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ORIGINAL ARTICLE

NDRG1 Controls Gastric Cancer Migration and Invasion through Regulating MMP-9

Xiaojing Chang[1,2,3] · Xiaoyang Xu[2] · Xiaoying Xue[3] · Jinguo Ma[2] · Zhenhua Li[2] · Peng Deng[2] · Jing Chen[1] · Shuanglong Zhang[2] · Yu Zhi[2] · Dongqiu Dai[1,2,4]

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Abstract The purpose of this study is to detect the clinical significance of NDRG1 and its relationship with MMP-9 in gastric cancer metastatic progression. 101 cases of gastric cancer specimens were utilized to identify the protein expression of NDRG1 and MMP-9 by immunohistochemistry, their clinical significance was also analyzed. The suppression by siRNA-NDRG1 was employed to detect the role of NDRG1 in gastric cancer progression and its relationship with MMP-9. NDRG1 expression was correlated inversely with the degree of tumor cell differentiation ($p < 0.01$), invasion depth ($p < 0.05$), lymph node metastasis ($p < 0.05$) and TNM stage ($p < 0.05$), whereas MMP-9 was positive correlated with the degree of tumor cell differentiation ($p < 0.01$), lymph node metastasis ($p < 0.05$) and TNM stage ($p < 0.05$), but not correlated with invasion depth ($p>0.05$). Furthermore, cell proliferation and invasion effect were remarkably enhanced when NDRG1 was silencing, but MMP-9 expression was increased. NDRG1 silencing enhances gastric cancer cells progression through upregulating MMP-9. It suggests that NDRG1 may inhibit the metastasis of gastric cancer via regulating MMP-9.

✉ Dongqiu Dai
daidq63@163.com

[1] Department of Gastrointestinal Surgery, The Fourth Affiliated Hospital, China Medical University, Shenyang, China

[2] Cancer Center, The Fourth Affiliated Hospital, China Medical University, Shenyang, China

[3] Department of Radiotherapy, The Second Hospital, Hebei Medical University, Shijiazhuang, China

[4] Department of Gastrointestinal Surgery and Cancer Center, The Fourth Affiliated Hospital, China Medical University, Shenyang 110032, China

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Introduction

Gastric cancer is one of the most common digestive malignancies in the world and over 70 % of new cases and deaths occur in developing countries, especially in Eastern Asia [1]. Its development is a major cause of mortality for gastric cancer patients. Therefore, inhibiting development and metastasis of gastric cancer is a critical aspect for future research.

NDRG1(N-myc downstream-regulated gene 1), a member of NDRG family which has four members, NDRG1–4 [2], is a 43 kDa protein which is ubiquitously expressed in human tissues and participates in cell growth, differentiation and organ formation [3, 4]. Being proved as tumor suppressor gene by abundant evidence, NDRG1 is involved in the metastatic progression of different types of tumors [3, 5, 6]. NDRG1 suppresses tumor growth, invasion and metastasis through arresting cell cycle, involving in p53-dependent apoptosis [7–9]. Nevertheless, NDRG1 over-expression could restore normal signaling to the cell and suppress metastasis via inhibiting the TGF-β-induced epithelial-mesenchymal transition (EMT) [10, 11]. Newly published studies reported that Wnt signaling way and ROCK1/pMLC2 pathway were involved in the NDRG1-mediated anti-metastatic process [12–14].

NDRG1 expression in prostate carcinoma tissues and gastric cancer cell line AGS is intensely closed to the expression of E-cadherin and MMP-2 [6, 15, 16]. To date, there is no report focusing on the relationship between NDRG1 and MMP-9 in gastric cancer tissues. MMP-9 belongs to MMPs family which is responsible

for degrading various components of extracellular matrix (ECM). The imbalance of MMPs results in many physiological and pathological processes, such as tumor metastasis and invasion. MMP-9 is the distinguished among MMPs, and promote tumor invasion [17, 18].

In the present study, we assessed NDRG1 and MMP-9 protein expression in 101 cases of gastric cancer specimens by immunohistochemistry, investigated their relationship and patients' clinical outcome. The suppression of NDRG1 by siRNA transfection was utilized to study the function of NDRG1 and its relationship with MMP-9 in gastric cancer progression.

Methods

Patients and Specimens

This study was supported by the institutional review board of China Medical University. All gastric cancer specimens were from 101 patients with gastric carcinoma who underwent curative resection with no chemotherapy or radiotherapy before surgery between April 2009 and January 2010 at Cancer Center of China Medical University. Specimens were collected immediately after tumor excision during surgery. All gastric cancer cases were pathologically confirmed. 30 patients were women and 71 were men.

Cell Culture and Transfection

Human Gastric cancer cell line SGC7901 was obtained from the Institute of Biochemistry and Cell Biology China Academy of Science (Shanghai, China) and maintained as recommended. Cells were cultured in RPMI 1640 (Invitrogen; USA) containing 10 % fetal calf serum and incubated in a humidified atmosphere of 5 % CO_2 at 37 °C. NDRG1-specific small interfering RNA (siRNA) and negative Control siRNA were purchased from GenePharma Co., Shanghai, China. The NDRG1 siRNA sequence was GGAGTCCTTCAACAGTTTG [19]. siRNA-Negative Control was GTTCTCCGAACGTGTCACGT. For following transfection experiments, RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA) was used according to manufacturers recommendations. NDRG1 expression was confirmed by Western Blotting.

Immunohistochemistry (IHC)

The slides (4 μm) were dewaxed, rehydrated and incubated in 0.3 % H_2O_2 for 10 min to inhibit endogenous peroxidase activity before blocking with 10 % normal goat serum (Maxin, China) for 30 min and incubating overnight at 4 °C with rabbit polyclonal antibody against NDRG1(1:100, Cell Signaling Technology, Danvers, MA, USA) and rabbit polyclonal antibody against MMP-9(1:500, Bioss, China). Slides were washed in phosphate-buffered saline (PBS), then incubated with biotinylated rabbit anti-rabbit IgG for 1h at room temperature. After incubation with avidin-biotin-peroxidase complex for 10 min, sections were stained with 3, 3-diaminobenzidine. Normal rabbit serum was used as a negative control.

The result of the immunohistochemistry for NDRG1 was judged based on the extent and intensity of staining as follows:(1) The extent of positive cells was estimated as 0 = ≤5%,1 = 6–25%,2 = 26–50%,3 = 51–75%,4 = >75%. (2) The intensity of staining was judged as 0 = achromatic, 1 = light yellow, 2 = yellow, 3 = brown. The score of the extent of positive cells was multiplied by the score of the intensity of staining, and the combined staining score as follows:(−) = 0, (+) = 1–4, (++) = 5–8, (+++) = 9–12 [20]. This semi-quantitative analysis was done by two indepent assessors without prior knowledge of the patient outcome.

Western Blotting

Whole-cell lysates were prepared from transfected cells. Samples were loaded onto 10 % SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore Corp, Bedford, MA, USA) and incubated with primary anti-NDRG1(1:1000, Cell Signaling Technology, Danvers, MA, USA), MMP-9(1:500, Bioss, Beijing, China) and GAPDH antibody overnight at 4°C. The next day, the membranes were washed with TBS thrice and incubated with a horseradish peroxidase-conjugated antibody against rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at room temperature and immunoreactive protein bands visualized with a ECL detection kit (Thermol Biotech Inc., Rockford, IL, USA). Each experiment was repeated three times.

Cell Inhibition Analyse

SGC7901 cells (3×10^3) were plated in per well of 96-well culture plates. After overnight incubation, SGC7901 cells were transfected with siRNA-NDRG1 or siRNA-Negative Control for 48 h, the untreated group as control. At the last 6 h of incubation, 10 μl Alamar Blue (Beyotime, China) was directly added to the medium resulting in a final concentration of 10%. The plate was re-incubated at 37°C 5 % CO_2 for 6h. The absorbance of 96-well plate was read at 570 and 600nm with a standard spectrophotometer. The number of viable cells correlates with the magnitude of dye reduction and is expressed as percentage of AB

reduction. The calculation of the percentage of AB reduction (%AB reduction) as follows [21]:

$$\%AB = (\varepsilon_{ox}\lambda_2)\,(A\lambda_1) - (\varepsilon_{ox}\lambda_1)\,(A\lambda_2) \Big/ (\varepsilon_{red}\lambda_1)\,(A'\lambda_2) - (\varepsilon_{red}\lambda_2)(\,A'\lambda_1) \times 100$$

In the formula, $\varepsilon\lambda_1$ and $\varepsilon\lambda_2$ are constants representing the molar extinction coefficient of AB at 570 and 600 nm, respectively, in the oxidized (εox) and reduced (εred) forms. $A\lambda_1$ and $A\lambda_2$ represent absorbance of test wells at 570 and 600 nm, respectively. $A'\lambda_1$ and $A'\lambda_2$ represent absorbance of negative control wells at 570 and 600 nm, respectively. The values of %AB reduction were corrected for background values of negative controls containing medium without cells.

Matrigel Invasion Assay

SGC7901 cells (1.0×10^4) in 200ul serum-free 1640 were seeded onto Matrigel-coated upper chambers of filter which was 8-μm pore size transwell chamber (Corning Life Sciences, Corning, NY, USA). In the lower chamber, 500 μl RPMI 1640 (Invitrogen, USA) containing 10 % fetal calf serum (FCS) was added as a chemoattractant. After 48 h of incubation, non-invading cells on the upper chambers were removed with a cotton-tipped swab, the lower side of the filters were fixed with 4% paraformaldehyde for 30 min and stained with methylrosanilinium chloride. The invaded cells were viewed under an Olympus microscope and counted in five fields of view at 200× magnification. The invasion ability of the cancer cells was expressed as the mean number of cells in five fields. The assay was carried out as three independent experiments.

Statistics

X^2-test, Fisher's exact test and the student's t-test were used for statistical analysis. SPSS ver.17.0 (SPSS, Chicago, IL, USA) was used throughout and *p*-values of <0.05 was considered significant.

Results

The Expression Status of NDRG1 and MMP-9 in Gastric Cancer

Results of immunohistochemistry showed both NDRG1 and MMP-9 protein expression were predominantly expressed in the cytoplasm of gastric cancer tissues. The positive staining of NDRG1 in gastric cancer tissues tended to decreased from well differentiation to poor differentiation, but it was increased for MMP-9 (Fig. 1). As shown in Table 1, the gastric cancer patients tended to decrease from negative to strong positive staining (+++) of NDRG1, whereas the gastric cancer patients increased from negative to strong positive (+++) of MMP-9. A significant correlation was found between the expression of NDRG1 and MMP-9 (Fig. 1, $p<0.001$).

The association of NDRG1 and MMP-9 expression with the clinicopathological parameters of gastric cancer patients were shown in Table 2. We found NDRG1 expression was significantly inverse correlated with the degree of tumor cell differentiation ($p < 0.001$), invasion depth ($p = 0.013$), lymph node metastasis ($p = 0.026$) and TNM stage ($p = 0.032$), whereas MMP-9 was statistically significantly positive correlated with the degree of tumor cell differentiation ($p < 0.001$), lymph node metastasis ($p < 0.001$) and TNM stage ($p = 0.044$), but not correlated with invasion depth ($p = 0.548$).

Results showed NDRG1 expression was highest in TNM grade I and lowest in grade IV, but the expression of MMP-9 was highest in TNM grade IV and lowest in grade I.

We also analyzed whether the expression of NDRG1 and MMP-9 were affected by other factors. However, there was no significant association between NDRG1, MMP-9 and the gender or age of the patients (Table 2).

The Effect of GC Cell Proliferation and Invasion after NDRG1-Silencing

To further examine the function of NDRG1 in GC cells, we used NDRG1-specific small interfering RNA to silencing NDRG1 expression in the highly metastatic SGC7901 cell line which expressed relatively higher level of NDRG1. Western blotting conformed NDRG1 was effectively down-regulated after transfection for 48h. The relationship between NDRG1 and MMP-9 was also further detected. Results showed the expression of MMP-9 was increased when NDRG1 was knocked down (Fig. 2a).

NDRG1-Silencing Enhances GC Cell Proliferation

Alamar Blue showed the reduction of SGC7901 cells that transfected with siRNA-NDRG1 was 86.58%, while it was 52.86% and 51.52% in transfected with siRNA- Negative Control and untreated group, respectively (Fig. 2b). The difference was significant ($p < 0.05$). The proliferation of gastric cancer cells was enhanced when NDRG1 was silencing.

NDRG1-Silencing Enhances GC Cell Invasion

Matrigel invasion assay showed the number of invading SGC7901 cells was significantly accelerated after transfected with siRNA-NDRG1(151.4 ± 6.88) compared with the siRNA-Negative Control transfected group (66.8 ± 5.17) and the control group (68.2 ± 4.55)($p < 0.05$) (Fig. 3). The

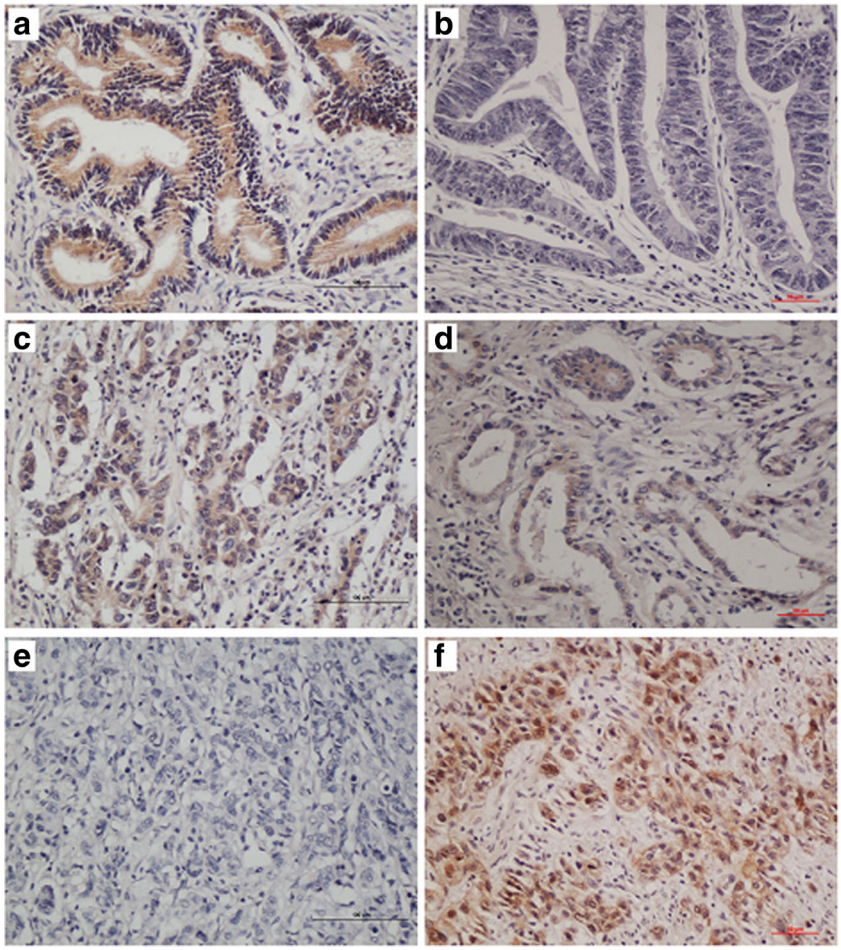

Fig 1 Immunohistochemistry analysis of NDRG1 and MMP-9 protein in primary gastric carcinomas tissue samples. NDRG1-specific staining was strong in well-differentiated gastric cancer cells with the patient who had no lymph nodes metastasis (**a**), while there was weak in moderate-differentiated cell line (**c**), no staining in poorly-differentiated gastric cancer cells with the patient who had lymph nodes metastasis (**e**). The staining results of MMP-9 was opposite (**b**, **d**, **f**)

invasion of gastric cancer cells was enhanced when NDRG1 was in the state of silencing.

Discussion

NDRG1 is also identical to DRG1, Cap43, rit42 and RTP, its expression appears to be regulated by multiple factors, including hypoxia [22], re-oxygenation [23], iron [24], tumor suppressor genes (P21, P53 and PTEN) [25, 26] and oncogenes (N-myc and c-myc) [27]. NDRG1 in partly interacts with Hsp70 and Hsp90 proteins and plays a pivotal biochemical function in human [28, 29]. To date, the determined function of NDRG1 in malignant tumors is still unclear. Many studies have showed that low NDRG1 expression in patients is associated with poor survival, and it has been proposed as an independent good factor for predicting prognosis and recurrence in many types of tumors [30–33]. But there have been also inconsistently reports in parvus carcinomas that find overexpression of NDRG1 in hepatocellular carcinoma [19], lung cancer [34] and breast cancer [35] promoting tumor progression and metastasis. Our previous studies showed that NDRG1 was down-regulated in gastric cancer cell lines and tissues and its loss expression was mainly due to DNA methylation, the overall survival rate of gastric cancer patients with high expression of NDRG1 was higher than those with low expression. [36, 37].

A newly published study reported that 20 out 65 patients with the intestinal type and 9 out of 64 (14.1%) patients with

Table 1 NDRG1 and MMP-9 protein level in gastric cancer tissues

Variable	Patients (n)	Immunostaining (%)				*P*-value
		–	+	++	+++	
NDRG1 (%)	101	50(49.5)	36(35.6)	12(11.9)	3(3.0)	<0.001*
MMP-9(%)	101	7(7.0)	26(25.7)	37(36.6)	31(30.7)	

*p-values <0.05

Table 2 Statistics of NDRG1 and MMP-9 protein expression in gastric cancer samples with clinicopathological parameters of gastric cancer patients

Variable	Patients (n)	NDRG1 immunostaining (%)				*P*-value	MMP-9 immunostaining (%)				*P*-value
		−	+	++	+++		−	+	++	+++	
Gender											
Female	30	18(26.0)	8(26.7)	2(6.7)	2(6.7)	0.183	1(3.3)	7(23.3)	11(36.7)	11(36.7)	0.708
Male	71	32(45.1)	28(39.4)	10(14.1)	1(1.4)		6(8.5)	19(26.8)	26(36.6)	20(28.2)	
Age (years)											
<60	45	24(53.3)	14(31.1)	6(13.3)	1(2.2)	0.800	3(6.7)	10(22.2)	16(35.6)	16(35.6)	0.789
≥ 60	56	26(46.4)	22(39.3)	6(10.7)	2(3.6)		4(7.1)	16(28.6)	21(37.5)	15(26.8)	
Tumor size											
<5cm	50	29(58.0)	16(32.0)	3(6.0)	2(4.0)	0.062	4(8.0)	14(28.0)	18(36.0)	14(28.0)	0.852
5-10cm	45	20(44.4)	18(40.0)	6(13.3)	1(2.2)		2(14.4)	10(22.2)	17(37.8)	16(35.6)	
≥ 10cm	6	1(16.7)	2(33.3)	3(50.0)	0(0.0)		1(16.7)	2(33.3)	2(33.3)	1(16.7)	
Location											
Upper	14	7(50.0)	6(42.9)	1(7.1)	0(0.0)	0.648	1(7.1)	6(42.9)	5(35.7)	2(14.3)	0.560
Middle	22	14(63.6)	6(27.3)	2(9.1)	0(0.0)		1(4.5)	4(18.2)	8(36.4)	9(40.9)	
Lower	62	27(43.5)	24(38.7)	8(12.9)	3(4.8)		5(8.1)	14(22.6)	23(37.1)	20(32.3)	
Whole	3	2(66.7)	0(0.0)	1(33.3)	0(0.0)		0(0.0)	2(66.7)	1(33.3)	0(0.0)	
Differentiation											
Well	8	1(12.5)	1(12.5)	5 (62.5)	1(12.5)	<0.001*	3(37.5)	4(50.0)	1(12.5)	0(0.0)	<0.001*
Moderate	35	8(22.9)	22(62.9)	4(11.4)	1(2.9)		4(11.4)	14(40.0)	13(37.1)	4(11.4)	
Poor	58	41(70.7)	13(22.4)	3(5.2)	1(1.7)		0(0.0)	8(13.8)	23(39.7)	27(46.6)	
Invasion depth											
T1 + T2	11	6(54.5)	2(18.2)	1(9.1)	2(18.2)	0.013*	1(9.1)	3(27.3)	2(18.2)	5(45.5)	0.548
T3 + T4	90	44(48.9)	34(37.8)	11(12.2)	1(1.1)		6(6.7)	23(25.6)	35(38.9)	26(28.9)	
Borrmann type											
I + II	10	6(60.0)	1(10.0)	2(20.0)	1(10.0)	0.190	0(0.0)	5(50.0)	3(30.0)	2(20.0)	0.278
III + IV	91	44(48.9)	34(37.8)	11(12.2)	1(1.1)		7(7.7)	21(23.1)	34(37.4)	29(31.9)	
Lymph node metastasis											
No	25	12(48.0)	5(20.0)	6(24.0)	2(8.0)	0.026*	5(20.0)	15(60.0)	4(16.0)	1(4.0)	<0.001*
Yes	76	38(50.0)	31(40.8)	6(7.9)	1(1.3)		2(2.6)	11(14.5)	33(43.4)	30(39.5)	
TNM stage											
I	18	10(55.6)	3(16.7)	3(16.7)	2(11.1)	0.032*	2(11.1)	7(38.9)	5(27.8)	4(22.4)	0.044*
II	23	14(60.9)	5(21.7)	4(17.4)	0(0.0)		3(13.0)	10(43.5)	4(17.4)	6(26.1)	
III	49	19(38.8)	26(53.1)	3(6.1)	1(2.0)		2(4.1)	8(16.3)	24(49.0)	15(30.6)	
IV	11	7(63.6)	2(18.2)	2(18.2)	0(0.0)		0(0.0)	1(9.1)	4(36.4)	6(54.5)	

*p-values <0.05

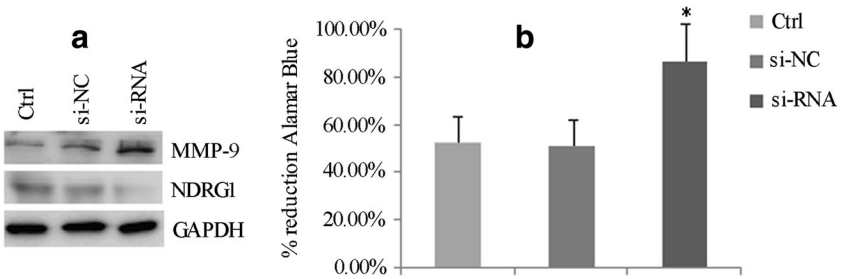

Fig 2 **a** NDRG1 and MMP-9 protein expression evaluation after transfected with siRNA-NDRG1 or siRNA-Negative Control for 48 h in SGC-7901 cell lines using Western Blot. **b** Alamar Blue showed the reduction of SGC-7901 cells that transfected with or without siRNA-NDRG1. *$p < 0.05$ compared with cells untransfection

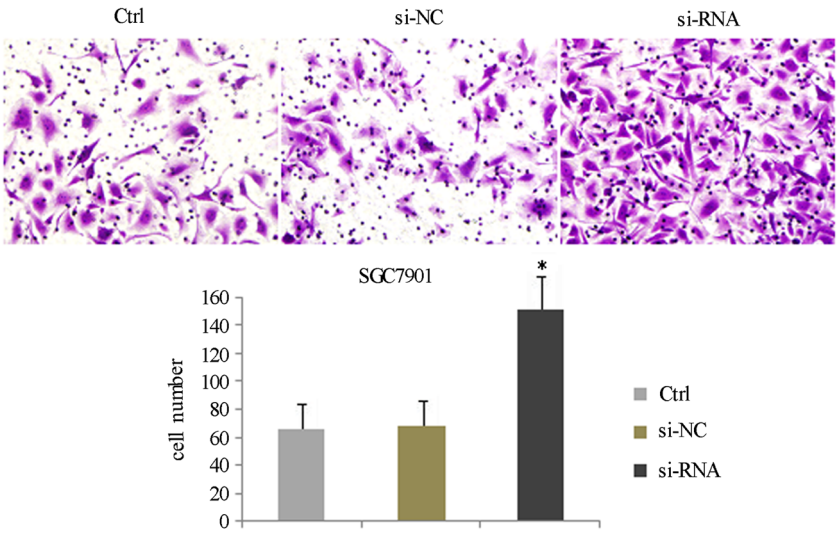

Fig 3 Effect of the suppression of NDRG1 on SGC7901 cells invasion. Representative photographs of suppression of NDRG1 in SGC7901 cells are presented (200× magnification). The columns indicate the number of cells invaded at the 48-h time point. *$p < 0.05$ compared with untransfected SGC7901 cells. The values represent the mean values ± standard deviation (SD)

the diffuse type of gastric cancer showed nuclear NDRG1/Cap43 expression, and nuclear NDRG1 expression was closely associated with infiltrating macrophages, tumor angiogenesis and poor prognosis of gastric cancer patients [38]. In the current study, we found only 2 cases showed NDRG1 expression in nuclear, therefore we did not further to analyze its clinical significance with nuclear expression. We analyzed the cytoplasm NDRG1 expression in gastric cancer patients and found that NDRG1 expression was statistically significantly inverse correlated with the degree of tumor cell differentiation ($p < 0.01$), invasion depth ($p < 0.05$), lymph node metastasis ($p < 0.05$) and TNM stage ($p < 0.05$). This was accordance with a previous study by Jiang K et al., who indicated NDRG1 expression had a inverse correlation with tumor stromal invasion, lymph node metastasis, pathological stage in 110 cases of gastric cancer specimens [39]. Whereas MMP-9 expression level was statistically significantly positive correlated with the degree of tumor cell differentiation ($p < 0.01$), lymph node metastasis ($p < 0.05$) and TNM stage ($p < 0.05$). Results showed NDRG1 expression was highest in TNM grade I and lowest in grade IV, but the expression of MMP-9 was highest in TNM grade IV and lowest in grade I. The positive staining of NDRG1 in gastric cancer tissues tended to decrease from well differentiation to poor differentiation, but it was increased for MMP-9. Furthermore, the strong positive staining of NDRG1 in patients who showed no lymph node metastasis was higher than those who showed lymph node metastasis, but it was opposite for MMP-9. MMP-9 could degrade components of extracellular matrix (ECM) and then promote tumor invasion [17, 18]. These findings suggest that NDRG1 may inhibit the invasion and metastasis of gastric cancer via regulating MMP-9. The result was further verified when NDRG1 was knocked down. We used the NDRG1-specific small inhibitory RNA (siRNA) to knock down NDRG1 expression in SGC7901 cell line which was showed relatively high level of NDRG1 in our previous study [36]. The results showed that MMP-9 expression was increased when NDRG1 was knocked down. A recent study had reported that MMP-2 was significantly increased in NDRG1 "silenced" cells, but the change of MMP-9 expression was not obvious [16]. The reason we thought may due to the different types of gastric cancer cell lines and their study limited in gastric cancer cells.

We further investigated the function of NDRG1 after transfection in gastric cancer cell line SGC7901. Alamar Blue (AB) and transwell analysis were used to detect the invasion and viability of SGC7901 cells. AB is a new method to measure quantitatively the viability of various cells and has been considered superior to other tests for cell viability such as MTT, XTT [21, 40]. Results showed cell invasion effect was remarkably enhanced as well as the proliferation status of SGC7901 cells when NDRG1 was silencing, but MMP-9 expression was increased. These findings were also supported by similar studies in other types of human tumors [13, 16, 30]. It suggests that NDRG1 expression may inhibit the growth and invasion of gastric cancer cells via modulating MMP-9 expression.

Taken together, results of our study suggest that NDRG1 is associated with biological aggressiveness in gastric cancer. NDRG1 silencing could promote the proliferation and invasion of gastric cancer, in turn, it means NDRG1 expression could inhibit gastric cancer progression. NDRG1 may suppress the invasion and metastasis of gastric cancer via regulating MMP-9. But further studies are needed to elucidate the precise mechanism of NDRG1 in tumors, especially in gastric cancer.

Conclusions

1. Expression of NDRG1 was significantly inverse correlated with the degree of tumor invasion depth, lymph node metastasis and TNM stage.

2. NDRG1 silencing could promote the proliferation and invasion of gastric cancer, NDRG1 may suppress the invasion and metastasis of gastric cancer via regulating MMP-9.

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Authors' Contributions Xiaojing Chang carried out the molecular genetic studies and drafted the manuscript. Xiaoyang Xu carried out the Western blotting. Jinguo Ma participated in the sequence alignment. Zhenhua Li carried out the IHC, Peng Deng and Jing Chen participated in Cell culture and transfection. Shuanglong Zhang carried out the Alamar Blue. Xiaoying Xue and Yu Zhi participated in the design of the study and performed the statistical analysis. Dongqiu Dai participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Competing Interests No conflicts of interest are declared by the authors.

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