In vitro and *in vivo* studies on the association of long non-coding RNAs H19 and urothelial cancer associated 1 with the susceptibility to 5-fluorouracil in rectal cancer

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Abstract. There is no predictive biomarker for response to 5-fluorouracil (5FU)-based neoadjuvant chemotherapy (NAC) in rectal cancer. In the present study, we examined potential long non-coding RNAs (lncRNAs) linked to the susceptibility to 5FU in cultured colorectal cancer cells, and in biopsy and resected tissues of 31 human rectal cancer cases treated with NAC. Candidate lncRNAs for the prediction of susceptibility to 5FU were investigated by comprehensive analysis of expression profiles of 84 lncRNAs in cultured cells using PCR array. Bioinformatic analysis identified H19 and urothelial cancer associated 1 (UCA1) as candidate biomarkers for 5FU susceptibility. Quantitative PCR of H19 and UCA1 in cultures of colorectal cancer cells demonstrated the notable variation in expression. The ratios of changes of H19 and UCA1 expression in response to 5FU were low in cells resistant to 5FU, whereas ratios were high in cells susceptible to 5FU. In 5FU-susceptible cells, cell proliferation was inhibited by 5FU. Upregulation of H19 and UCA1 were associated with the reduction in target molecule expression, including retinoblastoma and p27kip1. In 31 cases of rectal cancer, H19 and UCA1 expression levels in biopsy and resected tissue were comparable. The ratios of H19 and UCA1 expression in resected tissue compared with biopsy samples were low in 17 cases, whereas the ratios were high in 14 cases; 11 of the 17 cases (65%) with low ratios exhibited poor response to NAC, whereas 4 of the 14 cases (29%) with high ratios showed poor response (P=0.045). The increase in H19 and UCA1 expression may represent the response to impaired cell cycle in cells susceptible to 5FU. Our results indicate that changes in H19 and UCA1 expression may be considered for predicting the susceptibility to 5FU-based NAC in rectal cancer.

Introduction

Locally advanced rectal cancer is treated with pre-operative chemoradiotherapy, followed by the resection of rectal cancer (1,2). Pre-operative chemoradiotherapy (CRT) increases the possibility of complete resection and reduces the risk of local recurrence. However, radiation to the pelvic region may cause adverse effects, such as urogenital and anal dysfunctions (3,4). Recently, neoadjuvant chemotherapy (NAC) without radiation to the pelvic region has also been applied to locally advanced rectal cancer. However, approximately half of NAC-treated cases exhibit poor responses to initial treatment (5-7). Predicting the response to NAC from clinical and pathological features of rectal cancer remains difficult.

5-Fluorouracil (5FU) and its prodrugs, fluoropyrimidine derivatives, are key chemotherapy drugs for colorectal cancer. The 5FU agents are for adjuvant chemotherapy of rectal cancer, and also for pre-operative CRT and NAC (1,2,5-9). Previous studies reported that the expression levels of the target enzyme thymidine synthase (TS) and the enzymes involved in fluoropyrimidine metabolism are associated with the susceptibility of colorectal cancer to 5FU (10-13). The increased expression of TS (10,11) and dihydro pyrimidine dehydrogenase, which is a degradation enzyme of 5FU (10-13), and decreased expression of orotate phosphoribosyl transferase, which is a metabolic enzyme that convert 5FU into its active form (13), are suggestive for the resistance to 5FU. Previously, the association of resistance to 5FU with genetic features or non-coding RNAs has been reported. Micro-satellite instability status was also associated with the susceptibility to 5FU (14). Additional reports have indicated a link between microRNAs and long non-coding RNAs (lncRNAs) with the susceptibility of colorectal carcinoma cells to 5FU (15-22).

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LncRNAs are RNAs that >200 base pairs in length and serve significant roles in various biological functions, such as gene expression, protein expression and chromatin status (23). It has been noted that lncRNAs are involved in the proliferation, invasion and metastasis of carcinomas at various sites (24). Furthermore, an association between lncRNAs and the resistance to chemotherapeutic agents was reported in carcinomas (25). However, it is unclear which lncRNA is useful for the prediction of the susceptibility to 5FU.

In the present study, we examined the expression profile of lncRNAs in 5FU-susceptible and 5FU-resistant cultured colorectal cancer cells using PCR array analysis and identified possible candidate lncRNAs, H19 and urothelial cancer associated 1 (UCA1), by bioinformatic analysis. The expression levels of H19 and UCA1 and their alterations were examined in paired biopsy and resected specimens. Alterations in expression levels, rather than the basal expression levels of these lncRNAs, were suggested to be predictive for the susceptibility to NAC.

Materials and methods

Cell lines and culture. HCT116, DLD-1 and SW480 human colorectal carcinoma cell lines were employed in this study. HCT116 cells were obtained from the RIKEN BioResource Research Center; DLD-1 cells were obtained from Taiho Pharmaceutical Co., Ltd. SW480 cells were obtained from the American Type Culture Collection. To generate 5FU-resistant cell lines, the parental cells (designated as HCT116/p, DLD-1/p, and SW480/p) were repeatedly treated with 5FU (Sigma-Aldrich; Merck KGaA) at 37°C for 120 h. The concentration of 5FU started at $0.2 \mu M$ and was increased to 2, 5 and $10 \mu M$ and then by $10 \mu M$ until $100 \mu M$ (26). After each treatment step, cells were cultured in medium without 5FU until the cells proliferated to 80% confluency. The treated cells were designated as HCT116/5fu, DLD-1/5fu, and SW480/5fu. DLD-1/5fu cells were kindly provided by Taiho Pharmaceutical. The cells were cultured in RPMI 1640 (Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Nichirei Bioscience Inc.).

5FU susceptibility assay. Cells were plated on 6-well plates at $1.5x10^5$ cells/well, and cultured in medium without 5FU or with 1, 2, 5 or $10 \mu M$ 5FU at 37°C for 48 h. Then, the number of living cells was counted using a Cell Counter, model R1 (Olympus Corporation). The experiments were performed in triplicate. Survival was expressed as the percentage of living cells treated with 5FU, as calculated by dividing the mean numbers of living cells treated with 5FU by the mean numbers of living cells without 5FU. The half maximal-inhibitory concentration (IC_{50}) was calculated using GraphPad Prism, version 7 (GraphPad Software, Inc.).

Cell proliferation assay. Cells were plated in 6-well plates at $1.5x10^5$ cells/well in medium with or without $10 \mu M$ 5FU at 37°C until 96 h. At 24, 48, 72 and 96 h, the numbers of living cells were counted using a Cell Counter, model R1.

RNA extraction from cultured cells. Cells were plated on 60-mm dishes at $2.5x10^5$ cells/well, and cultured with or without $10 \mu M$ 5FU at 37°C for 48 h. After a wash with phosphate buffered saline (PBS), total RNA was extracted using TRIzol (Thermo Fisher Scientific, Inc.) according to the recommended protocol. The concentration of total RNA was measured.

Comprehensive analysis of lncRNA expression. DLD-1/p and DLD-1/5fu cells were cultured for 48 h without 5FU. Total RNA was extracted from these cells using TRIzol (Thermo Fisher Scientific, Inc.). Complementary DNA (cDNA) was synthesized from 1,000 ng total RNA using the RT^2 First Strand Kit (Qiagen, Inc.) according to the manufacturer's instructions. The expression profile of lncRNAs was obtained using the RT^2 lncRNA PCR Human Cancer PathwayFinder (Qiagen, Inc.). The PCR mixture was a $25 \mu l$ solution containing 1X RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, Inc.), cDNA synthesized from 8.5 ng total RNA, and primers. The expression of a total of 84 lncRNAs and 5 control housekeeping genes was analyzed; the list of analyzed lncRNAs is available at the manufacturer's website (cat. no. LAHS-002Z). PCR was performed using a 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.). The reaction was initiated with incubation at 95°C for 10 min, followed by 60 cycles of incubation at 95°C for 15 sec and at 60°C for 1 min. The expression levels of lncRNAs were standardized with levels of the housekeeping genes. The expression levels of lncRNAs in DLD-1/5fu cells were calculated as fold-change relative to the levels in DLD-1/p cells by the $2^{-\Delta\Delta Cq}$ method (27) and expressed as base-2 logarithm of fold-change.

Pathway analysis of lncRNA. Pathways of the lncRNAs were analyzed by Ingenuity Pathway Analysis (IPA; Qiagen, Inc.). The scores of networks were calculated by the numbers of focus molecules.

Estimation of doubling time of cultured cells. Cells were plated on 6-well plates at $1.5x10^5$ cells/well and cultured with or without $10 \mu M$ 5FU. At 0, 24, 48, 72 and 96 h after plating, the cell numbers were counted with Cell Counter, model R1. The doubling time was estimated from the linear phase of growth curve of cultured cells.

Cell cycle assay. Cells were plated on 100 mm dishes at $7.5x10^5$ cells/well and cultured with or without $10 \mu M$ 5FU. After 48 h, the cells were collected and counted using a Cell Counter, model R1. One million cells were transferred to 1.5 ml tube, and the cells were fixed in 1 ml of 70% ethanol at -20°C for 3 h. The cells were then washed once with PBS and resuspended in $200 \mu l$ of Cell Cycle reagents of Muse Cell Cycle Kit (cat. no. MCH 100106, Luminex Japan Corporation Ltd.). The cells were incubated for 30 min at room temperature in the dark. Cell cycle phases were determined by Muse Cell Analyzer (Luminex Japan Corporation Ltd.). Approximately 10,000 cells were analyzed, and the number of cells at cell cycle phases of G0/G1, S and G2/M were counted. The cell cycle phases were expressed as the percentage of cells at these phases.

Western blot analysis. Cells were plated on 100 mm dishes at $7.5x10^5$ cells/well and cultured with or without $10 \mu M$
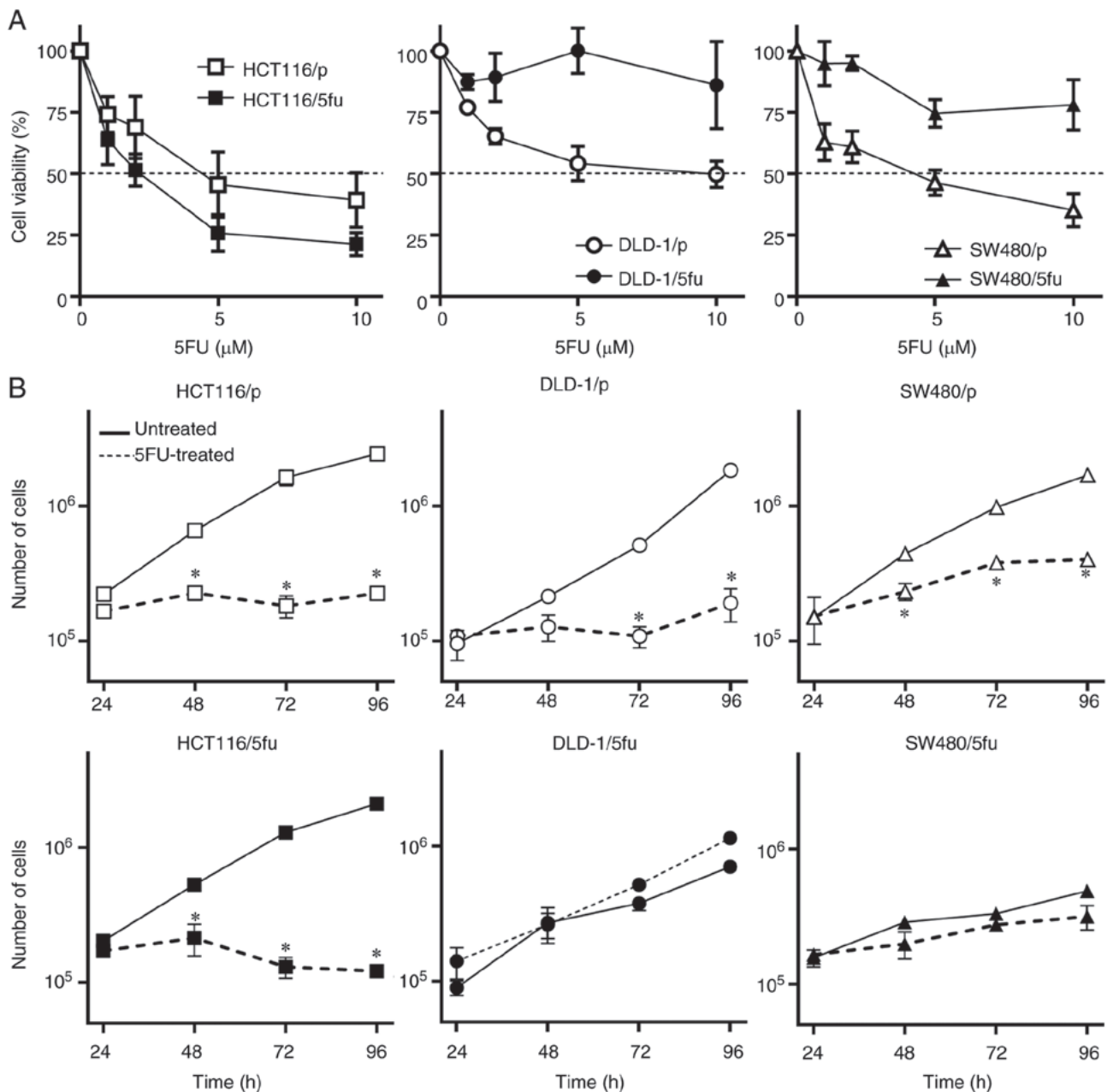
Figure 1. Biological features of cultured colorectal cancer cells. (A) Susceptibility of indicated cell lines to 5FU. (B) Proliferation of cultured cells untreated and treated with 10 μM 5FU. Proliferative ability of DLD-1/5fu and SW480/5fu cells was not affected by 5FU; the proliferation of HCT116/p, HCT116/5fu, DLD-1/p and SW480/5fu cells were inhibited by 5FU. *P<0.05 vs. untreated cells. 5FU, 5-fluorouracil.

5FU for 48 h. After a wash with PBS, the cells were lysed in 0.5% SDS/50 mM Tris-HCl (pH 7.6) and sonicated for 10 min. Protein samples were loaded onto 5-20% SDS-PAGE gradient gels and transferred to a polyvinylidene difluoride membrane. After blocking of the membrane with 5% skim milk in Tris-buffered saline/0.5% Tween 20 at room temperature for 30 min, the membrane was incubated with primary antibodies at 4˚C overnight: Anti-retinoblastoma (Rb) antibody (cat. no. 9309; 1:2,000; Cell Signaling Technology, Inc.), anti-p27kip1 antibody (cat. no. 610242; 1:5,000; Becton, Dickinson and Company), and anti-β-actin (cat. no. A5316; 1:10,000; Sigma-Aldrich; Merck KGaA). After washing the membrane with a buffer containing 250 mM Tris-HCl/150 mM NaCl/0.1% TritonX, the membrane was then incubated with

horseradish peroxidase-labeled anti-mouse IgG antibody (cat. no. A106PU; 1:10,000; American Qualex Scientific Products) at room temperature for 60 min. Peroxidase activity was detected with chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Inc.). The bands were quantified using Quantity One Software version 4. 6. 2 (Bio-Rad Laboratories, Inc.).

Rectal cancer cases. Cases of rectal cancer treated with NAC between January 2012 and April 2017 were retrieved from the records of the Department of Gastrointestinal and Hepato-Biliary-Pancreatic Surgery, Nippon Medical School Hospital. A total of 35 cases of rectal cancer, pre-operatively diagnosed as stage cT3 or cT4 without distant metastasis

and treated with six cycles of combination therapy with 5FU, leucovorin, and oxaliplatin, were identified. Molecular targeting drugs were not used and radiotherapy was not performed. After 1 month after the end of chemotherapy, the tumor was resected.

Histological subtypes, depth of the tumor invasion and histological therapeutic responses were evaluated in the resected tissue by two investigators in accordance with the criteria of the Japanese Classification of Colorectal Carcinoma (28): Grade 0, no denaturation and necrosis of the cancer; Grade 1a, denaturation and necrosis in less than one-third of the cancer tissue; Grade 1b, denaturation and necrosis in one-third or more to less than two-thirds of the cancer tissue; Grade 2, denaturation, necrosis and loss in two-thirds or more of the cancer tissue; and Grade 3, no residual cancer cells.

Two patients with Grade 3 response were excluded from the study as no cancer cells were identified in the resected tissue. Another two patients from whom biopsy specimens were not available were also excluded. Finally, 31 patients with rectal cancer who received NAC were studied. The present study was approved by the Ethics Committee of Nippon Medical School Hospital (approval no. 29-03-909). Written informed consent was obtained from all patients.

RNA extraction from biopsy and resected cancer tissues. Total RNA was extracted from formalin-fixed, paraffin-embedded tissue of biopsy specimens taken before NAC and resected tissue specimens taken after NAC treatment using RNeasy FFPE Kit (Qiagen, Inc.). Five slices of 10-μm-thick sections of biopsy tissue were deparaffinized using xylene and ethanol in 1.5 ml tubes and dried. Three slices of 10-μm-thick sections of resected tissue were deparaffinized, hydrated, and stained with hematoxylin at room temperature for 30 sec. Cancer tissue was dissected out, collected and then transferred to 1.5 ml tubes, and dried. Tissues were incubated at 56°C overnight in 240 μl of Buffer PKD provided with the kit. Total RNA was extracted according to the manufacturer's protocol, and the concentration of total RNA was determined by spectrophotometry.

Reverse transcription and quantitative PCR. cDNA was reverse-transcribed from total RNA using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. One microgram of total RNA extracted from cultured cells and 250 ng total RNA extracted from biopsy specimens and resected tissues were used as templates.

Quantitative PCR was performed in a 20-μl reaction mixture containing 1X TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific, Inc.), 1X TaqMan primers and probes; synthesized cDNA was then reverse transcribed. The TaqMan primers and probes were as follows: H19 (Hs00262142), UCA1 (Hs01909129), and 18S ribosome RNA (rRNA) (Hs03928990) (all from Thermo Fisher Scientific, Inc.). The reaction was initiated with incubation at 95°C for 20 sec, followed by 40 cycles of incubation at 95°C for 1 sec and at 60°C for 20 sec. cDNA that was reverse transcribed from a mixture of total RNA extracted from DLD-1/p, SW480/p, and HCT116/p cells were used for standardization. Alterations in fluorescence were monitored by the Step One Plus Real-Time PCR System
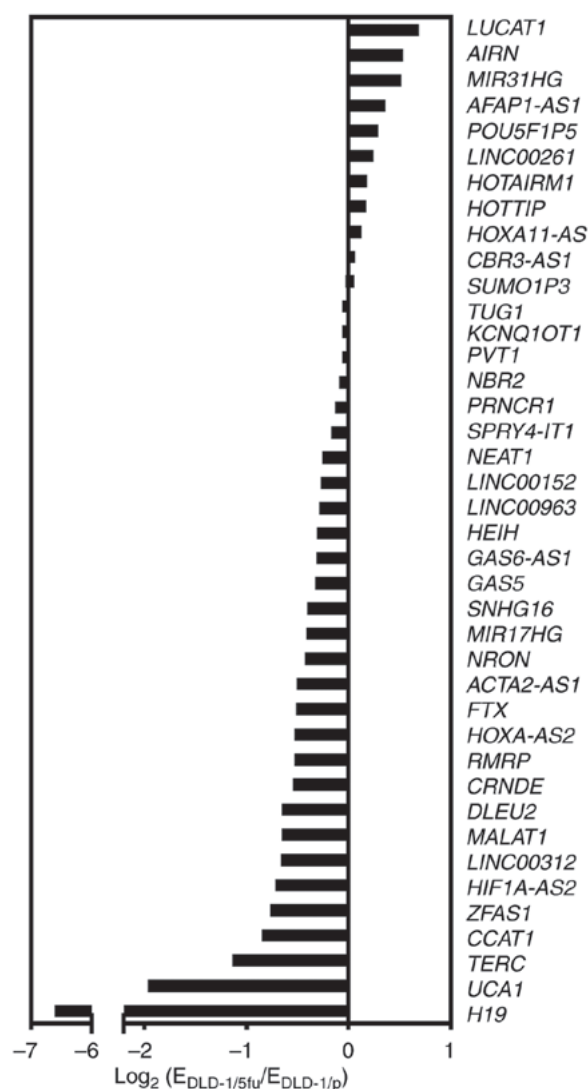

Figure 2. Expression profiling of lncRNAs in cultured cells. The base 2 logarithm of fold-changes of expression of lncRNAs in DLD-1/5fu ($E_{DLD-1/5fu}$) to those in DLD-1/p cells ($E_{DLD-1/p}$). H19, UCA1, and TERC lncRNAs were downregulated less than half in DLD-1/5fu cells compared with DLD-1/p cells. lncRNA, long non-coding RNA; TERC, telomerase RNA component; UCA1, urothelial cancer associated 1.

(Thermo Fisher Scientific, Inc.). The expression levels of H19 and UCA1 were standardized with those of 18S rRNA. The expression levels were calculated by the $2^{-\Delta\Delta Cq}$ method (27) and expressed as fold-change relative to the level of the mixture of total RNA extracted from DLD-1/p, SW480/p, and HCT116/p cells. The fold-changes in the expression levels of 5FU-treated cultured cells compared with untreated cultured cells were expressed as 5FU-treated/untreated ratio. The fold-changes in resected specimens compared with biopsy specimens were calculated as resection/biopsy ratio.

Statistical analysis. Data are expressed as the mean ± standard deviation. χ^2 and Mann-Whiney U tests, and two-way ANOVA with Sidak's post-hoc test were performed using GraphPad Prism, version 7 (GraphPad Software, Inc.). Hierarchical clustering was conducted using JMP software version 13.2 (SAS Institute Japan, Ltd.). $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. Networks identified by Ingenuity Pathway Analysis.

ID	Molecules in network	Score	Focus molecules	Top diseases and function
1	Akt, AR, COL2A1, corticosterone, CRNDE[a], CTNNB1, EGFR, FTX[a], GAS5[a], Gsk3, HMGA1, HNRNPA2B1, HOXA11-AS[a], IGF2, IL1, LINC00963[a], MIR17HG[a], MIR31HG[a], MMP14, MTDH, PARP1, PDGF BB, PGR, PRNCR1[a], progesterone, Rb, S100A8, SMARCA4, SOX2, SOX9, TWIST2, UCA1[a], ZBTB7A, ZEB1, ZFAS1[a]	22	10	Cellular movement, cell cycle, cellular development
2	CDKN3, COL2A1, corticosterone, CTCF, Cyclin D, NMT3L, FGF2, Gsk3, GSK3B, H19[a], H2BFM, HOTAIRM1[a], hydrogen peroxide, IGF2, KCNQ1OT1[a], LINC00261[a], MALAT1[a], Map3k7, NBR2[a], PDGF BB, PGR, POMC, PRKAA, Rb, RMRP[a], Srebp, SUMO1P3[a], TERC[a], TGFB1, TNF, tretinoin, TUG1[a], TWIST2, ZBTB7A, ZFP57	22	10	Cellular development, cellular growth and prolif eration, cellular movement
3	AFAP1-AS1[a], CASP8, CBR3-AS1[a], CCAT1[a], CCND1, CD28, CD3, CDC73, CHEK1, corticosterone, CYTOR[a], DLEU2[a], DNMT3A, ELAVL1, Gsk3, HMGA1, HNRNPU, HOXA-AS2[a], IGF1R, IGF2, IGF2BP3, MYC, NEAT1[a], NOS2, PARP1, PDGF BB, PI3K (complex), PVT1[a], Rb, RHOA, S100A8, SNHG16[a], SOX9, SPRY4-IT1[a], TWIST2	22	10	Cell cycle, cellular movement, cell death and survival
4	HOTTIP[a], HOXA13	3	1	Cell death and survival, developmental disorder, hereditary disorder
5	LRRK2, NFATC2, NRON[a]	2	1	Cellular development, cellular growth and, proliferation nervous system development and function

[a]Long non-coding RNAs identified in the present study.

Results

Susceptibility of cultured cells to 5FU. We treated a panel of parental and 5FU-resistant colorectal carcinoma cell lines with various concentrations of 5FU and determined the IC_{50} values (Fig. 1A). The IC_{50} values of the parental cells HCT116/p, DLD-1/p, and SW480/p were 4.5, 6.4 and 3.5 μM, respectively. The IC_{50} values of HCT116/5fu, DLD-1/5fu, and SW480/5fu cells were 2.0, 103.8 and 26.2 μM, respectively (data not shown).

We next examined the proliferation of cell lines treated with 10 μM 5FU. The proliferation of all three parental cell lines HCT116/p, DLD-1/p and SW480/p was significantly inhibited by 10 μM 5FU compared with the untreated cells (Fig. 1B). The proliferation of DLD-1/5fu and SW480/5fu cells were not notably inhibited by 5FU, whereas suppressed proliferation was exhibited by HCT116/5fu cells following treatment with 5FU.
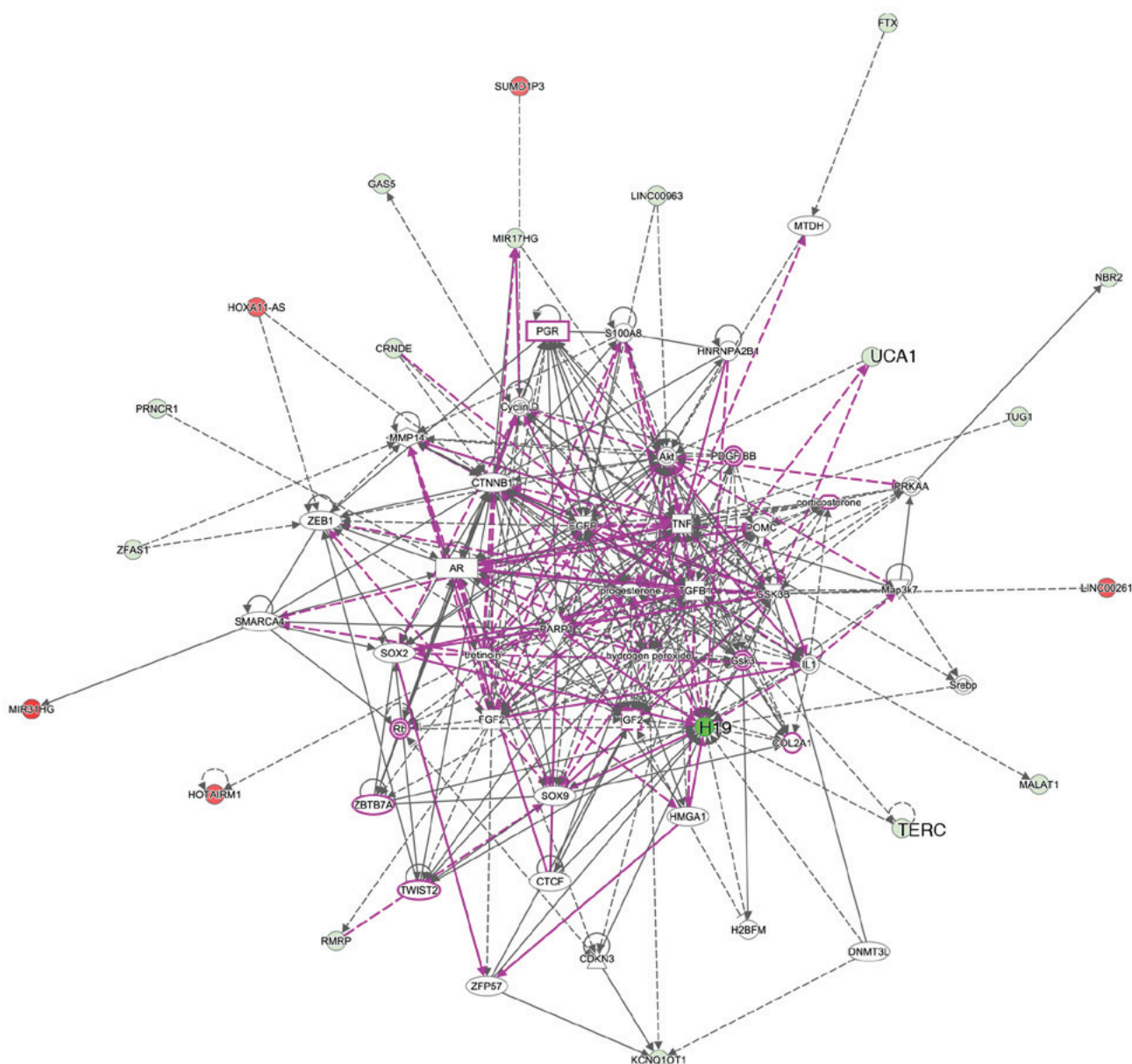
Collectively, these results indicated that the three parental cell lines and HCT116/5fu cells were susceptible to 5FU, while the DLD-1/5fu and SW480/5fu cell lines appeared to be resistant to 5FU.

Comprehensive and bioinformatics analysis of lncRNA expression in 5FU-resistant cells. We next performed a comprehensive analysis of the expression profile of lncRNAs in the 5FU-resistant line DLD-1/5fu compared with DLD-1/p parental cells. Of the 84 lncRNAs from the expression array, 40 lncRNAs were considered to be suitable for the analysis as the threshold cycle was <30 cycles in both DLD-1/p and DLD-1/5fu sets. Among the 40 lncRNAs, 11 lncRNAs were upregulated and 29 lncRNAs were downregulated in DLD-1/5fu cells compared with DLD-1/p cells (Fig. 2). No lncRNAs were upregulated by >2-fold. Among the downregulated lncRNAs, telomerase RNA component (TERC), UCA1, and H19 were decreased less than half.

Five networks were identified by IPA (Table I). Networks 1-3 contained 10 lncRNAs. UCA1 was categorized in network 1, and H19 and TERC were categorized in network 2. Pathway analysis revealed numerous connections of H19 with other molecules (Fig. 3). Although UCA1 showed fewer connections, UCA1 had connections with molecules in other networks. Only a few connections were observed with TERC. We thus suspected that H19 and UCA1 may be key molecules involved in the susceptibility of cultured cells to 5FU.

H19 and UCA1 expression in cultured cells. We next examined the expression levels of H19 and UCA1 in cultured cells. The expression levels of H19 and UCA1 markedly varied among the untreated cultured cells (Fig. 4A) and among the 5FU-treated cultured cells (Fig. 4B). The expression level of H19 was changed

Figure 3. Ingenuity Pathway Analysis of molecules in cultured DLD-1/p and DLD-1/5fu cells. Combined networks of 1 and 2 formed tight connections. Red lines indicate connection over networks 1 and 2. H19 had connections with numerous molecules within network 1, as well as connections with molecules in networks 2. In contrast, TERC had only two connections and had no connection across networks. Although UCA1 had only a few connections, fewer connections over networks 1 and 2 were observed. TERC, telomerase RNA component; UCA1, urothelial cancer associated 1.

>10-fold in HCT116/p and HCT116/5fu cells by treatment with 5FU, whereas the change of H19 in other cultured cells were <2-fold (Fig. 4C). The expression level of UCA1 was changed >3-fold in DLD-1/p and SW480/p cells by treatment with 5FU, whereas the change was less apparent in other cultured cells.

The association of between the 5FU-treated/untreated ratios of H19 and UCA1 expression and cell lines is presented in Fig. 4D. The ratios were low in the 5FU-resistant cell lines DLD-1/5fu and SW480/5fu. Conversely, the ratio of H19 or UCA1 expression was increased in the three parental cultured cells and HCT116/5fu cells, which were susceptible to 5FU.

Doubling time and cell cycle phases of cultured cells. With 5FU treatment, the doubling time was significantly increased in the parental cultured cells of HCT116/p, DLD-1/p and SW480/p

compared with their untreated counterparts (Table II). The doubling time of untreated HCT116/5fu cells was comparable with untreated parental cells, and the time was significantly increased following treatment with 5FU. The doubling time of untreated DLD-1/5fu and SW480/5fu was significantly increased than that of their parental cells. Treatment with 5FU did not affect the doubling time.

The cell cycle phase was determined with a Muse Cell Analyzer (Luminex Japan Corporation Ltd.). The cell cycle phases were expressed as percentage of the number of cells at the phases in total number of cells analyzed (Fig. S1). Following treatment with 5FU, the number of cells in S phase appeared to be increased for SW480/p and SW480/5fu (Fig. 5A). The change in cell cycle phases was subtle in HCT116/p, DLD-1/p, HCT116/5fu and DLD-1/5fu cells.
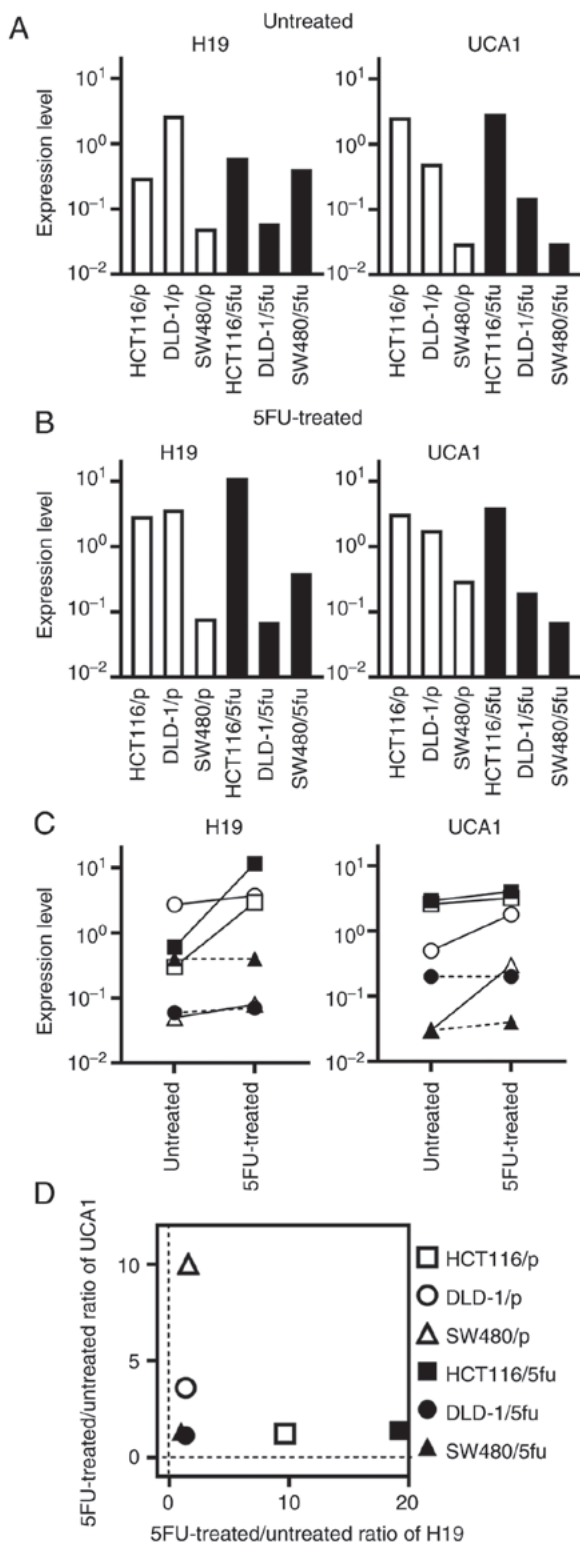
Figure 4. Expression levels of H19 and UCA1 in cultured cells. (A) Expression levels of H19 and UCA1 in untreated cultured cells. (B) Expression levels of H19 and UCA1 in 5FU-treated cultured cells. (C) Changes in H19 and UCA1 expression in the cultured cells in response to 5FU. (D) Association between 5FU-treated/untreated ratios of H19 and UCA1 expression in cultured cells. 5FU, 5-fluorouracil; UCA1, urothelial cancer associated 1.

Rb and p27kip1 expression in cultured cells. The expression of Rb and p27kip1, which are involved in the cell cycle, and are target molecules of H19 and UCA1. The expression of Rb was notably decreased in DLD-1/p and SW480/p cells in response

Table II. Effect of 5FU on doubling time of cultured cells.

Cell line	Doubling time (h)		Fold change
	Untreated	5FU-treated	
HCT116/p	20±5	200±46[b]	10.0
DLD-1/p	16±2	155±27[b]	9.6
SW480/p	22±2	67±4[b]	3.0
HCT116/5fu	24±1	301±17[b]	12.5
DLD-1/5fu	24±4[a]	25±4	1.0
SW480/5fu	68±3[a]	73±13	1.1

[a]$P<0.05$ vs. parental cells; [b]$P<0.05$ vs. untreated cells; 5FU, 5-fluorouracil.

to 5FU; Rb expression was also reduced in HCT116/p and HCT116/5fu cells (Fig. 5B). In contrast, the expression levels of Rb appeared to be unchanged in DLD-1/5fu and SW480/5fu cells treated with 5FU. The expression of p27kip1 was downregulated in three parental cells and HCT116/5fu cells, which were susceptible to 5FU, whereas the expression was notably unchanged in DLD-1/5fu and SW480/5fu cells, which were resistant to 5FU.

Clinicopathological features of rectal cancer cases. The clinicopathological features of the 31 patients with rectal cancer included in this study were summarized in Table III. Among the 31 cases, the numbers of cases with Grade 0, Grade 1a, Grade 1b and Grade 2 were 0, 15, 8 and 8, respectively. Cases with therapeutic responses of Grade 1b and 2 were designated as 'favorable response' cases, and cases with therapeutic responses of Grade 0 and 1a were designated as 'poor response' cases. There was no significant difference in the clinicopathological features between the two groups.

Expression of H19 and UCA1 in rectal cancer. The expression levels of H19 and UCA1 in the biopsy specimens of the cases with poor response were higher than those with favorable responses (Fig. 6A). In the resected tissue specimens, H19 and UCA1 expression levels appeared to be comparable between the cases with poor and favorable responses (Fig. 6B).

The expression level of H19 was altered >2-fold in 12 of 16 cases (75%) with favorable response and in 5 of 15 cases (33%) with poor response (Fig. 6C). Alterations in the expression levels of UCA1 markedly varied in cases with favorable response as well as those with poor response.

The association between the resection/biopsy ratios of H19 and UCA1 was shown in Fig. 6D. Cases with poor response tended to be located in the area in which both ratios were low. By hierarchical clustering, three major clusters were separated. Cluster 1 included the cases in which both ratios were low. Cases that showed a higher ratio of H19 were separated into cluster 2, and the cases that showed a higher ratio of UCA1 were separated into cluster 3. In cluster 1, 11 of the 17 cases exhibited a poor response (65%), whereas in clusters 2 and 3, 4 of the 14 cases were linked to a poor response (29%) (P=0.045, Table III).
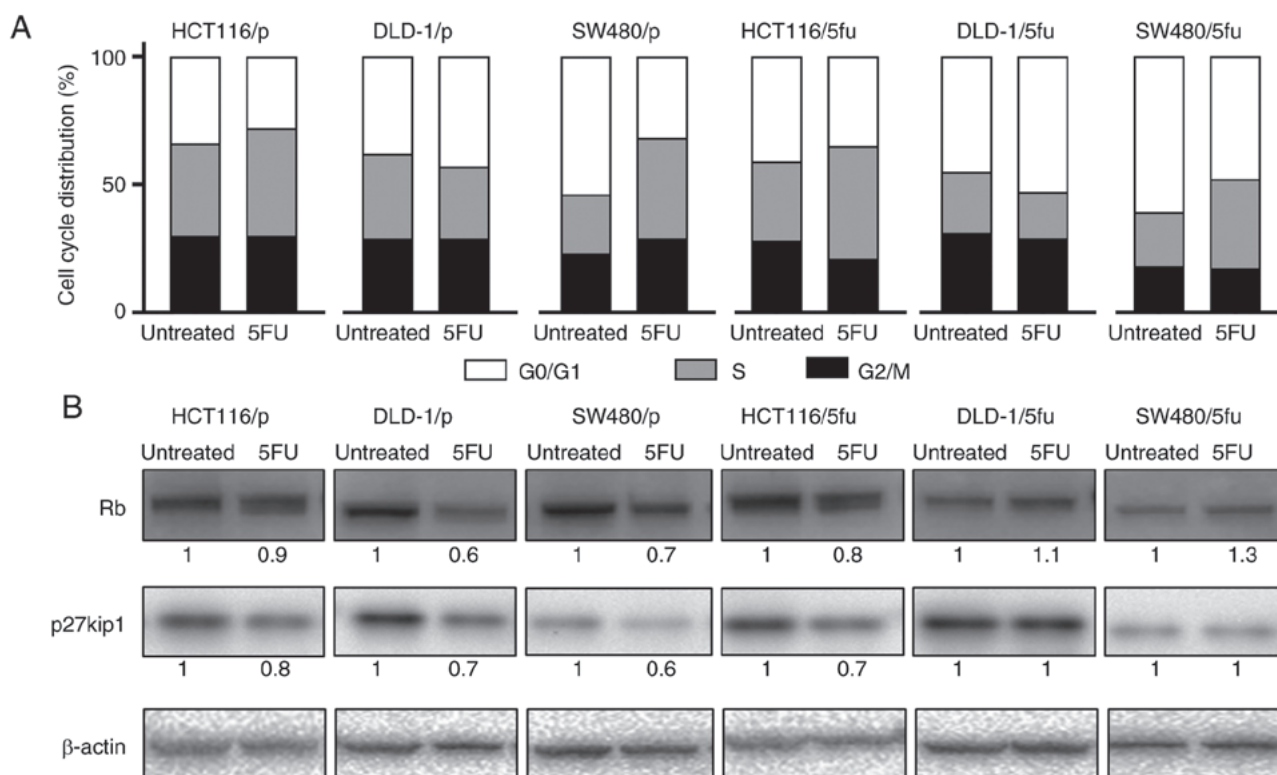
Figure 5. Cell cycle phase analysis and western blot analysis of the expression of Rb and p27kip1 in 5FU-treated and untreated cultured cells. (A) The cell cycle phases in 5FU-treated and untreated cells. (B) The expression levels of Rb and p27kip1. The number represents the fold-change of the expression levels in 5FU-treated cells, compared with that in untreated cells. 5FU, 5-fluorouracil; Rb, retinoblastoma.

Discussion

Several studies have examined the association between lncRNAs and the susceptibility to 5FU in colorectal cancer (15,16,18-22). However, a specific lncRNA that may be reliable for the prediction of susceptibility to 5FU has not yet been identified. In the current study, we examined the expression levels of 84 lncRNAs in 5FU-susceptible and 5FU-resistant cultured cells using PCR array analysis. Bioinformatic analysis revealed H19 and UCA1 as candidate molecules that may be involved in the susceptibility to 5FU. A previous study reported differentially expressed lncRNAs in 5FU-resistant cultured colorectal carcinoma cells compared with 5FU-susceptible cultured cells (15). Although the cultured cells were different from the cells used in the present study, H19 and UCA1 were included as differentially expressed lncRNAs. In addition, recent studies reported the association of H19 and UCA1 with the susceptibility to 5FU (16,22).

In the present study, the expression levels of H19 and UCA1 varied largely in 5FU-treated and untreated cultured cells, and their expression levels were notably higher in 5FU-susceptible cells compared with 5FU-resistant cells. In contrast with our findings, high H19 levels in 5FU-resistant cells were detected (22). This discrepancy may be due to the difference in the cultured cells used in this study and our investigation. Notably, the 5FU-treated/untreated ratios appeared to be more closely associated with the susceptibility compared with the expression levels in untreated or 5FU-treated cells. The 5FU-treated/untreated ratios of H19 and UCA1 were low in 5FU-resistant cells, whereas the ratios were high in

5FU-susceptible cells. To the best of our knowledge, we are the first to report on the association between alterations in the expression levels of lncRNAs in 5FU-treated cultured cells with the susceptibility to 5FU.

In human cases of rectal cancer treated with NAC, there was no significant difference in the expression levels of H19 and UCA1 between cases with favorable and poor responses both in biopsy tissue and resected tissue. The cases with low resection/biopsy ratios of H19 and UCA1 tended to exhibit a poor response to NAC. These results appear to be consistent with that in cultured cells. We proposed that changes in H19 or UCA1 expression in response to 5FU, rather than the stable expression before or after exposure to 5FU, may be useful for the prediction of the susceptibility to 5FU-based NAC in human rectal cancer. However, a small patient cohort was examined in the present study; alterations in the expression levels of H19 and UCA1 in response to NAC should to be verified in a larger number of patients.

In 5FU-susceptible cultured cells, cell proliferation was inhibited by 5FU. The doubling time was increased due to treatment with 5FU in 5FU-susceptible cells. In 5FU-resistant cells, the doubling time was significantly longer than their parental cells, and the doubling time was markedly unchanged by treatment with 5FU. The cell cycle phases appeared to be comparable in cultured cells. The inhibition in the proliferation in 5FU-susceptible cells due to impaired cell cycle progression was observed following inhibition of thymidylate synthase and DNA synthesis (10,29). The expression levels of Rb and p27kip1 were decreased in 5FU-susceptible cells treated with 5FU, whereas their expressions were unchanged

Table III. Clinicopathological features of rectal cancer cases.

Characteristic	Favorable (n=16)	Poor (n=15)	P-value
Age, years	64 (39-72)	66 (46-76)	0.178
Sex			0.354
Male	13	10	
Female	3	5	
Clinical depth of tumor invasion[a]			0.325
cT3	15	15	
cT4	1	0	
Pathological depth of tumor invasion[a]			0.573
pTis	1	0	
pT1	0	0	
pT2	6	5	
pT3	9	10	
pT4	0	0	
Tumor histology			0.376
Well differentiated	6	8	
Moderately differentiated	10	7	
Histological therapeutic response			<0.001
Grade 0	0	0	
Grade 1a	0	15	
Grade 1b	8	0	
Grade 2	8	0	
Hierarchical analysis			0.045
Cluster 1	6	11	
Cluster 2+3	10	4	

[a]Depth of invasion is defined as T-factor in the Japanese Classification of Colorectal Carcinoma (28).
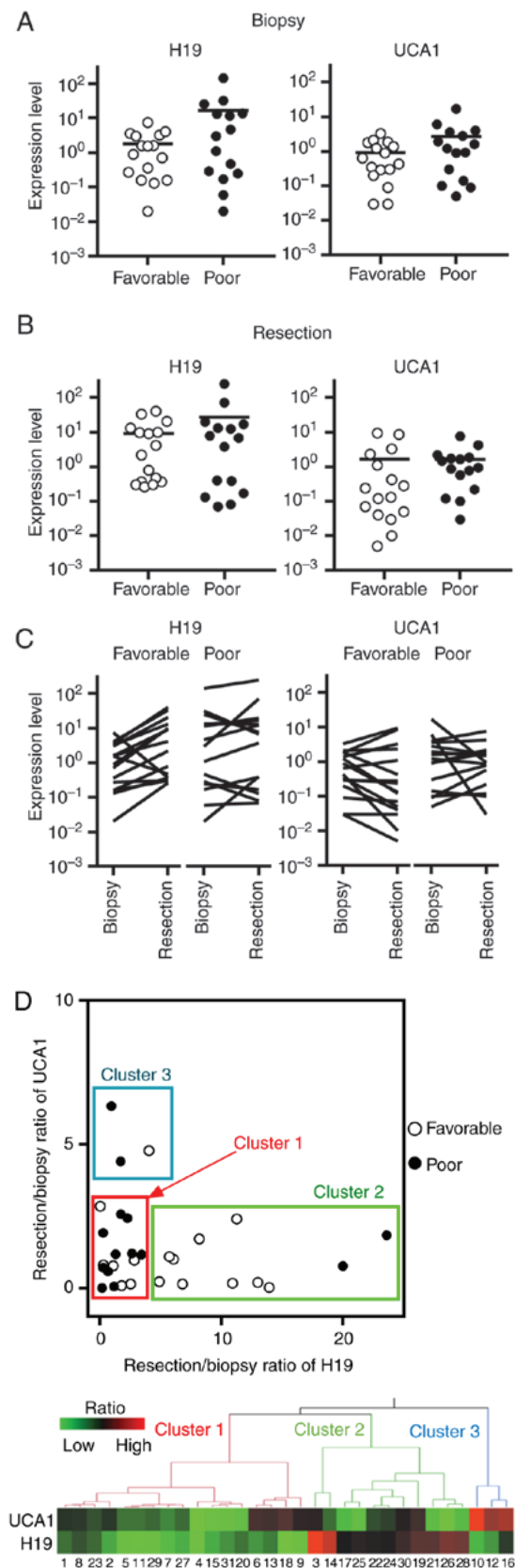

Figure 6. Expression levels of H19 and UCA1 in 31 cases of rectal cancer treated with neoadjuvant chemotherapy. (A) Expression levels of H19 and UCA1 in biopsy specimens. (B) Expression levels of H19 and UCA1 in resected tissue specimens. (C) Alterations in expression levels in neoadjuvant chemotherapy-treated rectal cancers. (D) Association between resection/biopsy ratios of H19 and UCA1 expression in rectal cancers. The numbers below the hierarchical map indicate the case number. Hierarchical clustering indicated that the cases were separated into three groups. UCA1, urothelial cancer associated 1.

in 5FU-resistant cells. Previous studies showed that elevated expression of H19 and UCA1 suppressed the expressions of Rb and p27kip1, respectively (30,31). Thus, we speculate that the reduction in Rb and p27kip1 levels may be, in part, caused by the upregulation of H19 and UCA1. These alterations in expression may represent the response to the impaired cell cycle caused by 5FU. Another study also showed that the expression levels of lncRNAs were upregulated in response to treatment with various chemical agents in human pluripotent stem cells (32). Elevations of H19 and UCA1 expression may also be linked to the cellular stress response. The precise mechanism underlying the elevation of H19 and UCA1 in response to 5FU requires further investigation.

　　Based on the results of present study, it is not plausible to predict the response to NAC by the quantitation of H19 and UCA1 only in biopsy specimens taken before NAC in human cases of rectal cancer. *In vitro* analyses demonstrated that the change of expression levels of H19 and UCA1 was noted in

5FU-susceptible cultured cells even at 48 h after the initiation of treatment with 5FU. It is thus expected that the response is predicted by the quantitation of H19 and UCA1 in paired biopsy specimens taken before NAC and early after the initiation of NAC. The early prediction of response to 5FU-based NAC may aid the identification of cases with poor responses, and facilitate the administration of intensive treatment or radiotherapy.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YY, TS, and RW were involved in the conception and design of the study. YY, KI, and MK performed the histological and biochemical experiments. MK, TY, and HY acquired the clinical data. YY prepared the figures. YY, TS, and RW drafted the manuscript. HY and ZN reviewed and edited the manuscript. ZN supervised the experiments and writing the manuscript and gave the final approval of manuscript to be published. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was conducted according to the Declaration of Helsinki and the Japanese Society of Pathology. The study was approved by the Ethics Committee of Nippon Medical School Hospital (approval no. 29-03-909). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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