Cellular Oncology 29 (2007) 335–349
IOS Press

Transcription factor E2F-1 is upregulated in human gastric cancer tissues and its overexpression suppresses gastric tumor cell proliferation

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Abstract. The E2F family members play a critical role in cell cycle regulation and other biological processes in the cell. To better understand the involvement of E2F-1 in the development and progression of gastric tumors, we investigated the mutation and expression of E2F-1 in human gastric cancer tissues and the effect of E2F-1 overexpression on the proliferation of gastric carcinoma cells. In this study, 80 pairs of gastric cancer specimens and paratumor tissues from different patients and 40 stomach mucosa specimens from healthy individuals were examined. PCR-SSCP analysis demonstrated that mutations were not detected in any of the gastric cancer and normal tissue specimens. In addition, the results of an immunohistochemistry assay revealed higher expression rates of E2F-1 ($P < 0.01$) in gastric cancer tissues (72.5%) than in paratumor tissues (30.0%) of the same individuals and stomach mucosa from healthy individuals (22.5%). However, no correlation was observed between the E2F-1 levels and patients' clinical features, such as sex, age, histological types, lymph node metastasis, and clinical stages ($P > 0.05$). Finally, the influence of E2F-1 overexpression on the growth of human gastric carcinoma MKN-45 cells *in vitro* was assessed by measuring colony formation, cell survival, and cell cycle progression. Our data clearly showed that cell growth and proliferation were significantly inhibited in MKN-45 tumor cells transfected with the expression vector encoding *E2F-1* in comparison with nontransfected cells or cells transfected with empty vector. These findings suggest that E2F-1, a stable and conservative gene during the oncogenesis and progression of stomach cancers, may potentially serve as a biomarker for clinical diagnosis of gastric carcinomas and as a target for the development of novel therapeutic interventions to treat this disease.

Keywords: E2F-1, mutation, gastric cancer, human tissues, MKN-45 cells, cell cycle, cell proliferation, cell growth, cell survival

1. Introduction

The E2F family of transcription factors was originally characterized by a sequence-specific DNA-binding factor bound to the adenovirus E2A promoter [1]. These factors are central regulators of cell cycle progression and play a crucial role in the control of cell proliferation by regulating the expression of the genes required for entry into and progression through the S phase of the cell cycle [2–9]. Thus far, eight members (E2F-1 to E2F-8) of the E2F family of DNA-binding proteins have been identified. They function as heterodimers with members of the DP family (DP1 and DP2), with the DNA-binding specificity being determined by the E2F subunit. The E2F family regulates overlapping sets of target genes, and all E2F family proteins contain related DNA-binding and dimerization domains.

E2F-1 is the prototype of the E2F family of transcription factors. In the cell, E2F-1 is associated pref-

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erentially with pRb (a product of the retinoblastoma susceptibility gene), and its transcriptional activity is negatively regulated by pRb. The growth suppression activity of pRb is dependent on its ability to interact with E2F [2,3]. Cyclin-cdk-dependent phosphorylation of pRb removes this inhibition, releasing E2F-1 transcriptional activity [2,10]. As a transcription factor, E2F-1 activates a number of genes responsible for DNA synthesis, repair, cell-cycle control, and apoptosis [5,6,8,11]. In addition, E2F-1 attracts numerous upstream signals in determining whether a cell advances through the cell cycle or dies via apoptosis [12,13].

Initially, E2F-1 was suggested to behave as an oncogene because it promotes cellular proliferation by stimulating the expression of a number of genes that promote the transition from G1 to S phase [2,14–17]. For instance, E2F-1 enhances the proliferation of cells in gastrointestinal carcinomas [18–20], and E2F-1 overexpression stimulates quiescent cells to enter into S phase [15]. In addition, the overexpression of E2F-1 in HNSCC cell lines can stimulate cell cycle re-entry but is also associated with increased invasiveness [21], suggesting a positive role of E2F-1 in cell cycle progression as well as tumor progression and metastasis.

Interestingly, the overexpression of E2F-1 has also been shown to induce apoptosis in several cell types, indicating that in addition to its critical role in regulating cell growth, E2F-1 also participates in coordinating programmed cell death [22–24]. Furthermore, recent studies with E2F-1 knockout mice have suggested that E2F-1 functions as a tumor suppressor [25–27]. The deregulated expression of an E2F-1 mutant that cannot be inactivated by cyclin A/cdk2 during S phase of the cell cycle was shown to lead to S phase arrest [28–30]. Similarly, the novel retinoid CD437, which induces E2F-1 activity but inhibits cyclin A/cdk2 activity, also stimulated S phase blockade [21,31]. Moreover, the constitutive expression of E2F-1 was shown to induce endogenous p21 expression, and p21 was responsible for E2F-1-dependent cell cycle S phase arrest [32]. These observations suggest that E2F-1 plays a double role in the regulation of cell growth and cell death.

Nevertheless, the role and importance of E2F-1 as an oncogene and tumor suppressor gene in cancers remain largely unknown at the present time. The relationship between E2F-1 and tumor kinetics has been studied in lung cancer [33,34], where the correlation of E2F-1 expression with the growth indices of non-small cell lung carcinomas (NSCLCs) was observed within the context of aberrant pRb and p53 status [33]. The relationships of E2F-1 with tumor kinetics in human carcinomas of the digestive system were also assessed recently. For example, Yamazaki et al. evaluated the E2F-1 status in 43 surgically resected oesophageal squamous cell carcinoma (OSCC) specimens and found that E2F-1 expression was higher in OSCC than in the corresponding adjacent non-tumorous squamous epithelium, and tumors with high E2F-1 positivity had significantly higher growth indices [39]. Additionally, Gorgoulis and colleagues examined the expression status of E2F-1 and its putative impact on tumor kinetics in four of the most common human malignancies including colon carcinoma, and their results revealed that E2F-1 has a growth-promoting effect in breast and bladder cancers, whereas the opposite seems to be the case for carcinomas of the colon and prostate [40]. However, relevant literature for gastric tumors is scarce [21,35–38].

Gastric cancer is one of the most frequent cancers and one of the most frequent causes of cancer-related mortality in China, with an incidence of 0.4 million new cases and 0.3 million deaths annually, and it ranks the third most common cancer in China [41]. However, the clinical significance and implication of E2F-1 expression in human gastric carcinoma are unclear. Therefore, we conducted this study to investigate the involvement of E2F-1 activity in the development and progression of gastric cancer. The objectives of this study were (i) to examine the mutation and expression status of E2F-1 at the protein and DNA levels in 80 patients with stomach cancer and 40 healthy control subjects; (ii) its relationship with the kinetic parameters of the tumors; (iii) to analyze the relationship between E2F-1 status and clinicopathological features of the patients; and (iv) to determine the influence of E2F-1 overexpression on cell proliferation or apoptosis in an *in vitro* cell model for gastric cancer. The long-term goals of our project are to exploit the potential use of E2F-1 as a marker for clinical diagnosis and prognosis of gastric carcinomas and as a target for the development of therapeutic approaches to treat this disease.

2. Patients and methods

2.1. Patient samples

Primary gastric carcinoma and adjacent nontumor tissues were obtained with informed consent from 80 patients diagnosed in the Department of Surgery, First Hospital of Guangxi Medical University (Nanning,

Guangxi, China), from 2001 to 2004, following Ethics Committee approval. All samples were examined histologically for the presence of tumor cells, and all paratumor tissues, taken at a distance of at least 5 cm from the tumor, were confirmed to be normal and free of tumor cells by HE staining. Each specimen of the patients was divided into two parts, one part for DNA extraction, and the other one for tissue sections. For controls, normal gastric mucosa tissues were obtained from 40 healthy individuals and were examined by histological sectioning.

2.2. Analysis of point mutations in the E2F-1 gene

Genomic DNA of the specimens was extracted using a DNA isolation kit (Huashun, Shanghai, China). The DNA sequence of the *E2F-1* gene was obtained from GeneBank (ID No.: AF516106). Primers were designed for all exons of *E2F-1*, and the primer sequences, PCR-amplified product sizes, and annealing temperatures are listed in Table 1. The E2F-1 exons were amplified by PCR using isolated genomic DNA as a template. For PCR of exon 1, LA Taq DNA polymerase (Takara Bio Inc., Shiga, Japan) was used; EX Taq DNA polymerase (Takara) was used for PCR of the other six exons. The amplification for exon 1 of *E2F-1* was done in the following amplification conditions: 25 μl reaction mixture contains LA PCR buffer II, dNTP, 2 units LA Taq polymerase, primer, and 100 ng genomic DNA at 94°C for 4 min, and then performed 30 cycles for amplification at 94°C for 30 s for denaturation, at 64°C for 40 s for annealing, at 72°C for 30 s for polymerization, and at 72°C for 10 min for balance. The amplification for exons 2–7 of *E2F-1* was done in the following conditions: 25 μl reaction mixture contains EX PCR buffer, 125 μm dNTP, 2.5 units EX Taq polymerase, primer, and 100 ng genomic DNA at 94°C for 4 min, and then performed 30 cycles for amplification at 94°C for 30 s for denaturation, at 54°C (or 55°C, 60°C or 62°C for different exons) for 40 s for annealing, at 72°C for 40 s for polymerization, and at 72°C for 10 min for balance. After PCR reaction, 2 μl of the amplified products (Table 1) were electrophoresed in 2% agarose gelatin gels buffered with 1 × TBE.

Single-strand conformational polymorphism (SSCP) was performed on SDS-PAGE gels (12%; acrylamide : bisacrylamide, 35:1; gel buffer: 0.112 M Tris/0.112 M acetate, pH 6.5; running buffer: 0.025 M Tris/0.088 M L-glycine, pH 8.8; gel size, 10 × 10 cm; gel thickness, 0.75 mm). This system requires a strict running temperature as calculated using the following formula: $T_s = [80 \times C/(A+1)]/\{2.71 + [C/(A+1)]\}$, where C is cytosine, A is adenine, and T_s is electrophoretic temperature. The T_s values of the E2F-1 exons are shown in Table 1. Argentous staining was performed after 3–4 h of electrophoresis at 300 V.

2.3. Immunohistochemical assay (immunohistochemistry)

Immunohistochemical assay was performed using a previously described method [42] with some modifications. In brief, 5-μm tissue sections were cut from formalin-fixed, paraffin-embedded specimens. The expression of E2F-1 protein was measured with

Table 1
PCR primers, annealing temperatures, and amplified products for the *E2F-1* gene

Exon	Primer sequence	Product length	Annealing temperature	T_s (°C)
1	Upstream 5′-CGT GAG CGT CAT GGC CTT GG-3′	291 bp	64°C	52.8
	Downstream 5′-GGC GTC CCT GGG GTC CGT AC-3′			
2	Upstream 5′-TCT TCT GGC CTC ACT CCT GGT T-3′	215 bp	55°C	26.2
	Downstream 5′-TCC CTA CAC TTG TCT GTT TGT ACG-3′			
3	Upstream 5′-CCA TCA TCC TGC TGC CCT GC-3′	350 bp	60°C	27.0
	Downstream 5′-TGT GCC TGC CCT CCT GTG-3′			
4	Upstream 5′-CCC TAC CCT CCC TGG TGC CT-3′	224 bp	55°C	29.0
	Downstream 5′-TGC TAA GCC TGC CTT CCACA-3′			
5	Upstream 5′-CCC CTA GAA GTC AAA GGT CAT G-3′	179 bp	54°C	26.7
	Downstream 5′-GCC CGG ATT CCC AGA T-3′			
6	Upstream 5′-CTG CCT GCT GCT TCC ACC-3′	275 bp	58°C	28.1
	Downstream 5′-CCA CCC CAC CCA CCT ACC-3′			
7	Upstream 5′-CCT GTG ATG CTC CCC GTC TCC-3′	293 bp	62°C	38.4
	Downstream 5′-CCT GGT CCC TCC AAG CCC TGT-3′			

UltraSensitiveTM S-P Kit (Maixin, Fuzhou, Fujian, China). Before labeling with primary antibody, the sections were deparaffinized, antigens were restored, and the sections were incubated with hydrogen peroxide to block endogenous peroxidase. The specimen sections were incubated overnight at 4°C with anti-E2F-1 primary antibody (1:2000) (Neo Markers, Fremont, CA, USA) and then with a secondary antibody (1:2000) (Neo Markers) conjugated with streptavidin-peroxidase. After color development with diaminobenzidine (DAB) peroxidase substrate solution, the sections were counterstained with hematoxylin. For the negative control, PBS was used instead of primary antibody. The immunohistochemical results were examined independently by two pathologists. All nuclei exhibiting a brown reaction product were considered positive. Cytoplasmic staining, which was seen occasionally, was considered non-specific. The sections were examined under a microscope at low power to identify evenly labeled areas, and an estimate of the cells positive for E2F-1 protein expression was scored as follows: 0–5%, negative (−); 5–50%, weak (+); and 50–100% strong (++).

2.4. Cell lines and cell culture conditions

The Poorly differentiated human gastric adenocarcinoma cell line MKN-45 and the African Green Monkey kidney fibroblast cell line COS-7 (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Gaithersburg, MD, USA). All media were supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were cultured in an incubator at 5% CO_2 and 37°C, with medium changes every 3 days.

2.5. Expression of E2F-1 in gastric carcinoma cells

(1) RT-PCR amplification of E2F-1:

The mRNA sequence of E2F-1 was obtained from GeneBank (ID No.: NM_005225.1). The first pair of primers (forward primer, 5′-GGACTTTGCAGGCAGCGGCG-3′; reverse primer, 5′-CTGGAAACCCTGGTCCCTCCAAGC-3′) was designed to amplify the entire coding sequence of E2F-1 and gave an amplified product of 1462 bp. The secondary primer set (forward primer, 5′-GAATTCATGGCCTTGGCCGGGGC-3′; reverse primer, 5′-GAATTCTCAGAAATCCAGGGGGGTG-3′), which included an EcoR I restriction site, was designed based on the product of the first primer pair and gave a product of 1362 bp. Two rounds of PCR were performed according to the TaKaRa LA Taq data sheet, followed by recovery of the 13-kb fragment from the agarose gel. The amplification for *E2F-1* was done in the following conditions: 25 μl reaction mixture contains PCR buffer II, 125 μm dNTP, 2.5 units EX Taq polymerase, primer, and 100 ng genomic DNA at 94°C for 5 min; then 94°C for 1 min, 72°C for 1 min, 72°C for 2 min for 5 cycles; 94°C for 1 min, 70°C for 1 min, 72°C for 2 min for 5 cycles; 94°C for 1 min, 68°C for 1 min, 72°C for 2 min for 5 cycles; 94°C for 1 min, 66°C for 1 min, 72°C for 2 min for 20 cycles, and finally at 72°C for 10 min for balance. After PCR reaction, 2 μl of the amplified product were electrophoresed in 1% agarose gelatin gels buffered with 1 × TBE.

(2) Construction of the E2F-1 expression vector:

The plasmid pCMV-HA, a gift from Professor Jin-De Zhu (Shanghai Cancer Institute, P. R. China), was used to construct the E2F-1 expression vector. The HA epitope tag has a sequence derived from influenza virus hemagglutinin and contains nine amino acid residues. The PCR product and pCMV-HA were digested with EcoR I. The fragment was isolated from agarose gels and ligated into the cut vector to form pCMV-E2F-1-HA, which was then transfected into competent cells. Positive clones were verified by EcoR I digestion.

(3) Plasmid transfections:

(i) Transient transfection of MKN-45 cells with *E2F-1*: One or two days before transfection, COS-7 cells and MKN-45 cells were seeded into 35-mm plates and cultured in DMEM with 10% fetal calf serum and without antibiotics. At 70–80% confluence, the COS-7 and MKN-45 cells were transiently transfected with pCMV-E2F-1-HA and pCMV-HA, respectively, using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. Each plate was transfected with a mixture containing 2 μg of plasmid DNA and 7.5 μl of liposome. At 5 h after transfection, the medium was replaced with fresh DMEM containing serum and without antibiotics, and the cells were harvested at 24 and 48 h. (ii) Establishment of stable transformants overexpressing *E2F-1*: The *E2F-1* expression vector (pCMV-E2F-1-HA) or the empty pCMV-HA vector was transfected to gastric cancer MKN-45 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. Each plate was transfected with a mixture containing 10 μg of plasmid DNA and 40 μl of liposome. At 5 h after transfection,

the medium was replaced with fresh DMEM containing serum and without antibiotics, and the cells were selected by G418 at 48 h following transfection. The concentration of G418 for selection was gradually decreased as follows: 1 mg/ml for 4 days; 750 μg/ml for 4 days; 500 μg/ml for 4 days; and 250 μg/ml as a sustaining dose. At day 20 after transfection, five transformants were established with the empty pCMV-HA vector and 15 transformants were established with the *E2F-1* expression vector. The selected cell colonies were transferred from 10-mm dishes to 96-well plates and then from 96-well plates to 24-well plates. The transformants overexpressing E2F-1 were further selected and validated by Western blot assay.

(4) Western blot analysis:

Cell lysates were prepared in a buffer containing 0.1 M NaCl, 0.01 M Tris-Cl (pH 7.6), 0.001 M EDTA (pH 8.0), 1 μg/ml aprotinin, 100 μg/ml PMSF, and 1% NP40. After protein quantitation using the Lowery protein assay, equal amounts of proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes by the semi-dry blotting method using a three-buffer system. The membrane was blocked with 5% BSA in PBST (PBS, pH 7.5, containing 0.1% Tween 20) and incubated with a 1:1500 dilution of primary antibody (anti-E2F-1) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. The membrane was then washed with PBST and incubated with a peroxidase-conjugated secondary antibody (1:2500) (Santa Cruz Biotechnology, Inc.) for 1 h. Specific antibody binding was detected using a chemiluminescence detection system (Pierce, USA), according to the manufacturer's recommendations. Western blot films were scanned, and the net intensities of the bands were quantified using Image-QuanT software (Molecular Dynamics, Sunnyvale, CA, USA). After development, the membrane was stripped and reprobed with antibody against β-actin (1:1000) (Santa Cruz Biotechnology, Inc.) to confirm equal sample loading.

2.6. Cell proliferation and survival assay

The viability and proliferation of MKN-45 cells were determined by MTS assay using a CellTiter 96 AQueous assay system (Promega, Madison, WI, USA), according to the manufacturer's instructions. The assay measures the dehydrogenase enzyme activity in metabolically active tumor cells, as reflected by the conversion of MTS to formazan, which is soluble in tissue culture medium and was detected by absorbance at 490 nm. The production of formazan is proportional to the number of living cells, with the intensity of the produced color serving as an indicator of the cell viability. Briefly, the MKN-45 cells stably transfected with pCMV-E2F-1-HA or empty vector were plated at 1×10^5 cells/well in 96-well plates and cultured for 7 days. MTS mixed with medium without serum was added to the cell cultures after removing the old medium. The plates were incubated at 37°C for 2 h, and the absorbance (A) at 490 nm was determined using a 96-well Opsys MR^{TM} microplate reader (ThermoLabsystems, Chantilly, VA, USA) and Revelation^{TM} QuickLink software. The blank control wells with medium only were set as zero absorbance. The percentage of cell survival was calculated using the background-corrected absorbance: % cell viability $= 100 \times (1 - \text{A of experimental well})/\text{A}$ of untreated control well. All experiments were performed at least three times, and representative data are presented in Fig. 5.

2.7. Cell cycle analysis by flow cytometry

MKN-45 cells (5×10^5) were seeded in a 10-cm tissue culture dish and incubated overnight. The cells were transiently transfected with pCMV-E2F-1-HA or pCMV-HA, cultured in medium for 48 h, and harvested. For cell cycle analysis, 1×10^6 cells were washed twice with ice-cold PBS, treated with trypsin, and then fixed in 70% cold ethanol at 4°C for 30 min. The cell pellet was incubated in a solution containing 50 ng/ml propidium iodide, 0.2 mg/ml RNase, and 0.1% Triton X-100 at room temperature for 30 min, and then analyzed by flow cytometry using a FACscan (Becton Dickinson, Mountain View, CA, USA). The data were analyzed with the CellFit cell-cycle analysis program (version 2.01.2).

2.8. Statistical analysis

Data are shown as the mean ± SD. To analyze the correlation between E2F-1 protein expression and the patients' parameters, including histopathological findings, the χ^2 test or Fisher's exact test were used. The data of immunohistochemical assays were statistically analyzed with the SPSS 10.0 statistical package, followed by variance analysis for comparison among different groups. The Student's t-test was used to analyze the statistical significance of the differences between the control and E2F-1 groups in cell survival assay, colony formation assay, and cell cycle study. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Mutation of the E2F-1 gene in human gastric tumor, paratumor, and normal gastric tissues

First, we searched for the mutation of the *E2F-1* gene in human gastric tumor, paratumor, and normal gastric tissues. We extracted genomic DNA from specimens of the gastric tumor, paratumor, and normal gastric mucosa tissues and analyzed for point mutations in all seven exons of *E2F-1* by polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP). As shown in Fig. 1A, the exon lengths (bp) of the *E2F-1* gene were 291 (exon 1), 215 (exon 2), 350 (exon 3), 224 (exon 4), 179 (exon 5), 275 (exon 6), and 293 (exon 7), respectively. No insertions, deletions, or mutations were identified in any of the *E2F-1* exons in the gastric tumor, paratumor, or normal stomach mucosa tissues examined in this study. Fig. 1B, C and D show a representative PCR-SSCP experiment, demonstrating that no mutations were detected in exon 2, exon 3 and exon 5 of *E2F-1*, respectively, in the tumor or nontumor tissues from four different patients with gastric carcinomas.

3.2. Comparison of E2F-1 protein expression among human gastric tumor, paratumor and normal stomach tissues

About 80% of cases and control subjects in the study were male, with a mean age at diagnosis of 62 years (range, 25 to 82 years). Higher levels of E2F-1 expression were detected in gastric tumor tissues. The positive rates of E2F-1 protein expression in the tumor group, paratumor group, and control subjects were 72.5% (58/80), 30.0% (24/80), and 22.5% (9/40), respectively. The positive rate was significantly higher in the tumor group than in the paratumor or healthy control group ($P < 0.001$). On the contrary, the negative rate of E2F-1 expression was considerably higher in the paratumor and healthy control groups than in the tumor group ($P < 0.01$), with a percentage of 27.5% (22/80) in gastric tumor, 70% (56/80) in paratumor, and 77.5% (31/40) in normal stomach mucosa tissues.

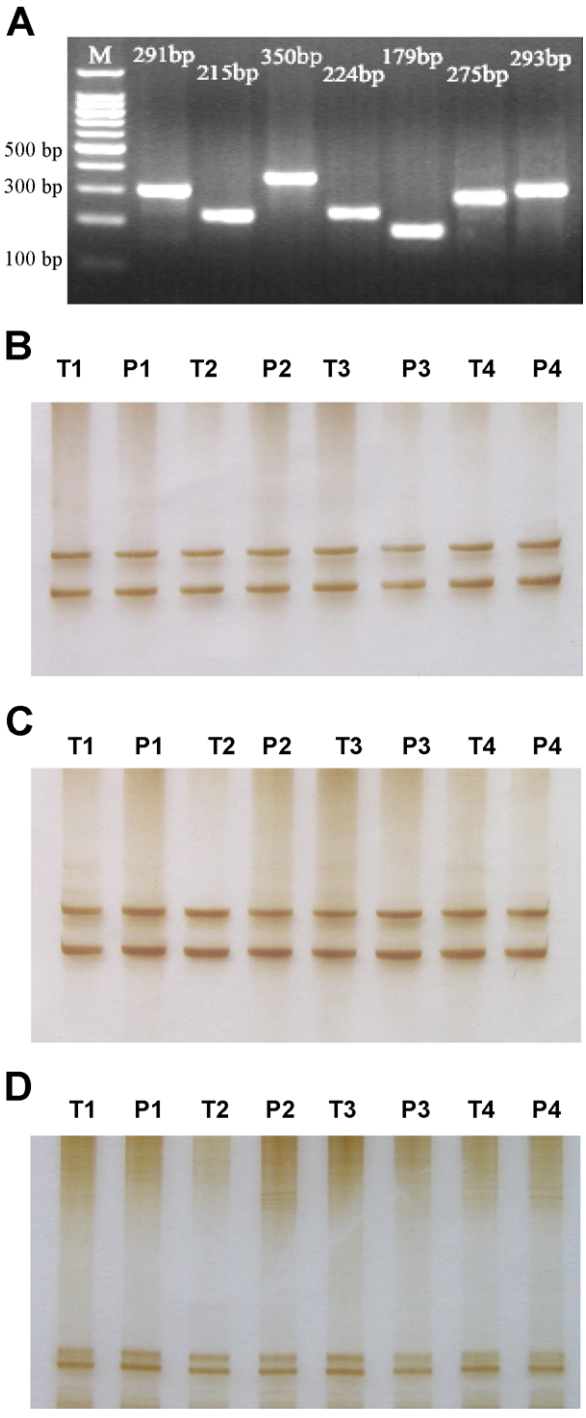

Fig. 1. PCR-SSCP analysis to detect *E2F-1* gene mutations in gastric tumor and nontumor tissues. (A) Polymerase chain reaction (PCR) amplification of the human *E2F-1* gene in healthy stomach tissues. The 2% agarose gel shows the lengths (bp) of the *E2F-1* exons: exon 1, 291; exon 2, 215; exon 3, 350; exon 4, 224; exon 5, 179; exon 6, 275; exon 7, 293. M, DNA markers ranging from 100 to 2000 bp. (B, C and D) Based on PCR-single-strand conformational polymorphism (PCR-SSCP), no insertions, deletions, or mutations are detectable in *E2F-1* exon 2 (B), exon 3 (C) and exon 5 (D) of gastric tumor and paratumor tissues from four different patients with stomach cancers. All seven exons were validated for sequence by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. T, tumor tissue; P, paratumor tissue.

Table 2
Detection of E2F-1 protein expression in gastric tumor, paratumor, and normal gastric tissues by immunohistochemistry

Tissue group	Total No. of specimens	No. of − specimens	No. of + specimens	No. of ++ specimens	Positive rate (%)	P-value
Normal	40	31	7	2	22.5	$<0.0001^{*}$
Tumor	80	22	43	15	72.5	$<0.0001^{**}$
Paratumor	80	56	20	4	30.0	0.3850^{***}

−, negative; +, weak; ++, strong expression of E2F-1 protein. $^{*}P$, normal group *vs*. tumor group. $^{**}P$, tumor group *vs*. paratumor group. $^{***}P$, normal group *vs*. paratumor group.

However, there was no statistical difference in E2F-1 protein expression between the paratumor group and the healthy control subjects ($P > 0.05$) (Table 2 and Fig. 2).

The relationships between E2F-1 expression and the clinical features of gastric cancer patients are shown in Tables 3 and 4. In the tumor group, the mean age of E2F-1-positive patients was 62.8 years (range, 46–82 years), and that of E2F-1-negative patients was 61.2 years (range, 38–78 years). Of the 58 patients (59.8%) who were positive for E2F-1, 47 (81.0%) were male and 11 (19.0%) were female. The rate of E2F-1 expression in the tumor group was not correlated with gender ($P = 0.936$) or age ($P = 0.783$). Higher levels of E2F-1 expression by immunostaining did not correlate positively and significantly to histological type ($P = 0.687$), lymph node metastasis ($P = 0.303$), or tumor stage ($P = 0.584$). In the paratumor group, E2F-1 protein expression was not associated with gender ($P = 0.755$), age ($P = 0.769$), histological type ($P = 0.955$), lymph node metastasis ($P = 0.243$), or tumor stage ($P = 0.655$), which was similar to the result in the tumor group (Tables 3 and 4).

3.3. *Construction and transfection of pCMV-E2F-1-HA recombinant vector*

To examine the effect of E2F-1 on gastric tumor cell growth *in vitro*, we used the plasmid pCMV-HA to construct the vector pCMV-E2F-1-HA encoding E2F-1. As shown in Fig. 3, pCMV-E2F-1-HA contained a 1.3-kb EcoR I fragment, representing the

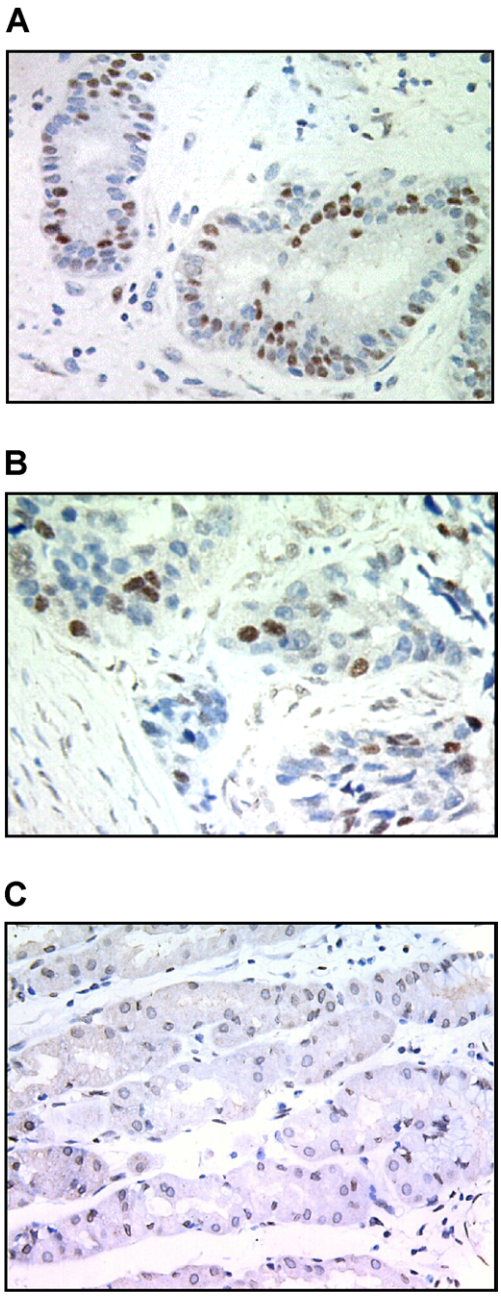

Fig. 2. Immunohistochemical assessment of E2F-1 protein expression in gastric cancer tissues. Five-μm tissue sections were cut from formalin-fixed, paraffin-embedded stomach tumor tissue specimens. Immunohistochemical staining with anti-E2F-1 antibody shows that E2F-1 protein is strongly positive (A), weakly positive (B), or negative (C) in human gastric carcinoma tissues. Representative sections of gastric tumors stained by immunohistochemistry for E2F-1 are shown (original magnification, ×200).

Table 3
Relationship between E2F-1 protein expression and clinicopathological features of gastric carcinoma patients

Clinicopathological features	No. of patients	−	+	++	Positive rate (%)	P-value
Gender						
Male	65	18	34	13	72.3	0.936
Female	15	4	9	2	73.3	
Age (years)						
<65	42	11	23	8	73.8	0.783
⩾65	38	11	20	7	71.1	
Histological subtype[a]						
Tubular adenocarcinoma	16	4	10	2	75.0	0.687
Papillary adenocarcinoma	13	2	8	3	84.6	
Poorly differentiated adenocarcinoma	47	15	22	10	68.1	
Signet-ring cell carcinoma	4	1	3	0	75.0	
Lymph node metastasis[b]						
(+)	51	16	25	10	68.6	0.303
(−)	29	6	18	5	79.3	
Tumor stage[b]						
Early stage (I–II)	33	8	18	7	75.8	0.584
Late stage (III–IV)	47	14	25	8	70.2	

−, negative; +, weak; ++, strong expression of E2F-1 protein.
[a] These parameters were determined by pathological analysis.
[b] These parameters were determined by pathological analysis according to the TNM system. (+), with and (−), without lymph node metastasis.

Table 4
Relationship between E2F-1 protein expression and clinicopathological features in surgical samples of paratumor stomach tissues

Clinicopathological features	No. of patients	−	+	++	Positive rate (%)	P-value
Gender						
Male	65	45	17	3	30.8	0.755
Female	15	11	3	1	26.7	
Age (years)						
<65	42	30	9	3	28.6	0.769
⩾65	38	26	11	1	31.6	
Histological subtype[a]						
Tubular adenocarcinoma	16	12	3	1	25.0	0.955
Papillary adenocarcinoma	13	9	2	2	30.8	
Poorly differentiated adenocarcinoma	47	32	14	1	31.9	
Signet-ring cell carcinoma	4	3	1	0	25.0	
Lymph node metastasis[b]						
(+)	51	38	11	2	25.5	0.243
(−)	29	18	9	2	37.9	
Pathological stage[b]						
Early stage (I–II)	33	24	8	1	27.3	0.655
Late stage (III–IV)	47	32	12	3	31.9	

−, negative; +, weak; ++, strong expression of E2F-1 protein.
[a] These parameters were determined by pathological analysis.
[b] These parameters were determined by pathological analysis according to the TNM system. (+), with and (−), without lymph node metastasis.

exogenous E2F-1 insertion. This fragment was not present in the empty pCMV-HA after EcoR I digestion.

COS-7 cells and MKN-45 cells were transiently transfected with pCMV-E2F-1-HA for 24 and 48 h, and E2F-1 expression was analyzed by Western blot-

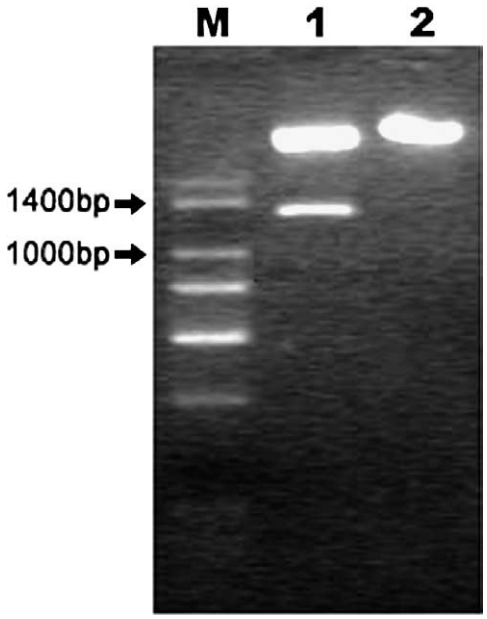

Fig. 3. Verification of the pCMV-E2F-1-HA recombinant vector construct. EcoR I digestion of pCMV-E2F-1-HA revealed a 1.3-kb fragment, representing the exogenous E2F-1 insert; no fragment resulted from digestion of the empty vector, pCMV-HA. M, DGL2000 DNA marker; Lane 1, cloned vector pCMV-E2F-1-HA; Lane 2, empty vector pCMV-HA.

ting with antibody against E2F-1. Markedly higher levels of E2F-1 protein were expressed in both COS-7 and MKN-45 cells transfected with pCMV-E2F-1-HA (Fig. 4). In contrast, no E2F-1 expression was observed in the MKN-45 cells transfected with empty vector pCMV-HA (Fig. 4).

3.4. *Overexpression of E2F-1 inhibits cell growth and proliferation in the human gastric tumor cell line MKN-45*

Next, we determined the *in vitro* survival rates of gastric tumor cells stably transfected with pCMV-E2F-1-HA, using the human gastric carcinoma cell line MKN-45 as a model for gastric cancer. As shown in Fig. 5, the transfection of MKN-45 cells with pCMV-E2F-1-HA significantly reduced cell survival ($P < 0.01$), as assessed by MTS assay. Additionally, we observed that tumor cells stably transfected with E2F-1 obviously grew slower than cells stably transfected with empty vector, which was consistent with the elevated levels of E2F-1 in MKN-45 cells and indicated a suppressive effect of E2F-1 on MKN-45 cell growth and survival.

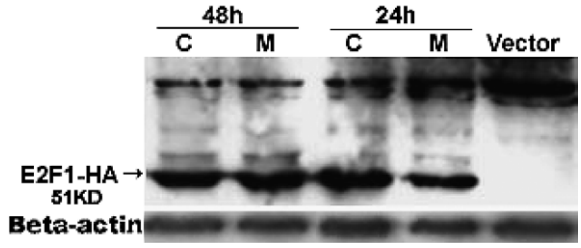

Fig. 4. Western blot analysis of E2F-1 protein levels in human gastric carcinoma cells transfected with pCMV-E2F-1-HA. MKN-45 and COS-7 cells were transiently transfected *in vitro* with pCMV-E2F-1-HA. Cellular proteins were extracted from the cells at 24 and 48 h after transfection, and the level of E2F-1 protein was analyzed by Western blotting. β-Actin was utilized as a control to verify equal protein loading and transfer. Shown are Western blot data representative of those obtained from three separate experiments. C, COS-7 cells transiently transfected with pCMV-E2F-1-HA; M, MKN-45 cells transiently transfected with pCMV-E2F-1-HA; *Vector*, MKN-45 cells transiently transfected with empty vector pCMV-HA. E2F-1-HA (51 kD) = E2F-1 (46.9 kD) + HA (4.1 kD).

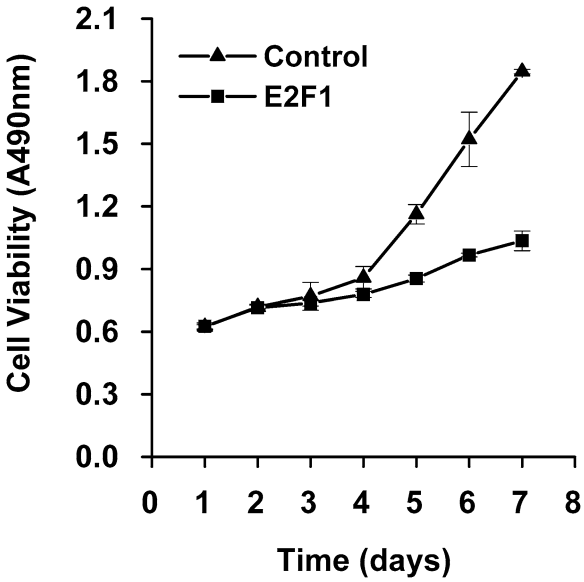

Fig. 5. Survival of human MKN-45 gastric cancer cells stably transfected *in vitro* with *E2F-1*. MKN-45 cells were stably transfected with either empty vector pCMV-HA (*Control*) or pCMV-E2F-1-HA (*E2F1*). Cell survival was assessed by MTS assay, as described in *Patients and methods*. The results represent the means of at least three independent experiments. The inhibition of proliferation was observed after 24 h of MTS treatment in MKN-45 cells stably transfected with pCMV-E2F-1-HA. $P < 0.01$ for the control group *vs.* E2F-1 group.

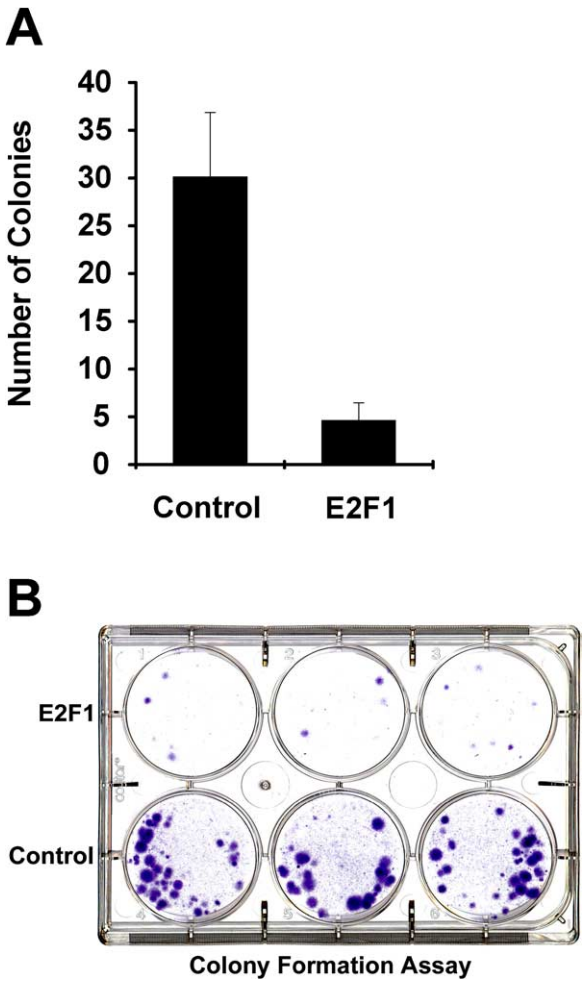

Fig. 6. Effect of E2F-1 overexpression on cell growth in cultured human stomach cancer cells, as determined by the colony growth assay. (A) MKN-45 cells were plated in six-well plates, transiently transfected with either pCMV-E2F-1-HA or the empty vector pCMV-HA, and grown in G418 for 7 days. The surviving fraction of cells (visible colonies) was stained with gentian violet, photographed, and counted manually. Each column presents the mean ± SD from three separate determinations. $P < 0.001$ for control *vs*. E2F-1 group. (B) A representative result of the colony formation assay obtained from three independent experiments. *E2F1*, MKN-45 cells transiently transfected with pCMV-E2F-1-HA; *Control*, MKN-45 cells transiently transfected with pCMV-HA.

To confirm the inhibitory effect of E2F-1 on the growth of MKN-45 cells, we performed colony formation assays. Fig. 6 shows that a proportion of human gastric carcinoma cells transiently transfected with pCMV-HA proliferated again in 7 days after transfection, whereas regrowth was significantly diminished in human stomach cancer cells transiently transfected with *E2F-1*. The MKN-45 cells transfected with pCMV-E2F-1-HA exhibited 7.5-fold fewer colonies than did cells transfected with pCMV-HA ($P < 0.001$). Together, these data suggest that E2F-1 inhibited cell growth and proliferation in our gastric cancer cell system.

3.5. Effect of E2F-1 overexpression on cell cycle control in human MKN-45 gastric carcinoma cells

Finally, we used flow cytometry to determine whether the inhibitory effect of E2F-1 on MKN-45 cell proliferation was mediated, at least in part, through affecting cell cycle progression. We found that MKN-45 cells transiently transfected with pCMV-E2F-1-HA were 71.4% at G0/G1 phase and 18.7% at S phase, with a 19.6% increase in G1-phase cell population and a 39.1% decrease in S-phase cell population at 48 h after transfection with *E2F-1*, as compared to cells transiently transfected with empty vector ($P < 0.05$) (Table 5 and Fig. 7). These data indicate that cell growth inhibition by E2F-1 was associated with significant cell cycle arrest in G1 phase and suggest that E2F-1 suppresses cell proliferation by controlling the G1 checkpoint and inducing a specific block in cell cycle progression.

4. Discussion

In the current study, we investigated the mutation and expression of *E2F-1* in human gastric cancer tissues and assessed the effect of E2F-1 overexpression on the proliferation of human gastric carcinoma MKN-45 cells *in vitro*. We showed that (i) no insertion, deletion, or mutation was present in any of the seven *E2F-1* exons in any of the gastric cancer or normal tissues examined; (ii) the rate of E2F-1 expression was higher in gastric tumor tissues than in paratumor mucosa and normal gastric mucosa; (iii) the level of E2F-1 ex-

Table 5
Cell cycle analysis by flow cytometry in human MKN-45 gastric carcinoma cells transfected with *E2F-1*

Group	Cell cycle phase		
	G0/G1 (%)	S (%)	G2/M (%)
Control	59.7*	30.7	9.6
E2F-1	71.4*	18.7	9.9

Control, MKN-45 cells transiently transfected with pCMV-HA. E2F-1, MKN-45 cells transiently transfected with pCMV-E2F-1-HA.
* $P < 0.05$, E2F-1 group *vs*. control group for G0/G1 cells.

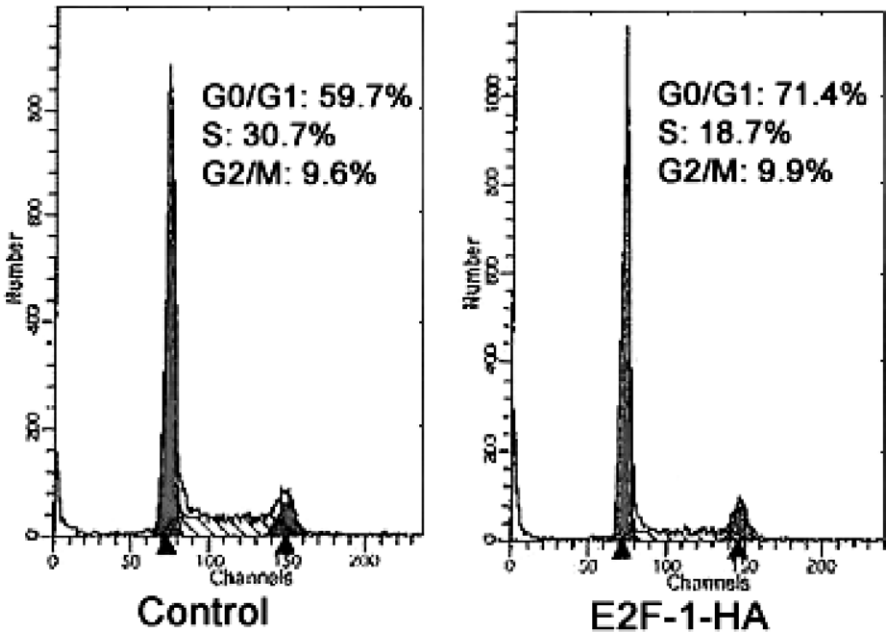

Fig. 7. Cell cycle analysis by flow cytometry in MKN-45 human gastric carcinoma cells transfected with *E2F-1*. MKN-45 cells were transiently transfected with pCMV-E2F-1-HA (*E2F-1-HA*) or pCMV-HA (*Control*). The cells were harvested and stained at 48 h after transfection. The cell cycle distribution of the propidium iodide-labeled cells was analyzed by flow cytometry, as described in *Patients and methods*. The data are representative of three independent experiments. $P < 0.05$ for the G0/G1 population in control *vs*. E2F-1-HA group.

pression was not correlated with clinicopathological features, including gender, age, histological subtype, lymph node metastasis, and clinical stage in gastric tumors; and (iv) the overexpression of E2F-1 in MKN-45 cells inhibited cell growth and proliferation and blocked entry into S phase of the cell cycle.

E2F-1 activates a number of genes responsible for DNA replication and for S phase progression [5]. It has a dual role in cancer development, with the capacity to act as both an oncogene and a tumor suppressor gene [43–45]. E2F-$1^{(-/-)}$ mice exhibited a bimodal phenotype, facilitating tumor development in some tissues, and inhibiting tumor formation in others [23,25,46]. The molecular basis of this behavior is undetermined, and it is not clear whether it also applies in human tumors.

Several lines of evidence suggest that E2F-1 has the potential to function as an oncogene, promoting the proliferation of cells beyond their normal constraints [14–20]. Yang and colleagues [47] demonstrated that the overexpression of E2F-1 can transform rat fibroblasts into tumor cells. E2F-1 loses this transformation ability when it is mutated [48]. In the present study, a large number of DNA samples from stomach tissues of gastric cancer patients and healthy individuals were analyzed, and no mutation of E2F-1 was evidenced. These results indicate that E2F-1 might be a stable and conservative gene with a low mutation rate during oncogenesis. Although *E2F-1* mutations and deletions were not detected in gastric carcinoma in this study, other genetic and epigenetic alterations of the *E2F-1* gene are not ruled out. Therefore, further investigation is necessary to determine the amplification and modifications (acetylation, methylation, phosphorylation, and ubiquitylation) of the *E2F-1* gene in stomach cancer and other tumors, as well as the relationship between these changes in *E2F-1* and tumor growth and development.

Opinions differ concerning the association of E2F-1 expression with clinical pathological features. Ho et al. [49] found that E2F-1 expression was lower in primary breast cancers than in breast cancers with metastases. Zhang and coworkers [50] showed that E2F-1 expression was higher in invasive duct cancer than in duct cancer *in situ*. Both groups proposed that E2F-1 expression was higher in poorly differentiated tumors than in well-differentiated tumors, such that faster growing and more poorly differentiated tumors had higher levels of E2F-1 expression. In the current study, we found that the E2F-1 level was considerably higher in gastric tumors (72.5%) than in paratumor mucosa and normal gastric mucosa (30.0% and

22.5%, respectively) and that the difference in the E2F-1 level between the two groups reached statistical significance. Similar findings were reported recently in five of the six tumor types examined, including carcinomas of the bladder, breast, colon, lung, and oesophagus, with the exception of prostate tumors [33,39,40]. This demonstrates that E2F-1 expression is positively correlated with tumor progression. However, there were no correlations between clinical pathological features and E2F-1 expression in surgical specimens of gastric carcinoma or paratumor normal mucosa in the present study. Rabbani et al. [48] reported similar results, with an E2F-1 expression rate of 60% (80/133) and E2F-1 mutation rate of 5.3% (7/133) in bladder tumors. Moreover, they did not find any correlations between the E2F-1 expression level and different types of bladder cancers or lymph node metastasis. In contrast to the results of Rabbani et al., Yamazaki and coworkers provided evidence showing that OSCC patients with increased E2F-1 expression had a significantly shorter recurrence free survival time, suggesting the influence of E2F-1 expression on clinical outcome in patients with OSCC [39]. Gorgoulis and colleagues studied the relationship between the expression status of E2F-1 and tumor kinetic parameters in four of the most common human malignancies and found that E2F-1 expression correlated with proliferation and growth indices in breast and bladder carcinomas, whereas the opposite seems to be the case for colon and prostate cancers [40]. In a separate study investigating a panel of 87 NSCLCs, Gorgoulis *et al.* also found that squamous cell lung carcinomas exhibited significantly higher percentages of E2F-1-positive cells than adenocarcinomas [33], reflecting the different biological profiles of these NSCLC histological subtypes. In addition, they demonstrated that the lung carcinomas with increased E2F-1 positivity have significantly raised growth indexes and were associated with poor patient outcome [33]. These observations indicate that the correlation between E2F-1 expression and clinical characteristics may vary among tumor types.

We obtained a stable cell line expressing E2F-1 by transfecting MKN-45 gastric tumor cells with the expression vector pCMV-E2F-1-HA. Our results clearly showed that E2F-1 overexpression in MKN-45 cells could inhibit cell growth and block the cell cycle transition from G1 to S phase, indicating that the overexpression of E2F-1 can suppress tumor progression. Recently, several studies reported an association between E2F-1 overexpression and tumor cell growth and survival. Hunt and colleagues [51] found that adenovirus-mediated E2F-1 overexpression induced apoptosis in human breast and ovarian carcinoma cells. Fueyo and coworkers [52] showed that the overexpression of E2F-1 could trigger apoptotic cell death in human gliomatous cells. After the colonic adenocarcinoma cell lines HT-29 and SW620 were transfected with E2F-1, Elliott et al. [53] demonstrated that E2F-1 overexpression could inhibit tumor cell growth and induce apoptosis and that it was accompanied by the upregulation of c-Myc and $p14^{ARF}$ and the downregulation of MCL-1 in these cells. Furthermore, Elliott et al. [54] also investigated the correlation between E2F-1 overexpression and chemotherapy sensitivity in primary pancreatic cancer, showing that E2F-1 overexpression mediated by adenoviruses not only induced apoptosis but also enhanced the sensitivity of the cells to chemotherapeutic drugs. Together, these studies demonstrate that E2F-1 overexpression can inhibit cell growth and induce apoptosis in different types of human cancers.

However, the molecular mechanisms underlying the effect of E2F-1 overexpression on cell cycle arrest, cell growth inhibition, and cell survival reduction in MKN-45 cells remain unknown. Accumulating evidence suggests that E2F-1 can induce cell cycle arrest and trigger apoptosis by both p53-dependent and p53-independent pathways through several mechanisms. First, a recent report demonstrated that the increased expression of E2F-1 caused cell cycle arrest in S phase of the cell cycle through a p53-independent induction of endogenous p21 [32]. p21 is a potent inhibitor of cyclin-dependent kinases capable of arresting cell cycle progression. Second, E2F-1 augments p53-dependent apoptosis through the transcriptional activation of the alternative reading frame (ARF) gene, which in turn modulates the activity of MDM2 and leads to the release of p53 [55]. Third, E2F-1 signals apoptosis independently of p53 via direct transcriptional activation of the apoptotic protease-activating factor-1 (Apaf-1) gene [56] and the p53 family member p73 [57,58], which are direct targets for E2F-1. Apaf-1 mechanistically regulates cytochrome c release from mitochondria and subsequently causes apoptosis by activation of caspase-9 and caspase-3, while increased p73 levels can induce cell cycle arrest and apoptosis [57]. E2F-1 has also been shown to participate directly in apoptotic pathways by stimulating the accumulation of caspases through a direct transcriptional mechanism [59], and E2F-1-dependent apoptosis is significantly compromised by the inhibition

of caspase activity or inactivation of the Apaf-1 gene [56,60]. Fourth, one mechanism through which E2F-1 can promote apoptosis is by inhibiting the activity of NF-κB, which regulates cell survival by activating various anti-apoptotic genes [61]. Moreover, E2F-1 can inhibit NF-κB DNA-binding activity, thereby providing another mechanism of inhibition of NF-κB activity by E2F-1 [62]. Finally, in addition to the role of E2F-1 in cell cycle arrest, apoptosis, and cell differentiation, studies in recent years have identified E2F-1 as an important transcriptional regulator of genes involved in cell growth arrest and cell proliferation suppression through an E2F-dependent self-inhibitory loop [63–65]. For example, the nuclear factor CDCA4 is induced by E2F-1 and regulates E2F-dependent transcriptional activation and cell proliferation. Thus, CDCA4 forms a negative feedback loop in which CDCA4 is first induced by E2F-1 and then inhibits E2F-1-dependent transcriptional activity. It is possible that the effect of E2F-1 overexpression on cell cycle G1 arrest and cell growth inhibition observed in our cell model system may be mediated partly through the important role of CDCA4 and other molecules in the regulation of cell cycle progression via their effects on the transcriptional activity of E2F-responsive promoters during the G1 and S phases. It is likely that E2F-1 plays opposing roles in regulating cell growth and proliferation in MKN-45 cells, depending upon the level and activity of E2F-1 in the cells. It promotes cell growth when the level and activity of E2F-1 are increased, whereas it suppresses cell proliferation through the E2F-dependent self-inhibitory loop when E2F-1 is overexpressed. Thus, the present study supports a role for E2F-1 in the control of cell cycle progression and cell proliferation through the transcriptional regulation of E2F-responsive genes in tumor and normal cells.

In conclusion, this study demonstrates that *E2F-1* might be a stenoplastic and conservative gene involved in the development of gastric carcinomas and indicates that the low mutation rate in *E2F-1* supports its crucial role in this tumor. The higher level and rate of E2F-1 protein expression in gastric cancer tissues relative to those in paratumor and normal stomach tissues suggest that E2F-1 may function as an oncogene to enhance tumor development. However, this function and the cellular and molecular basis for E2F-1 in promoting tumor cell growth remain to be experimentally determined. Conversely, we found that E2F-1 serves as a suppressor of tumor progression by causing growth inhibition and cell cycle arrest at G1 phase. Our findings strengthen the proposal of a role for E2F-1 in the control of cell proliferation and cell survival in tumor and normal cells. Whether E2F-1 contributes to proliferation or apoptosis may depend on the E2F-1 level in a cell; thus, the level and activity of E2F-1 could help determine the fate of a cell. Although E2F-1 is well known for its role in cell cycle progression, its role and underlying mechanism in tumor cell cycle arrest, cell growth suppression, and apoptotic cell death have not been clearly defined.

Further studies will assess how E2F-1 serves its role in coordinately regulating cell growth and cell death under certain circumstances, and will elucidate the molecular basis for the effect of E2F-1 overexpression on cell cycle control and cell survival in gastric tumor and normal cells. Furthermore, the observation that the deregulation of the E2F family of transcription factors is a common event in most, if not all, human cancers, together with its role in cell growth arrest and cell survival control, make E2F-1 an attractive target for designing new types of cancer therapeutics aimed at modulating E2F-1 activity. The pharmacological activation of E2F-1 in tumor cells could provide a potent apoptotic signal, thus limiting the extent of tumor cell proliferation. Our present study suggests that gene transfer of *E2F-1* may be a successful strategy in stomach cancer gene therapy. Considering the current lack of effective treatments against malignant gastric tumors, we advocate using E2F-1 as a drug or *E2F-1* gene therapy as a new therapeutic approach for this disease in the clinic and recommend further investigation.

Acknowledgements

This work was partly supported by grants from the Natural Science Foundation of Guangxi, China (Nos. 0135012 and 0640085).

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