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E2F1 promote the aggressiveness of human colorectal cancer by activating the ribonucleotide reductase small subunit M2

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ABSTRACT

As the ribonucleotide reductase small subunit, the high expression of ribonucleotide reductase small subunit M2 (RRM2) induces cancer and contributes to tumor growth and invasion. In several colorectal cancer (CRC) cell lines, we found that the expression levels of RRM2 were closely related to the transcription factor E2F1. Mechanistic studies were conducted to determine the molecular basis. Ectopic overexpression of E2F1 promoted RRM2 transactivation while knockdown of E2F1 reduced the levels of RRM2 mRNA and protein. To further investigate the roles of RRM2 which was activated by E2F1 in CRC, CCK-8 assay and EdU incorporation assay were performed. Overexpression of E2F1 promoted cell proliferation in CRC cells, which was blocked by RRM2 knockdown attenuation. In the migration and invasion tests, overexpression of E2F1 enhanced the migration and invasion of CRC cells which was abrogated by silencing RRM2. Besides, overexpression of RRM2 reversed the effects of E2F1 knockdown partially in CRC cells. Examination of clinical CRC specimens demonstrated that both RRM2 and E2F1 were elevated in most cancer tissues compared to the paired normal tissues. Further analysis showed that the protein expression levels of E2F1 and RRM2 were parallel with each other and positively correlated with lymph node metastasis (LNM), TNM stage and distant metastasis. Consistently, the patients with low E2F1 and RRM2 levels have a better prognosis than those with high levels. Therefore, we suggest that E2F1 can promote CRC proliferation, migration, invasion and metastasis by regulating RRM2 transactivation. Understanding the role of E2F1 in activating RRM2 transcription will help to explain the relationship between E2F1 and RRM2 in CRC and provide a novel predictive marker for diagnosis and prognosis of the disease.

1. Introduction

Colorectal cancer (CRC) is the third most common type of cancer reported in men and is the second reported in women, with 1,360,600 new cases being diagnosed and 693,900 people dying of it in 2012 worldwide [1], and the incidence rates continue to increase rapidly in China and other economically transitioning countries [2,3]. In most cases, lethality in CRC patients is resulted from metastasis that contributes to tumor resistance to conventional therapies and an overall poor prognosis [4,5].

Ribonucleotide reductase (RR) is the only rate-limiting enzyme which catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates in the metabolic process of nucleotides. RR is composed of two identical large subunits (RRM1) and two identical small subunits (RRM2 or RRM2B). RRM1 contains a catalytic site and two allosteric effector-binding sites. RRM2 and RRM2B contain a diiron-tyrosyl radical required for the enzyme activity [6]. The RR holoenzyme constitutes two forms: RRM1-RRM2 and RRM1-RRM2B which provide dNTP for DNA replication and repair, respectively [7]. The balance of deoxyribonucleotide triphosphate pool is dependent on the strict regulation of RR. Thus

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dysregulated RR is closely related with instability of genome, cancer initiation and development in many types of malignancy.

Interestingly, the three subunits of RR display entirely different roles in the occurrence and development of cancer. RRM1 functions like a cancer suppressor gene during the process of cell transformation and neoplasm metastasis by activating PTEN signaling network [8,9]. However, In contrast to RRM1, the high expression of RRM2 induces cancer and contributes to tumor growth and invasion. Several cancers such as oral squamous cell carcinoma, cervical carcinoma and gastric carcinoma have been reported to elevate the expression of RRM2 [10–12]. RRM2 is not only an essential component in nucleotide metabolism but that it is also capable of acting in cooperation with a variety of oncogenes [13]. Altered RRM2 cooperates with Ras, resulting in the activation of a major Ras pathway involves the Raf-1 and MAPK2 which increases transformation and tumorigenic potential [14]. In pancreatic adenocarcinoma, overexpression of RRM2 increases cellular invasiveness by promoting MMP-9 expression in a NF-κB-dependent manner [15]. Furthermore, RRM2-overexpression significantly decreases anti-angiogenic thrombospondin-1 (TSP-1) while proangiogenic vascular endothelial growth factor (VEGF) mRNA and protein increase. So cancer cells which overproduced RRM2 possess more angiogenic potential [16].

As the cell cycle-driver, E2F is a very important transcription factor in regulating RRM2 gene. S-phase specific transcription of RRM2 is the result of E2F1 mediated transcriptional activation and releasing E2F4 mediated transcriptional repression [17]. Compared to gemcitabine-sensitive pancreatic cancer cells Panc-1, the resistant Panc-1GemR cells respond to gemcitabine by increasing the expression of RRM2 through E2F1 mediated transcriptional activation [18]. Several reports in colorectal carcinogenesis have shown that overexpression of E2F1 is associated with the progression of adenomas to adenocarcinomas and metastasis [19–22]. Hence, various natural and synthetic drugs prevent colon cancer cell growth by inhibiting the expression of important cell cycle protein [23].

In the present study, we demonstrated that E2F1 promoted the proliferation, migration, invasion and metastasis of CRC cells by transactivating RRM2. Clinical specimen analyses confirmed that both E2F1 and RRM2 were increased in most colorectal cancer tissues compared to the paired normal tissues, while the level of E2F1 was correlated with that of RRM2 in cancer tissues. Furthermore, we found that higher RRM2 and E2F1 levels correlated with significantly worse survival. The possible biological significance and clinical relevance of the relationship between E2F1 and RRM2 in colorectal cancer development should be considered in the process of diagnosis and therapy.

2. Materials and methods

2.1. Cell cultures

DLD1, SW480, HCT116, HCT8, HT29 and RKO were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Transfection and siRNA interference

The cmyc-E2F1 and Flag-RRM2 were transfected with X-treme GENE HP DNA Transfection Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. E2F1 small interfering RNA (siRNA), RRM2 siRNA, and scrambled siRNA (Santa Cruz Biotechnology, TX, USA) were transfected with Lipofectamine™ RNAiMAX (Invitrogen, NY, USA) according to the manufacturer's instructions.

2.3. Quantitative real time RT-PCR

Total RNA was prepared using the RNAiso™ Plus reagent (Takara, Otsu, Japan) and reverse-transcribed using a PrimeScript™RT reagent kit (Takara). Quantitative PCR was performed with SYBR Green mix (Takara) according to the manufacturer's instructions. β-Actin was used as loading control.

2.4. Western blot analysis

The whole cell lysate were analyzed with the antibodies mouse anti-human RRM2, rabbit anti-human E2F1, GAPDH (Santa Cruz Biotechnology), IRDye® 800CW- or IRDye® 680-conjugated secondary antibodies (LI-COR, Lincoln,NE) were used for staining and then detected by an Odyssey® infrared imaging system (LI-COR).

2.5. EdU incorporation assay

The EdU (5-ethynyl-2-deoxyuridine) incorporation assay was used to represent DNA synthesis in cells. Cells were transfected with the indicated siRNAs and expression plasmids. Next, cells were washed 3 times with PBS, and then incubated in serum-free RPMI 1640 with 10 μM EdU for 2 h. After extensive washing with PBS, cells were blocked with 10% FBS in PBS for 30 min. Incorporated EdU was detected by the fluorescent azide coupling reaction (Invitrogen). Images of the cells were captured with a fluorescence microscope (Nikon, Tokyo, Japan) and analyzed by ImageJ (National Institutes of Health, Bethesda, MD).

2.6. CCK-8 assay

Cells in logarithmic growth were plated and transfected with the indicated siRNAs and expression plasmids. After indicated time of culture, CCK-8 (Cell Counting Kit-8) (Dojindo Molecular Technologies, Maryland, USA) was added, and the OD_{450} was measured using an automatic plate reader.

2.7. Reporter gene assay

The promoter sequences of the human RRM2 gene (−2465/+23) was amplified by PCR and inserted into the pGL3-Basic luciferase reporter vector (Promega, Madison, WI). Cells were plated onto 24-well plates the day before transfection. The cells were cotransfected with 0.5 μg of firefly luciferase reporter constructs, 0.02 μg of pRL-SV40 Renilla luciferase reporter plasmids (Promega), and 0.5 μg cmyc-E2F1 using the X-treme GENE HP DNA Transfection Reagent. The luciferase activity was measured by a dual-luciferase reporter assay system (Promega).

2.8. Wound healing assay

Cells were seeded in six-well plates. After transfection for 24 h, the monolayer was gently and slowly scratched with a pipette tip across the center of the well. After scratching, the well was gently washed several times with PBS to remove detached cells. The well was replenished with fresh medium without serum, and the cells allowed growing for additional 48 h, when images of the stained monolayer were captured on a microscope. The wound was evaluated using ImageJ.

2.9. Cell invasion assays

Cell invasion were assessed in Boyden chambers with Matrigel according to the manufacturer's protocol (Invitrogen). First, an 8-mm-porosity polycarbonate membrane was covered with 200 μL

of serum-free medium containing 1×10^5 cells per well. The plates were then incubated with 10% FBS medium for 48 h at 37 °C in a 5% CO_2 incubator. The invasion cells on the bottom surface of the filter were fixed, stained, and counted using optical microscopy.

2.10. Immunohistochemistry

A total of 154 human CRC samples were collected at the Sanmen People's Hospital of Zhejiang after informed consent had been given by all patients. The immunohistochemistry was performed using an Envision Detection System (DAKO, Carpinteria, CA, USA) according to the manufacturer's instructions. To estimate the score for each slide, at least 8 individual fields at 200 × were chosen, and 100 cancer cells were counted in each field. The immunostaining intensity was divided into four grades: 0, negative; 1, weak; 2, moderate; and 3, strong. The proportion of positive-staining cells was divided into five grades: 0, <5%; 1, 6–25%; 2, 26–50%; 3, 51–75%; and 4, >75%. The staining results were assessed and confirmed by two independent investigators blinded to the clinical data. The percentage of positivity of the tumor cells and the staining intensities were then multiplied in order to generate the IHC score, and graded as low expression (score 0–6) and high expression (score 7–12). Cases with a discrepancy in scores were discussed to obtain a consensus.

2.11. Statistics

A database was created and transferred to SPSS 22.0 for Windows. Statistical data analysis was performed using the two-tailed Student's t-test, chi-squared, one-way ANOVA, and the results are presented as the mean ± SD of three separate experiments.

3. Results

3.1. E2F1 promotes RRM2 transactivation in CRC cells

To determine the relationship between E2F1 and RRM2, we tested the level of E2F1 and RRM2 in several CRC cell lines. The high expression levels of E2F1 and RRM2 were observed in HCT116, HT29 and SW480, while low levels of E2F1 and RRM2 were found in RKO, HCT8 and DLD1 (Fig. 1a). When E2F1 was overexpressed in RKO and HCT8 cells, the mRNA and protein expression of RRM2 were significantly upregulated (Fig. 1b,c,d). Knocking down E2F1 expression by its specific siRNA in SW480 and HT29 cells, the mRNA and protein level of RRM2 decreased as expected (Fig. 1e,f). Reporter gene analyses also showed that overexpression of E2F1 induced transcriptional activity of the RRM2 promoter in RKO and HCT8 cells (Fig. 1g). These results indicate that E2F1 promote RRM2 transactivation in CRC cells.

3.2. E2F1 promotes the proliferation of CRC cells by activating RRM2

Considering E2F1 and RRM2 as indispensable S phase-drivers in cell cycle, CCK-8 assay and EdU incorporation assay were performed to characterize the function of E2F1 and RRM2 in cell proliferation. In HT29 cells, silencing E2F1 repressed cell proliferation and additional ectopic expression of RRM2 reversed the effect to some extent (Fig. 2a). Overexpression of E2F1 promoted cell proliferation in the transfected RKO cells while RRM2 knockdown attenuated the E2F1 induced cell proliferation (Fig. 2b). In EdU incorporation assay, E2F1 knockdown blocked DNA synthesis in HT29 cells whereas further overexpressed RRM2 increased EdU incorporation (Fig. 2c). Consistently, increased DNA synthesis was observed in E2F1 transfected RKO cells, and RRM2 silencing had a destructive effect on DNA synthesis stimulated by E2F1 (Fig. 2d). These results suggest that E2F1 plays a significant role in boosting cell proliferation through activating RRM2 in CRC cells.

3.3. E2F1 promotes the migration and invasion of CRC cells by activating RRM2

As the high expression of RRM2 may contribute to tumor invasion and metastasis, we next investigated the functional role of E2F1 and RRM2 in CRC cell migration and invasion. The results showed that knockdown of either E2F1 or RRM2 weakened the acquisitive capacity of migration and invasion in HT29 cells while overexpression of RRM2 rescued the wound healing and invading abilities induced by siE2F1 (Fig. 3a,c). Similarly, overexpression of either E2F1 or RRM2 increased migration and invasion in RKO cells, and RRM2 knockdown with siRNA interference partly retarded the E2F1 effects (Fig. 3b,d). The above results indicate that RRM2 transactivation by E2F1 is essential for migration and invasion in CRC cells.

3.4. E2F1 expression is upregulated in CRC tissues and positively associated with RRM2 level at the advanced cancer stage

Immunohistochemical staining was carried out to analyze the clinical relevance of E2F1 and RRM2 expressions in 154 human CRC specimens. The expression levels of RRM2 and E2F1 were increased in the tumor compared with the normal intestinal tissue (Fig. 4a and Table 1). Further analyses showed that the RRM2 and E2F1 staining were positively correlated with lymph node metastasis (LNM), distant metastasis and advanced TNM stages ($p < 0.05$) but not with tumor size ($p > 0.05$) (Table 2). Moreover, the E2F1 expression levels paralleled the changes of RRM2 in the CRC cases as shown by immunohistochemical analyses ($p < 0.05$) (Fig. 4b). The 5-year overall survival (OS) rate of the RRM2 and E2F1 double-high expression group was significantly lower than that of the RRM2 and E2F1 double-low expression group (27.7% vs 55.4%, $p < 0.001$). Of note is that patients with low levels of RRM2 have a better prognosis than RRM2-high expression patients regardless of the E2F1 expression status (Fig. 4c). These data suggest that E2F1 is positively correlated with RRM2 expression, which is involved in CRC progression and metastasis.

4. Discussion

Abnormal expression of ribonucleotide reductase is closely relative to several types of cancer. As the small subunit of RR, upregulation of RRM2 increases RR activity, which provides extra dNTPs in cancer cells. Furthermore RRM2 could serve as an independent prognostic factor and predict poor survival of CRCs [24,25]. Here we found that the increased RRM2 contributed to the proliferation, migration, invasion and metastasis of CRC cells. Previous observation has shown that the RRM2 is not only a rate-limiting component for ribonucleotide reduction, but is also capable of acting in cooperation with a variety of oncogenes to promote transformation and tumorigenesis [14]. Overexpression of RRM2 in KB and PC-3 cells could induce the migration ability of HUVECs (human umbilical vein endothelial cells) [26]. Besides, it has been reported that RRM2 could increase the MMP-9 expression and enhance the cell invasion ability in cancer cell lines [15]. Therefore, dNTPs pool expansion, acceleration of cell proliferation, and improvement of metastasis ability may partly explain why RRM2 increases the aggressiveness and causes poor survival in CRCs.

The regulatory mechanisms which control the gene expression of RR subunits depend on different conditions. Due to its long half-life, the level of RRM1 is virtually constant throughout the cell cycle

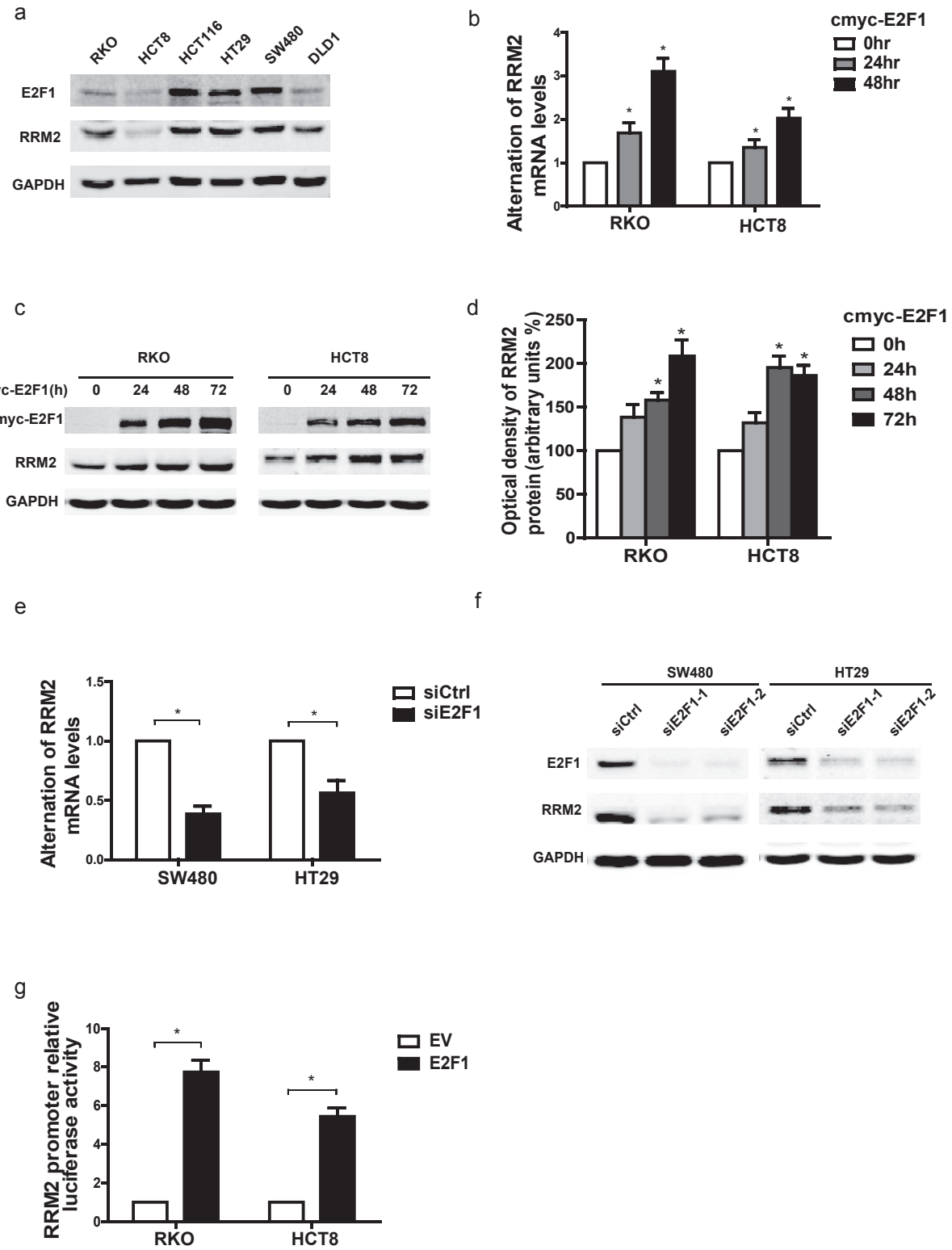

Fig. 1. E2F1 promotes RRM2 transactivation in CRC cells. a Expression of E2F1 and RRM2 proteins in 6 CRC cell lines were analyzed by western blot. b The mRNA level of RRM2 was analyzed by qRT-PCR in CRC cells after transfection with the E2F1 expression plasmid at different time points. c and d The protein level of RRM2 were analyzed by western blot in CRC cells after transfection with the E2F1 expression plasmid at different time points. e and f The mRNA and protein levels of RRM2 in CRC cells after transfected with the siRNA targeting E2F1. g Relative luciferase activity in CRC cells co-transfected with with EV or E2F1 expression plasmid, RRM2 promoter reporter (−2465/+23) and an internal control reporter pRL-TK. *$P < 0.05$.

and always in excess of the level of RRM2 [27,28]. Therefore, the activity of RR is mainly regulated by the synthesis and degradation of the RRM2 protein. As an important transcription factor, E2F1 is involved in S-phase specific expression of RRM2 in physiological situations [29,30]. On the other hand, the transcription of RRM2 was also activated by E2F1 through ATM/ATR-CHK1 signal pathway

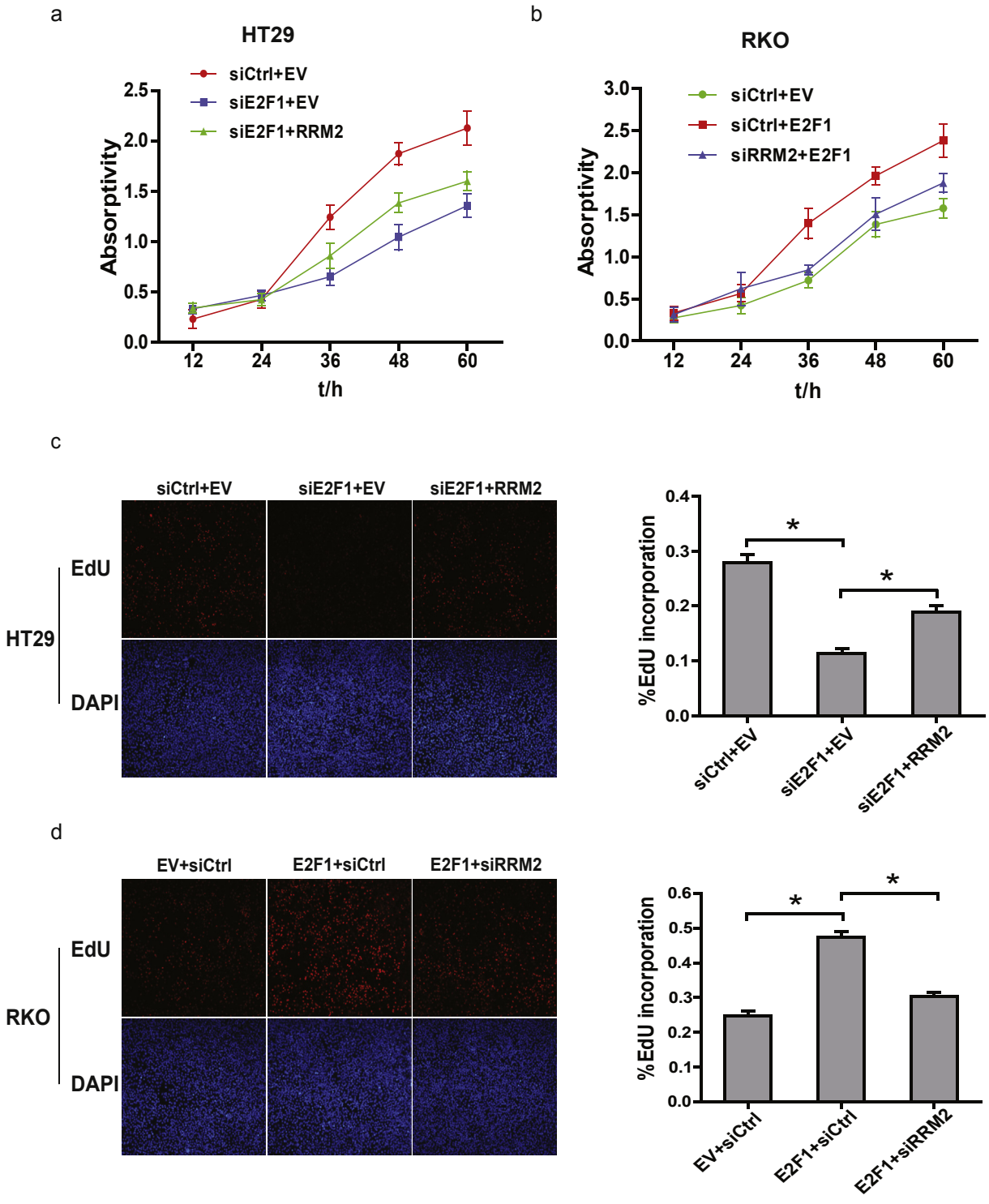

Fig. 2. E2F1 promotes the proliferation of CRC cells by activating RRM2. a and b At 12–60 h after the indicated siRNAs and expression plasmids transfection, the viability of HT29 (a) or RKO (b) cells were determined by the CCK-8 assay. c and d DNA synthesis of HT29 (c) or RKO (d) cells were measured by EdU incorporation assay after the indicated transfection. $^{*}P < 0.05$.

in response to DNA damage [31]. As a potential predictor for response to fluropyrimidines in colon cancer, E2F1 controls the transcription of several genes encoding proteins involved in DNA synthesis, namely thymidylate synthase, dihydrofolate reductase, and thymidine kinase [29,32]. A high correlation between E2F1 and TS was recently shown in colorectal cancer metastases to the lung and the liver [21]. Our results suggested that overexpressed E2F1 upregulated the expression of RRM2 by promoting its transcriptional activation in CRC cell lines. Further investigations showed that E2F1 promoted the proliferation, migration and invasion of CRC cells which were attenuated by knockdown of RRM2. So RRM2 may function as an important target gene of E2F1, which plays a vital role in the progress of CRC.

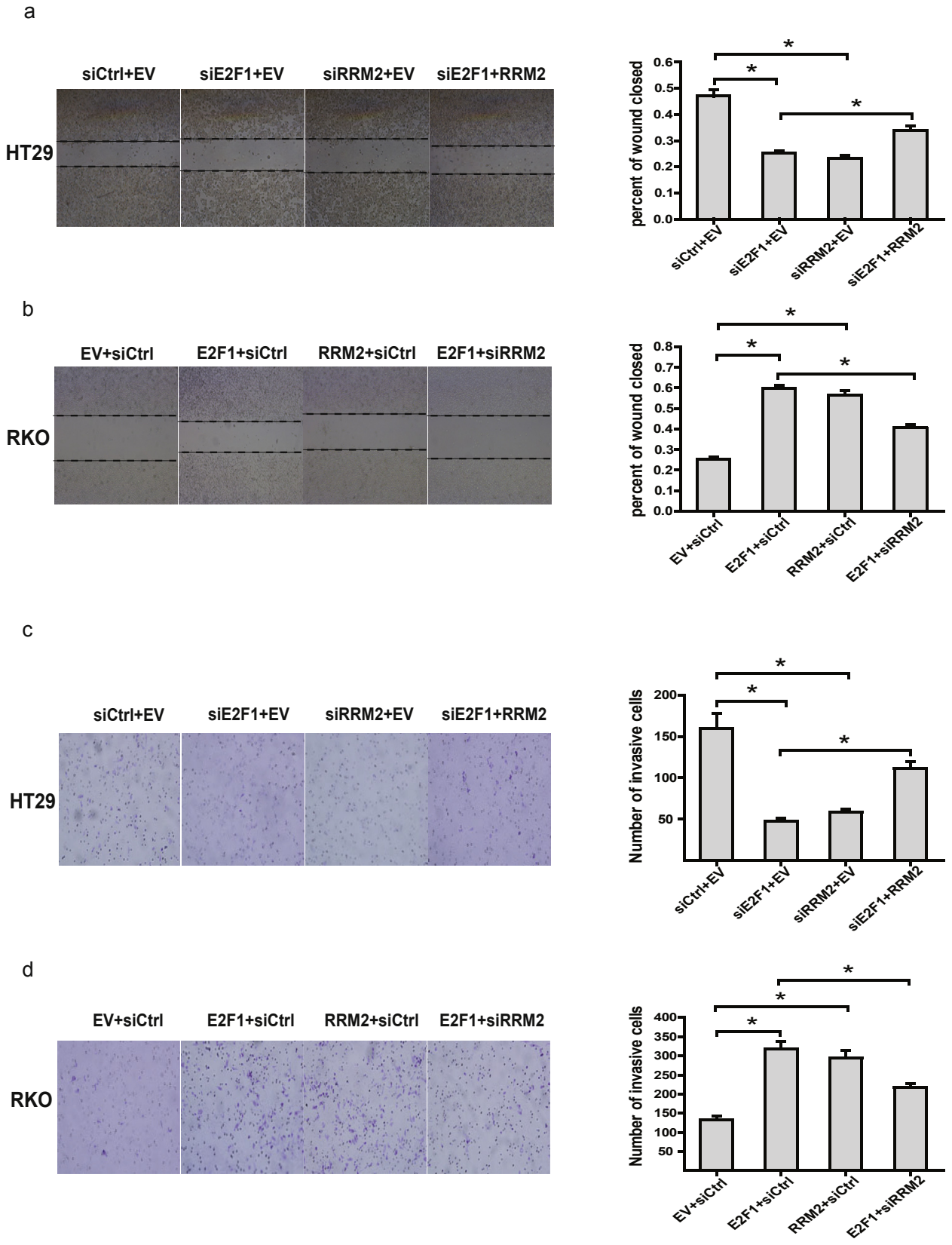

Fig. 3. E2F1 promotes the migration and invasion of CRC cells by activating RRM2. a and b Left panels: images from scratch assays with HT29 (a) or RKO (b) cells transfected with indicated siRNAs and expression plasmids. Right panels: percentage wound closure 48 h after the indicated transfection. $^{*}P < 0.05$. c and d Left panels: representative images of HT29 (c) or RKO (d) cells penetrating the Matrigel in invasion assays. Right panels: numbers of invasive cells transfected with the indicated siRNAs and expression plasmids for 48 h $^{*}P < 0.05$.

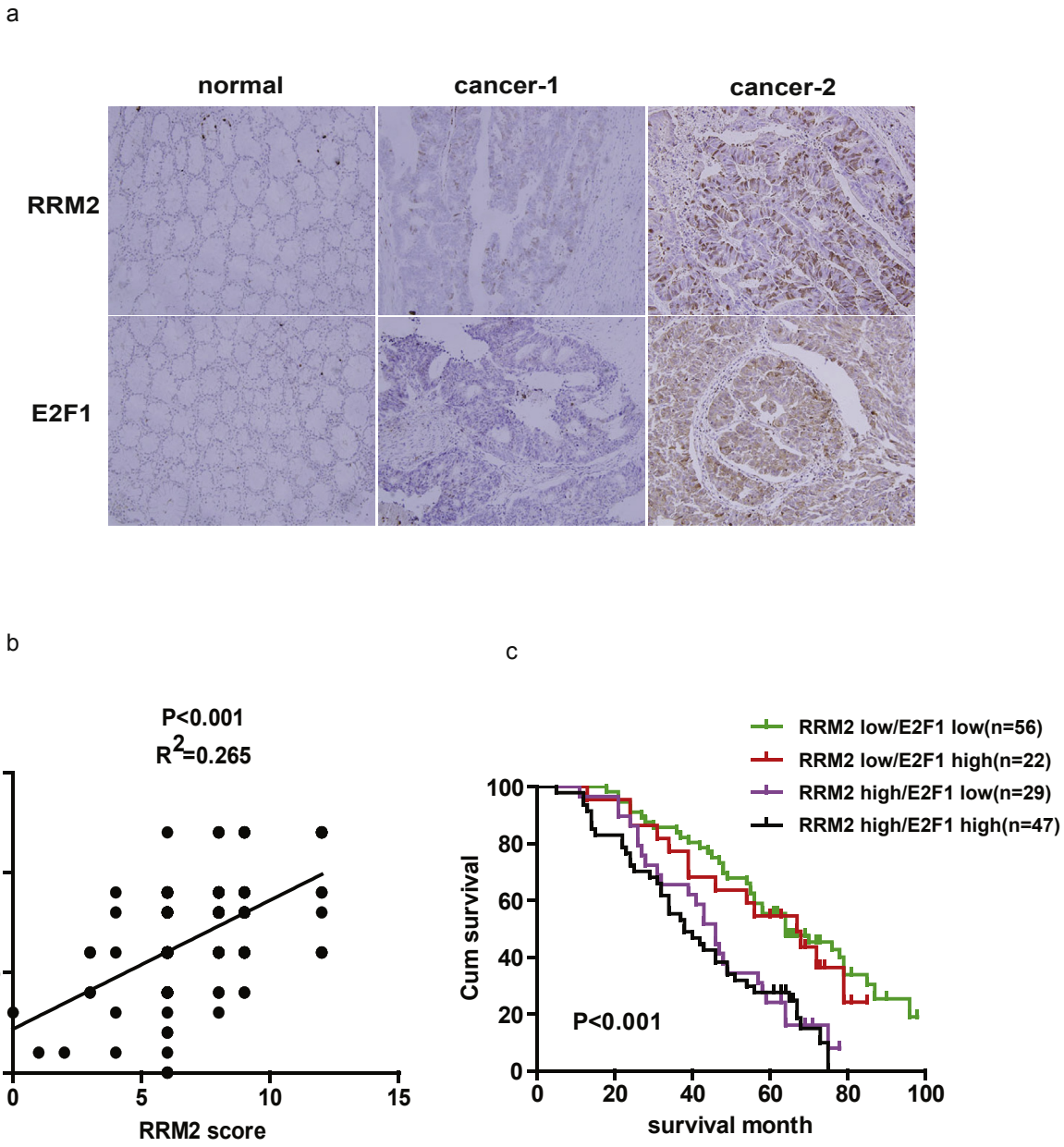

Fig. 4. E2F1 expression is upregulated in CRC tissues and positively associated with RRM2 level at the advanced cancer stage. a Representative images of E2F1 and RRM2 immunostaining in cancer and adjacent non-cancerous tissues. b The correlation of concurrent immunostaining scores of RRM2 and E2F1 in CRC tissues. c The survival rate plot of indicated expression groups in CRC.

Analyses of clinical specimens from CRC patients showed that both E2F1 and RRM2 expressions were upregulated in most cancer tissues compared to paired normal tissues. The high expression of E2F1 in CRC might be achieved by phosphorylation or other post-translational modifications and the mechanism should be further investigated [33]. The increased RRM2 protein levels were positively correlated with TNM stage and metastasis in parallel with the E2F1 expression. Therefore, the clinical evidence further support

Table 1
Expression of RRM2 and E2F1 in CRC and adjacent normal tissues.

	Cases	RRM2 expression (cancer)			E2F1 expression (cancer)		
		Low Cases	High Cases	*P* Value	Low Cases	High Cases	*P* Value
CRC	154	78	76	<0.001*	85	69	<0.001*
Adjacent normal tissues	154	147	7		139	15	

*p < 0.05.

Table 2
Correlation of the expression of RRM2 and E2F1 with clinicopathological features in CRC.

		Cases	RRM2 expression (cancer)			E2F1 expression (cancer)		
			Low	High	*P* Value	Low	High	*P* Value
			Cases	Cases		Cases	Cases	
		154	78	76		85	69	
Tumor location					0.988			0.830
	Colon	65	33	32		35	30	
	Rectum	89	45	44		50	39	
Gender					0.788			0.296
	male	83	41	42		43	40	
	female	71	37	34		42	29	
Age					0.365			0.645
	≤65	64	36	28		35	29	
	>65	90	42	48		50	40	
Tumor size					0.592			0.426
	<5 cm	72	36	36		41	31	
	≥5 cm	82	42	40		44	38	
LNM					0.004*			0.013*
	N0	87	51	36		54	33	
	N1/2	67	27	40		31	36	
TNM					0.032*			0.014*
	I	23	15	8		15	8	
	II	59	29	30		32	27	
	III	60	32	28		35	25	
	IV	12	2	10		3	9	
Distant metastasis					0.002*			0.001*
	M0	142	76	66		82	60	
	M1	12	2	10		3	9	

LNM: lymph node metastasis; $^{*}P < 0.05$.

the hypothesis that E2F1 performs as the transcriptional activator of RRM2 in promoting CRC development: the increased RRM2 protein activated by E2F1 contributes to tumor initiation and metastasis. Moreover, we analyzed the survival rate of the patients with different E2F1 and RRM2 expression. For most patients, the expression level of RRM2 and E2F1 was negatively correlated with the survival rate. However, the survival rate of some patients with inverse correlation between RRM2 and E2F1 depended on RRM2 levels. So it is reasonable to suggest that the role of E2F1 in the initiation and progress of CRC is dependent on the expression level of RRM2. Thus, integrative assessments of RRM2 and E2F1 protein expression in clinical samples would help reach a proper prognosis as well as the rational use of different RR inhibitors: enzyme inactivators or gene expression suppressors of RRM2 for personalized cancer therapy.

In summary, our molecular and clinical evidence demonstrated the relationship between E2F1 and RRM2 and their roles in promoting CRC invasion and metastasis. Still, the underlying mechanism of regulating RRM2 in CRC cells has not been clearly elucidated. Further understanding of the upstream regulatory pathway of RRM2 may help to provide new biomarkers and therapeutic targets for human colorectal cancer.

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