The c-MYC-ABCB5 axis plays a pivotal role in 5-fluorouracil resistance in human colon cancer cells

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Abstract

c-MYC overexpression is frequently observed in various cancers including colon cancer and regulates many biological activities such as aberrant cell proliferation, apoptosis, genomic instability, immortalization and drug resistance. However, the mechanism by which c-MYC confers drug resistance remains to be fully elucidated. In this study, we found that the c-MYC expression level in primary colorectal cancer tissues correlated with the recurrence rate following 5-fluorouracil (5-FU)-based adjuvant chemotherapy. Supporting this finding, overexpression of exogenous c-MYC increased the survival rate following 5-FU treatment in human colon cancer cells, and knockdown of endogenous c-MYC decreased it. Furthermore, c-MYC knockdown decreased the expression level of ABCB5, which is involved in 5-FU resistance. Using a chromatin immunoprecipitation assay, we found that c-MYC bound to the ABCB5 promoter region. c-MYC inhibitor (10058-F4) treatment inhibited c-MYC binding to the ABCB5 promoter, leading to a decrease in ABCB5 expression level. ABCB5 knockdown decreased the survival rate following 5-FU treatment as expected, and the ABCB5 expression level was increased in 5-FU-resistant human colon cancer cells. Finally, using a human colon cancer xenograft murine model, we found that the combined 5-FU and 10058-F4 treatment significantly decreased tumorigenicity in nude mice compared with 5-FU or 10058-F4 treatment alone. 10058-F4 treatment decreased the ABCB5 expression level in the presence or absence of 5-FU. In contrast, 5-FU treatment alone increased the ABCB5 expression level. Taken together, these results suggest that c-MYC confers resistance to 5-FU through regulating ABCB5 expression in human colon cancer cells.

Keywords: c-MYC ● ABCB5 ● 5-fluorouracil resistance ● colon cancer

Introduction

Colorectal cancer is the most common malignant solid tumour worldwide, and one of the leading causes of cancer-related deaths. 5-fluorouracil (5-FU) is one of the main chemotherapeutic agents used to kill colon cancer cells. In patients with advanced colon cancer, 5-FU treatment reduces tumour size and prolongs survival. However, the frequent emergence and survival of 5-FU-resistant colon cancer cells often causes recurrence following 5-FU-based adjuvant chemotherapy. Therefore, uncovering the mechanism underlying the acquisition of 5-FU resistance might facilitate the identification of a predictive marker of poor prognosis for 5-FU treatment, and provide a new potential therapeutic target for the treatment of colorectal cancer.

The MYC family genes encode transcription factors that regulate cell cycle, cell growth, differentiation, apoptosis, transformation, genomic instability and angiogenesis [1, 2]. In particular, overexpression of c-MYC has been found in various cancer cells [2] including colorectal cancer cells [3, 4] and is often associated with poor prognosis [5]. Furthermore, c-MYC has been found to be involved in drug resistance. Tumour cells resistant to cisplatin chemotherapy in vivo display elevated c-myc expression [6], and c-myc antisense oligonucleotides sensitize human colorectal cancer cells to chemotherapeutic drugs [7]. Recent study has been shown that c-MYC overexpression decreased the expression level of the bridging integrator 1, leading to increased poly (ADP-ribose) polymerase 1 (PARP1) activity and resistance to cisplatin [8]. However, the mechanism by which c-MYC regulates drug resistance remains to be fully elucidated.

ATP-binding cassette (ABC) transporters are a family of transporter proteins that contribute to drug resistance via
ATP-dependent drug efflux pumps. Some ABC transporters confer chemoresistance by causing the efflux of anti-cancer drugs [9, 10], and their expression levels correlate with the disease-free survival rate of colorectal cancer patients after adjuvant chemotherapy [11]. Interestingly, recent studies have revealed that MYCN regulates the expression levels of some ABC transporter genes in neuroblastoma [12], and c-MYC regulates the expression levels of some ABC transporter genes in chronic myelogenous leukaemia [13].

In this study, we identified ABCBS5 as a novel c-MYC target gene and examined the role of the c-MYC-ABCB5 axis in 5-FU resistance in human colon cancer cells.

Materials and methods

Clinical colorectal cancer specimens

Patients with colorectal cancer who underwent surgical treatment at Yamaguchi University and affiliated hospitals between April 2012 and September 2012 were enrolled in this study. Detailed information about these patients is presented in Table 1. Resected tumour specimens were immediately taken from resected colons and kept at −80°C until total RNA extraction. These samples were used in accordance with institutional guidelines and the Helsinki Declaration after obtaining informed consent from all patients.

Preparation of nuclear extracts and whole cell lysates

Nuclear extracts were prepared from COLO205 and COLO-320 cells using Nuclear Complex Co-IP Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s protocol. Whole cell lysates from COLO320, Caco-2 and 5-FU-resistant Caco-2 cells were prepared using 1% (v/v) NP-40, 0.5% sodium deoxycholate, 1% (v/v) Nonidet P-40 (NP-40) lysis buffer as described previously [16]. Caco-2 xenografts were lysed with RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM protease inhibitor cocktail) and homogenized with shaking stainless steel beads (Qiagen) using Mixer Mill MM300 (Qiagen).

Western blotting

Western blotting was performed as described previously [16] using nuclear extracts or whole cell lysates. The following primary and secondary antibodies were used. The primary antibodies were mouse monoclonal c-MYC antibody (9E10; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), goat polyclonal ABCBS5 antibody (46-620; ProSci Inc., Powder, CA, USA), goat polyclonal MRP5 antibody (Abcam), rabbit monoclonal PCNA antibody (D3H8P XP; Cell Signaling, Danvers, MA, USA), goat polyclonal ABCB5 antibody (46-620; ProSci Inc., Poway, CA, USA), and mouse monoclonal PARP1 antibody (F-2; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit monoclonal BCRP/ABCG2 antibody (Abcam, Cambridge, MA, USA), goat polyclonal MRP5 antibody (Abcam, Cambridge, MA, USA), goat polyclonal ABCB5 antibody (46-620; ProSci Inc., Poway, CA, USA), and mouse monoclonal p-Tubulin antibody (B-7; Santa Cruz Biotechnology, Inc.). The secondary antibodies were antirabbit and antimouse.
Table 1 Relationship between recurrence and clinicopathological characteristics of colorectal patients treated with 5-FU-based adjuvant chemotherapy after curative surgery

| No. of patients | Non-Recurrence | Recurrence | P   |
|-----------------|----------------|------------|-----|
|                 | 13             | 7          |     |
| Gender          |                |            |     |
| Male            | 7 (53.8%)      | 3 (42.9%)  | N.S.|
| Female          | 6 (46.2%)      | 4 (57.1%)  |     |
| Age             | 71.1 ± 8.1     | 75.0 ± 4.0 | N.S.|
| Location        |                |            |     |
| Right           | 7 (53.8%)      | 2 (28.6%)  | N.S.|
| Left            | 2 (15.4%)      | 2 (28.6%)  |     |
| Rectum          | 4 (30.8%)      | 3 (42.8%)  |     |
| Histological grade |            |            |     |
| Well            | 1 (7.7%)       | 1 (14.3%)  | N.S.|
| Moderate        | 12 (92.3%)     | 5 (71.4%)  |     |
| Poor            | 0 (0%)         | 1 (14.3%)  |     |
| Invasion depth  |                |            |     |
| T2              | 1 (7.7%)       | 0 (0%)     | N.S.|
| T3              | 12 (92.3%)     | 6 (85.7%)  |     |
| T4              | 0 (0%)         | 1 (14.3%)  |     |
| Lymphatic metastasis |         |            |     |
| Positive        | 7 (53.8%)      | 5 (71.4%)  | N.S.|
| Negative        | 6 (46.2%)      | 2 (28.6%)  |     |
| Lymphatic invasion |            |            |     |
| Positive        | 13 (100%)      | 5 (71.4%)  | N.S.|
| Negative        | 0 (0%)         | 2 (28.6%)  |     |
| Venous invasion |                |            |     |
| Positive        | 8 (61.5%)      | 3 (42.8%)  | N.S.|
| Negative        | 5 (38.5%)      | 4 (57.1%)  |     |
| Stage (UICC, 2009) |            |            |     |
| IIA             | 3 (23.1%)      | 0 (0%)     | N.S.|
| IIB             | 3 (23.1%)      | 2 (28.6%)  |     |
| IIIA            | 1 (7.7%)       | 0 (0%)     |     |
| IIIB            | 2 (15.4%)      | 3 (42.8%)  |     |
| IIIC            | 4 (30.8%)      | 2 (28.6%)  |     |
immunoglobulins conjugated to horseradish peroxidase (Igs-HRP), anti-rabbit Igs-HRP and anti-goat Igs-HRP (Dako, Carpenteria, CA, USA).

**Cell viability assay (WST-8 assay)**

Cell viability was evaluated using Cell Count Reagent SF (Nacalai Tesque; Kyoto, Japan) according to the manufacturer’s protocol. Cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA).

**Semi-quantitative RT-PCR**

Total RNA isolation, synthesis of cDNA and subsequent PCR were performed as described previously [15]. DNA sequences of the primers used for RT-PCR are listed in Table S2. Reaction conditions for each primer set were 50°C for 30 min. and 94°C for 2 min. followed by 20 cycles (MDR1, GAPDH), 24 cycles (c-MYC, ABCB5), 25 cycles (ABCC4, ABCC5) or 27 cycles (MRP1) of the following reaction: denaturing step at 94°C for 30 sec.; annealing at 54.5°C (GAPDH), 55°C (GAPDH), 57.5°C (c-MYC) or 60°C (MDR1, MRP1, ABCC4, ABCC5) for 30 sec.; and extension at 72°C for 30 sec. PCR products were analyzed on a 1% agarose gel containing ethidium bromide and detected using a UV transilluminator.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was performed with ChIP-IT™ Express Enzymatic (Active Motif) according to the manufacturer’s protocol. Normal mouse IgG (sc-2027) and mouse monoclonal anti-c-MYC (N-262) antibody were used for ChIP (Santa Cruz Biotechnology, Inc.). Each region containing a c-MYC-binding site within the (N-262) antibody were used for ChIP (Santa Cruz Biotechnology, Inc.).

**Nude mouse xenograft model and in vivo experiments**

Seven-week-old female BALB/c athymic Nu/Nu mice were purchased from Japan SLC Inc. (Shizuoka, Japan). A mixture of 1 × 10⁶ Caco-2 cells and Matrigel (BD Biosciences, San Jose, CA, USA) was subcutaneously injected to the right side of the back of the mice under anaesthesia. Tumour size was calculated using the following formula: volume = length × width × height. When the tumour size was about 1000 mm³, the mice were daily treated with DMSO as a vehicle control, 5-FU (10 mg/kg), 10058-F4 (20 mg/kg) or 5-FU (10 mg/kg) and 10058-F4 (20 mg/kg) by intraperitoneal administration for 2 weeks. The tumour volume was measured on day 0, 3, 5, 7, 10 and 14 following each treatment. Tumour tissues were used for immunofluorescent and western blotting analyses. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Yamaguchi University.

**Immunofluorescence staining**

The excised human colon cancer xenografts were fixed with a 10% formalin neutral buffer solution (Wako; Osaka, Japan) for 24 hrs at room temperature and were placed in 10% sucrose, 20% sucrose and 30% sucrose for 6 hrs each at 4°C with shaking. The samples were embedded in OCT compound, and the embedded samples were sectioned (5 μm) for immunofluorescence staining. The sections were first incubated with blocking reagent including 1% Triton X-100 for 1 hr at room temperature. After aspirating the blocking solution, the sections were incubated with a rabbit monoclonal antibody against K67 (SP6) (Abcam) or a goat polyclonal antibody against ABCB5 (Abcam) at 4°C overnight. The sections were rinsed three times with 1× PBS for 5 min. each and were incubated with Alexa Fluor 555F(ab)₂ fragment of goat anti-rabbit IgG (H+L) (Life Technologies) or donkey anti-goat IgG-TR (Santa Cruz Biotechnology, Inc.) for 1 hr at room temperature in the dark. The sections were rinsed three times with 1× PBS for 5 min. each. Nuclei were detected using DAPI staining. After washing three times with 1× PBS for 5 min. each, the sections were mounted with Fluorescent Mounting Medium (Dako). The images were acquired using the 360 ± 20 nm (blue) and 545 ± 12.5 nm (red) excitation filters on a B2-X710 All-in-One fluorescence microscope (KEYENCE Japan, Osaka, Japan).

**Detection of apoptotic cells by TUNEL method**

The paraffin tissue sections were deparaffinized by heating at room temperature for 5 min. to melt the wax, then immersed in xylene and washed in a graded ethanol. The sections were rinsed three times with 1× PBS for 5 min. each and were incubated with proteinase K at 37°C for 30 min., and then with PBS including 0.1% Triton X-100 for 2 min. After washing with PBS, apoptotic cells were detected with TUNEL method using In situ Cell Death Detection Kit, TMR red (Roche Applied Science, Waltham, MA, USA) according to the manufacturer’s protocol. Nuclei were detected using DAPI staining. After washing three times with 1× PBS for 5 min. each, the sections were mounted with Fluorescent Mounting Medium (Dako). The images were acquired using the 360 ± 20 nm (blue) and 545 ± 12.5 nm (red) excitation filters on a B2-X710 All-in-One fluorescence microscope (KEYENCE Japan).

**Detection of apoptotic dead cells by flow cytometric analysis**

Caco-2 cells were treated with DMSO, 5-FU (10 μM), 10058-F4 (32 μM), 5-FU (10 μM) and 10058-F4 (32 μM) for 72 hrs. The cells were resuspended in binding buffer containing Annexin V-FITC and PI using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer’s protocol. Annexin V-FITC and PI staining were quantified using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).
Statistical analysis

Statistical analyses were performed with SPSS for Windows ver. II. Data are expressed as means ± SD. To compare the experimental groups, Student’s *t*-test was performed. Data from clinical colorectal cancer specimens were analysed using Pearson correlation calculations. Kaplan–Meier curves were compared using the log-rank test. *P* < 0.05 (•) or *P* < 0.01 (••) was considered to be statistically significant.

Results

High expression of *c-MYC* in surgically resected primary colorectal cancer tissues correlates with recurrence following 5-FU-based adjuvant chemotherapy

To investigate whether *c-MYC* expression in surgically resected primary colorectal cancer tissues correlates with recurrence following 5-FU-based adjuvant chemotherapy, we selected 20 patients who had received 5-FU-based adjuvant chemotherapy after curative surgery. Total RNAs were purified from surgically resected tumours before 5-FU-based adjuvant chemotherapy and used for real-time RT-PCR. As shown in Figure 1A, the *c-MYC* expression level in surgically resected primary colorectal cancer tissues of patients with recurrence following 5-FU-based adjuvant chemotherapy was significantly higher than that of patients without recurrence (*P* = 0.002). Clinicopathological characteristics of these colorectal cancer patients are presented in Table 1. As shown in Table 1, all clinicopathological characteristics did not correlate with recurrence following 5-FU-based adjuvant chemotherapy. Furthermore, ROC curve analysis was used to obtain the optimal cut-off value of *c-MYC* mRNA levels in primary surgical cancer tissues of colorectal cancer patients. As shown in Figure 1B and Table 2, the Kaplan–Meier plot showed that patients with the high expression level of *c-MYC* mRNA had a poorer recurrence-free survival rate than that of the patients with low expression level of *c-MYC* mRNA (*P* = 0.0004). These results suggest that the high *c-MYC* expression level in primary colorectal cancer tissues correlates with the recurrence rate following 5-FU-based adjuvant chemotherapy.

c-MYC modulates 5-FU resistance in human colon cancer cells

To determine whether *c-MYC* is involved in 5-FU resistance, we investigated the effect of *c-MYC* overexpression or knockdown on the survival rate following 5-FU treatment in human colon cancer cells. We selected COLO205 cells for exogenous *c-MYC* overexpression, because parental COLO205 cells had low *c-MYC* expression level in the nucleus, but not in the cytoplasm (data not shown). COLO205 cells were transfected with pcDNA3-empty vector or pcDNA3-*c-MYC*, and exogenous *c-MYC* overexpression was confirmed by western blotting (Fig. 2A). As shown in Figure 2B, *c-MYC* overexpression in COLO205 cells significantly increased the survival rate following 5-FU treatment compared with control cells. Furthermore, we selected COLO-320 cells for knockdown of endogenous *c-MYC*, because parental COLO-320 cells presented a high *c-MYC* expression level in the nucleus, but not in the cytoplasm (data not shown). COLO-320
### Table 2 Relationship between c-MYC expression and clinicopathological characteristics of colorectal patients treated with 5-FU-based adjuvant chemotherapy after curative surgery

| c-MYC expression | Low | High | \(P\) |
|------------------|-----|------|------|
| **No. of patients** | 11  | 9    |      |
| **Gender**       |     |      |      |
| Male             | 7 (63.6%) | 3 (33.3%) | N.S. |
| Female           | 4 (36.4%) | 6 (66.7%)  |      |
| **Age**          | 70.8 ± 8.7 | 74.3 ± 3.7 | N.S. |
| **Location**     |     |      |      |
| Right            | 6 (54.5%) | 3 (33.3%) | N.S. |
| Left             | 1 (9.1%)  | 3 (33.3%)  |      |
| Rectum           | 4 (36.4%) | 3 (33.3%)  |      |
| **Histological grade** |     |      |      |
| Well             | 1 (9.1%)  | 1 (11.1%)  | N.S. |
| Moderate         | 10 (90.9%) | 7 (77.8%)  |      |
| Poor             | 0 (0%)    | 1 (11.1%)  |      |
| **Invasion depth** |     |      |      |
| T2               | 0 (0%)    | 1 (11.1%)  | N.S. |
| T3               | 11 (100%) | 7 (77.8%)  |      |
| T4               | 0 (0%)    | 1 (11.1%)  |      |
| **Lymphatic metastasis** |     |      |      |
| Positive         | 6 (54.5%) | 6 (66.7%)  | N.S. |
| Negative         | 5 (45.5%) | 3 (33.3%)  |      |
| **Lymphatic invasion** |     |      |      |
| Positive         | 11 (100%) | 7 (77.8%)  | N.S. |
| Negative         | 0 (0%)    | 2 (22.2%)  |      |
| **Venous invasion** |     |      |      |
| Positive         | 7 (63.6%) | 4 (44.4%)  | N.S. |
| Negative         | 4 (36.4%) | 5 (55.6%)  |      |
| **Stage (UICC, 2009)** |     |      |      |
| IIA              | 2 (18.2%) | 1 (11.1%)  | N.S. |
| IIB              | 3 (27.3%) | 2 (22.2%)  |      |
| IIIA             | 1 (9.1%)  | 0 (0%)     |      |
| IIIB             | 2 (18.2%) | 3 (33.3%)  |      |
| IIIC             | 3 (27.3%) | 3 (33.3%)  |      |
cells were transfected with scrambled siRNA or c-MYC siRNA, and the knockdown of endogenous c-MYC in nuclear extracts of COLO-320 cells was confirmed by western blotting (Fig. 2C). As shown in Figure 2D, c-MYC knockdown significantly decreased the survival rate following 5-FU treatment when compared with control cells. As expected, the survival rate following 5-FU treatment in parental COLO-320 cells was higher than that in parental COLO205 cells, and there was correlation between c-MYC expression level and the survival rate following 5-FU treatment (data not shown). These results indicate that c-MYC modulates 5-FU resistance in human colon cancer cells.

c-MYC regulates ABCB5 expression through its binding to the ABCB5 promoter

To identify the ABC transporter gene regulated directly by c-MYC, we investigated the effect of c-MYC knockdown on the expression levels of 5 ABC transporter genes, which are closely involved in resistance to chemotherapeutic agents including 5-FU [17–23]. Total RNAs were purified from COLO-320 cells transfected with scrambled siRNA or c-MYC siRNA and used for RT-PCR to assess MDR1, MRP1, ABCB5, ABCC4 and ABCC5 expression levels. The results showed that c-MYC knockdown decreased ABCB5, ABCC4 and ABCC5 expression levels, whereas MDR1 and MRP1 expression levels increased or nearly remained unchanged (Fig. 3A). Among ABCB5, ABCC4, and ABCC5, ABCB5 expression level was decreased the most by c-MYC knockdown. Therefore, we focused on ABCB5 as a possible c-MYC target gene. To investigate whether c-MYC directly binds to the ABCB5 promoter, a ChIP assay was performed with COLO-320 cells. As shown in Figure 3B, there were four locations of c-MYC-binding sites (CAT-GTG) within the ABCB5 promoter region, spanning from −5000 to +1500 base pairs (bp) with respect to the transcription start site. They were localized at −3415/−3410, −1812/−1807, −87/−82 and +1101/+1106 bp (Fig. 3B). We also searched other c-MYC-binding sites (CACGTG and CACGCG), but these binding sites were not present in this region. The results showed that c-MYC bound to two binding sites within the ABCB5 promoter (−3415/−3410 and −87/−82) (Fig. 3C). These results suggest that ABCB5 is a c-MYC target gene.

**c-MYC inhibitor, 10058-F4, decreased the ABCB5 expression level through the inhibition of c-MYC binding to the ABCB5 promoter**

It is well known that c-MYC protein oncogenic activity requires dimerization with MAX [24]. To assess the importance of c-MYC-MAX complex-targeted DNA binding on the induction of ABCB5 expression, we used the c-MYC small molecule inhibitor, 10058-F4, to inhibit the binding of c-MYC to target DNA by preventing c-MYC-MAX heterodimerization [25]. A ChIP assay, RT-PCR and western blotting were performed with COLO-320 cells treated with DMSO or 10058-F4 for 48 hrs. 10058-F4 treatment decreased the amount of c-MYC binding to the ABCB5 promoter (Fig. 4A). As expected, 10058-F4 treatment decreased ABCB5 expression at mRNA and protein levels (Fig. 4B and C). Furthermore, we investigated the effect of 10058-F4 treatment on the expression levels of other 5-FU resistance-involved ABC transporters, ABCC5 and ABCG2 [26]. Although 10058-F4 treatment decreased ABCC5 expression, the expression level of ABCC5 protein was very low in COLO-320 cells (Fig. 4D, upper panel). ABCG2 protein was expressed in COLO-320 cells, and 10058-F4 treatment did not decrease ABCG2 expression (Fig. 4D, middle panel). These results suggest that c-MYC positively regulates the expression of ABCB5 through c-MYC binding to the ABCB5 promoter.

**ABCB5 is closely involved in 5-FU resistance**

To elucidate the role of ABCB5 in 5-FU resistance, we examined the effect of ABCB5 knockdown on the survival rate following 5-FU treatment in COLO-320 cells. The knockdown of endogenous

| c-MYC expression | Low | High |
|------------------|-----|------|
| No. of patients  | 11  | 9    |
| Chemotherapy regimen | | |
| UFT             | 4 (36.4%) | 4 (44.4%) | N.S. |
| Xeloda          | 2 (18.2%) | 2 (22.2%) |
| TS-1            | 5 (45.5%) | 3 (33.3%) |
| Recurrence      | | |
| +               | 0 (0%) | 7 (77.8%) | 0.0002 |
| −               | 11 (100%) | 2 (22.2%) |

Table 2. Continued

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ABCB5 was confirmed in COLO-320 cells transfected with ABCB5 siRNA by western blotting (Fig. 5A). In ABCB5-silenced COLO-320 cells, the survival rate following 5-FU treatment was significantly decreased compared with that in control cells, and ABCB5 knockdown had no effect on the survival rate following cisplatin treatment (Fig. 5B). Moreover, 5-FU-resistant Caco-2 cells presented a high ABCB5 expression level and a significantly high survival rate following 5-FU treatment compared with those of parental Caco-2 cells (Fig. 5C and D). These results suggest that ABCB5 is closely involved in 5-FU resistance in human colon cancer cells.

**The combined treatment of 5-FU and 10058-F4 decreased tumorigenicity in nude mice**

To investigate the effect of the combined treatment of 5-FU and 10058-F4 on tumorigenicity in nude mice, a human colon cancer...
xenograft murine model was developed. As shown in Figure 6A and B, 5-FU or 10058-F4 treatment decreased tumour volumes compared with the control. Furthermore, the combined treatment of 5-FU and 10058-F4 significantly decreased tumorigenicity in nude mice compared with 5-FU or 10058-F4 treatment ($P < 0.05$). The ratio of Ki67-positive cells was decreased, and the ratio of TUNEL-positive cells was increased in 5-FU, 10058-F4 and the combined 5-FU and 10058-F4 treatment groups compared with the control (Fig. 6C–F). The combined treatment of 5-FU and 10058-F4 tended to decrease the Ki67-positive ratio and to increase the TUNEL-positive ratio the most. Furthermore, 10058-F4 treatment alone increased the ABCB5 expression level when compared with the control (Fig. 6G and H). Conversely, 5-FU treatment alone decreased the ABCB5 expression level when compared with the control (Fig. 6G and H).

Discussion

Previous reports indicating that c-MYC was overexpressed in many types of cancers and contributed to drug resistance motivated us to investigate the role of c-MYC expression in 5-FU resistance in this study. First, we performed real-time RT-PCR using surgically resected tumours from 20 patients with colorectal cancer who were subsequently treated with 5-FU-based adjuvant chemotherapy after curative surgery. Our results showed that the c-MYC expression level in resected colorectal cancer tissues markedly correlated with recurrence following 5-FU-based adjuvant chemotherapy. Although this study was limited by the small number of cases and short follow-up periods after operation, the results indicate that c-MYC expression might be an early recurrence predictive marker for colorectal cancer patients following 5-FU-based adjuvant chemotherapy. Recent studies have indicated that MYCN regulates the transcription of specific ABC transporter genes in neuroblastoma [12], and that c-MYC regulates the expression of ABC transporter genes in chronic myelogenous leukaemia [13]. Given that ABC transporters contribute to the resistance to chemotherapeutic agents by causing the efflux of anti-cancer drugs, we have been suggested that c-MYC increases 5-FU resistance in human colon cancer cells by regulating the expression of ABC transporters. To identify the ABC transporter genes regulated by c-MYC in human colon cancer cells, we investigated the effect of c-MYC knockdown on MDR1, MRP1,
The effect of the c-MYC inhibitor, 10058-F4, on ABCB5 expression. (A) ChIP analysis was performed on COLO-320 cells treated with DMSO or the c-MYC inhibitor, 10058-F4. The number on the left corresponds to the arrow number in Figure 3B. The number under the band in the second lane from the left shows the ratio of ChIP with c-MYC to INPUT band intensity normalized to the value obtained in control cells. c-MYC, mouse monoclonal anti-c-MYC antibody; IgG, normal mouse IgG; INPUT, chromatin DNA of 1/100 quantity used in ChIP. (B) Total RNAs were purified from COLO-320 cells treated with DMSO (vehicle) or 10058-F4 at various concentrations (32, 64 and 100 µM) for 48 hrs, and ABCB5 and GAPDH expression was detected by RT-PCR. The number under each band in the upper panel shows the ratio of ABCB5 to GAPDH band intensity normalized to the value obtained in control cells. (C and D) Whole cell lysates were prepared from COLO-320 cells treated with DMSO (vehicle) or 10058-F4 at a concentration of 64 µM for 48 hrs, and ABCB5, ABCG2, and α-Tubulin expression in whole cell lysates was detected by western blotting. α-Tubulin was used to assess the total amount of whole cell lysates loaded on the gel. The number under the band shows the ratio of ABCB5, ABCG2 or α-Tubulin to α-Tubulin band intensity normalized to the value obtained in control cells.

ABCB5, ABCC4 and ABCC5 expression levels, which are closely involved in the resistance to chemotherapeutic agents including 5-FU [17–23]. Interestingly, c-MYC knockdown markedly decreased the expression level of ABCB5, which promotes doxorubicin transport [20] and is involved in 5-FU resistance [21]. Moreover, ChIP assays revealed that c-MYC binds to two binding sites within the ABCB5 promoter region. c-MYC inhibitor, 10058-F4, decreased the amount of c-MYC binding to its binding site within the ABCB5 promoter region and led to the decrease in ABCB5 expression at mRNA and protein levels. Furthermore, we examined the change in the expression levels of other 5-FU resistance-involved ABC transporters, ABCC5 and ABCC2 proteins following 10058-F4 treatment. Unexpectedly, COLO-320 cells had very low ABCC5 expression at protein level. Although 10058-F4 treatment decreased ABCC5 expression, it appeared that the decrease in ABCB5 expression had no effect on 5-FU resistance because of its low expression in COLO-320 cells. As expected, ABCC5 knockdown did not decrease survival rate following 5-FU treatment in COLO-320 cells (Fig. S1). ABCC2 was expressed in COLO-320 cells at protein level, and 10058-F4 treatment did not decrease ABCC2 expression. ABCB5-silenced COLO-320 cells showed a decreased survival rate following 5-FU treatment, and 5-FU-resistant Caco-2 cells presented a high ABCB5 expression level and high survival rate following 5-FU treat-
ment compared with those in parental Caco-2 cells. These results suggest that c-MYC confers resistance to 5-FU through regulating ABCB5 expression in human colon cancer cells. Furthermore, *in vivo* study showed that the combined treatment of 10058-F4 and 5-FU significantly decreased tumorigenicity in nude mice compared with 5-FU or 10058-F4 treatment alone. The combined treatment of 5-FU and 10058-F4 tended to decrease the Ki67-positive ratio and to increase the TUNEL-positive ratio the most. *In vitro* study also
showed that the combined treatment of 5-FU and 10058-F4 increased the ratio of Annexin V- and PI-positive cells and decreased the expression level of PCNA (Fig. S2A and B). As expected, 10058-F4 treatment decreased the ABCB5 expression level in spite of the presence or the absence of 5-FU treatment. Remarkably, 5-FU treatment alone increased the ABCB5 expression level. This finding is consistent with a recent study demonstrating that the ABCB5 expression level is increased in rectal cancer specimens from patients following 5-FU-based chemotherapy and human colorectal cancer murine xenografts following 5-FU treatment [21]. Importantly, we observed that ABCB5 expression level in primary colorectal cancer tissues did not correlate with the recurrence rate following 5-FU-based adjuvant chemotherapy (Fig. S3). As expected, there was no correlation between ABCB5 and c-MYC expression levels in primary colorectal cancer tissues (Fig. S4). Combined with the results that ABCB5 expression level is increased following 5-FU treatment, we consider that low expression level of ABCB5 in primary colorectal cancer tissues is because of a lack of 5-FU-based adjuvant chemotherapy, and ABCB5 expression level may be increased in recurrent colorectal cancer tissues survived following 5-FU-based adjuvant chemotherapy. Although the mechanism by which 5-FU treatment increases ABCB5 expression level remains unknown, we propose two possibilities. One possibility is that some population of colon cancer cells which originally have high ABCB5 expression survive following 5-FU treatment. Another possibility is that 50-FU treatment increases ABCB5 expression in some population of colon cancer cells, and the colon cancer cells with the increased ABCB5 expression can survive. A recent report supports the latter possibility, showing that HIF-1α is up-regulated in tumour cells in response to doxorubicin [27]. There is a possibility that 5-FU treatment enhances c-MYC activity and leads to the induction of ABCB5 expression. Further studies are needed to elucidate the mechanism by which 5-FU treatment increases the ABCB5 expression level.

In this study, we showed that c-MYC confers resistance to 5-FU by regulating ABCB5 expression in human colon cancer cells. Our results suggest that the c-MYC-ABCB5 axis could be a potential therapeutic target in 5-FU-resistant colorectal cancer cells.

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Conflicts of interest
The authors declare no conflict of interest.

Supporting information
Additional Supporting Information may be found in the online version of this article:

**Figure S1** Effect of ABCC5 knockdown on the survival rate following 5-FU treatment in COLO-320 cells.

**Figure S2** Effect of the combined treatment of 5-FU and 10058-F4 on Caco-2 cells in vitro.

**Figure S3** Relationship between ABCB5 expression level in surgically resected primary colorectal cancer tissues and recurrence following 5-FU-based adjuvant chemotherapy.

**Figure S4** Relationship between c-MYC and ABCB5 expression levels in surgically resected primary colorectal cancer tissues.

**Table S1** DNA sequence of primers used for real-time RT-PCR.

**Table S2** DNA sequence of primers used for RT-PCR.

**Table S3** DNA sequence of primers used for ChiP assay.
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