF2C-AS1 or FENDRR. (d) Colony formation after knock-down of MEF2C-AS1 or FENDRR. (e) Cell migration in transwell assay after knock-down of MEF2C-AS1 or FENDRR.

patients' tumor tissues. However FENDRR levels were not associated with other clinicopathological features, such as age, gender, tumor size, location and invasion depth. However, no significant difference of FENDRR expression of plasma was found between DGC patients and healthy controls ( $P = .822$ ) (Figure 5f).

#### MEF2C-AS1 and FENDRR Modulate Target Gene Expression and In Vitro Tumor Behaviors

AGS and SNU-1 cells were transfected with MEF2C-AS1 siRNA, MEF2C-AS1 scramble, FENDRR siRNA or FENDRR scramble, respectively. Cell lysates were analyzed by qRT-PCR and the results



show that endogenous MEF2C-AS1 or FENDRR was efficiently down-regulated by their siRNA (Figure 6a). The effects of MEF2C-AS1 or FENDRR knock-down on the expression of *FAT3*, *NTN1* and *LYVE1* were measured by western blot. The results showed that MEF2C-AS1 or FENDRR knock-down reduced the protein levels of *FAT3*, *NTN1* and *LYVE1* (Figure 6b). Then, we investigated the effects of MEF2C-AS1 or FENDRR knock-down on proliferation, colony formation, in-vitro migration in two gastric cell lines. The effect of MEF2C-AS1 or FENDRR knock-down on cell proliferation was measured by MTT assay and the results show that MEF2C-AS1 or FENDRR knock-down promoted cell proliferation (Figure 6c). In the colony formation assay, MEF2C-AS1 or FENDRR knock-down increased colony numbers (Figure 6d). In addition, MEF2C-AS1 or FENDRR knock-down enhanced cell migration (Figure 6e). Taken together, these results suggest that MEF2C-AS1 or FENDRR down-regulation reduced *FAT3*, *NTN1*, *LYVE1* gene expression and promoted aggressive tumor phenotypes.

## Discussion

In this study, we comprehensively analyzed the expression patterns of transcriptome of paired tissues from six diffuse gastric cancer patients using high-throughput sequencing technology. We identified 112 lncRNAs differentially expressed in diffuse gastric cancer. The identified lncRNAs were considered to represent a group of recurrently deregulated lncRNAs potentially associated with tumorigenesis. Most of these lncRNAs' function is unknown, but some have been reported to play a role in gastric cancer, including PVT1 [22], GAPLINC [11] and FENDRR [23]. For instance, high expression of PVT1 indicated a poor prognosis of gastric cancer and promotes cell proliferation [22]. In our study, the fold change of PVT1 in gastric cancer versus normal tissue is 3.48 and adjusted *P*-value of 0.0103. Notably, we identified some newly assembled lncRNA candidates potential related to DGC tumorigenesis.

In addition, 467 mRNAs were identified to be differently expressed in DGC cancer. To illustrate the function of lncRNAs, we constructed a co-expression network for deregulated lncRNAs and mRNAs. Pathway analysis revealed genes in co-expression network were enriched in cell cycle, cytokine-cytokine receptor interaction, cellular senescence, p53 signaling pathway and so on. In the co-expression network, MEF2C-AS1 and FENDRR were co-expressed with 27 and 17 protein-coding genes, respectively. These genes were enriched to RAS and TGF-beta signaling pathway. So among these co-expressed protein-coding genes, *FHL1* [19], *NTN1*, *EGR1* [24], *CITED2* [25] and *LYVE1* [26] were reported to be related with gastric cancer. In gastric cancer cell lines, knock-down of MEF2C-AS1 or FENDRR reduced the expression of *FAT3*, *NTN1* and *LYVE1*. In addition, knock-down of MEF2C-AS1 or FENDRR promoted aggressive tumor behaviors in in-vitro assays. So we speculated MEF2C-AS1 and FENDRR were involved in invasion and/or metastasis of gastric cancer. Interestingly, previous study reported decreased expression of FENDRR is associated with poor prognosis in gastric cancer and FENDRR regulates gastric cancer cell metastasis by affecting fibronectin1 expression [23]. But MEF2C-AS1 was rarely reported in gastric cancer.

Validation data showed MEF2C-AS1 and FENDRR were significantly downregulated in tumor tissues compared with normal tissues. And decreased FENDRR are associated with aggressive tumor characteristics including more advanced stage (*P* = .030), poor differentiation (*P* = .043) and lymphatic metastasis (*P* = .001), which

was agree with previous study [23]. No association was found between MEF2C-AS1 expression and clinical characteristics. While the expression level MEF2C-AS1 was significantly higher in DGC patients' plasma than that in healthy controls' plasma. There was no significant difference of FENDRR expression level in plasma between DGC patients and healthy controls. This results suggested that FENDRR might be a useful marker of prognostic and progression in DGC, while MEF2C-AS1, a marker of diagnosis and screening in DGC.

## Conclusions

Taken together, we presented an integrative analysis of transcriptome in diffuse gastric cancer. We identified a panel of recurrently deregulated lncRNAs and mRNAs that maybe potential drivers and diagnostic, therapeutic biomarkers of DGC, although further targeted validation of these lncRNAs is still needed in future. Two lncRNAs MEF2C-AS1 and FENDRR were proved to be potential biomarkers of diagnosis and prognosis, respectively. This study provided a valuable resource of further functional research of lncRNAs in DGC.

## Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 81472277, 81671886).

## Competing Interests

The authors declare that they have no competing interests.

## Acknowledgements

Not applicable.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2018.06.007>.

## References

- [1] Tan P and Yeoh KG (2015). Genetics and molecular pathogenesis of gastric adenocarcinoma. *Gastroenterology* **149**, 1153–1162.e1153.
- [2] Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, and He J (2016). Cancer statistics in China, 2015. *CA Cancer J Clin* **66**, 115–132.
- [3] Lauren P (1965). The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An Attempt at a Histo-Clinical Classification. *Acta Pathol Microbiol Scand* **64**, 31–49.
- [4] Van Cutsem E, Sagaert X, Topal B, Haustermans K, and Prenen H (2016). Gastric cancer. *Lancet* **388**, 2654–2664.
- [5] Guttman M and Rinn JL (2012). Modular regulatory principles of large non-coding RNAs. *Nature* **482**, 339–346.
- [6] Schmitt AM and Chang HY (2016). Long Noncoding RNAs in Cancer Pathways. *Cancer Cell* **29**, 452–463.
- [7] Cancer Genome Atlas Research N (2014). Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* **513**, 202–209.
- [8] Han W, Zhang Z, He B, Xu Y, Zhang J, and Cao W (2017). Integrated analysis of long non-coding RNAs in human gastric cancer: An in silico study. *PLoS One* **12**e0183517.
- [9] Zhang ZZ, Shen ZY, Shen YY, Zhao EH, Wang M, Wang CJ, Cao H, and Xu J (2015). HOTAIR long noncoding RNA promotes gastric cancer metastasis through suppression of poly r(C)-binding protein (PCBP) 1. *Mol Cancer Ther* **14**, 1162–1170.
- [10] Li H, Yu B, Li J, Su L, Yan M, Zhu Z, and Liu B (2014). Overexpression of lncRNA H19 enhances carcinogenesis and metastasis of gastric cancer. *Oncotarget* **5**, 2318–2329.
- [11] Hu Y, Wang J, Qian J, Kong X, Tang J, Wang Y, Chen H, Hong J, Zou W, and Chen Y, et al (2014). Long noncoding RNA GAPLINC regulates CD44-

- dependent cell invasiveness and associates with poor prognosis of gastric cancer. *Cancer Res* **74**, 6890–6902.
- [12] Zhang EB, Kong R, Yin DD, You LH, Sun M, Han L, Xu TP, Xia R, Yang JS, and De W, et al (2014). Long noncoding RNA ANRIL indicates a poor prognosis of gastric cancer and promotes tumor growth by epigenetically silencing of miR-99a/miR-449a. *Oncotarget* **5**, 2276–2292.
- [13] Li Y, Wu Z, Yuan J, Sun L, Lin L, Huang N, Bin J, Liao Y, and Liao W (2017). Long non-coding RNA MALAT1 promotes gastric cancer tumorigenicity and metastasis by regulating vasculogenic mimicry and angiogenesis. *Cancer Lett* **395**, 31–44.
- [14] Sun M, Jin FY, Xia R, Kong R, Li JH, Xu TP, Liu YW, Zhang EB, Liu XH, and De W (2014). Decreased expression of long noncoding RNA GAS5 indicates a poor prognosis and promotes cell proliferation in gastric cancer. *BMC Cancer* **14**, 319.
- [15] Peng W, Si S, Zhang Q, Li C, Zhao F, Wang F, Yu J, and Ma R (2015). Long non-coding RNA MEG3 functions as a competing endogenous RNA to regulate gastric cancer progression. *J Exp Clin Cancer Res* **34**, 79. doi:10.1186/s13046-015-0197-7.
- [16] Han Y, Ye J, Wu D, Wu P, Chen Z, Chen J, Gao S, and Huang J (2014). LEIGC long non-coding RNA acts as a tumor suppressor in gastric carcinoma by inhibiting the epithelial-to-mesenchymal transition. *BMC Cancer* **14**, 932.
- [17] Ru GQ, Han Y, Wang W, Chen Y, Wang HJ, Xu WJ, Ma J, Ye M, Chen X, and He XL, et al (2017). CEACAM6 is a prognostic biomarker and potential therapeutic target for gastric carcinoma. *Oncotarget* **8**, 83673–83683.
- [18] Padua D and Massague J (2009). Roles of TGFbeta in metastasis. *Cell Res* **19**, 89–102.
- [19] Xu Y, Liu Z, and Guo K (2012). Expression of FHL1 in gastric cancer tissue and its correlation with the invasion and metastasis of gastric cancer. *Mol Cell Biochem* **363**, 93–99.
- [20] Sakashita K, Mimori K, Tanaka F, Kamohara Y, Inoue H, Sawada T, Hirakawa K, and Mori M (2008). Clinical significance of loss of Fhl1 expression in human gastric cancer. *Ann Surg Oncol* **15**, 2293–2300.
- [21] Kai Y, Wang L, Xuan Z, He Z, Xia Y, Xu J, Song W, Li B, Zheng L, and Sun G (2017). Netrin-1 promotes gastric cancer cell proliferation and invasion via the receptor neogenin through PI3K/AKT signaling pathway. *Oncotarget* **8**, 51177–51189.
- [22] Kong R, Zhang EB, Yin DD, You LH, Xu TP, Chen WM, Xia R, Wan L, Sun M, and Wang ZX, et al (2015). Long noncoding RNA PVT1 indicates a poor prognosis of gastric cancer and promotes cell proliferation through epigenetically regulating p15 and p16. *Mol Cancer* **14**, 82. doi:10.1186/s12943-015-0355-8.
- [23] Xu TP, Huang MD, Xia R, Liu XX, Sun M, Yin L, Chen WM, Han L, Zhang EB, and Kong R, et al (2014). Decreased expression of the long non-coding RNA FENDRR is associated with poor prognosis in gastric cancer and FENDRR regulates gastric cancer cell metastasis by affecting fibronectin1 expression. *J Hematol Oncol* **7**, 63.
- [24] Wang T, Zhao L, Yang Y, Tian H, Suo WH, Yan M, and Fu GH (2013). EGR1 is critical for gastrin-dependent upregulation of anion exchanger 2 in gastric cancer cells. *FEBS J* **280**, 174–183.
- [25] Regel I, Merkl L, Friedrich T, Burgermeister E, Zimmermann W, Einwachter H, Herrmann K, Langer R, Rocken C, and Hofheinz R, et al (2012). Pan-histone deacetylase inhibitor panobinostat sensitizes gastric cancer cells to anthracyclines via induction of CITED2. *Gastroenterology* **143**, 99–109.e110.
- [26] Ozmen F, Ozmen MM, Ozdemir E, Moran M, Seckin S, Guc D, Karaagaoglu E, and Kansu E (2011). Relationship between LYVE-1, VEGFR-3 and CD44 gene expressions and lymphatic metastasis in gastric cancer. *World J Gastroenterol* **17**, 3220–3228.