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Contents

ABOUT COVER

Editorial Board of *World Journal of Gastrointestinal Oncology*, Salem Youssef Mohamed, MD, Professor, Internal Medicine, Zagazig University, Zagazig 44516, Egypt. salemyousefmohamed@gmail.com

AIMS AND SCOPE

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RESPONSIBLE EDITORS FOR THIS ISSUE

ORIGINAL ARTICLE

Basic Study

N-myc downstream regulated gene 1 inhibition of tumor progression in Caco2 cells

Yi-Xiao He, Hong Shen, Yu-Zhu Ji, Hai-Rong Hua, Yu Zhu, Xiang-Fei Zeng, Fang Wang, Kai-Xin Wang

Yi-Xiao He, Yu-Zhu Ji, Department of Pathology, Mianyang Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Mianyang 621000, Sichuan Province, China

Yi-Xiao He, Hai-Rong Hua, Fang Wang, Department of Pathology and Pathophysiology, Faculty of Basic Medical Sciences, Kunming Medical University, Kunming 650500, Yunnan Province, China

Hong Shen, Department of Pathology, Zhaotong First People's Hospital, Zhaotong 657000, Yunnan Province, China

Yu Zhu, School of Nursing, Henan Vocational College of Applied Technology, Kaifeng 450000, Henan Province, China

Xiang-Fei Zeng, Department of Clinical Pathology, West China Hospital, Sichuan University, Chengdu 610000, Sichuan Province, China

Kai-Xin Wang, Department of Pathology, Huazhong University of Science and Technology Union Shenzhen Hospital (Nanshan Hospital), Shenzhen 518052, Guangdong Province, China

Corresponding author: Kai-Xin Wang, MD, Associate Chief Physician, Department of Pathology, Huazhong University of Science and Technology Union Shenzhen Hospital (Nanshan Hospital), No. 89 Taoyuan Road, Nanshan District, Shenzhen 518052, Guangdong Province, China. 313683968@qq.com

Abstract

BACKGROUND
Invasion and migration are the irreversible stages of colorectal cancer (CRC). The key is to find a sensitive, reliable molecular marker that can predict the migration of CRC at an early stage. N-myc downstream regulated gene 1 (NDRG1) is a multifunctional gene that has been tentatively reported to have a strong relationship with tumor invasion and migration, however the current molecular role of NDRG1 in CRC remains unknown.

AIM
To explore the role of NDRG1 in the development of CRC.

METHODS
NDRG1 stably over-expressed Caco2 cell line was established by lentiviral

infection and NDRG1 knock-out Caco2 cell line was established by CRISPR/Cas9. Furthermore, the mRNA and protein levels of NDRG1 in Caco2 cells after NDRG1 over-expression and knockout were detected by real-time polymerase chain reaction and western blot. The cell proliferation rate was measured by the cell counting kit-8 method; cell cycle and apoptosis were detected by flow cytometry; invasion and migration ability were detected by the 24-transwell method.

RESULTS
NDRG1 over-expression inhibited Caco2 proliferation and the cell cycle could be arrested at the G1/S phase when NDRG1 was over-expressed, while the number of cells in the G2 phase was significantly increased when NDRG1 was knocked out. This suggests that NDRG1 inhibited the proliferation of Caco2 cells by arresting the cell cycle in the G1/S phase. Our data also demonstrated that NDRG1 promotes early cell apoptosis. Invasion and migration of cells were extensively inhibited when NDRG1 was over-expressed.

CONCLUSION
NDRG1 inhibits tumor progression in Caco2 cells which may represent a potential novel therapeutic strategy for the treatment of CRC.

Core Tip: This study investigated the molecular functions of N-myc downstream regulated gene 1 (NDRG1) in the process of colorectal cancer (CRC) migration through stable over-expression or knockout of NDRG1 in the Caco2 CRC cell line. Our results showed that NDRG1 over-expression arrested the cell cycle at the G1/S phase, while its knock-out significantly increased the number of G2 phase cells. Altogether, our results highlight the fact that NDRG1 inhibits tumor progression in Caco2 cells which may provide a novel diagnostic or therapeutic tool in inhibiting the migration of CRC.

INTRODUCTION

Malignant neoplasms are currently the leading cause of death in the world and the biggest obstacle to longer lives. Among new cancer cases in the world in 2018[1], colorectal cancer (CRC) accounted for 10.2% of new cancer cases and 9.2% of cancer deaths. CRC has become the third most common cancer and the incidence of CRC is increasing year by year.

Invasion and metastasis are two malignant features of CRC, involving the spreading of tumor cells from the primary tumor, penetrating into blood and lymphatic vessels and extravasation to the metastatic site. These are the main issues affecting the efficacy of treatment and the prognosis of CRC, and are the main cause of death in patients with CRC. Recent studies on the molecular biological mechanism of CRC indicate that the invasion and migration of CRC are associated with genes as well as microRNA[2]. It is the result of the combined action of multiple transfer-related genes and transfer-inhibitory genes. Many new biological indicators of prognosis and potential therapeutic targets related to CRC are being studied, however, early and sensitive biological indicators that can predict the migration of CRC at an early stage have not yet been discovered.

N-myc downstream regulated gene 1 (NDRG1) is a multifunctional gene. Recent studies have shown that NDRG1 is related to tumor invasion and migration[3], apoptosis[4], tumor cell proliferation[5], drug response and drug resistance of tumor cells[6]. Its expression in tumors is tissue-specific; it acts as a metastasis suppressor gene in prostate cancer[7] and ovarian cancer[8], but in lung cancer and esophageal squamous cell carcinoma, NDRG1 promotes tumor development[9]. The current role of NDRG1 in CRC is controversial. Many researchers have found that NDRG1 inhibits tumor invasion and migration in CRC[10-12], however, Wang *et al*[13] and Shah *et al*[14] found that NDRG1 promoted the development of CRC. Koshiji *et al*[15] found that the expression of NDRG1 differed with race and pathological stage of CRC patients.

To further investigate the role of NDRG1 in the development of CRC, we over-expressed and knocked out NDRG1 in the Caco2 CRC cell line, and then assessed cell proliferation, apoptosis, invasion and migration *in vitro*. We conducted these experiments to analyze the role of the NDRG1 gene in CRC, thus providing a theoretical basis for the early diagnosis and prognosis of CRC metastasis and a potential new molecular target for treatment of CRC.

MATERIALS AND METHODS

Cell culture
A Caco2 CRC cell line was purchased from the Kunming Cell Bank of the Kunming Institute of Zoology, Chinese Academy of Sciences. The Caco2 cell line was derived from a 72-year-old Caucasian male with colorectal adenocarcinoma. The 293T cells were provided by the Kunming Institute of Zoology, Chinese Academy of Sciences. The media required for culturing Caco2 and 293T cells was a Dulbecco's Minimum Essential Medium media (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, United States) containing 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin. All cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Establishment of cell lines
The lentiviral plasmid used for NDRG1 over-expression was GV358-NDRG1 (Genechem, Shanghai, China). The plasmid vector used for NDRG1 knockout was pL-CRISPR.EFS.GFP, which was kindly provided by the Kunming Institute of Zoology, Chinese Academy of Sciences. We designed three single-guide RNAs (sgRNAs) which were both located in exon 3 of the NDRG1 gene: sgRNA1, sgRNA2 and sgRNA3 (Table 1), inserting these three sgRNAs into the pL-CRISPR.EFS.GFP plasmid. All constructed plasmids were identified by sequencing. The empty GV358 and pL-CRISPR.EFS.GFP plasmids were used as a control. The plasmid (GV358-NDRG1, pL-CRISPR.EFS.GFP-sgRNA1, pL-CRISPR.EFS.GFP-sgRNA2, pL-CRISPR.EFS.GFP-sgRNA3, pL-control, GV358-control) and its corresponding packaging plasmid (Pspax2, PMD2.G) were co-transfected into 293T cells for virus packaging. Virus supernatants were then collected, concentrated and purified and finally, cells were infected with the virus. The cells successfully infected with the virus were sorted by flow cytometry. Then, the monoclonal NDRG1 knockout cells were sorted and isolated using flow cytometry. The monoclonal cells were expanded and cultured to extract DNA. The third exon of NDRG1 was amplified by polymerase chain reaction (PCR), the PCR product was sequenced and identified and the successfully identified cells were subjected to T-A cloning. The T-A cloning product was also sent for sequencing. All the primers used are shown in Table 1.

RNA extraction and quantitative PCR
The total cellular RNA was extracted (miRNeasy Kit; Qiagen, Hilden, Germany) and converted into cDNA (RevertAid First Strand cDNA Synthesis Kit; Thermo Fisher Scientific) according to the kit instructions. The ABI qPCR instrument was used for amplification of the reactants. The reaction procedure was: 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s for 40 cycles and 60 °C for 1 min. The results were normalized using GAPDH, and relative gene expression levels were calculated by the $\triangle \triangle$ Ct method. Three replicate wells were repeated for each sample. All the primers used are shown in Table 2.

Western blotting
High-efficiency RIPA lysate was used to extract total cellular protein and protein concentrations were quantified using the BCA assay. An aliquot of 80 μg protein was separated by electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were electrotransferred from the gel to a PVDF membrane, and then blocked with 5% non-fat milk solution for 2 h. Membranes were incubated with an anti-human NDRG1 monoclonal antibody (1:500, Cat. No. 9485S; CST, Danvers, MA, United States), β-tubulin (1:5000, Cat. No. 6046; Abcam, Cambridge, United Kingdom) was incubated for 1 h, and then incubated at 4 °C overnight. The next day, the corresponding secondary antibody (NDRG1 1:500, β-tubulin 1:10000, Cat. No. AS014; ABclonal, Woburn, MA, United States) was incubated for 1 h at room temperature. The membrane was washed and underwent detection using an enhanced enterochromaffin-like detection system.

Cell counting kit-8 assay
GV358-control, GV358-NDRG1, pL-control, and pL-NDRG1-knockout cells were seeded in 96-well plates at 2×10^3 cells per well for the cell counting kit-8 (CCK-8) cell proliferation assay (CCK-8 kit; Beyotime, Beijing, China), then cultured for 1, 2, 3, and 4 d, respectively. According to the manufacturer's instructions, the cells were incubated with the CCK-8 reagent at 37 °C for 1 h, with the absorbance of each sample scanned on a microplate reader equipped to read absorbance values at 450 nm.

Table 1 Primers used for the establishment of cell lines

Gene	Forward primer	Reverse primer
sgRNA1	5'-ATCCTCACCTACCATGACAT-3'	5'-ATGTCATGGTAGGTGAGGAT-3'
sgRNA2	5'-ACGCTGTGTGGGACTCCCAA-3'	5'-TTGGGAGTCCCACACAGCGT-3'
sgRNA3	5'-GTTCATGCCGATGTCATGG-3'	5'-ACCATGACATCGGCATGAAC-3'
NDRG1	5'-TTTGGTGCATTTAACAGCGCAGTCT-3'	5'-CAGGAAGTCCCAGGCAAAAAGAAAC-3'

NDRG1: N-myc downstream regulated gene 1; sgRNA: Single-guide RNA.

Table 2 Primers used for quantitative polymerase chain reaction

Gene	Forward primer	Reverse primer
GAPDH	5'-GCACCGTCAAGGCTGAGAA-3'	5'-TGGTGAAGACGCCAGTGGA-3'
NDRG1	5'-ATGTCTCGGGAGATGCAGGATGTAG-3'	5'-CTAGCAGGAGACCTCCATGGACTTG-3'

NDRG1: N-myc downstream regulated gene 1.

Assessment of cell cycle by flow cytometric analysis

For cell cycle synchronization, 5×10^5 cells were plated in 6-well plates. After the cells were completely adherent, the cells were cultured in the serum-free medium for 24 h and then cultured in a serum medium for 24 h. Cells were collected and fixed with pre-cooled 75% ethanol at 4 °C for 14-24 h. After fixation, propidium iodide and RNAase were added to the stained cells for 30 min in the dark, then detected by flow cytometry.

Assessment of apoptosis by flow cytometric analysis

Cells were trypsinized without EDTA then washed twice with cell staining buffer and resuspended with Annexin V Binding Buffer to adjust the cell concentration to $(0.25\text{-}1.0) \times 10^7$/mL. 100 µL of the cell suspension was transferred into a new 1.5 mL centrifuge tube and 5 µL of APC Annexin V and 10 µL of propidium iodide solution were added, then incubated at room temperature for 15 min in the dark and tested by flow cytometry.

24-transwell for invasion and migration

Invasion experiments were done using the Chamber Matrigel Invasion 24-well DO (Cat. No. 354480; Biocoat Inc., Horsham, PA, United States), and migration experiments were done using a Transwell Chamber (Cat. No. 3422; Corning Inc., Corning, NY, United States). All experimental steps were carried out according to the given protocols. Cells were seeded at a concentration of 1×10^5 in the upper chamber. The lower chamber was filled with a DMEM medium containing 20% FBS. After 48 h, chambers were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet, and the number of cells that passed through the Filter were observed. Cells stained with crystal violet were eluted using 33% acetic acid, and the absorbance of the eluate was measured by a microplate reader at 570 nm.

Statistical analysis

The data were statistically analyzed using GraphPad Prism 7.0 (La Jolla, CA, United States). Student's *t*-test was used for statistical analysis. Data are presented as a mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Flow cytometry sorting of cells

After viral infection of cells, positive cells (GFP-positive) were sorted by flow cytometry. The results showed that positive rates of GV358-control cells, GV358-NDRG1 cells, pL-control cells, and pL-NDRG1-knockout cells were 58.3%, 54.1%, 0.506%, and 0.461%, respectively (Figures 1A-D). The cells in the selected NDRG1 over-expression group (GV358-control cells, GV358-NDRG1 cells) were expanded and cultured. For knockout cells (pL-NDRG1-knockout cells), we used flow cytometry to sort for monoclonal cells with an efficiency of 4.73% (Figure 2).
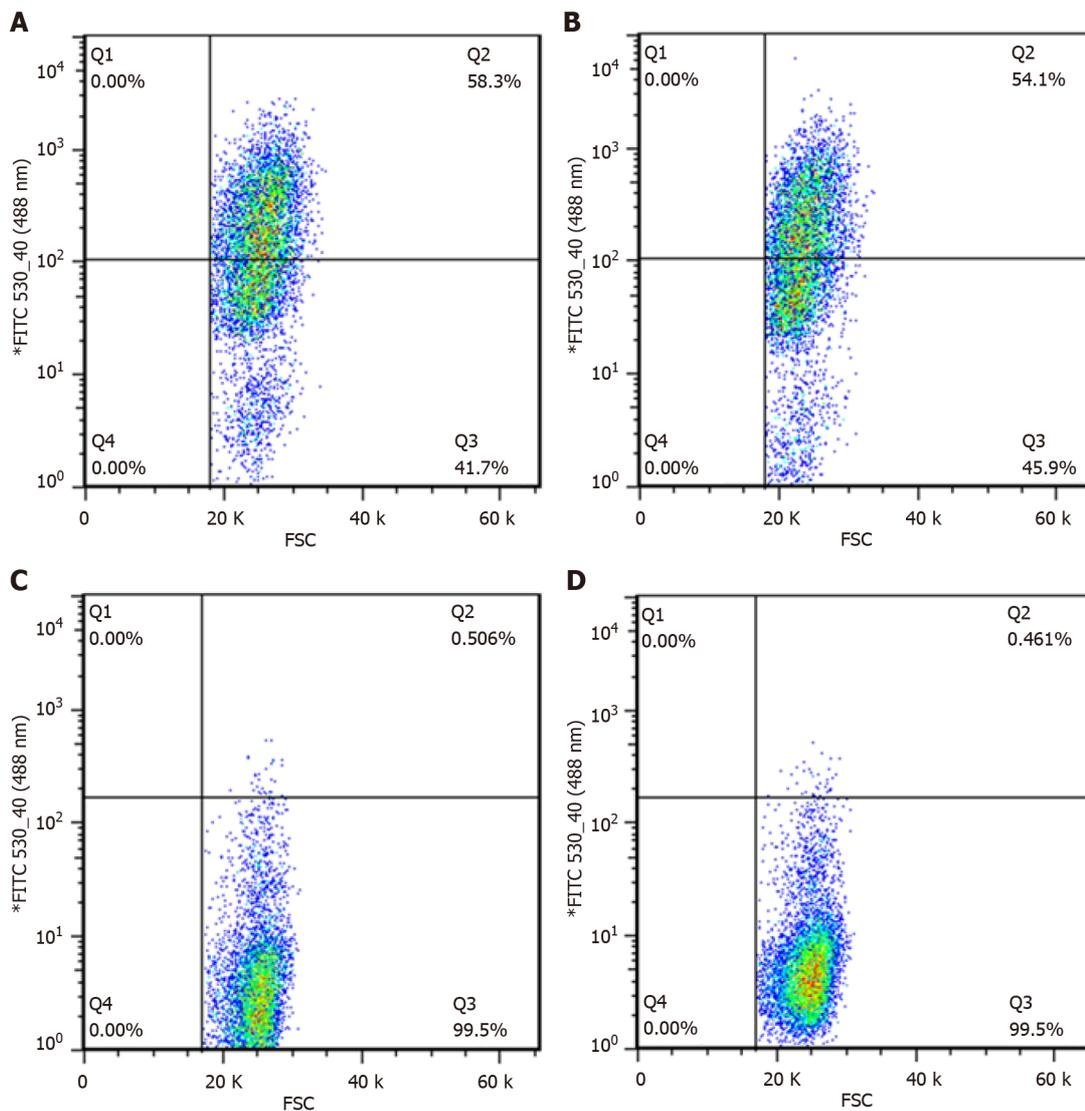
DOI: 10.4251/wjgo.v14.i12.2313 **Copyright** ©The Author(s) 2022.

Figure 1 Sorting efficiency of cells. A: Sorting efficiency of GV358-control cells (58.3%); B: Sorting efficiency of GV358-N-myc downstream regulated gene 1 (NDRG1) cells (54.1%); C: Sorting efficiency of pL-control cells (0.506%); D: Sorting efficiency of pL-NDRG1-knockout cells (0.461%).

Identification of gene knockout cell sequences

After monoclonal cells were expanded and cultured, cell DNA was extracted, the third exon of NDRG1 was amplified and products were sent for sequencing. Sequencing results showed that a DNA strand nick was generated on the third exon of NDRG1, then an A base was inserted. An analysis of the NDRG1 sequence in which the A base was inserted was performed using SnapGene software, and it was found that a TGA stop codon was formed after the inserted A base, thereby terminating the translation of NDRG1. The amplified exon of NDRG1 was identified by T-A cloning and sent for sequencing again. The sequencing results were consistent with previous results (Figure 3).

Verification of NDRG1 stable over-expression and knockout of the Caco2 cell line

After sorting GFP-positive cells by flow cytometry and sequencing identification, we detected the changes in NDRG1 mRNA and protein expression levels by quantitative PCR (qPCR) and western blotting. The relative expression level of NDRG1 mRNA of GV358-NDRG1 cells increased about three-fold compared with the control group (P = 0.0006). In the pL-NDRG1-knockout cells, NDRG1 mRNA levels were clearly diminished relative to the controls (P < 0.0001) (Figure 4A). Western blotting revealed that the protein levels in the over-expressed cells approximately doubled (P < 0.0001). After NDRG1 gene knockout, the level reduced significantly (P < 0.0001) (Figures 4B-D). All cells were expanded (Figure 5).

NDRG1 arrested the cell cycle in the G1/S phase, which inhibited the proliferation of Caco2 cells

In different tumor cells, NDRG1 can either promote or inhibit the proliferation of tumor cells. To
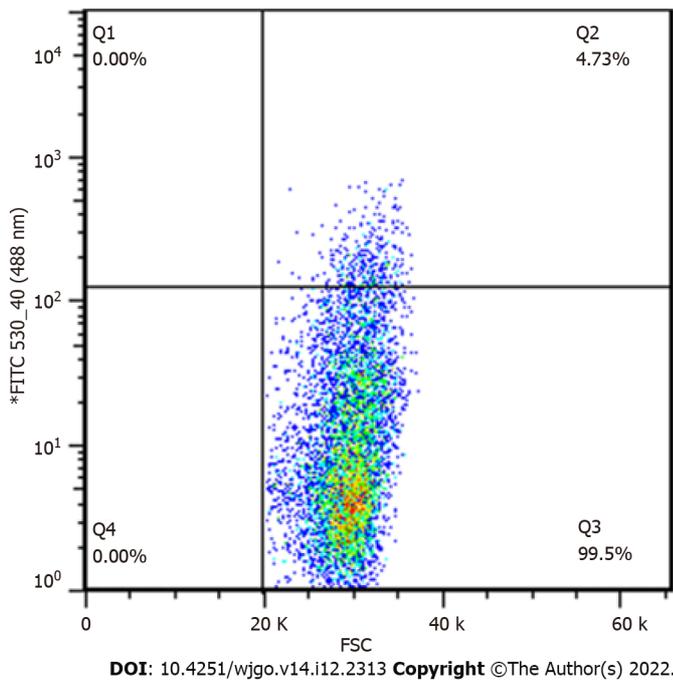
Figure 2 Sorting efficiency of monoclonal cells. Sorting efficiency of pL-N-myc downstream regulated gene 1-knockout cells for monoclonal cells (4.73%).
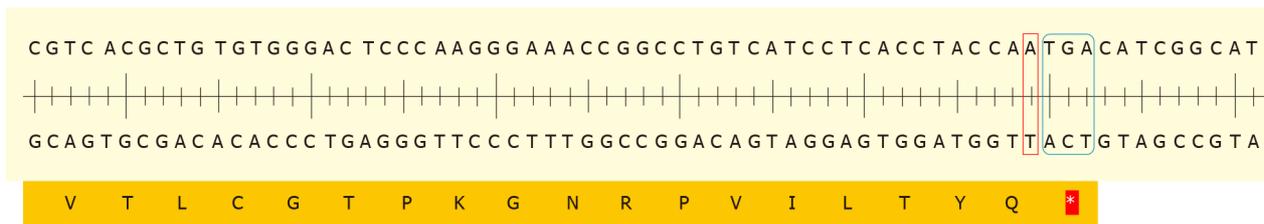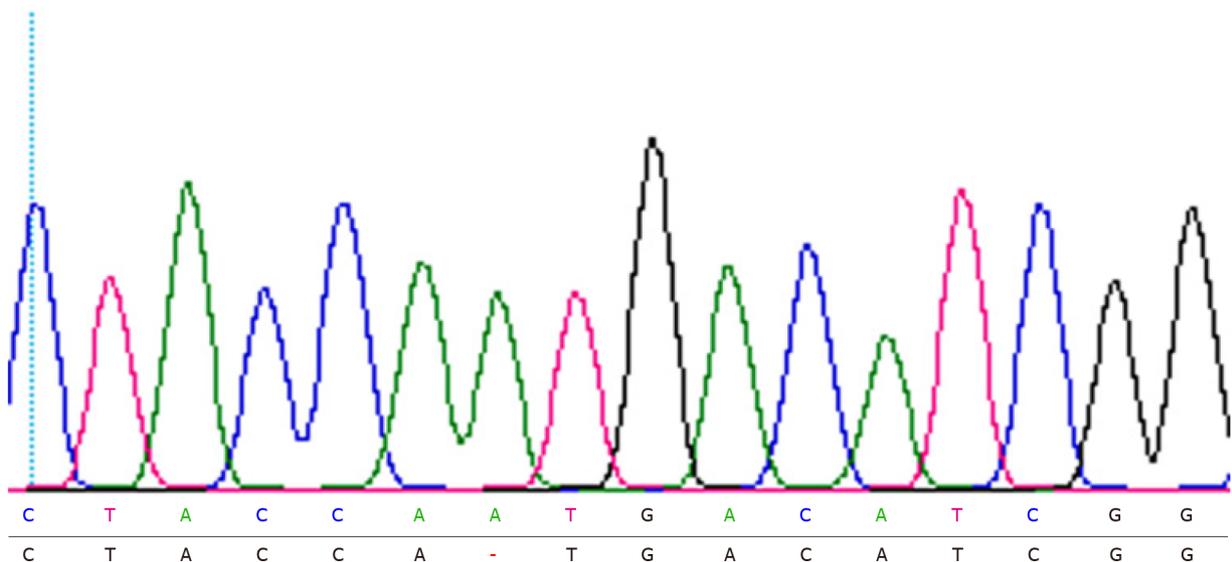
Figure 3 Sequencing results of N-myc downstream regulated gene 1 exon3 T-A clone of N-myc downstream regulated gene 1 knockout cell. A: DNA strand nick was generated on the third exon of N-myc downstream regulated gene 1 (NDRG1), then an A base was inserted. An analysis of the NDRG1 sequence was performed using SnapGene software; B: Sequencing results of NDRG1 exon 3 of NDRG1 knockout cells.

A: mRNA expressiom

B

C

D: Protein expression

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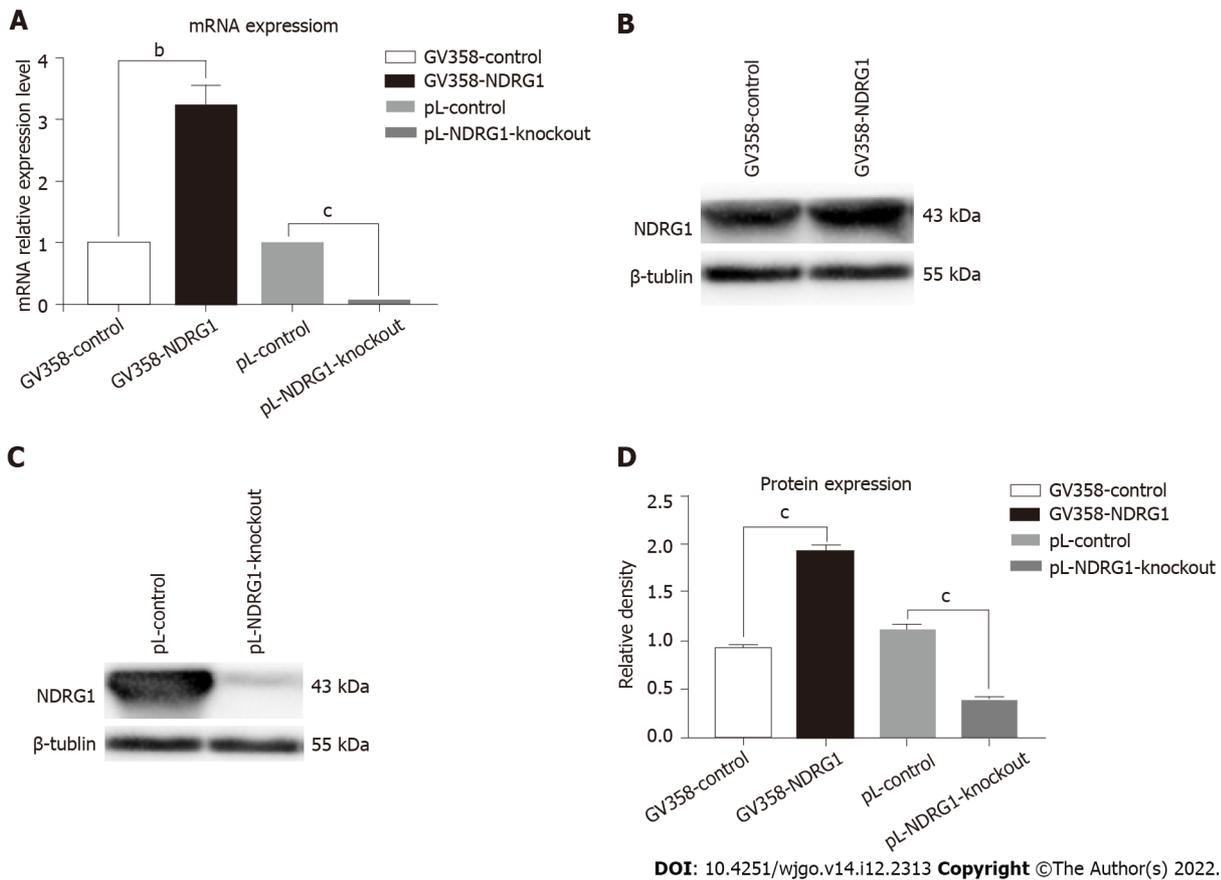
Figure 4 The results of quantitative polymerase chain reaction and western blotting after N-myc downstream regulated gene 1 over-expression and knockout. A: The mRNA levels of N-myc downstream regulated gene 1 (NDRG1) were detected using real-time polymerase chain reaction. The relative levels of NDRG1 mRNA were calculated using the $\triangle\triangle$Ct method, and normalized to GAPDH expression; B-D: Representative western blot bands and statistical results of NDRG1 protein expression normalized to β-tubulin. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ *vs* control cells. Experiments were repeated three times.
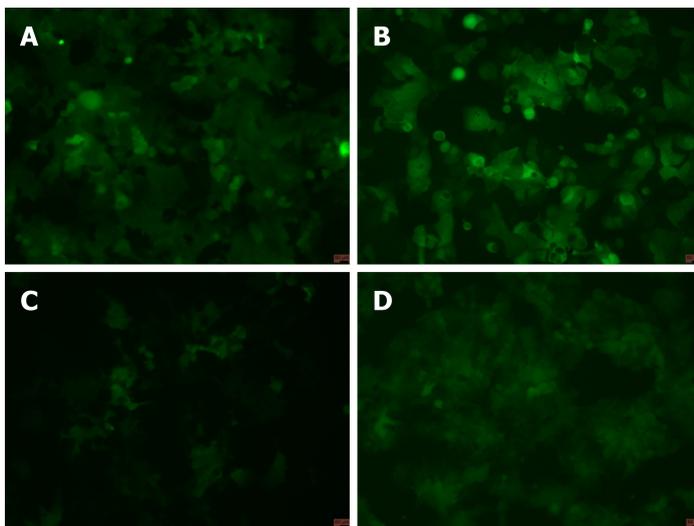
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Figure 5 Cells under a fluorescence microscope. A: GV358-Control cells (Scale bar: 60 μm); B: GV358-N-myc downstream regulated gene 1 (NDRG1) cells (Scale bar: 60 μm); C: pL-control cells (Scale bar: 60 μm); D: pL-NDRG1-knockout cells (Scale bar: 60 μm).

confirm the role of NDRG1 in Caco2 cells, we detected cell proliferation using the CCK-8 assay and flow cytometry. As shown in Figure 6, the relative proliferative activity of GV358-NDRG1 cells was decreased at 48 h, 72 h and 96 h compared with the GV358-control cells ($P < 0.001$) (Figures 6A and 6B), while pL-NDRG1-knockout cells were higher than pL-control cells at 24 h, 48 h, 72 h and 96 h ($P < 0.01$)

(Figures 6C and 6D), which indicates that over-expression of NDRG1 could inhibit the proliferation of Caco2 cells. Flow cytometry may illuminate the underlying mechanism. The proportion of G1 phase cells was significantly increased ($P < 0.0001$) (Figures 6E-G) after over-expression of NDRG1, while G2 phase cells were increased after NDRG1 knockout ($P < 0.0001$) (Figures 6H-J). The above results show that NDRG1 inhibits the proliferation of Caco2 cells by arresting the cell cycle in the G1/S phase.

NDRG1 inhibited migration and invasion of Caco2 cells

NDRG1 was also involved in tumor invasion and metastasis. Metastatic ability is also called exercise ability. Invasion and metastasis are two complementary processes. Invasive ability can be regarded as the basis of tumor cell metastasis. Thus, we also detected changes in the invasion and migration ability of cells using a 24- well transwell chamber. The number of cells passing through the chamber decreased after over-expression of NDRG1 ($P = 0.0001$) but increased after NDRG1 knockout ($P = 0.0001$). These results indicate that NDRG1 inhibited migration and invasion of Caco2 cells (Figure 7).

NDRG1 promoted early apoptosis of Caco2 cells

In order to further investigate the effect of NDRG1 on Caco2 cells, we used flow cytometry to detect cell apoptosis. The early apoptosis of the cells was increased after the over-expression of NDRG1 ($P = 0.0002$) (Figures 8A-C), and the rate of pL-NDRG1-knockout cells was decreased compared with pL-control cells ($P = 0.0013$) (Figures 8D-F), indicating that NDRG1 promoted early apoptosis of Caco2 cells.

DISCUSSION

According to the 2018 Global Cancer Statistics Report[1], CRC is the third most common cancer after lung cancer and breast cancer, and represents a serious threat to life and health worldwide. In addition, the incidence and mortality of CRC is on the rise, with the mortality rate reaching 95% for people over 45-years-old. CRC is a common malignant tumor which seriously threatens the life and health of patients. Unfortunately, the cause of this malignant disease is still unclear. Recent studies have shown that an excessive intake of red meat and processed meat increases the risk of CRC[16-18]. A large alcohol intake is also a risk factor[17-18] and a high-fat diet may also be related to CRC[19].

Invasion and metastasis are two malignant features of CRC. Tumor invasion and metastasis are multi-step, multi-stage, complex and orderly processes of interaction between tumor and host. They are the synergistic results of a variety of factors that promote the transfer mechanism of tumor cells in response to changes in the host environment. In addition, in recent years the molecular biology mechanisms of tumor invasion and metastasis research has shown that these processes are related to metastasis promoter gene activation and metastasis suppressor gene inactivation, as mutations of a variety of oncogenes and tumor suppressor genes can induce or enhance the metastatic potential of cancer cells. Therefore, tumor invasion and metastasis are the result of the combined action of multiple genes that promote and inhibit metastasis[20]. Clinically, the vast majority of CRC patients die, not from the primary tumor, but from multiple organ damage caused by tumor invasion and metastasis. Thus, the search for CRC metastasis suppressor genes is particularly important in current research. Further research on metastasis-associated genes in CRC cells will guide the early diagnosis of CRC, predicting early metastasis of cancer which is important for reducing the mortality of patients with CRC.

NDRG 1 is a member of the NDRG family. Human NDRG genes include NDRG1, NDRG2, NDRG3 and NDRG4, which are located on different chromosomes. The NDRG family belongs to an α/β hydrolase superfamily, but there is no hydrolase catalytic site. NDRG1 is located on human chromosome 8q24.3, is about 60 kb in length, and contains 16 exons and 15 introns. The full length of the mRNA is about 3 kb, and is composed of 394 amino acids and is highly conserved. The protein encoded by the gene is 43 kDa, and exists mainly in the cytoplasm, and to a lesser extent in the nucleus. NDRG1 is a multifunctional protein involved in cell growth[21], apoptosis[4], cell cycle regulation[22], tumor cell proliferation[5,23] and tumor invasion and metastasis[3]. It can be induced by a variety of drugs such as induced differentiation agents[24,25], and not only regulates homeostatic and genomic stability[26], but is also involved in the regulation of epidermal growth factor[27]. In addition, NDRG1 is related to the regulation of multiple signaling pathways, including the noncanonical nuclear factor-kappaB pathway [12,25], the PI3K/AKT/mTOR pathway[28,29], the RAS/RAF/MEK/ERK pathway[28,29], the transforming growth factor-beta pathway[30], and the Wnt/β-catenin pathways[3,9], suggesting it plays an important role in the development of tumors.

Recent studies have shown that NDRG1 may promote or inhibit the development of cancers, but its exact function in the process remains undefined. Some scholars believe that NDRG1 promotes the progression of liver cancer[31-33], lung cancer[34-36], bladder cancer[37], and gastric cancer[38,39]. However, other researchers have found that NDRG1 inhibits the development of prostate cancer[7,40], nasopharyngeal carcinoma[41], oropharyngeal squamous cell carcinoma[42], and ovarian cancer[8]. The role of NDRG1 in CRC is not conclusively understood.
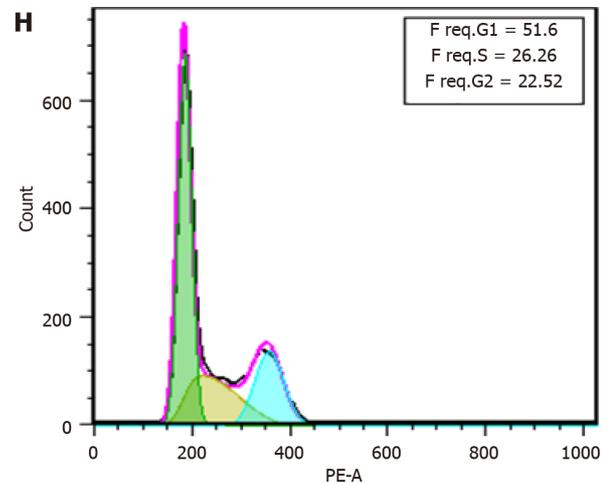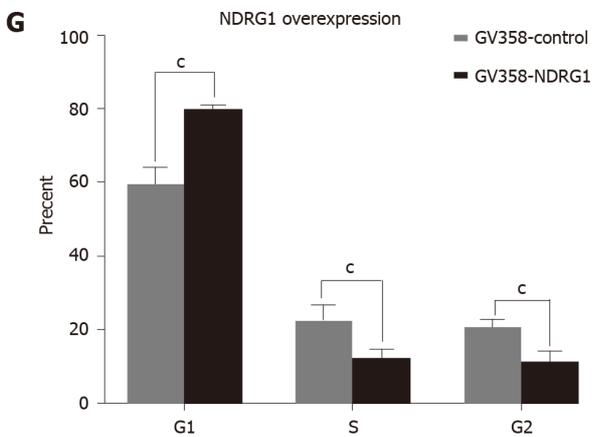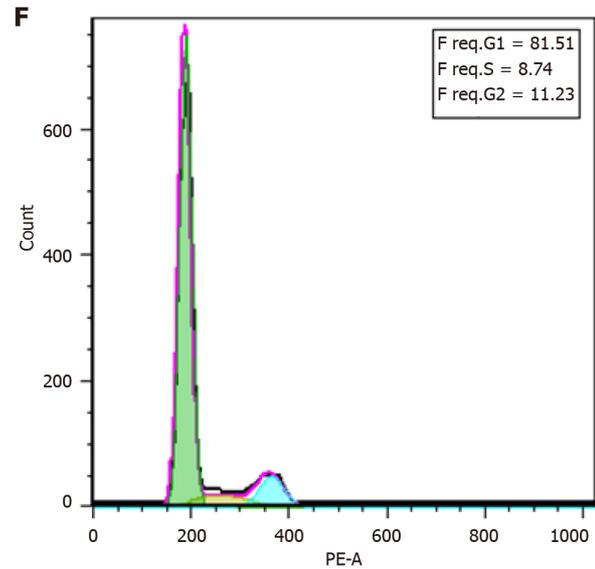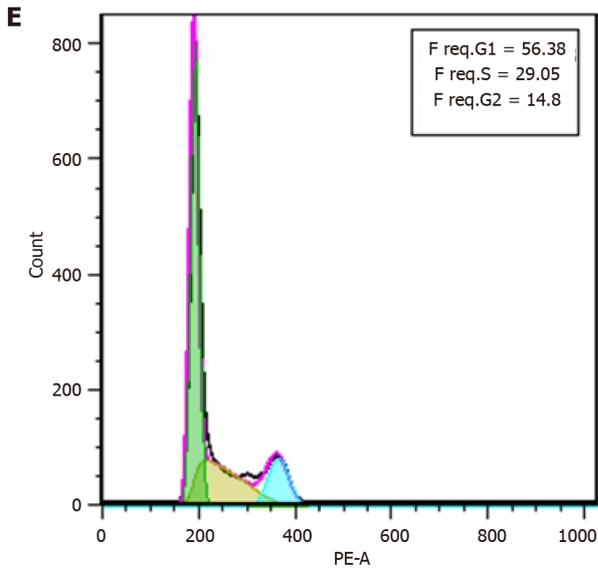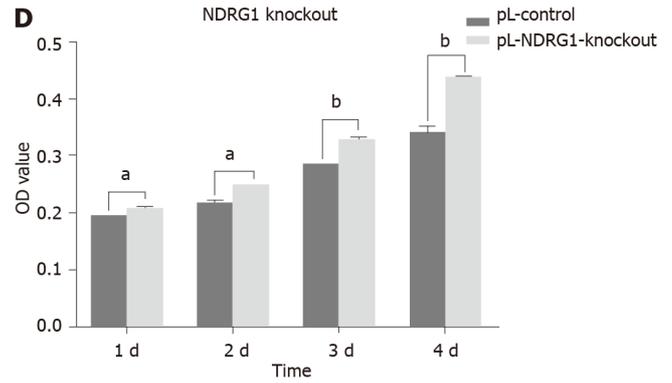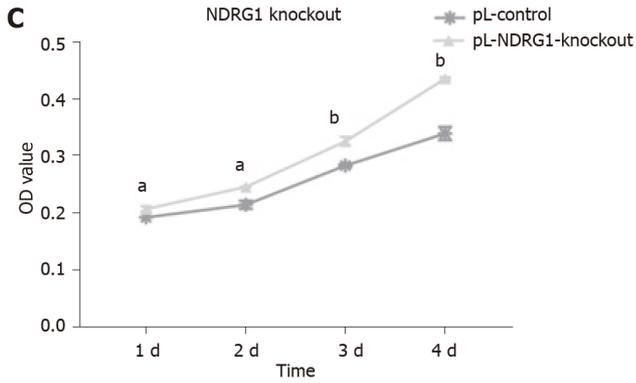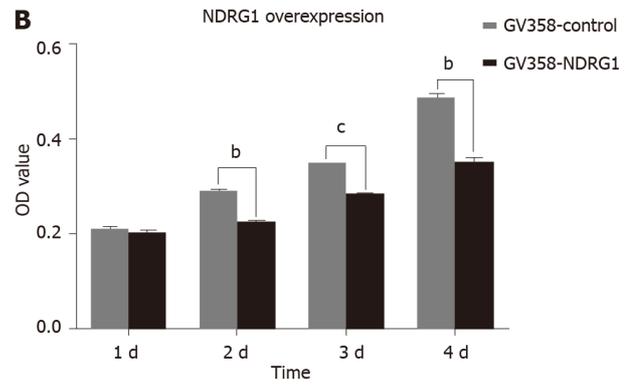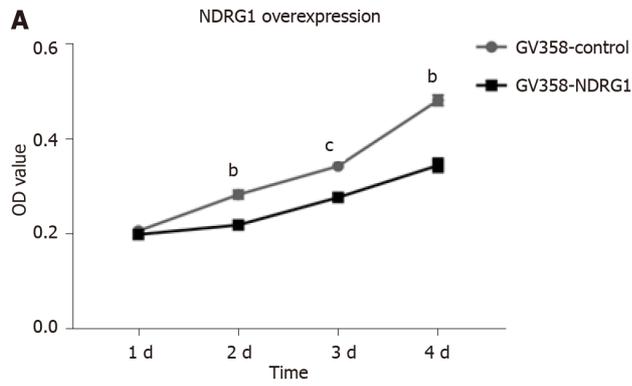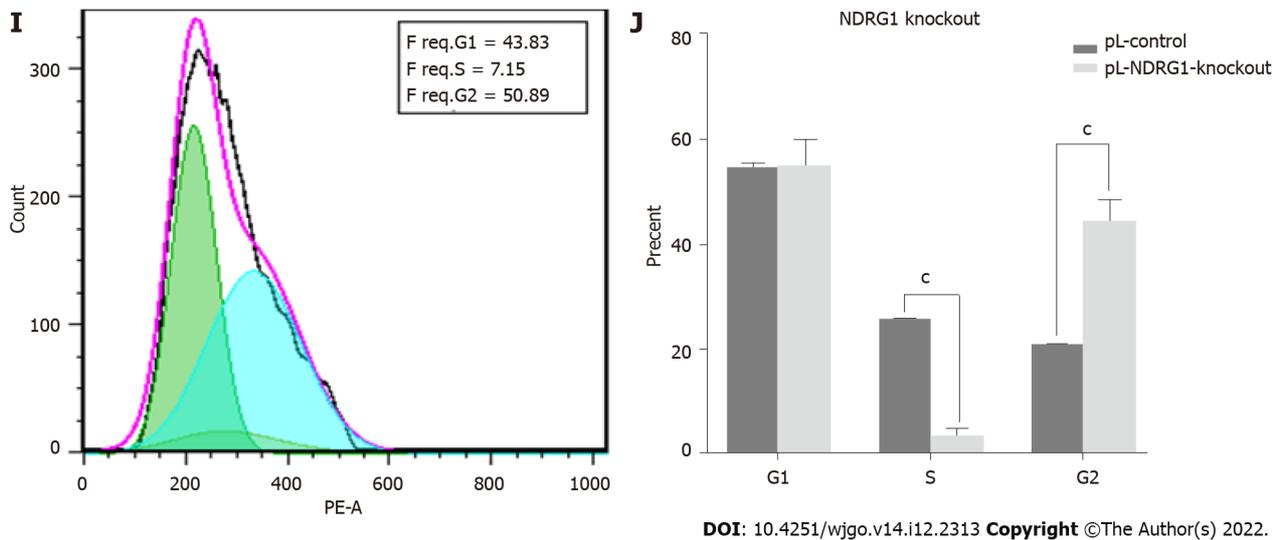
Figure 6 N-myc downstream regulated gene 1 inhibits the proliferation of Caco2 cells. Cell viability was measured using the cell counting kit-8 assay. The cell cycle was measured using flow cytometry. A: Proliferation curves of Caco2 cells at 24 h, 48 h, 72 h and 96 h after N-myc downstream regulated gene 1 (NDRG1) over-expression; B: Statistical histogram of proliferation activity of Caco2 cells at 24 h, 48 h, 72 h and 96 h after NDRG1 over-expression; C: Proliferation curves of Caco2 cells at 24 h, 48 h, 72 h and 96 h after NDRG1 knockout; D: Statistical histogram of proliferation activity of Caco2 cells at 24 h, 48 h, 72 h and 96 h after NDRG1 knockout; E: Cell cycle diagram of GV358-control group; F: Cell cycle diagram of GV358-NDRG1-over-expression group; G: Statistical histogram of proportions of G1, S, and G2 cells after NDRG1 over-expression; H: Cell cycle diagram of pL-control group; I: Cell cycle diagram of pL-NDRG1-knockout group; J: Statistical histogram of proportions of G1, S, and G2 cells after NDRG1 knockout. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ *vs* control. Experiments were repeated three times.

Vaes *et al*[43] found that NDRG1 mRNA levels were significantly reduced in CRC tissues compared to normal colon tissue. Wang *et al*[10] showed that silencing NDRG1 expression in CRC cells increased cell growth, invasion and migration. Mi *et al*[11] also found that NDRG1 inhibited epithelial-mesenchymal transition, invasion and migration of CRC cells by promoting ubiquitination of caveolin-1 (Cav1). These studies indicate that NDRG1 is a positive regulator of CRC. Interestingly, Koshiji *et al*[15] found that the expression of NDRG1 was different in CRC patients of different races and at different pathological stages, which resulted in different clinical outcomes. Some research also supports the premise that NDRG1 is a transfer-promoting gene[13,44]. It has also been observed that NDRG1 locates at the centrosome of CRC cells and participates in the cell cycle as a microtubule-associated protein and mitotic site. In p53 deficient tumor cell lines, NDRG1 inhibits polyploid development and increases the number of cells by inducing cell cycle arrest. Therefore, NDRG1 can protect cells from uncontrolled proliferation, suggesting that NDRG1 possesses different effects in different cancer cells, and may be related to specific cell types and pleiotropic functions of genes.

To further investigate the role of NDRG1 in the development of CRC, lentivirus infection was used to establish stable NDRG1 over-expression in the Caco2 CRC cell line, and CRISPR/Cas9 was used to establish stable NDRG1 knock down in the Caco2 cells. Relative mRNA expression and protein expression of the cloned cells were detected by flow cytometry, qPCR and western blot. The results showed that NDRG1 mRNA levels increased by 3.218 times on average after over-expression, and western blot results showed that protein expression increased by about 2 times on average after NDRG1 over-expression. The relative mRNA expression level after NDRG1 was knockout and was only about 0.07, and the protein expression of NDRG1 after knockout was about 0.3. Following verification of over-expression and knock down, changes in cell proliferative ability, cell cycle, apoptosis, invasion and migratory ability were measured.

The results showed that the rate of cell proliferation was slowed down after NDRG1 over-expression, while the rate of cell proliferation was increased after NDRG1 gene knockout, both of which were statistically significant ($P < 0.01$). Cell cycle assay results showed that after NDRG1 over-expression, the proportion of cells in G1 phase increased significantly ($P < 0.0001$), while the proportion of cells in S and G2 phase decreased. After NDRG1 was knocked out, the proportion of cells in S phase decreased significantly, however, there was an increased number of cells in G2 phase indicating that the proportion of cells in the proliferation phase increased, and cell growth remained active. Combining the results of the two experiments, we concluded that NDRG1 over-expression arrested the cell cycle in G1/S phase and sequentially inhibited the cell growth rate of Caco2 cells. In contrast, NDRG1 knockout could enhance the mitosis of Caco2 cells, thus promoting cell growth. Then, flow cytometry was used to analyze the effect of NDRG1 on apoptosis of Caco2 cells. After NDRG1 over-expression, both the rates of early apoptosis and total apoptosis of cells increased ($P < 0.05$), indicating that NDRG1 over-expression promoted the apoptosis of Caco2 cells. In contrast, after NDRG1 was knocked out, both the rates of early apoptosis and total apoptosis of Caco2 cells decreased ($P < 0.05$), indicating that NDRG1
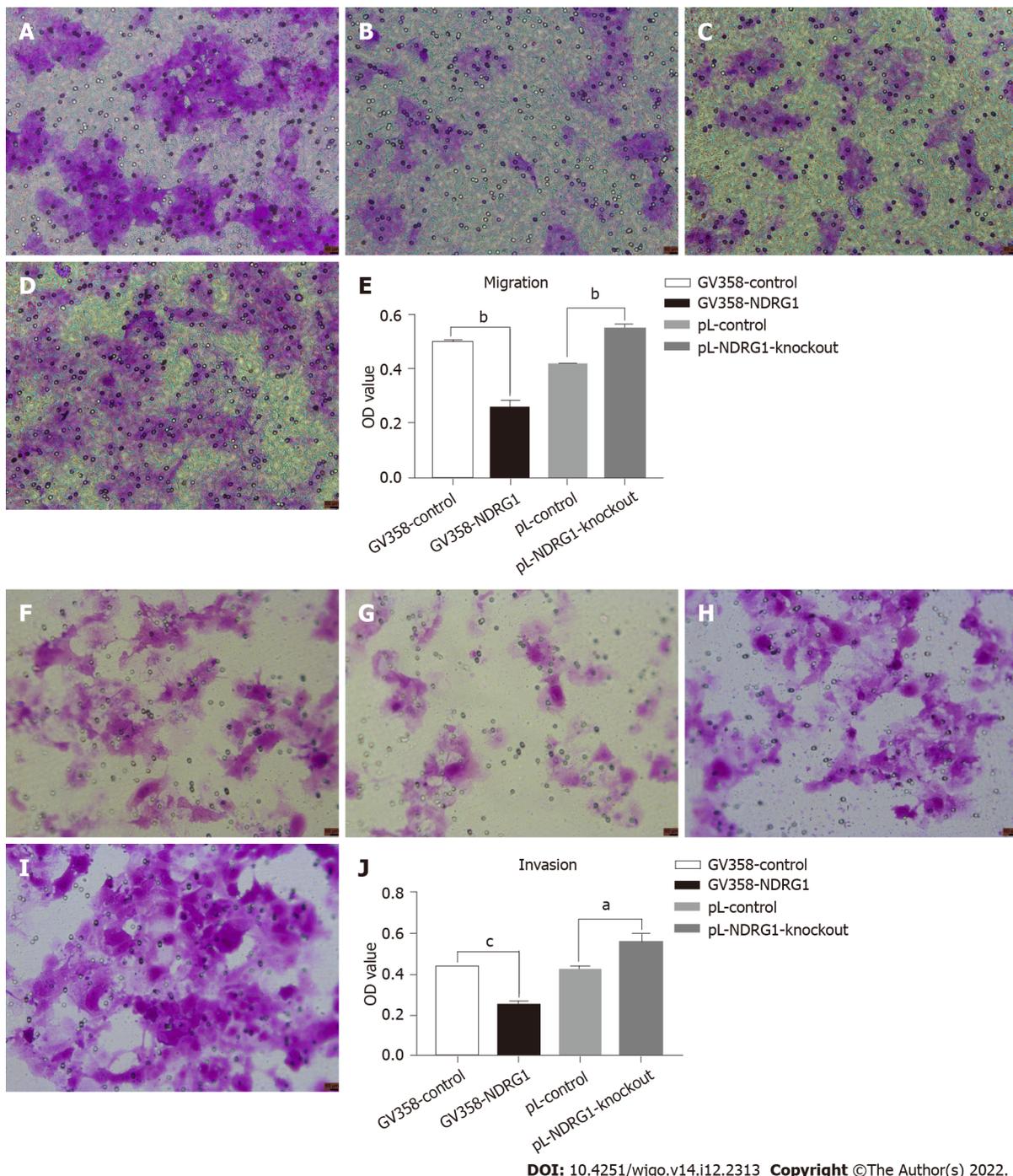
Figure 7 N-myc downstream regulated gene 1 inhibited migration and invasion of Caco2 cells. 24-well transwell chambers were used to detect migration and invasion. Cells were stained with crystal violet, eluted with 33% acetic acid, and the absorbance of the eluate was measured at 570 nm. The number of cells passing through the chamber at 48 h of the migration experiment (A-E). The number of cells passing through the chamber at 48 h of the invasion experiment (F-J). A: Number of cells passing through the chamber at 48 h of migration assay in GV358-control group (Scale bar: 60 μm); B: Number of cells passing through the chamber at 48 h of migration assay in GV358-N-myc downstream regulated gene 1 (NDRG1)-over-expression group (Scale bar: 60 μm); C: Number of cells passing through the chamber at 48 h of migration assay in pL-control group (Scale bar: 60 μm); D: Number of cells passing through the chamber at 48 h of migration assay in pL-NDRG1-knockout group (Scale bar: 60 μm); E: Statistical histograms of OD values eluted by crystal violet after NDRG1 over-expression and knockdown at 48h of migration assay; F: Number of cells passing through the chamber at 48 h of invasion assay in GV358-control group (Scale bar: 60 μm); G: Number of cells passing through the chamber at 48 h of invasion assay in GV358-NDRG1-over-expression group (Scale bar: 60 μm); H: Number of cells passing through the chamber at 48 h of invasion assay in pL-control group (Scale bar: 60 μm); I: Number of cells passing through the chamber at 48 h of invasion assay in pL-NDRG1-knockout group (Scale bar: 60 μm); J: Statistical histograms of OD values eluted by crystal violet after NDRG1 over-expression and knockdown at 48h of invasion assay. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ *vs* control. Experiments were repeated three times.

knockout can inhibit the apoptosis of Caco2 cells. Invasion and migration are two complementary processes, and migratory ability (also known as motor ability) can be regarded as the basis for invasion of tumor cells. In this experiment, we used a 24-well transwell chamber to detect the invasion and migration of cells at 48 h. The results showed that NDRG1 over-expression inhibited cell invasion and
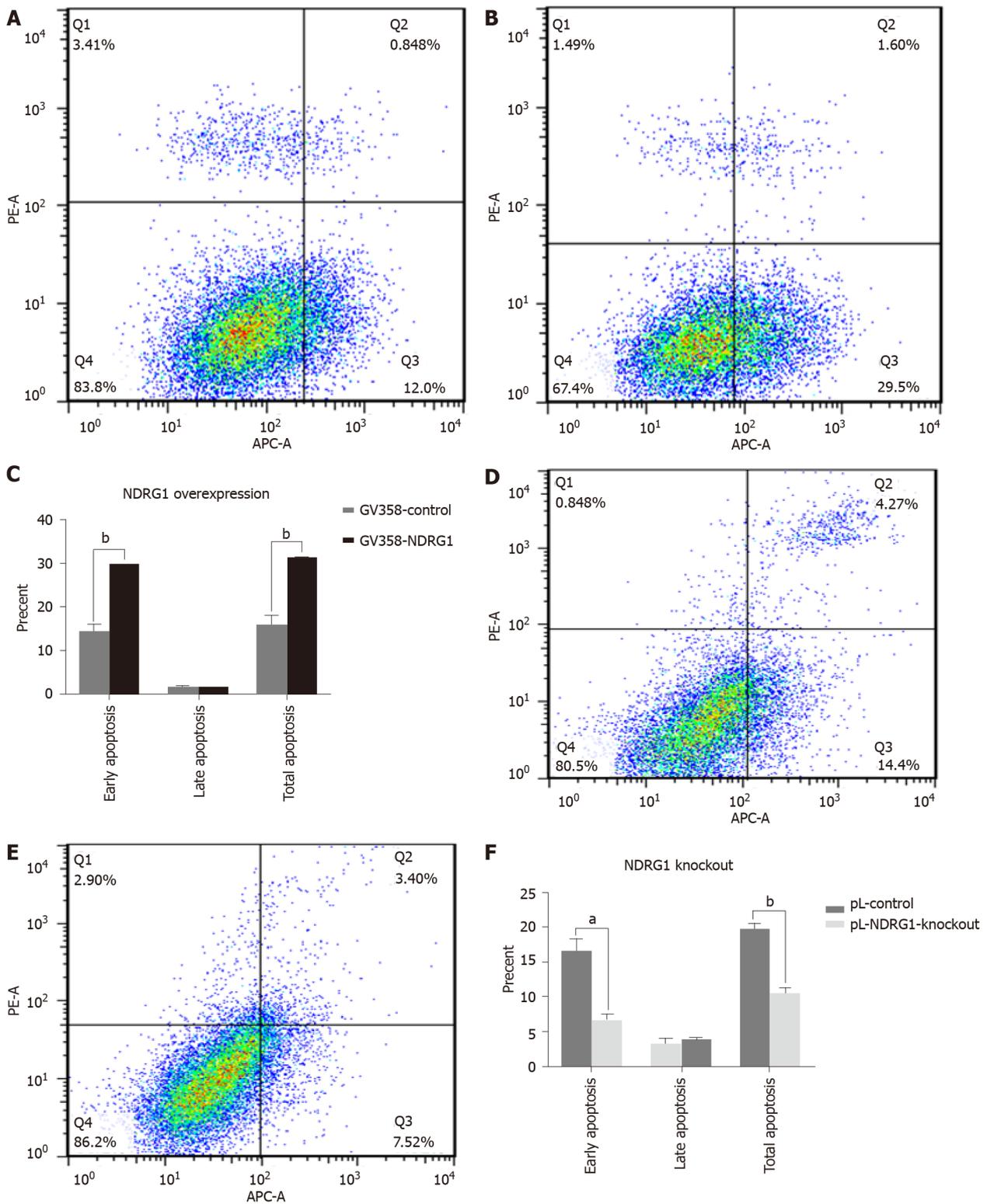
Figure 8 N-myc downstream regulated gene 1 promoted early apoptosis of Caco2 cells. Annexin V-APC/propidium iodide (PI) staining by flow cytometry was performed to detect the apoptosis of cells. Early apoptosis (Annexin V-APC+/PI-), late apoptosis (Annexin V-APC+/PI+), and necrosis (Annexin V-APC-/PI+). A: Apoptosis rate of Caco2 cells in GV358-control group; B: Apoptosis rate of Caco2 cells in GV358-N-myc downstream regulated gene 1 (NDRG1)-over-expression group; C: Statistical histogram of apoptosis rate of Caco2 cells after NDRG1 over-expression; D: Apoptosis rate of Caco2 cells in pL-control group; E: Apoptosis rate of Caco2 cells in pL-NDRG1-knockout group; F: Statistical histogram of apoptosis rate of Caco2 cells after NDRG1 knockout. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ *vs* control. Experiments were repeated three times.

migration, while cell invasion and migration were promoted after knockout. In conclusion, NDRG1 can inhibit the proliferation of Caco2 cells by arresting the cell cycle in G1/S phase, promoting early apoptosis of cells, and inhibiting the invasive and migratory ability of cells. Thus, NDRG1 is a tumor

metastasis suppressor gene in Caco2 cells.

NDRG1 has been shown to be a key player in the spread of cancer and the proliferation of cancer cells. However, the effects of NDRG1 on tumor invasion and metastasis, along with the mechanisms behind it are poorly understood. Aikemu *et al*[45] provided *in silico* evidence that NDRG1 plays a crucial role in actin reorganization in CRC. They found that NDRG1 loss disrupts the binding between RhoGDI α and CDC42, triggers the activation of CDC42 and the downstream PAK1/Cofilin cascade, thereby promoting the formation of filopodia and invasion of CRC. The knockdown of NDRG1 led to enhanced mobility of CRC cells *in vivo* and correlates with active CDC42 expression. In addition, Aikemu *et al*[45] found an elevated level of active CDC42 in patients with advanced T stage cancer that was negatively correlated with NDRG1 expression. In sum, these results uncover a mechanism utilized by NDRG1 to regulate CDC42 activity in coordinating cytoskeleton reorganization, which is crucial in cancer invasion. Claudin-2 (CLDN2), a well-defined component of cellular tight junction, has also been suggested to be associated with CRC progression. Wei *et al*[46] demonstrated that CLDN2 is up-regulated in CRC samples and associated with poor survival. Additionally, CLDN2 depletion significantly promoted NDRG1 transcription, leading to termination of CRC growth and metastasis *in vitro* and *in vivo*. NDRG1 is a key regulator that interacts with many classic tumor signaling pathways, including some molecules downstream of the epidermal growth factor receptor (EGFR). Yang *et al*[47] demonstrated that NDRG1 inhibited the expression of EGFR, blocked EGFR phosphorylation, and reduced the distribution of EGFR distribution in the cell membrane, cytoplasm and nucleus. NDRG1 suppression of EGFR subsequently suppressed pathways downstream of EGFR, including the RAS/RAF/ERK and PI3k/AKT/mTOR pathways. NDRG1 was also able to attenuate endocytosis and degradation of EGFR induced by Cav1. NDRG1 could be a promising biomarker to predict optimum responses to cyclophos-phamide (commonly known as CTX) and a key target to enhance CTX activity in the treatment of metastatic CRC.

To further clarify the mechanism by which NDRG1 inhibits tumorigenesis, we are proceeding to observe the relevant signal pathway and the abilities of NDRG1 in nude mice. This preliminary basic research is useful in providing new potential targets for molecular therapy in CRC.

CONCLUSION

In conclusion, we conducted analyses on the effect of NDRG1 on the Caco2 cell line. Through the analyses of the NDRG1 on the biological behaviors of Caco2 cell line, we found that NDRG1 would reduce the proliferation, apoptosis, migration and invasion abilities of Caco2 cell line. NDRG1 may have potential application value as a molecular biological index to predict the early invasion and metastasis of CRC.

ARTICLE HIGHLIGHTS

Research background
In American colorectal cancer patients, the expression of N-myc downstream regulated gene 1 (NDRG1) in primary colorectal cancer was always less than that in adjacent normal tissues. Our study on clinical samples showed the opposite. Therefore, the effect of NDRG1 in cancer may be related to the ethnic backgrounds of colorectal cancer. The future research direction is to find out whether the role of NDRG1 in the development of colorectal tumors is related to ethnic differences through *in vivo* and *in vitro* experiments.

Research motivation
This study identifies the *in vitro* role of NDRG1 in Caucasian large intestine tumors as inhibition of tumor cell invasion and migration.

Research objectives
This study only showed the *in vitro* effect of NDRG1 in Caucasian large intestine tumors, and the question remains to be solved about the effect of NDRG1 in large intestine tumors of other races.

Research methods
RNA extraction and quantitative polymerase chain reaction, western blotting, cell counting kit-8 assay, assessment of cell cycle by flow cytometric analysis, assessment of apoptosis by flow cytometric analysis, 24-transwell for invasion and migration were used in the experiments. GraphPad Prism 7.0. student's *t*-test was used for statistical analysis.

Research results

The primary objective was to investigate the role of NDRG1 in the development and progression of colorectal tumors. The objective has been to investigate the *in vitro* role of NDRG1 in the development of Caucasian human bowel tumors. The significance of achieving these goals for future research in this area is to provide clinicians with a theoretical basis for selecting therapies for colorectal cancer.

Research conclusions

The research topic is NDRG1 and the key problem to be solved is the role of NDRG1 in the occurrence and development of colorectal tumors. The results can provide a certain basis for the selection of treatment options for colorectal tumors.

Research perspectives

In 2004, our research group published an article in the *World Journal of Gastroenterology* on our research data with a large clinical sample (150 cases): "Correlation of N-myc downstream-regulated gene 1 over-expression with progressive growth of colorectal neoplasm.", which carried significance on the association of NDRG1 with colorectal cancer progression.

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