Decreased expression of ubiquitin-specific protease 10 correlates unfavorable prognosis in colorectal cancer

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Abstract

Background: Ubiquitin-specific proteases (USPs) play an important role in fundamental cellular processes. Among these, USP10 is known for its association with tumor development and progression of multiple cancers. Here, we found a potential link between USP10 and p14ARF in colorectal cancer. Methods: USP10 and p14ARF protein expression was assessed via immunohistochemistry (IHC) on a tissue microarray from 280 colorectal cancer cases. IHC scores were evaluated by digital image analysis and compared with patients' outcomes. In addition, we examined DNA hypermethylation in colorectal cancer cell lines and tissues, which were matched with adjacent normal colon samples. Results: USP10 expression (USP10 loss) was lost in 18.6% of samples (52/280 cases), which was linked to lymphovascular invasion (p =0.019) and distant metastases (p <0.001). Similarly, loss of p14ARF expression (p14ARF loss) was associated with more advanced tumors. USP10 expression correlated positively with p14ARF expression (r =0.617, p <0.001). USP10 loss , p14ARF loss , and loss of both USP10 and p14ARF (USP10 loss /p14ARF loss) were significantly associated with shorter disease-free survival and overall survival in comparison to USP10 intact , p14ARF intact , and USP10 intact /p14ARF intact , respectively. Multivariate analysis revealed that USP10 loss (Hazard ratio=2.07, p =0.046) and USP10 loss /p14ARF loss (Hazard ratio=1.41, p =0.010) are independent prognostic factors for disease-free survival in colorectal cancer patients. Furthermore, aberrant hypermethylation of the USP10 promoter region was found in colorectal cancer cell lines and tissues. Conclusions: The present results suggest that USP10 loss is a potential prognostic marker for colorectal cancer.

Background

Colorectal cancer is the fourth cause of cancer-related death worldwide [1]. The 5-year survival rate of metastatic colorectal cancer remains at a disappointing 10–20% despite significant improvements in metastatic colorectal cancer management via targeted therapies in combination with chemotherapy [2, 3]. In the past two decades, many molecular markers and clinicopathological factors of colorectal cancer have been suggested as predictors of the postoperative prognosis of colorectal cancer patients to help clinicians determine appropriate treatments [4]. However, personalized therapy era requires that the heterogeneous group of colorectal cancer patients be classified in more detail. Thus, further research into new molecular biomarkers and clinical prognostic factors is essential to developing novel therapeutic strategies.

Ubiquitin-specific proteases (USPs) degrade substrates and modulate transcriptional regulation, which plays a key role in tumorigenesis [5, 6]. Among the USPs, ubiquitin-specific proteases 10 (USP 10) is a primary cytoplasmic deubiquitinase that acts as a tumor suppressor gene or oncogene as it regulates different substrates, including p53 [7], AMP-activated protein kinase (AMPK) [8], fms-like tyrosine kinase 3 (FLT3) [9], and phosphatase and tensin homolog (PTEN) [10]. Furthermore, the elevation or loss of USP10 expression has already been linked to poor prognosis in various cancers, including stomach [11], breast [12], prostate [13], glioblastoma [14], hepatocellular [15], and non-small cell lung cancer [16]. Ko et al. recently reported that USP10 plays a crucial role in oncogene-induced senescence by regulating the stability of p14ARF and that dual loss of USP10 and p14ARF is a factor indicating an negative outcome in patients with non-small cell lung cancer [17]. We have also recently observed that the dual loss of USP10 and p14ARF expression predicts a worse prognosis for small intestinal adenocarcinoma patients [18]. The evaluation of oncogene-induced senescence-related proteins in clinical samples could lead to the development of novel therapeutic strategies that could improve patient outcomes. Currently, the clinical and prognostic value of USP10 and p14ARF expression in patients with colorectal cancer is largely unknown. In this study, we examined the clinicopathological features of USP10 and p14ARF in colorectal cancer patients. Moreover, we examined the hypermethylation of the USP10 promoter region, in colorectal cancer cell lines and primary tumors, for its down-regulating epigenetic mechanism in protein expression.

Methods

Patients and tumor specimens

We initially included 300 colorectal cancer specimens resected at Kangbuk Samsung Hospital between January 2006 and December 2008. Twenty cases with distant metastases at operation were excluded, for a final population of 280 patients. Clinical information, including age, gender, survival time, and survival status, were obtained from medical records. All tumor tissues were examined by a pathologist (KEK), and collected after obtaining informed consent from patients, according to the guidelines of the regional Institutional Review Board of Kangbuk Samsung Hospital (approval no. 2019-07-022; Seoul, South Korea).

Pathologic diagnosis and generation of tissue microarray

The resection specimens were fixed in neutrally buffered 10% formalin solution and grossly examined by pathologists. Representative sections of tumor were embedded in paraffin blocks and the glass slides were used for pathologic diagnosis. During the microscopic diagnosis, tumor differentiation, pT stage, pN stage, lymphovascular invasion, and peritumoral dysplasia were recorded. After microscopic review, the tumor area was marked on each glass slide. Representative tumor cores 2 mm in diameter were obtained from the 280 colorectal cancer tissues, and 5 tissue microarray (TMA) blocks were generated.

Immunohistochemical staining

Immunohistochemistry was performed using the Dako EnVision^{+} Dual Link System-HRP (Dako, Carpinteria, CA) as previously described [18]. In brief, TMA sections were deparaffinized and rehydrated. Heat-mediated antigen retrieval was subsequently performed using pH 6 (USP10) or 9 (p14ARF) citrate antigen retrieval buffer (Dako). The slides were incubated with anti-USP10 rabbit polyclonal antibodies (Abcam, Cambridge, MA; cat. ab72486) at a 1:5000 dilution for 1 h at room temperature and anti-p14ARF mouse monoclonal antibodies (Cell Signaling Technology, Danvers, MA; clone no. 4C6/4) at a 1:500 dilution for 1 h at room temperature. The antigen-antibody reaction visualized with DAB^{+} (3, 3’-diaminobenzidine; Dako). The negative control omitted the primary antibody and rabbit immunoglobulin.

The stained TMA slides were made into images using a NanoZoomer 2.0 HT scanner (Hamamatsu Photonics K.K., Hamamatsu, Japan). The immunohistochemical staining images were analyzed using Visiopharm software v6.9.1 (Visiopharm, Hørsholm, Denmark) to determine the percentage of positive cells.

DNA methylation analysis

For the methylation analyses, we used genomic DNA from colon cancer cell lines (HCT116, RKO, HT29, SW480, DLD1, COLO 320, SW48, Lovo, Caco-2, and SW620) from a previous study [19]. Other genomic DNA was isolated from normal colon tissues (n = 13) and colorectal cancer primary tissues (n = 13) (Supplementary Table S1) using a standard phenol-chloroform method. A methylation-specific polymerase chain reaction was used for to analyze methylation, as previously described [18]. Briefly, the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA) was used to modify 2ug of DNA. Polymerase chain reaction (PCR) amplification of the bisulfite-converted DNA was performed with previously reported primer pairs specific for either the unmethylated or methylated sequence [18]. PCR amplicons (262-bp) were separated on 2% agarose gels and visualized by ethidium bromide staining. For the bisulfite sequence analysis, PCR amplicons were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated and purified from positive clones (10–15 from each sample), using a NucleoSpin Plasmid Isolation Kit (Macherey-Nagel, Düren, Germany), and subsequently sequenced using the M13F primer.

Statistical analysis

Data were analyzed using IBM SPSS 23.0 statistical software (IBM Corp, Armonk, IL). The expression values for USP10 and p14ARF were dichotomized (intact vs. loss) using the cut-off values that showed the most discriminative power (immunohistochemistry score of 68.2 for USP10 and 80.0 for p14ARF). Crosstabs, Pearson's chi-square test, and Fisher's exact test were employed. For the survival analysis, a Kaplan Meier analysis was used. Cox regression analyses were utilized to calculate the hazard ratios (HRs) of risk factors in univariate and multivariate conditions.

A modified random survival forest (RFS) analysis was conducted as described previously [20, 21]. Briefly, we built a clinical RFS model using lymph node (LN) metastasis and pT stage, and then combined it with molecular-level features (USP10 and p14ARF). The RSF models were built using R software ver. 3.1.3 (R Foundation, Vienna, Austria; http://www.R-project.org). The concordance-index (C-index) was calculated using the "surv-comp" R package. A C-index of 0.5 is as good as a random guess. $p < 0.05$ was considered significant.

Results

Clinicopathological characteristics

The patients were 173 males (61.8%) and 107 females (38.2%) and had a mean age of 62.4 years (range: 33 to 90). The most common tumor location was the rectum (145 cases, 51.8%), followed by the left colon (71 cases, 25.3%) and right colon (64 cases, 22.9%). The patients were followed up for a mean of 55.4 months (range: 1.0-120.6 months) postoperatively. Distant metastasis and local recurrence occurred in 52 (18.6%) and 9 (3.2%) patients, respectively, and 42 (15.0%) patients died from colorectal cancer.

USP10 and p14ARF protein expression

In the immunohistochemical staining, the normal colonic crypt showed cytoplasmic expression of USP10 and cytoplasmic and nuclear expression of p14ARF (Fig. 1). Colorectal cancer tissue revealed the same expression pattern as normal colonic tissue. Among the colorectal cancer cases, 52 (18.6%) showed a loss of USP10, 80 (28.6%) showed a loss of p14ARF, and 35 (12.5%) showed a loss of both proteins. A significant correlation was found between USP10 and p14ARF expression in cancer tissues ($r = 0.617$, $p < 0.001$) (Fig. 2A). Furthermore, subgroup analysis revealed a stronger correlation between USP10 and p14ARF expression in patients without LN metastasis ($r = 0.680$, $p < 0.001$) (Figs. 2B and C).

The loss of USP10 protein expression ($USP10^{loss}$) significantly correlated with lymphovascular invasion ($p = 0.019$), distant metastasis ($p < 0.001$), DFS ($p < 0.001$), and OS ($p = 0.004$) (Table 1). The clinicopathological features of the loss of p14ARF protein ($p14ARF^{loss}$) cases were significantly associated with aggressive tumor behavior, including advanced pT stage ($p = 0.001$), advanced pN stage ($p = 0.044$) and more distant metastases ($p = 0.024$). Furthermore, the $p14ARF^{loss}$ correlated with worse DFS ($p = 0.018$) and OS ($p < 0.001$). $USP10^{loss}/p14ARF^{loss}$ cases also exhibited more distant metastases ($p < 0.001$) and shorter DFS ($p < 0.001$) and OS ($p < 0.001$), compared to $USP10^{intact}/p14ARF^{intact}$ cases (Table 1).

Table 1 The association between clinicopathological features and USP10 and p14ARF protein expression

	USP10			p14ARF			Dual of USP10 and p14ARF		
	Intact	Loss	*p*-value	Intact	Loss	*p*-value	Intact	Loss	*p*-value
	No (%)	No (%)		No (%)	No (%)		No (%)	No (%)	
Age			0.204			0.891			0.309
< 60 years	81 (35.5)	24 (46.2)		76 (38.0)	29 (36.2)		69 (37.7)	17 (48.6)	
≥ 60 years	147 (64.5)	28 (53.8)		124 (62.0)	51 (63.8)		114 (62.3)	18 (51.4)	
Gender			0.453			0.167			0.353
Male	138 (60.5)	35 (67.3)		118 (59.0)	55 (68.8)		107 (58.5)	24 (68.6)	
Female	90 (39.5)	17 (32.7)		82 (41.0)	25 (31.2)		76 (41.5)	11 (31.4)	
Tumor size			0.951			0.145			0.536
< 5 Cm	109 (47.8)	24 (46.2)		101 (50.5)	32 (40.0)		92 (50.3)	15 (42.9)	
≥ 5 Cm	119 (52.2)	28 (53.8)		99 (49.5)	48 (60.0)		91 (49.7)	20 (57.1)	
Tumor location			0.321			0.633			0.326
Right colon	48 (21.0)	16 (30.8)		48 (24.0)	16 (20.0)		43 (23.5)	11 (31.4)	
Left colon	59 (25.9)	12 (23.1)		48 (24.0)	23 (28.8)		47 (25.7)	11 (31.4)	
Rectum	121 (53.1)	24 (46.1)		104 (52.0)	41 (51.2)		93 (50.8)	31 (37.2)	
Tumor differentiation			0.580			1.000			0.926
Well + moderate	217 (95.2)	51 (98.1)		191 (95.5)	77 (96.2)		174 (95.1)	34 (97.1)	
Poor	11 (4.8)	1 (1.9)		9 (4.5)	3 (3.8)		9 (4.9)	1 (2.9)	
pT			0.093			0.001*			0.066
pT_1+ pT_2	53 (23.2)	6 (11.5)		53 (26.5)	6 (7.5)		51 (27.9)	4 (11.4)	
pT_3+ pT_4	175 (76.8)	46 (88.5)		147 (73.5)	74 (92.5)		132 (72.1)	31 (88.6)	
pN[a]			0.121			0.044*			0.070
pN_0	124 (54.4)	21 (41.2)		112 (56.0)	33 (41.8)		104 (56.8)	13 (38.2)	

pN_1+ pN_2	104 (45.6)	30 (58.8)		88 (44.0)	46 (58.2)		79 (43.2)	21 (61.8)	
Lymphovascular invasion[b]			0.019*			0.610			0.121
Absent	112 (58.9)	13 (36.1)		94 (56.5)	31 (51.7)		90 (58.8)	9 (39.1)	
Present	78 (41.1)	23 (63.9)		72 (43.4)	29 (48.3)		63 (41.2)	14 (60.9)	
Peritumoral dysplasia[c]			0.171			0.084			0.135
Absent	150 (87.2)	33 (63.5)		132 (86.3)	51 (96.2)		120 (85.7)	21 (100.0)	
Present	22 (12.8)	1 (2.9)		21 (13.7)	2 (3.8)		20 (14.3)	0 (0.0)	
Local recurrence			0.470			0.957			1.000
Absent	222 (97.4)	49 (94.2)		193 (96.5)	78 (97.5)		178 (97.3)	34 (97.1)	
Present	6 (2.6)	3 (5.8)		7 (3.5)	2 (2.5)		5 (2.7)	1 (2.9)	
Distant metastasis[d]			<0.001*			0.024*			<0.001*
Absent	195 (85.5)	33 (63.5)		170 (85.0)	58 (72.5)		158 (86.3)	21 (60.0)	
Present	33 (14.5)	19 (36.5)		30 (15.0)	22 (27.5)		25 (13.7)	14 (40.0)	
Disease-free survival status			<0.001*			0.018*			<0.001*
Alive	194 (85.1)	32 (61.5)		169 (84.5)	57 (71.2)		158 (86.3)	21 (60.0)	
Expire	34 (14.9)	20 (38.5)		31 (15.5)	23 (28.8)		25 (13.7)	14 (40.0)	
Overall survival status			0.004*			<0.001*			
Alive	201 (88.2)	37 (71.2)		180 (90.0)	58 (72.5)		166 (90.7)	23 (65.7)	<0.001*
Expire	27 (11.8)	15 (28.8)		20 (10.0)	22 (27.5)		17 (9.3)	12 (34.3)	

[a]Calculated only 279 cases with available information of lymph node metastasis

[b]Calculated only 226 cases with available information of lymphovascular invasion

[c]Calculated only 206 cases with available information of peritumoral dysplasia

[d]Calculated only 252 cases with available information of distant metastasis

*Statistically significant ($p < 0.05$)

Hypermethylation of the promoter region of USP10

It is well known that promoter hypermethylation regulates p14ARF gene silencing [22–24]. Interestingly, the methylation level of the USP10 promoter region has not been actively investigated in human cancers. Accordingly, we examined USP10 promoter methylation levels in colon cancer cell lines, normal tissues, and primary colorectal cancer tissues using methylation-specific PCR and bisulfite sequencing. MSP analysis showed that USP10 was frequently hypermethylated in 10 colon cancer cell lines and primary colorectal cancer tumor tissues but not in normal colon tissues (Fig. 3A). These data suggest that promoter hypermethylation of USP10 is cancer-specific. We also confirmed the methylation status of USP10 with bisulfite sequencing analysis. The USP10 promoter region exhibited dense hypermethylation in both colon cancer cell lines and colorectal cancer tissues (77–83% of all CpG sites), but normal tissues exhibited relatively low methylation levels (30–35% of total CpG sites) (Fig. 3B).

Prognostic value of USP10 and p14ARF protein expression

Estimated five-year DFS and OS rates were 82.1% and 85.0%, respectively. The patients who lost USP10 expression (mean survival months: 69.13 ± 6.52) had a lower DFS rate than those with intact USP10 expression (mean survival months: 102.42 ± 2.86) ($p < 0.001$) (Fig. 4A). Losing p14ARF expression (mean survival months: 77.58 ± 5.04) produced a similar prognostic effect on DFS, compared to $p14ARF^{intact}$ patients ($p = 0.007$) (Fig. 4B). Notably, $USP10^{loss}/p14ARF^{loss}$ (mean survival months: 67.37 ± 8.01) correlated with a significantly poor DFS rate compared to $USP10^{intact}/p14ARF^{intact}$ cases (mean survival months: 103.79 ± 3.10) ($p < 0.001$) (Fig. 4C). OS analysis produced similar results. The patients who lost USP10 or p14ARF had a shorter mean OS time than those with intact expression of each protein (81.68 vs. 107.55 months for USP10, $p = 0.003$ and 82.19 vs. 109.60 months for p14ARF, $p < 0.001$) (Figs. 4D and E), and the patients who lost both the USP10 and p14ARF proteins had a significant decrease in OS time compared with the intact expression group (76.69 vs. 110.37 months of mean OS, $p < 0.001$) (Fig. 4F).

In subgroup analysis according to LN metastasis at the time of surgery, $USP10^{loss}/p14ARF^{loss}$ was a significant factor indicating a worse prognosis for both DFS ($p = 0.025$) and OS ($p < 0.001$) times in the group without LN metastasis (Supplementary Figs. S1A and C). Notably, no one in the subgroup without LN metastasis and with dual intact protein expression died from colorectal disease (Supplementary Fig. S1C). However, in the subgroup that had LN metastasis at the time of surgery, $USP10^{loss}/p14ARF^{loss}$ was only significantly correlated with DFS ($p = 0.041$), not OS (Supplementary Figs. S1B and D).

The relationship between clinicopathological variables and survival are summarized in Table 2. The HRs for the mortality rate based on $USP10^{loss}$ ($p < 0.001$) and $p14ARF^{loss}$ ($p = 0.009$) expression were 2.88 and 2.06, respectively. Furthermore, $USP10^{loss}/p14ARF^{loss}$ was also significantly associated with worse patient survival [$p < 0.001$; HR, 3.5; 95% confidence interval (CI) 1.82 to 6.75]. The clinicopathological factors associated with shorter patient survival were lymphovascular invasion ($p < 0.001$), pT classification ($p = 0.005$), and LN metastasis ($p < 0.001$) (Table 2). Multivariate analysis revealed that $USP10^{loss}$ ($p = 0.046$; HR, 2.07; CI 1.01 to 4.24) and $USP10^{loss}/p14ARF^{loss}$ ($p = 0.010$; HR, 1.41; CI 1.09 to 1.82) were independent factors for a poor DFS prognosis (Table 3). Notably, $USP10^{loss}/p14ARF^{loss}$ was also a strong negative prognostic factor for OS ($p = 0.011$; HR, 2.81; CI 1.09 to 7.27) (Supplementary Table S2). LN metastasis

was significantly associated with unfavorable DFS and OS, while lymphovascular invasion was significantly correlated with worse DFS (Table 3 and Supplementary Table S2).

Table 2
Cox proportional univariate analysis of prognostic variables affecting patient disease-free survival with colorectal cancer

Variable	Mean survival (95% CI)	Hazard ratio (95% CI)	p value
Age			0.784
< 60 years	97.12 (88.60-105.64)	1-	
≥ 60 years	92.46 (85.93–98.98)	0.93 (0.54–1.59)	
Sex			0.895
Male	91.19 (84.71–97.66)	1-	
Female	98.84 (90.12-107.57)	0.90 (0.51–1.57)	
Tumor size			0.170
< 5 Cm	91.01 (84.91–97.10)	1-	
≥ 5 Cm	93.53 (85.39-101.67)	1.458 (0.85–2.51)	
Tumor location			0.352
Right colon + Left colon	89.42 (81.81–97.03)	1-	
Rectum	100.34 (93.20-107.48)	0.78 (0.45–1.32)	
Tumor differentiation			0.119
Well + Moderate	98.44 (92.95-103.92)	1-	
Poor	61.96 (37.11–86.80)	2.25 (0.81–6.24)	
Lymphovascular invasion			< 0.001*
Absent	89.46 (84.83–94.09)	1-	
Present	80.75 (70.91–90.58)	3.33 (1.73–6.42)	
Peritumoral dysplasia			0.113
Absent	79.64 (74.32–84.97)	1-	
Present	91.59 (82.31-100.87)	0.20 (0.28–1.46)	
pT classification			0.005*
$pT_1 + pT_2$	102.22 (97.39-107.04)	1-	
$pT_3 + pT_4$	92.85 (86.28–99.42)	5.28 (1.65–16.93)	
pN classification [b]			< 0.001*
pN_0	102.36 (96.96-107.77)	1-	
$pN_1 + pN_2$	86.43 (77.62–95.24)	3.47 (1.88–6.39)	

*Statistically significant (p < 0.05)

Variable	Mean survival (95% CI)	Hazard ratio (95% CI)	p value
USP10 expression			< 0.001*
Intact	102.42 (96.83-108.02)	1-	
Loss	69.13 (56.35–81.90)	2.88 (1.66–5.01)	
p14ARF expression			0.009*
Intact	101.83 (95.81-107.85)	1-	
Loss	77.58 (67.70-87.45)	2.06 (1.20–3.53)	
Dual of USP10 and p14ARF expression			< 0.001*
Intact	103.79 (97.71-109.87)	1-	
Loss	67.37 (51.68–83.06)	3.50 (1.82–6.75)	
*Statistically significant (p < 0.05)			

Table 3
Multivariate analysis for prediction factors for disease-free survival

Variables	USP10 or p14ARF		Dual of USP10 and p14ARF	
	Hazard ratio [95% CI]	p value	Hazard ratio [95% CI]	p value
pT stage (≥ pT_3)	1.46 [0.90–2.38]	0.124	1.46 [0.90–2.37]	0.126
pN stage (≥ pN_1)	2.70 [1.28–5.67]	0.009*	2.69 [1.28–5.67]	0.009*
Lymphovascular invasion	2.22 [1.12–4.40]	0.023*	2.24 [1.14–4.41]	0.019*
$USP10^{loss}$	2.07 [1.01–4.24]	0.046*	NA	
$p14ARF^{loss}$	1.32 [0.66–2.65]	0.439	NA	
$USP10^{loss}/p14ARF^{loss}$	NA		1.41 [1.09–1.82]	0.010*
CI, confidence interval; NA, not applicable				
*Statistically significant (p < 0.05)				

Prognostic value of combined clinical and molecular model

The C-index for the prediction of recurrence and death was compared between the clinical model and the combined clinical and molecular model. The combined clinical and USP10/p14ARF-RFS model offered significantly better recurrence prediction (median C-index, 0.740) than the clinical variable-only-RFS model (median C-index, 0.722; p = 0.001; Fig. 5A). Furthermore, the combined OS model displayed significantly improved predictive power (median C-index, 0.787) in comparison to the clinical-only OS model (median C-index, 0.735; p < 0.001; Fig. 5B).

Discussion

We found that loss of USP10 and p14ARF protein expression correlates with worse prognosis in colorectal cancer patients, and we confirmed a positive correlation between the molecules. Interestingly, $USP10^{loss}$ and $USP10^{loss}/p14ARF^{loss}$ were significant markers of poor prognosis in patients without LN metastasis at the time of surgery, suggesting that $USP10^{loss}/p14ARF^{loss}$ could predict disease progression in node-negative colorectal cancer. In addition, the methylation analysis revealed that the promoter region for USP10 is frequently hypermethylated in colorectal cancer cell lines and colorectal cancer primary tumors but not in normal colon tissues.

USP10 deubiquitinates p53 via MDM2, which regulates cellular p53 via reverse translocation and degradation of the protein [7]. Also involved in the deubiquitination and stabilization of PTEN in lung cancer cells, USP10 has been reported to be a tumor suppressor [10]. In addition, is an important mediator in the c-Myc-USP10-p14ARF axis because it deubiquitinates and stabilizes p14ARF [17]. Lu et al. also suggested that USP10 behaves as a tumor suppressor in hepatocellular carcinoma [15]. They demonstrated that USP10 inhibits the mTOR signaling pathway to deter cell growth in hepatocellular carcinoma. Further evidence that corroborates our results indicates that USP10 is a tumor suppressor in various cancer types, including gastric cancer [11], hepatocellular carcinoma [15], non-small cell lung cancer [17], small intestinal adenocarcinoma [18], and ovary cancer [25]. These studies suggest that the loss of USP10 expression is significantly correlated with poor patient outcomes. On the contrary, Ouyang et al. proposed that USP10 plays an oncogenic role in colon cancer [26]. They reported that USP10 promotes the expression of the oncogenic factor Musashi-2 (MSI2) by preventing its proteasome-dependent degradation. In addition, they demonstrated that USP10 promotes colon cell proliferation by deubiquitinating MSI2. Other evidence supports USP10 as an oncogenic factor in other cancers, including prostate cancer [13], glioblastoma multiforme [14], and breast cancer [12]. Thus, the role of USP10 in cancer progression is controversial. One of the main reasons for this inconsistency in the data could be that cell conditions, such as the genetic mutation of tumor protein 53 (TP53), dictate the role of USP10 as a tumor suppressor or oncogenic factor[7]. It is well-known that TP53 encodes a 53-kDa phosphoprotein and is commonly inactivated in a wide range of tumors, including colorectal cancer. Although we did not assess it in this study, the mutation rate of TP53 is reported to be about 50% in colorectal cancer. Notably, the prevalence of the TP53 mutation in colorectal patients depends on multiple factors, including tumor stage, location, and the status of hypermutation [27]. Therefore, further studies are needed to clarify the association between the functional role of USP10 and the TP53 genotype in colorectal cancer.

Although several previous studies reported that USP10 is associated with tumor suppression in colon cancer, it remains unclear why USP10 expression is often downregulated in human cancers. In terms of the tumor suppressive role of USP10 in cancer, USP10 could contribute indirectly to regulating cell proliferation or tumor formation by interacting with other tumor suppressor proteins such as p53 or SIRT6 [28, 29]. Recently, overwhelming evidence suggests that promoter methylation is a key epigenetic mechanism that regulates gene expression, and that tumor-suppressor gene silencing in most cancers is caused by aberrant hypermethylated gene promoter regions. We had previously studied whether the CpG islands of the USP10 promoter region were hypermethylated in small intestinal adenocarcinoma [18], and our results imply that USP10 methylation occurs in an early stage of colorectal cancer development. Thus, the results of our previous and present studies strongly support that the downregulation of USP10 is epigenetically regulated in CRC.

The alternative reading frame (ARF) protein is frequently mutated in human cancer, and the CDKN2a locus encodes two different proteins (p14ARF and p16INK4a) [30]. ARF is involved in regulating cell cycle arrest and apoptosis through p53-dependent and -independent pathways and is a potent tumor suppressor [31]. The promoter region of p14ARF is known to be hypermethylated in a wide spectrum of human cancers, including colorectal cancer [32]. According to one recent meta-analysis studying the prognostic value of p14ARF, the hypermethylation of p14ARF was more frequently observed in right side colon cancer and microsatellite instability (MSI)-associated cancer than in left side colon cancer and non-MSI associated cancer [33]. In addition, methylation was not associated with tumor differentiation or

colorectal cancer stage [33]. Although a CpG island within the promoter region of p14ARF has been widely studied, p14ARF protein expression has not been thoroughly assessed via immunohistochemistry in colorectal cancers. A previous study reported that intact p14ARF expression was found in 36.9% (17/46) of colorectal cancers in Korean patients [34]. On the other hand, we observed that p14ARF expression was not decreased in 71.4% (200/280) of colorectal cancer cases (Table 1). This discrepancy might be explained by the lack of well-defined p14ARF cut-off values for immunohistochemical scoring methods. Recently, Ko et al. demonstrated a positive correlation between USP10 and p14ARF expression in non–small cell lung cancer and found that the loss of both molecules correlated with poor prognosis. The role of USP10 and p14ARF as tumor suppressors has also been shown in small intestinal adenocarcinoma [18] and epithelial ovarian cancer [25]. Corroborating previous studies, we we found that downregulation of both molecules was associated with poor patient prognosis. Interestingly, the prognostic significance of $USP10^{loss}$/$p14ARF^{loss}$ was especially prominent in the group of patients that was LN-negative at the time of surgery (Supplementary Fig. S1). Therefore, to predict patients' prognoses, immunohistochemical staining for both USP10 and p14ARF is recommended if no LN metastasis is identified during surgery.

Our study has a few limitations. First, we used combined conventional immunohistochemistry and digital image analysis to quantitate the USP10 and p14ARF markers. Although the conventional manual scoring of immunohistochemical staining provides reasonable intra- and inter-observer reproducibility, this methodology has issues that make optimal scoring and deciding standard cut-off values for positivity difficult. We previously demonstrated that continuous immunohistochemical scoring via digital image analysis could improve the identification of optimal cut-off points compared with manual visual scoring [35], but that methodology needs to be standardized. For instance, a previously validated, commercially available antibody clone and a well-defined algorithm and cut-off value are essential for clinical utility. Second, although drug resistance in neoadjuvant therapy is a major factor contributing to patients’ negative outcomes, this single-institution study considered patients who did not undergo chemotherapy or radiation therapy prior to surgery. Interestingly, a previous study reported that the loss of USP10 correlated significantly with chemoresistance in epithelial ovarian cancer [25]. Therefore, further multiple-institution studies considering a broad spectrum of diseases, including chemosensitivity response and disease recurrence cases, is warranted to validate optimal cut-offs and prognostic values. Third, our study could not include the results of various molecular tests, including MSI. The molecular characterization of patients with colorectal cancer has become a routine examination in clinical practice because genetic information about oncogenes and tumor suppressor genes provides insight into disease progression and response to therapy. However, we could not evaluate the association between molecular variation and USP10 or p14ARF protein expression. Further investigation with a larger number of cases and more molecular profiling data could be necessary to maximize the clinical value of USP10 or p14ARF information in colorectal cancer.

Conclusions

In conclusion, we found that the $USP10^{loss}$ correlates with worse patient outcomes. In addition, USP10 and p14ARF protein expression exhibit a significant positive correlation in colorectal cancer patients, suggesting that the USP10-p14ARF axis is implicated in cancer progression. Furthermore, $USP10^{loss}$/$p14ARF^{loss}$ could be an independent prognostic marker for poor DFS and OS times in colorectal cancer patients. Notably, $USP10^{loss}$ is linked to the hypermethylation of its promoter region in colorectal cancer. The results from this study suggest that $USP10^{loss}$/$p14ARF^{loss}$ patients should be considered for intensified clinical management. Also, further studies need to be conducted to elucidate the mechanistic role of USP10 in tumor progression of different molecular subtypes of colorectal cancer.

List Of Abbreviations

USP10: ubiquitin-specific protease 10; AMPK: AMP-activated protein kinase; FLT3: fms-like tyrosine kinase 3; PTEN: phosphatase and tensin homolog; TMA: tissue microarray; IHC: immunohistochemistry; DFS: disease-free survival; OS: overall survival; HR: hazard ratio; RFS: random survival forest; LN: lymph node; MSI2: Musashi-2; TP53: tumor protein 53; ARF: alternative reading frame; MSI: microsatellite instability; MSP: methylation specific PCR; C-index: concordance-index; CI: confidence interval.

Declarations

Ethics approval and consent to participate

This study was approved by the regional Institutional Review Board of Kangbuk Samsung Hospital (approval no. 2019-07-022; Seoul, South Korea), and each patient provided informed consent. All procedures were conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

Data is available in the supporting files.

Competing interests

The authors declare that there is no conflict of interest.

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Authors' contributions

KK, JMY, and J-YC conceived the study and devised the experimental design. KK, TH, YP, and JMY performed experiments. KK, D-HK, HK, IH, CHC, JMY, and J-YC performed data analysis for experiments or clinical records. KK, JMY, and J-YC wrote the final version of the manuscript and figure legends. All authors read and approved the final manuscript.

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Figures

Figure 1

Microscopic features of USP10 and p14ARF immunohistochemical staining. The normal colonic crypt shows USP10 positivity in the cytoplasm (a) and p14ARF positivity in the cytoplasm and nucleus (d). The colorectal cancer case with intact USP10 and p14ARF expression reveals diffuse cytoplasmic and nuclear staining (b and e). A case with the loss of USP10 and p14ARF is presented in c and f.

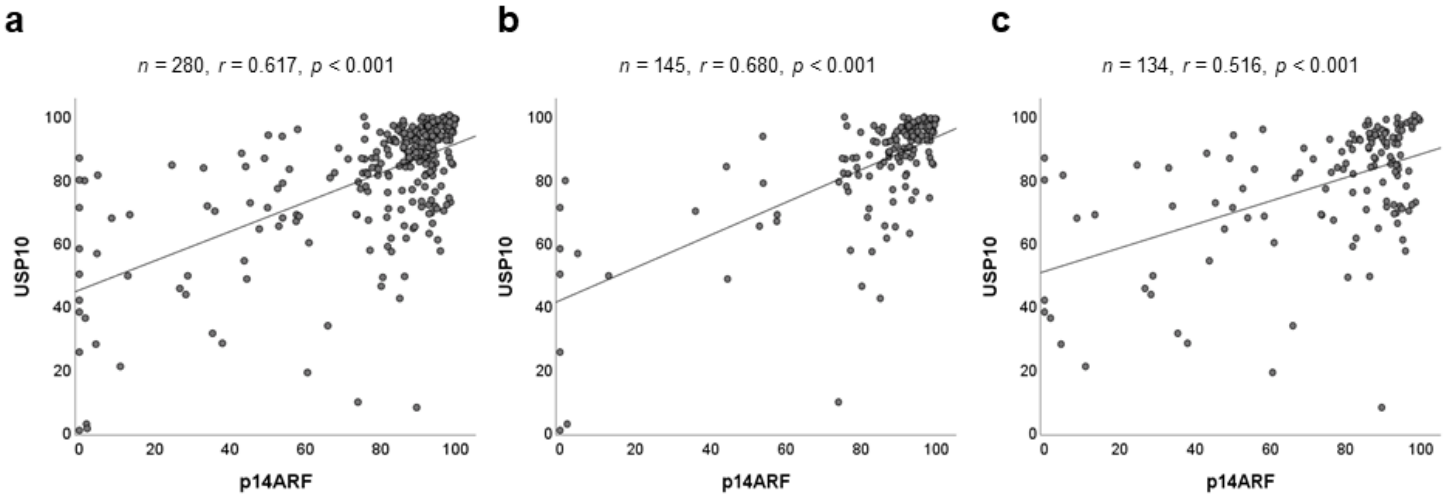

Fig. 2.

Figure 2

Correlation analysis between USP10 and p14ARF expression in patients with colorectal cancer. (a) USP10 expression correlated significantly with the expression of p14ARF (r=0.617, p<0.001). (b and c) A stronger correlation between USP10 and p14ARF expression was observed in patients without lymph node (LN) metastasis by in the subgroup analysis (r=0.680 and r=0.516, both p<0.001, respectively).

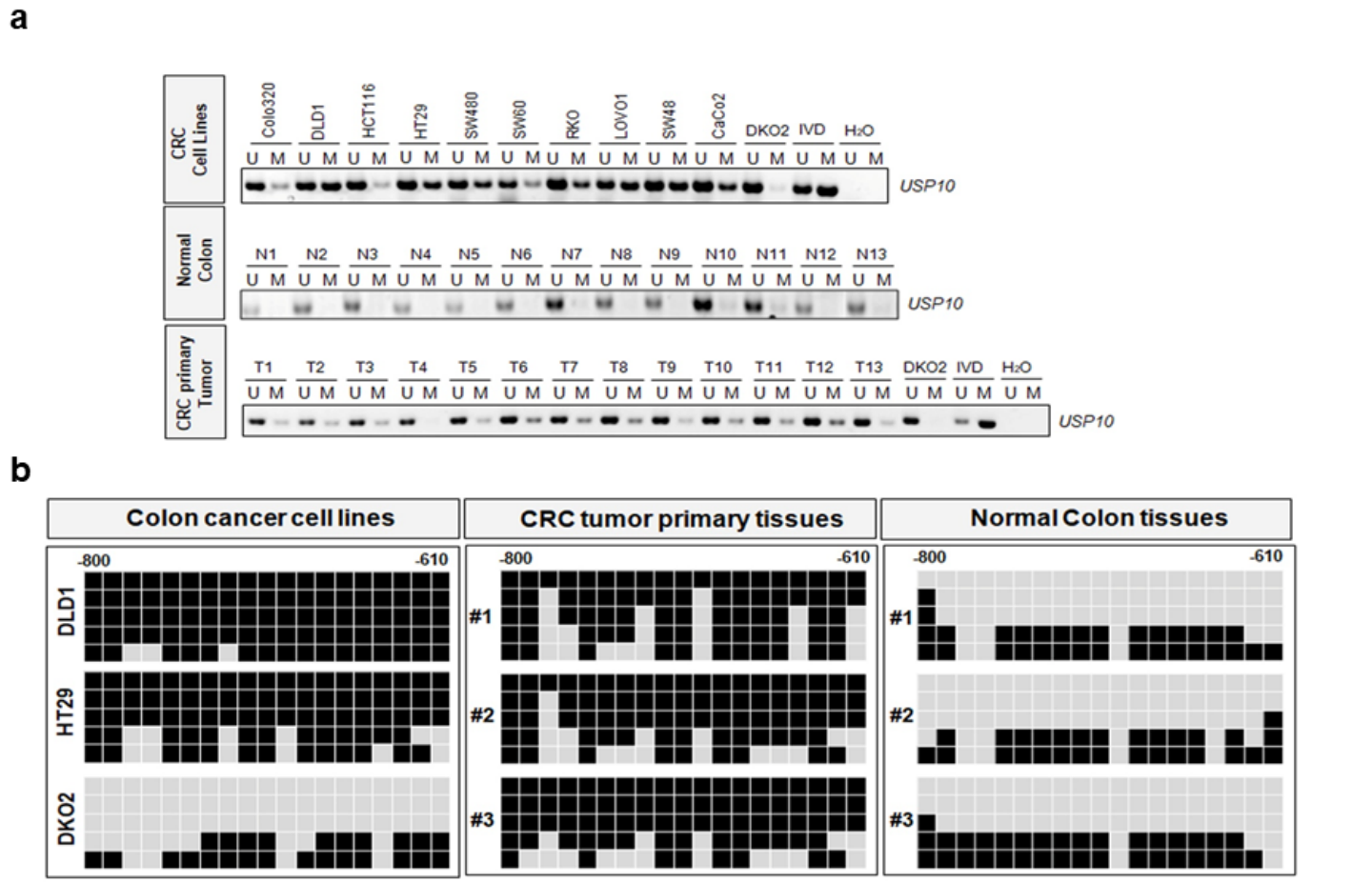

Fig. 3.

Figure 3

Methylation analysis of the USP10 promoter regions in colorectal cancer (CRC) cell lines, tumor tissues, and normal colon tissue. (a) Methylation specific PCR (MSP) analysis of the USP10 promoter region in colon cancer cell lines, normal colon tissues (n=13), and colorectal cancer primary tumor tissues (n=13). DNA is hypermethylated in CRC cell lines and primary tumor tissues, whereas the PCR product was absent from methylated lane of USP10 in normal colon tissues. The PCR products distinguish between unmethylated (U) and methylated (M). DKO cells [36] were used as an unmethylated control. IVD: in-vitro methylated control, H2O: water control containing no DNA. (b) Bisulfite sequencing analysis of USP10. Representative bisulfite sequencing analyses were performed for the USP10 gene in representative three colon cancer cell lines, three colorectal cancer primary tumor tissue samples and three normal controls. Each square represents a CpG dinucleotide. Black squares represent methylated cytosines, and white squares represent unmethylated cytosines.

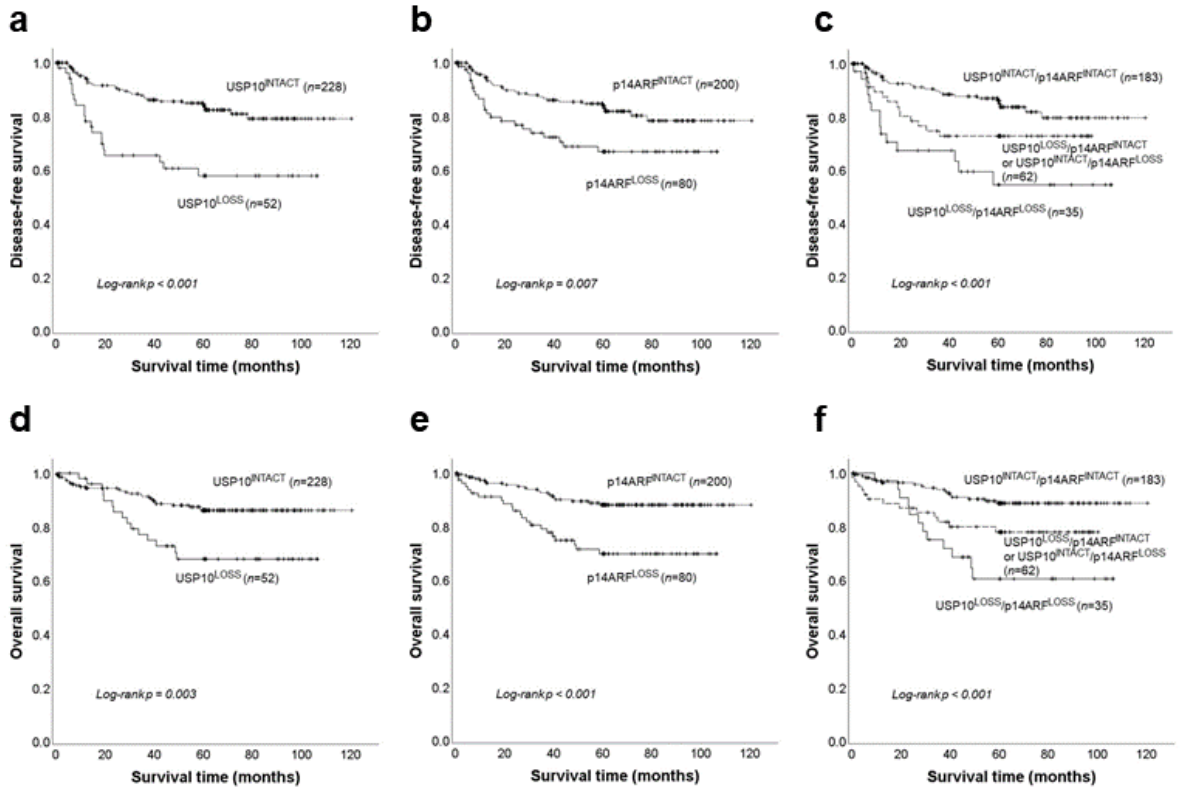

Fig. 4.

Figure 4

Kaplan-Meier plots for disease-free survival and overall survival according to the expression of USP10 and p14ARF in colorectal cancer. (a and b) Patients who lost of USP10 or p14ARF expression showed poorer disease-free survival (DFS) than patients with intact USP10 or p14ARF expression (log-rank test, p<0.001 and p=0.007, respectively). (c) Notably, patients with dual loss of USP10 and p14ARF expression showed worse DFS than patients with dual intact USP10 and p14ARF expression (log-rank test, p<0.001). (d and e) Patients who lost of USP10 or p14ARF expression showed worse overall survival (OS) than patients with intact USP10 or p14ARF expression (log-rank test, p=0.003 and p<0.001, respectively). (f) Furthermore, patients with dual loss of USP10 and p14ARF expression showed significantly poorer OS than patients with dual intact USP10 and p14ARF expression (log-rank test, p<0.001). The cut-off value for intact USP10 expression was an IHC score greater than 68.2, and the cut-off for intact p14ARF expression was an IHC score greater than 80.0.

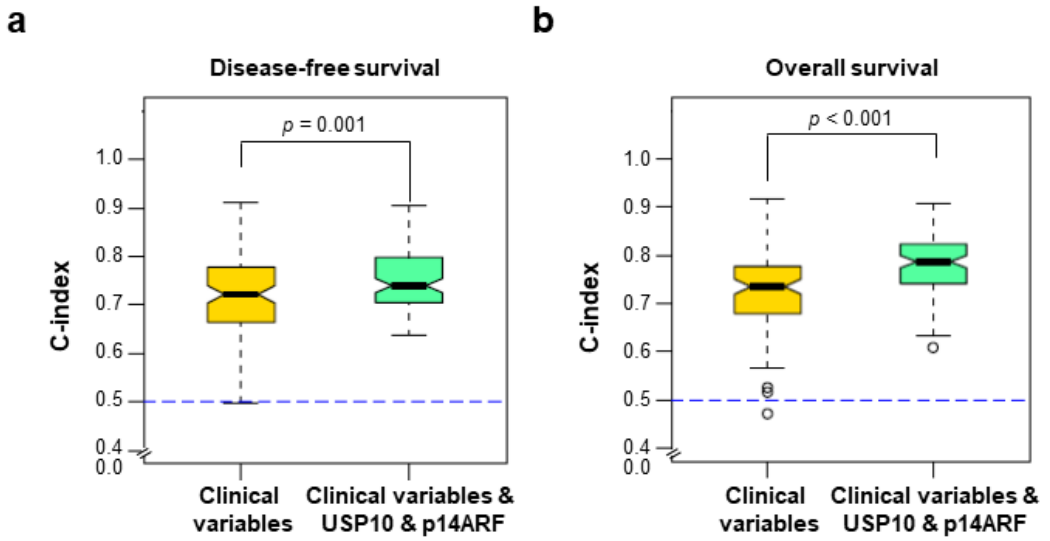

Fig. 5.

Figure 5

Prediction of survival by the clinical- and the combined clinical and molecular- random survival forest (RFS) models. The plots represent the distribution of the concordance-index (C-index) from 100 rounds of cross-validation. (a) The combined clinical and molecular (USP10/p14ARF) RFS model (median C-index=0.740) has more power to predict recurrence yield than the clinical RFS model (median C-index=0.722). (b) The combined clinical and molecular model showed better performance than the clinical only model in predicting death (p<0.001). The dashed line marks the C-index equivalent to a random guess (C-index=0.5).

Supplementary Files

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- USP10inCRCSupplFig.S1..pptx