

miR-31 promotes proliferation of colon cancer cells by targeting E2F2

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Abstract MicroRNA-31 (miR-31) plays important roles in colon cancer development. However, the underlying mechanism is still not clear. We have explored the functions of miR-31 on proliferation of colon cancer cells as well as the underlying mechanism. E2F2 was identified as a direct target of miR-31. miR-31 regulated the proliferation of colon cancer cells by targeting E2F2. Moreover, in the present study, E2F2 acted as a tumor suppressor in colon cancer by repressing the expression of survivin and regulating the expression of CCNA2, C-MYC, MCM4 and CDK2. A possible mechanism for the function of miR-31 on colon cancer proliferation is presented and

indicates that miR-31 might become a target for anti-cancer drug design.

Keywords Anti-cancer drug design · Colon cancer cells · E2F2 · MicroRNA-31 · Proliferation · Survivin · Tumor suppression

Introduction

MicroRNAs (miRNAs) are small endogenous non-coding RNA ranging from 19 to 22 nt. They occur in animals, plants and viruses. miRNA genes are first transcribed to pri-miRNAs, then cut to shorter hairpin pre-miRNAs by Drosha and, finally, cleaved into mature single-stranded miRNAs by Dicer. miRNAs frequently bind to “seed matches” in the 3′-

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untranslated region (3'UTR) of target mRNAs resulting in translational inhibition or degradation of protein-coding transcripts. miRNAs regulate more than 30 % of all human genes (Carrington and Ambros 2003).

Expression of microRNA-31 (miR-31) is frequently altered in cancer cells. miR-31 is expressed at high level in carcinomas of the tongue, head, neck, colon, hepatocellular and lung and is down-regulated in tumors of bladder, prostate, gastric, breast and ovarian. miR-31 also participates in many process including proliferation, migration, differentiation and apoptosis. Here, we have found that miR-31, which is over-expressed in colon cancer, is related to the proliferation of colon cancer cells, but the underlying mechanism is unclear.

Members of E2F family play important roles in proliferation, apoptosis, differentiation. E2Fs usually bind to Rb protein to form the Rb/E2F complex which regulates the expression of genes involved in cell cycle progress, DNA synthesis, checkpoint control and apoptosis. E2F2 is one of the E2F family members and belongs to the promotor group. However, there are several reports about its suppressor activity, E2F2 suppresses the proliferation of T lymphocytes (Azkargorta et al. 2010; Infante et al. 2008; Opavsky et al. 2007; Pusapati et al. 2010). Here in this study, we explored whether E2F2 can act as a suppressor in colon cancer.

In our study, we have verified that E2F2 is a direct target of miR-31 using western blotting and luciferase reporter assay. miR-31 is also shown to regulate the proliferation of colon cancer by targeting E2F2 using siRNA technology and E2F2 acts as a suppressor in colon cancer by over-expression of E2F2.

Materials and methods

Cell culture

Human colon cancer cell SW-620 and human embryonic kidney cell HEK-293T were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10 % (v/v) fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5 % (v/v) CO₂. Human colon cancer cell HCT-116 was cultured in RPMI 1640 media with 10 % (v/v) FBS at 37 °C in a humidified atmosphere with 5 % CO₂.

Transfection

Cells were seeded in six-well plates at 10⁵/well. 4 µg DNA or 100 pmol RNA were transfected into the cells using Lipofectamine 2000 reagent according to the manufacturer's protocol.

Cell viability assay

Cell viability assay was performed to test the effects of miR-31 or E2F2 on the proliferation of colon cancer cells. After treatment with transfection, 6,000 cells of each sample were plated in 96-well plates in triplicate, cells proliferation was tested every 24 h using MTT.

EdU incorporation assay

EdU incorporation assay was used to determine the speed of DNA synthesis with a Cell-light EdU Apollo 567 In Vitro Kit (Ribobio, Guangzhou, China) according to the manufacturer's protocol. 48 h after transfection, 50 mM EdU was added to each well, and the cells were cultured for additional 2 h at 37 °C. Then the cells were fixed with 4 % (w/v) paraformaldehyde for 30 min and permeabilized with 0.5 % (v/v) Triton X-100 for 10 min at room temperature. After washing with PBS, 100 µl Apollo staining solution was added to each well, and the cells were incubated for 30 min at room temperature. The cells were subsequently stained with 100 µl Hoechst 33342 for 30 min and visualized under a laser scanning confocal microscope.

Total RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was extracted from each sample after transfection using Trizol according to the manufacturer's protocol. The miR-31 expression level was detected by SYBR Green qRT-PCR method with a Hsa-miR-31 Detection Kit and EzOmics SYBR qPCR Kit (Biomics, Jiangsu, China) using StepOne real-time PCR system, miR-31 expression level was normalized to U6 snRNA and the miR-31 relative expression level of each group was calculated using the $2^{-\Delta\Delta C_t}$ method. mRNA expression levels of E2F2, CCND3, CCNA2, CDC2, CDK2, MCM4, MCM7 and c-myc were detected by SYBR Green qRT-PCR method with a EzOmics SYBR qPCR kit using the primers given in Supplementary Table 1. The expression level was

normalized to GAPDH and the relative expression level was calculated using the $2^{-\Delta\Delta C_t}$ method.

Luciferase reporter assay

Luciferase reporter assay was used to test whether miR-31 bound to the 3'UTR of E2F2 mRNA directly. E2F2 mRNA containing wild type (WT), mutant type (MUT) or deleted type (DEL) miR-31 binding site were cloned respectively into the pMIR-REPORT luciferase reporter vector. HEK-293T cells were seeded in a 24-well plate and 0.2 μ g of either WT, MUT or DEL, together with 0.2 μ g β -galactosidase (β -gal) control plasmid plus either 20 pmol miR-31 mimics (31 M) or its corresponding negative control (MNC) were co-transfected into HEK-293T cells using Lipofectamine 2000 reagent. 48 h after transfection, luciferase activity and β -gal activity was measured using luciferase assay system and β -galactosidase enzyme assay system (Promega, Madison, WI, USA), respectively, according to the manufacturer's protocols. The luciferase activity was normalized to β -gal.

Western blotting

Cells were harvested 48 h after transfection and lysed in RIPA lysis buffer on ice. Protein concentration was measured using BCA method. 40 μ g protein of each sample were subjected to 12 % SDS-PAGE. The separated proteins were transferred to PVDF membranes. Membranes were blocked with 5 % (w/v) skim milk and then incubated with primary antibodies against E2F2 (Epitomics, Burlingame, CA), survivin (Bioworld, Atlanta, Georgia, USA) and β -actin (Bioworld) following by incubating with horseradish peroxidase-conjugated secondary antibodies. The protein expression level was normalized to β -actin.

Plasmids construct

Total RNA was extracted from colon cancer cells using Trizol reagent according to the manufacturer's protocol. The total RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega) and random primer. The coding sequence (CDS) and 3'UTR of E2F2 were amplified by PCR using the primers listed in Supplementary Table 2 and cDNA as templates. Then the CDS and 3'UTR of E2F2 were digested with KpnI and EcoRI and inserted into

pcDNA 3.1. After sequencing confirmation, they were named as E2F2-CDS and E2F2-3'UTR.

Statistics

All experiments were performed at least three times. The data was presented as the mean \pm SD. Student's *t* test was performed for comparisons between two groups. $P < 0.05$ was considered to be significant.

Result

miR-31 promotes proliferation of colon cancer

To investigate the effect of miR-31 on proliferation of colon cancer, a cell viability assay was performed. miR-31 mimics (31 M) was used to increase the expression level of miR-31 and miR-31 inhibitor (31I) was used to decrease miR-31 expression level. 31 M or its corresponding negative control (MNC), 31I or its corresponding negative control (INC) were transfected into colon cancer cell line HCT-116 and SW-620. Before the cell viability assay, the expression level of miR-31 was determined by qRT-PCR. As shown in Fig. 1, after transfection with 31 M, the miR-31 expression level increased more than 19-fold and 34-fold respectively in HCT-116 and SW-620 compared with those transfected with MNC (Fig. 1a). After transfection with 31I, the miR-31 expression level decreased by 48 and 84 % respectively in HCT-116 and SW-620 compared with those transfected with INC (Fig. 1b).

When transfected with 31 M, the proliferation capability of cells significantly increased comparing to cells transfected with MNC in both HCT-116 and SW-620 (Fig. 1c, e). After transfection with 31I, the proliferation capability of HCT-116 and SW-620 decreased significantly compared to cells transfected with INC (Fig. 1f, h). These results demonstrates that miR-31 increases the cell viability of colon cancer.

To explore the effect of miR-31 on proliferation of colon cancer, we performed EdU incorporation assay. Data showed that after transfection with 31 M, EdU-positive cells (red) increased to 1.72-fold and 1.76-fold, respectively, in HCT-116 and SW-620 compared with those transfected with MNC (Fig. 1d). Whereas, when transfection with 31I, EdU-positive cells was decreased by 58 and 55 % compared with cells transfected with INC in both HCT-116 and SW-620 (Fig. 1g). The cell viability

assay and EdU incorporation assay demonstrates that miR-31 promotes the proliferation of colon cancer.

E2F2 is a direct target of miR-31

To explore the mechanism how miR-31 regulates the proliferation of colon cancer, we predicted that miR-31 regulated the proliferation of colon cancer by regulating its potential target E2F2 which was predicted by online software TargetScan (<http://www.targetscan.org/>). Western blotting was used to

explore the relationship between the expression level of miR-31 and E2F2. After transfection with 31 M, the E2F2 expression level decreased by 71 and 84 %, respectively, in HCT-116 and SW-620 comparing to that of cells transfected with MNC. After transfection with 31I, E2F2 expression level increased 1.5-fold and 1.3-fold, respectively, in HCT-116 and SW-620 comparing to that of cells transfected with INC (Fig. 2c). These results demonstrates that E2F2 expression is negatively regulated by miR-31.

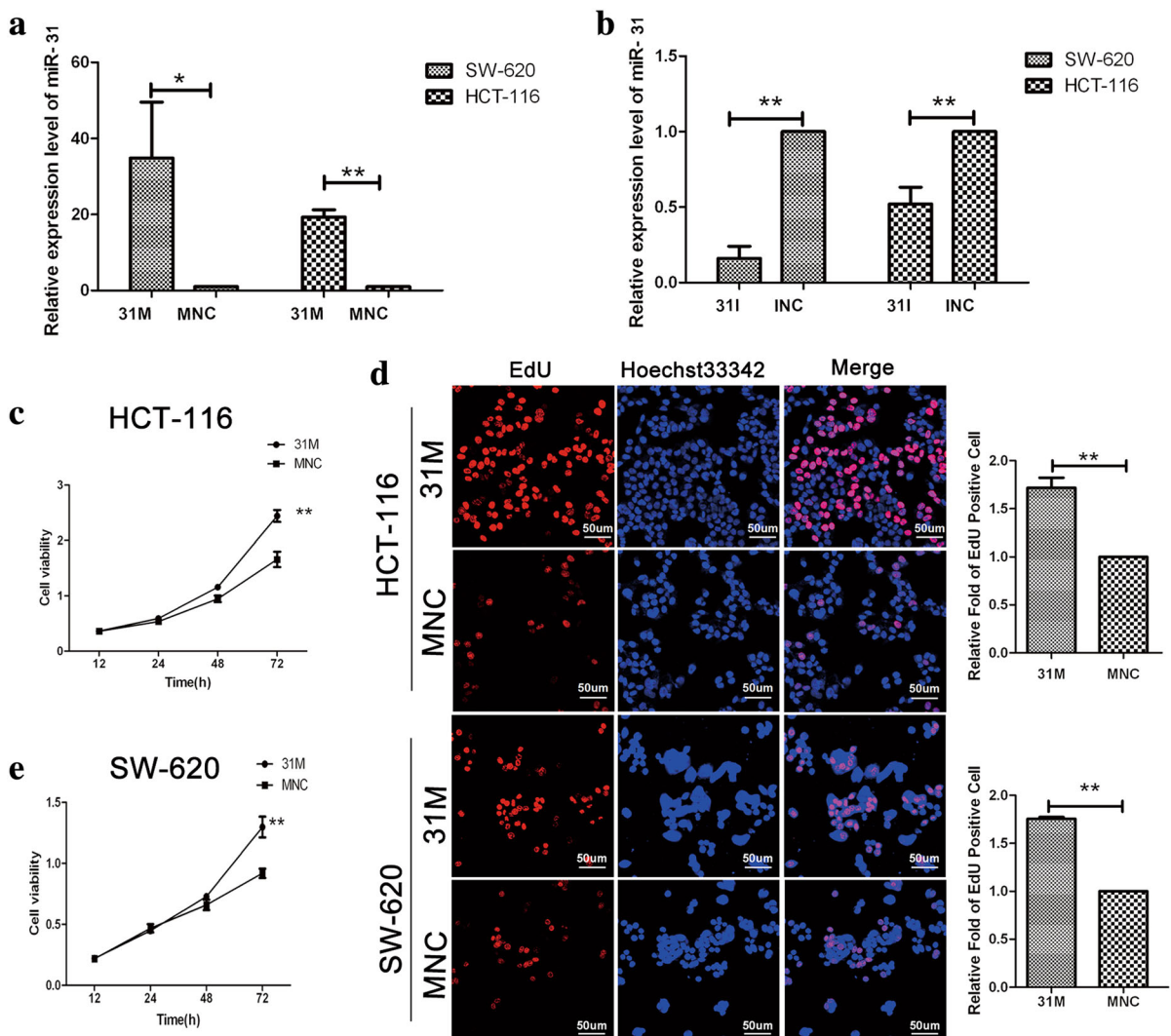


Fig. 1 miR-31 promotes the proliferation of colon cancer. **a**, **b** qRT-PCR detected the expression level of miR-31 in HCT-116 and SW-620 cells transfected with miR-31 mimics (31 M) or miR-31 inhibitor (31I) compared with those transfected with its corresponding negative control (MNC or INC). The result

were presented as fold changes of expression level. **c**, **e** Cell viability detected by MTT after transfection with 31 M. **f**, **h** Cell viability after transfection with 31I. **d** Cells in S phase detected by EdU after transfection with 31 M. **g** Cells in S phase after transfection with 31I. (* $P < 0.05$, ** $P < 0.01$)

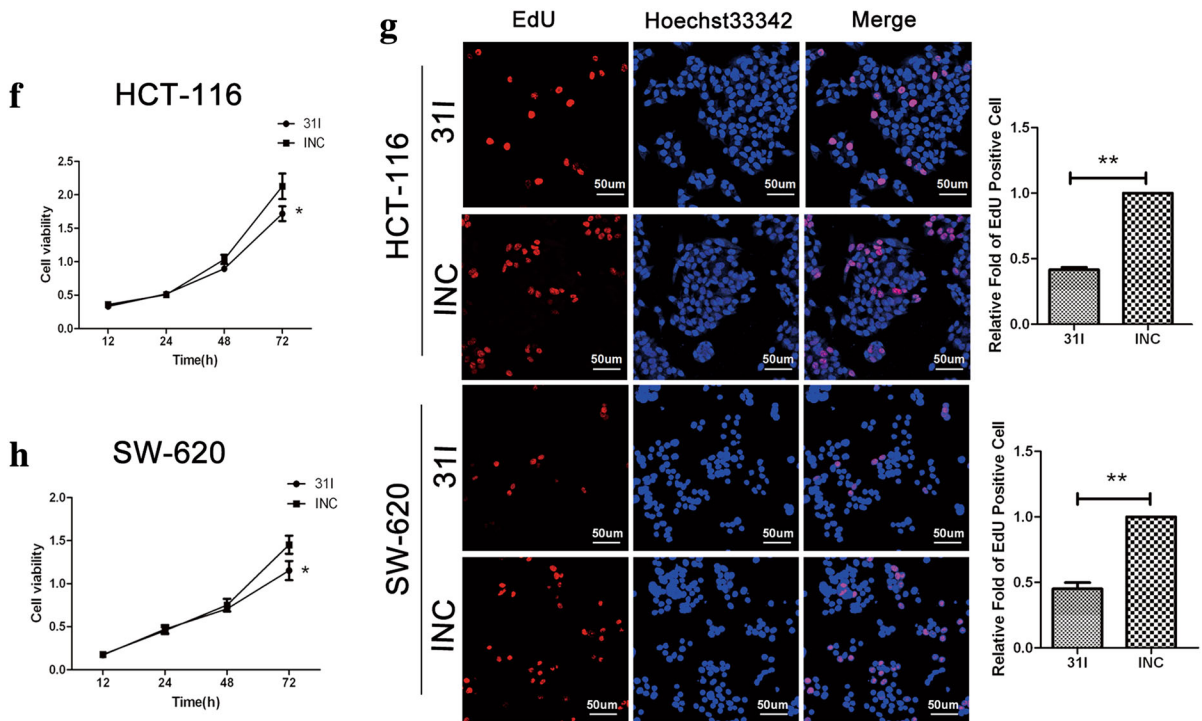


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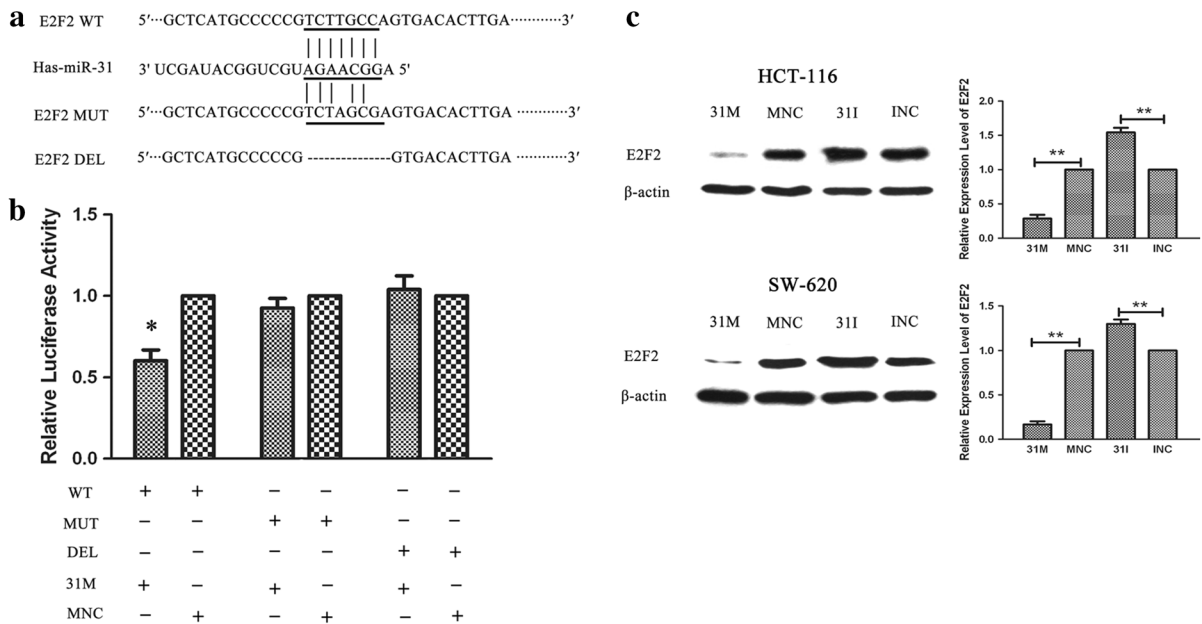


Fig. 2 E2F2 is a direct target of miR-31. **a** The sequence of wild type (WT), mutant type (MUT) and deleted type (DEL) luciferase reporter plasmid. **b** Luciferase reporter assay after transfection. Luciferase activities were normalized to β-gal.

c Western blot analysis for E2F2 protein expression after transfection with 31 M or 31I, the expression level of E2F2 was normalized to β-actin. (*P < 0.05, **P < 0.01)

To investigate whether E2F2 is a direct target of miR-31, a luciferase reporter assay was performed. According to the predicted binding site in the 3'UTR of E2F2, a WT and MUT and DEL binding site in the 3'UTR were constructed (Fig. 2a). The WT, MUT, DEL was co-transfected with 31 M or MNC plus β -gal control plasmid. The relative luciferase activity of cells co-transfected with WT and 31 M decreased by

52.8 % comparing to that co-transfected with WT and MNC (Fig. 2b). There were no significant differences between the relative luciferase activity of cells co-transfected with MUT plus either 31 M or MNC. There were also no significant differences between the relative luciferase activity of cells co-transfected with DEL plus either 31 M or MNC. These results demonstrates that mutating or deletion the miR-31 binding

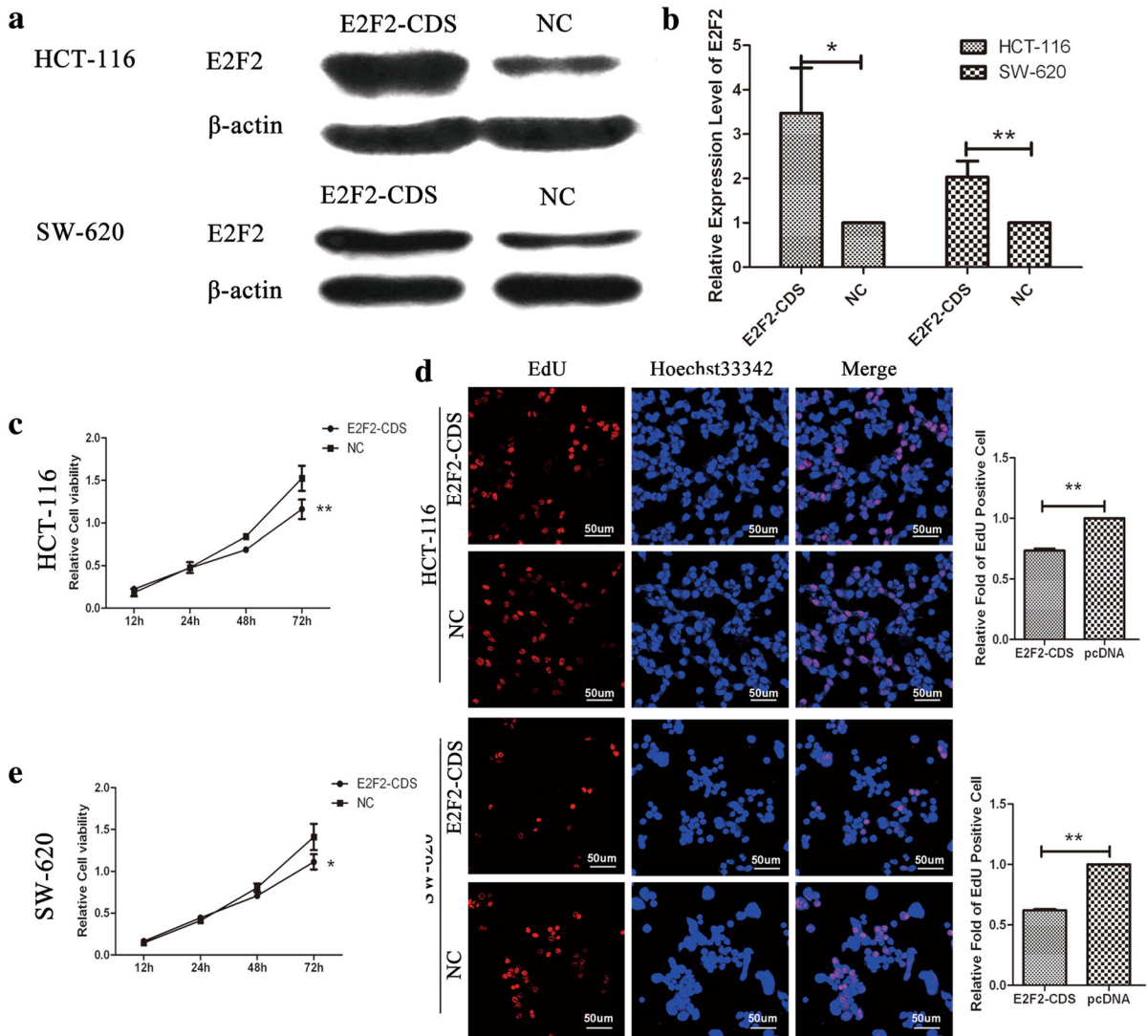


Fig. 3 E2F2 acts as a tumor suppressor in colon cancer. **a**, **b** Western blot analysis for E2F2 protein expression after transfection with E2F2-CDS, the expression level of E2F2 was normalized to β -actin. **c**, **e** Cell viability detected by MTT after transfection with E2F2-CDS or its negative control (NC). **d** Cells in S phase detected by EdU after transfection with E2F2-CDS or NC. **f**, **g** Western blot analysis for survivin protein

expression after transfection with E2F2-CDS or NC, the expression level of survivin was normalized to β -actin. **h** qRT-PCR detected the expression level of CCDN3, CCNA2, CDC2, C-MYC, CDK2, MCM4, MCM7 after transfection with E2F2-CDS, the result were presented as fold changes of expression level. (* $P < 0.05$, ** $P < 0.01$)

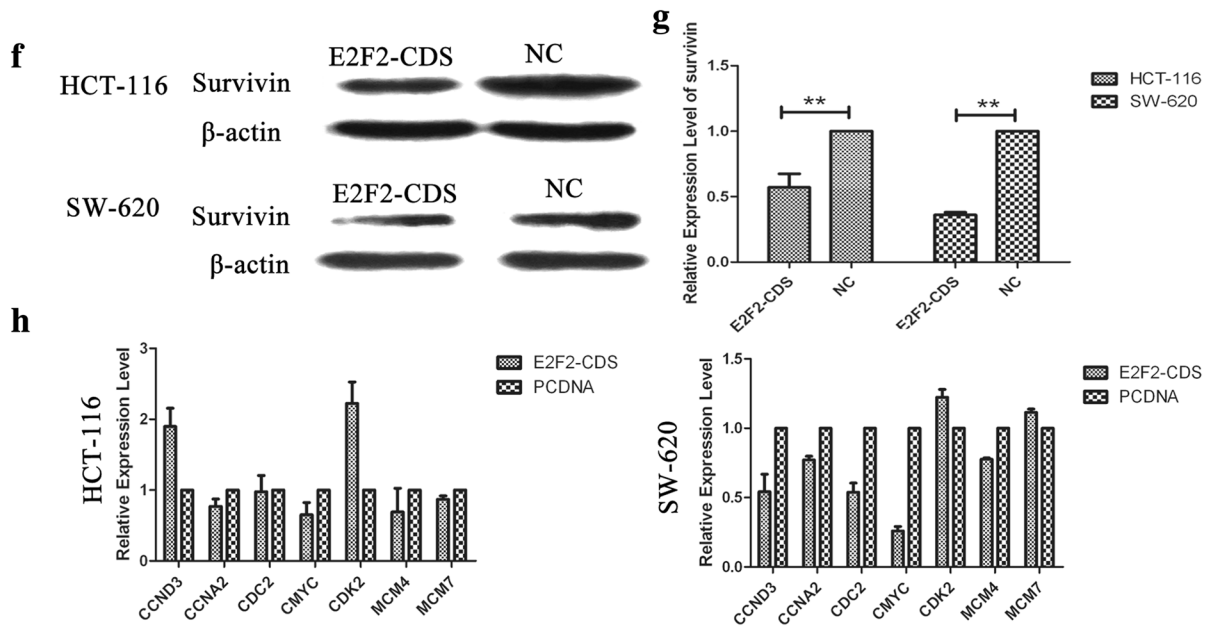


Fig. 3 continued

sites abolishes the repressive effect of 31 M. Data of luciferase reporter assay and western blot demonstrates that E2F2 is a direct target of miR-31.

E2F2 inhibits proliferation of colon cancer

E2F2 plays important roles in cell cycle progress and DNA synthesis. To further investigate the role of E2F2 in the proliferation of colon cancer, we constructed a plasmid named E2F2-CDS which contained E2F2 CDS to over-express E2F2 in colon cancer. When transfected with E2F2-CDS, E2F2 expression level increased to 3.5-fold and twofold in HCT-116 and SW-620 respectively (Fig. 3a). Then cell viability assay and EdU incorporation assay were performed to investigate the effect of E2F2 on colon cancer cells proliferation. Data showed that cells Fig. 3 transfected with E2F2-CDS had low cell viability (Fig. 3c, e) and less EdU positive cells (Fig. 3d) comparing to that transfected with its corresponding negative control (pcDNA). This means that E2F2 acts as a tumor suppressor in colon cancer.

To validate that E2F2 acts as a tumor suppressor in colon cancer, we detected the expression level of survivin which is very important for cell survival and growth. After transfection with E2F2-CDS, the expression level of survivin decreased by 43 and

64 % respectively in HCT-116 and SW-620 comparing to that transfected with NC (Fig. 3f, g). These result demonstrates that E2F2 actually inhibits the proliferation capability of colon cancer.

To investigate how E2F2 acts as a tumor suppressor in colon cancer, we detected the expression level of CCND3, CCNA2, CDC2, C-MYC, CDK2, MCM4 and MCM7 by qRT-PCR. Data showed that after transfection with E2F2-CDS, the expression level of CCNA2 and C-MYC and MCM4 decreased in both HCT-116 and SW-620 and the expression level of CDK2 increased (Fig. 3h). The expression level of CCND3, CDC2 and MCM7, however, changed differently in HCT-116 and SW-620. These results demonstrate that E2F2 acts as a tumor suppressor in colon cancer by regulating the expression of CCNA2, C-MYC, MCM4 and CDK2.

miR-31 regulates the proliferation of colon cancer by targeting E2F2

To investigate whether miR-31 promotes proliferation through regulation of E2F2, we used E2F2 specific siRNA to knockdown E2F2. The effect of E2F2 siRNA was detected by qRT-PCR (Fig. 4a). Then we investigated the role of miR-31 in colon cancer cells proliferation after knockdown of E2F2 by E2F2 siRNA. When 31 M was co-transfected with negative control of E2F2

siRNA (SINC), the cell viability and EdU positive cells were increased compared with that of cells co-transfected with MNC and SINC, which was consistent with the results above. However, when 31 M was co-transfected with E2F2 siRNA, it failed to increase the cell viability and EdU-positive cells compared with that of cells co-transfected with MNC and SINC (Fig. 4c–f). This means that knockdown of E2F2 weakens the effect of miR-31. In other words, knockdown of E2F2 only partly reverses the effect of miR-31. This demonstrates that miR-31 regulates the proliferation of colon cancer by targeting E2F2.

We also constructed a plasmid containing 3'UTR of E2F2, named E2F2-3'UTR. When transfected with E2F2-3'UTR, the expression level of miR-31 decreased by 80 and 97.6 %, respectively, in HCT-116 and SW-620 compared with that transfected with its corresponding negative control (Fig. 4b). These

results demonstrates that miR-31 regulates the proliferation of colon cancer by targeting E2F2.

Discussion

miR-31 is a classic multi-functional miRNA, its functions are diverse and depending on the context. In breast cancer and ovarian cancer, miR-31 acts as a tumor suppressor inhibiting proliferation, migration and invasion (Creighton et al. 2010; Valastyan and Weinberg 2010) but, in cancers of epithelial cells, such as lung cancer and colon cancer, it acts as an oncogene promoting proliferation and tumorigenesis (Cekaite et al. 2012; Xi et al. 2010).

Colon cancer is one of the world leading causes of cancer-related death. It is a complex genetic disease

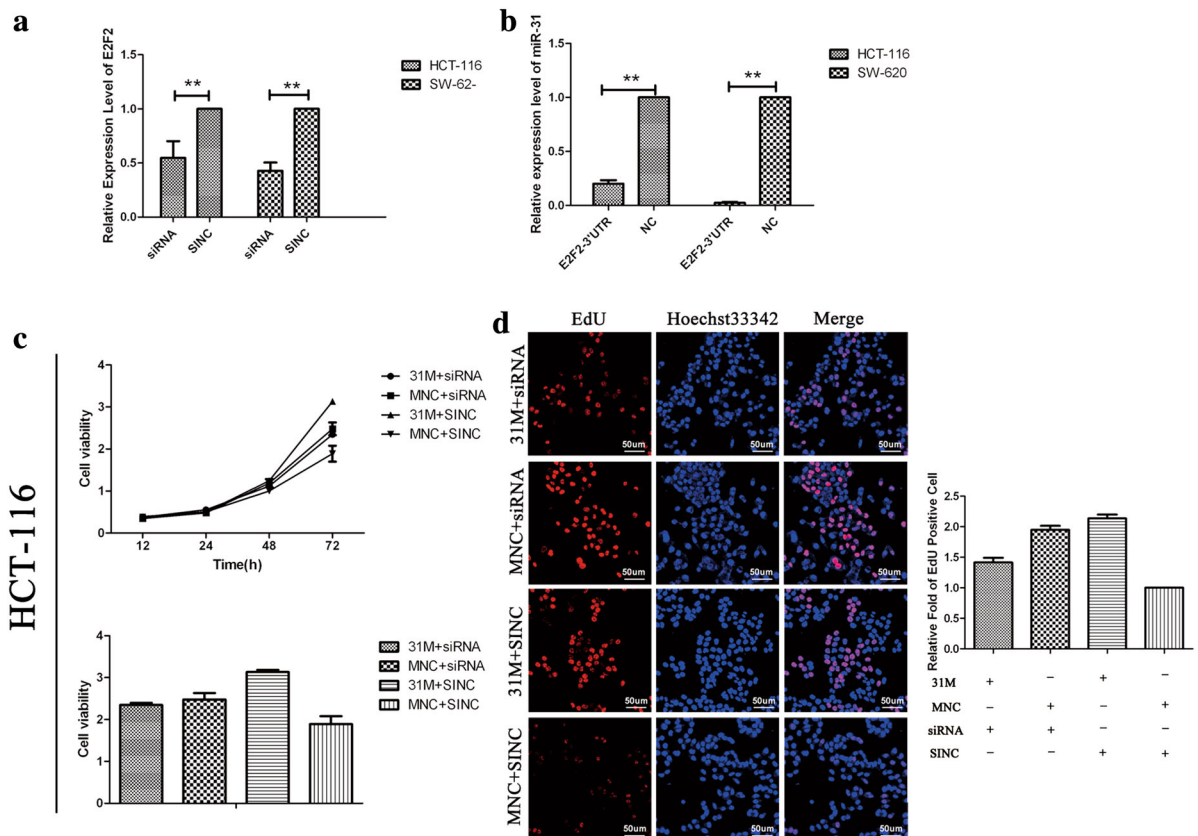


Fig. 4 miR-31 regulates the proliferation of colon cancer by targeting E2F2. **a** qRT-PCR detected the expression level of E2F2 after transfection with E2F2 specific siRNA or its negative control (SINC), the result were presented as fold changes of expression level. **b** qRT-PCR detected the expression level of miR-31 after transfection with E2F2-3'UTR or its negative

control (NC), the result were presented as fold changes of expression level. **c, e** Cell viability detected by MTT after transfection with 31 M or MNC plus siRNA or SINC. **d, f** Cells in S phase detected by EdU after transfection with 31M or MNC plus siRNA or SINC. (* $P < 0.05$, ** $P < 0.01$)

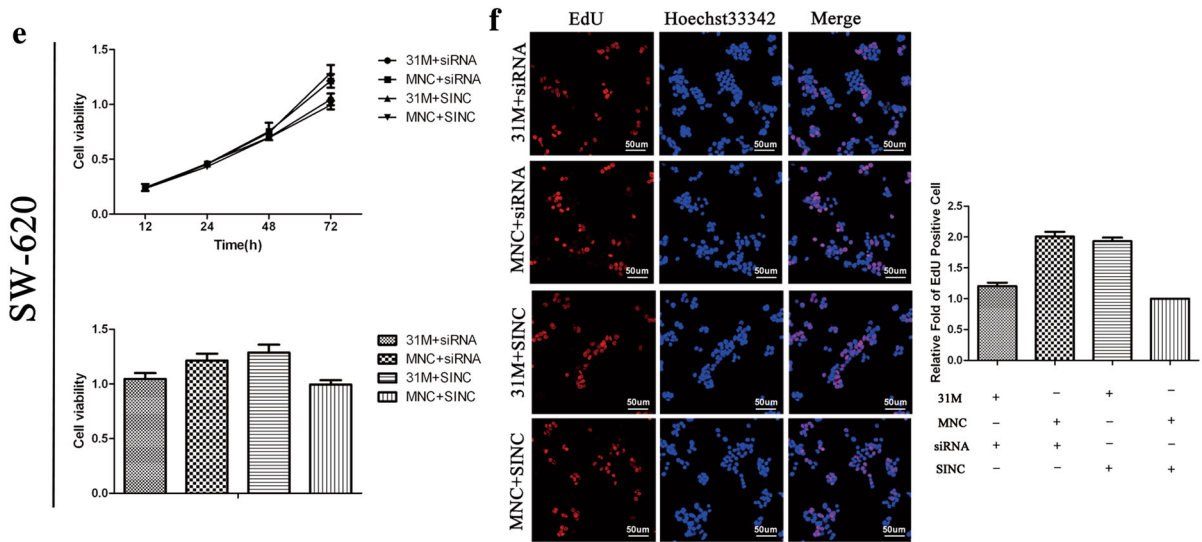


Fig. 4 continued

caused by abnormalities in gene structure and expression. miR-31 is expressed at a very high level in colon cancer and its expression level is correlated with the stage of colon cancer (Laurila and Kallioniemi 2013). As colon cancer develops slowly from premalignant lesions to a malignant tumor and miR-31 increases the resistance to chemotherapy (Dong et al. 2014), miR-31 may become an excellent biomarker of colon cancer and a target of anti-cancer drug designing (Luo et al. 2011).

Members of E2F family play important roles in the regulation of cell cycle by repression or transactivation of cell cycle regulators including cyclins, cyclin-dependent kinases (CDKs) and checkpoint regulators. E2F family members can be grouped into promoters and repressors. The so-called promoters included E2F1, E2F2, E2F3 because of their similar structure. The E2F promoter also has tumor suppressive properties. (Johnson and Degregori 2006) hypothesized that E2F1 may function as a tumor suppressor when it is absent and as an oncogene when it is over-expressed. We assume that E2F2, which is classified as a promotor group, acts as an oncogene when it is over-expressed and as a suppressor when it is absent, just like E2F1. E2F2 can truly act as an “activator” to promote the expression of its targets. E2F2 can also act as a suppressor by repressing cell cycle regulators to maintain quiescence (Infante et al. 2008) and suppressing myc-induced proliferation and tumorigenesis (Opavsky et al. 2007; Pusapati et al. 2010).

E2F2 expresses at very low level in colon cancer (Xanthoulis and Tiniakos 2013). However, whether E2F2 acts as a suppressor in colon cancer is still unknown. Our previous study indicates that knock-down of E2F2 promotes proliferation and cell cycle of colon cancer (Li et al. 2014). In present study, we explored the roles of E2F2 in colon cancer by over-expression of E2F2 and found that E2F2 inhibited the proliferation of colon cancer by inhibiting the expression of survivin and regulating the expression of CCNA2, C-MYC, MCM4 and CDK2. These results, together with our previous study, declare that E2F2 acts as a tumor suppressor in colon cancer cells.

Survivin is a multifunctional protein that controls cell proliferation, cell cycle and apoptosis. It is expressed in most human cancers, but is absent in normal and differentiated tissues. It has a close relationship with tumor survival. Repression of survivin by E2F2 provides further evidence for the hypothesis that E2F2 acts as a tumor suppressor in colon cancer.

There are also other miRNAs that regulates proliferation by targeting E2F2. let-7 (Dong et al. 2010), miR-24 (Lal et al. 2009), miR-125b (Wu et al. 2012) inhibit the proliferation of leukocythemia cells, glioblastoma stem cells and prostate cancer cells respectively by targeting E2F2. OncomiR is miRNA that promotes the tumorigenesis. In our study we demonstrate that miR-31, as an oncomiR, promotes proliferation of colon cancer by targeting E2F2 and we also

demonstrate that E2F2 acts as a tumor suppressor in colon cancer by inhibiting survivin and regulating the expression of CCNA2, C-MYC, MCM4 and CDK2.

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Supporting information Supplementary Table 1 - Primers for quantitative real-time PCR.

Supplementary Table 2 - Primers for plasmids construct.

References

- Azkargorta M, Fullaondo A, Laresgoiti U, Aloria K, Infante A, Arizmendi JM, Zubiaga AM (2010) Differential proteomics analysis reveals a role for E2F2 in the regulation of the Ahr pathway in T lymphocytes. *Mol Cell Proteomics* 9:2184–2194
- Carrington JC, Ambros V (2003) Role of microRNAs in plant and animal development. *Science* 301:336–338
- Cekaite L et al (2012) MiR-9, -31, and -182 deregulation promote proliferation and tumor cell survival in colon cancer. *Neoplasia* 14:868–879
- Creighton CJ et al (2010) Molecular profiling uncovers a p53-associated role for microRNA-31 in inhibiting the proliferation of serous ovarian carcinomas and other cancers. *Cancer Res* 70:1906–1915
- Dong Q et al (2010) MicroRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2. *PLoS ONE* 5:e10147
- Dong Z, Zhong Z, Yang L, Wang S, Gong Z (2014) MicroRNA-31 inhibits cisplatin-induced apoptosis in non-small cell lung cancer cells by regulating the drug transporter ABCB9. *Cancer Lett* 343:249–257
- Infante A et al (2008) E2F2 represses cell cycle regulators to maintain quiescence. *Cell Cycle* 7:3915–3927
- Johnson DG, Degregori J (2006) Putting the oncogenic and tumor suppressive activities of E2F into context. *Curr Mol Med* 6:731–738
- Lal A et al (2009) miR-24 Inhibits cell proliferation by targeting E2F2, MYC, and other cell-cycle genes via binding to “seedless” 3′UTR microRNA recognition elements. *Mol Cell* 35:610–625
- Laurila EM, Kallioniemi A (2013) The diverse role of miR-31 in regulating cancer associated phenotypes. *Genes Chromosom Cancer* 52:1103–1113
- Li T, Yang J, Lv X, Liu K, Gao C, Xing Y, Xi T (2014) miR-155 regulates the proliferation and cell cycle of colorectal carcinoma cells by targeting E2F2. *Biotechnol Lett* 36:1743–1752
- Luo X, Burwinkel B, Tao S, Brenner H (2011) MicroRNA signatures: novel biomarker for colorectal cancer? *Cancer Epidemiol Biomark Prev* 20:1272–1286
- Opavsky R et al (2007) Specific tumor suppressor function for E2F2 in Myc-induced T cell lymphomagenesis. *Proc Natl Acad Sci USA* 104:15400–15405
- Pusapati RV, Weaks RL, Rounbehler RJ, McArthur MJ, Johnson DG (2010) E2F2 suppresses Myc-induced proliferation and tumorigenesis. *Mol Carcinog* 49:152–156
- Valastyan S, Weinberg RA (2010) miR-31: a crucial overseer of tumor metastasis and other emerging roles. *Cell Cycle* 9:2124–2129
- Wu N et al (2012) miR-125b regulates the proliferation of glioblastoma stem cells by targeting E2F2. *FEBS Lett* 586:3831–3839
- Xanthoulis A, Tiniakos DG (2013) E2F transcription factors and digestive system malignancies: how much do we know? *World J Gastroenterol* 19:3189–3198
- Xi S et al (2010) Cigarette smoke induces C/EBP-beta-mediated activation of miR-31 in normal human respiratory epithelia and lung cancer cells. *PLoS ONE* 5:e13764