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High Expression of UBE2B as a Poor Prognosis Factor in Patients With Rectal Cancer Following Chemoradiotherapy

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Abstract. *Background/Aim: Neoadjuvant concurrent chemoradiotherapy (CCRT) is the standard therapeutic strategy for rectal cancer. However, 15-20% of patients undergoing neoadjuvant CCRT progress to recurrence or distant metastases. Therefore, identifying a predictive biomarker is necessary for treating CCRT. Materials and Methods: We investigated the relationship between the levels of histone ubiquitination enzyme and clinicopathological outcomes in patients with rectal cancer who were administered CCRT and confirm the role of histone ubiquitination enzyme in regulating the cell response to ionizing radiation (IR). Results: Clinical data indicated that UBE2B expression was significantly correlated with tumor regression grade. Inhibition of UBE2B elevated the genotoxicity of IR to radioresistant cell lines. In contrast, UBE2B over-expression reduced cell sensitivity to IR. Importantly, the recruitment of 53BP1 and Rad51 was remarkably prolonged in cells after pre-treatment with UBE2B inhibitor, TZ9, suggesting a defective DNA repair pathway in UBE2B-deficient cells. Conclusion: These results indicate that over-expression of UBE2B correlates with poor response and low survival rate in patients who are administered preoperative CCRT.*

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The incidence of colorectal cancer (CRC) has been related to the human development index (1). In 2018, approximately 704,376 rectal cancer cases were diagnosed worldwide, resulting in 310,394 deaths (2). In Taiwan, both the incidence and mortality of rectal cancer have been increasing each year, with 5,751 new rectal cancer cases and 1,687 deaths reported in 2016 (3). Generally, CRCs include both colon cancer and rectal cancer. Given the different locations of the cancer in the human body, colon cancer patients and rectal cancer patients are treated using different methods. Concurrent chemoradiotherapy (CCRT) is used as a neoadjuvant therapy for locally advanced rectal cancer showing lymph node metastases or peri-rectal extension before radical surgery, or as a definitive therapy for unresectable cases (4, 5). Nevertheless, 15-20% of patients administered neoadjuvant CCRT eventually develop local recurrence or distant metastases (6). Furthermore, the pathological complete response (pCR) rate is low, with only 4-33% of patients administered fluoropyrimidine-based CCRT achieving a pCR (7-9). Evidence suggests that patients who achieved pCR after

CCRT have superior outcomes compared to those who show an incomplete pathologic response (10). Therefore, prognostic biomarkers are needed to predict the outcomes of patients administered CCRT and to improve the response to ionizing radiation (IR).

IR exerts lethal effects by causing DNA damage. Radiation-induced DNA lesions include base pair damage, single-strand breaks, and double-strand breaks (DSBs). To maintain genome integrity, cells have evolved several response systems for repairing numerous DNA lesions. Homologous recombination and non-homologous end-joining are two major approaches used to repair DSBs. Cells with a defective DNA damage response (DDR) display sensitivity to IR exposure. Therefore, investigating the detailed mechanisms of the DDR may provide important clues for clinical rectal cancer treatment.

Increasing evidence has indicated that histone ubiquitination plays an important role in modulating the interplay among DNA repair systems. For example, UBE2A and UBE2B are ubiquitin-conjugating enzymes involved in RAD18-mediated ubiquitination of H2B and proliferating cell nuclear antigen (PNCA) to bypass DNA replication-mediated single-strand breaks by activating the DNA damage tolerance of DNA polymerase (11-13). USP16, a histone H2A deubiquitinase, interacts with the ubiquitin E3 ligase HERC2 to negatively regulate DDR (14). Ubiquitin-specific protease 21 (USP21) can deubiquitinate and stabilize BRCA2, and further trigger the recruitment of Rad51 to the DNA damage site. Knockdown of USP21 decreases the cell's ability to perform HR repair, increases the levels of γH2AX, and reduces tumor cell survival (15). USP22 is one of the Spt-Ada-Gcn5-acetyltransferase (SAGA) deubiquitination modules required for the deubiquitination of histone H2B in initiating DDR. Knockdown of USP22 not only blocks the deubiquitination of H2BK120ub, but also decreases ATM- and DNAPK-induced γH2AX formation following DNA damage (16). Thus, the cellular repair process is tightly controlled and regulated by the interplay between ubiquitination and deubiquitination of histone and non-histone proteins at damaged loci. Therefore, targeting aberrant histone ubiquitination may provide a personalized strategy for predicting the response of patients to CCRT.

This study aimed to investigate the relationship between the levels of histone ubiquitination enzyme and clinicopathological outcomes in patients with rectal cancer who were administered CCRT. In vitro cell-based assays were conducted to confirm the role of histone ubiquitination enzyme in regulating the cell response to IR.

Materials and Methods

Cell culture. SW48 (ATCC, Manassas, VA, USA), HCT-116 and Caco-2 (BCRC, HsinChu, Taiwan) colon cancer cell lines were used in this study. Cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and antibiotics and incubated at 37˚C in a humidified incubator in a 5% CO_2 atmosphere. HCT-116-R cells are HCT-116 cells that survived exposure to 8 Gy IR.

Plasmid and antibodies. Anti-tubulin, anti-actin, anti-UBE2A, and anti-UBE2B antibodies were purchased from Genetex (San Antonio, TX, USA). Specific RNA interference expression plasmids sh-UBE2B (#1 sequence: GCATGGTGTGAACTAAGTTAT; #2 sequence: GCAGTTATATTTGGACCAGAA; #3 sequence: CGGG ATTTCAAGCGGTTACAA) and shRNA directed against luciferase (sequence: CTTCGAAATGTCCGTTCGGTT) as a control shRNA were purchased from the National RNAi Core Facility (Academia Sinica, Taiwan).

UBE2B transfection. To establish the stable UBE2B-depleted HCT-116-R cells, HCT-116-R cells were transfected with media containing sh-UBE2B lentivirus particles and polybrene. After 24 h, the medium was replaced with fresh medium containing 2.5 μg/ml of puromycin to select successfully infected cells. The Myc-DDK-tagged UBE2B plasmid was purchased from OriGene (Rockville, MD, USA). TZ9, a specific inhibitor of UBE2B, was obtained from Cayman Chemical (Ann Arbor, MI, USA).

Colony formation assay. About 1×10^3 cells seeded in 6-cm dish were treated with 4 Gy of IR and then incubated for 14 days to allow the colony formation. Colonies were stained with Giemsa (Sigma Aldrich Co., St. Louis, MI, USA) and counted. Relative survival was defined as the number of colonies counted after IR, divided by the number of colonies without IR. The experiments were repeated at least three times.

Real-time RT-qPCR analysis. Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Equal amounts of RNA were used to synthesize first-strand cDNA using the RT2 first strand kit (Qiagen). Reverse transcription (RT)-PCR was performed using SYBR Green and the Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers used were: UBE2A-forward, 5'-CCTCATGCGGGACTTCAAGA-3'; UBE2A-reverse, 5'-CCTGGC TGTTTGCTGGACTA-3'; UBE2B-forward, 5'-AAGCGGTTACAA GAGGACCC-3'; UBE2B-reverse, 5'-CCTGATAAAGCTGTGC TGCC -3'; RNF2-forward, 5'-TTCAGGCCTCATCCCACACT-3'; RNF2-reverse, 5'-CAGCAGCTCCGCCACTC-3'; RNF20-forward, 5'-GAACAGCGACTCAACCGACA-3'; RNF20-reverse, 5'-GGAA TTCACCCGTTCTAGGACTT-3'; USP16-forward: 5'-GGACAA CTGGAGTGTATGG TGT-3', USP16-reverse: 5'-ATCTTTCT CTGGCTTTGGAGTTG-3'; USP22-reverse, 5'-AAGAAGTCCCG CAGAAGTGG-3'; GAPDH-forward, 5'-AAGGCTGGGGCTCA TTTGC-3' and GAPDH-reverse, 5'-GCTGA TGATCTTGAGGCT-3'.

Immunoblot analysis. Protein extraction and immunoblotting were performed as previously described (17). Briefly, cells were lysed and then cellular debris were removed by centrifugation. Proteins were quantified, separated on an SDS-polyacrylamide gel, transferred to nitrocellulose membranes and then immunoblotted using the indicated antibodies.

Patient eligibility and follow-up. Paraffin-embedded tissue blocks and follow-up medical records of 172 patients with rectal cancer in

Chi-Mei hospital between 1998 and 2004 were retrieved (18). All patients in the study were histologically confirmed as having a rectal adenocarcinoma and no distant metastasis. None of the 172 patients had been treated with pelvic irradiation. The patients were regularly followed-up after diagnosis until death or their last appointment. This study was approved by the institutional review board of Chi Mei Medical Center (IRB10801001). All patients were pathologically diagnosed as having rectal adenocarcinoma by endoscopic or surgical biopsy.

Histopathologic evaluation. Post-CCRT surgical specimens and Posttreatment (Post-Tx) T and N stages were evaluated by two pathologists. To objectively evaluate tumor regression after neoadjuvant CCRT, we performed tumor regression grading as described by Dworak *et al*. (19). 'Grade 0' were tumors with no observed regression; 'grade 1'- cancer cells with severe fibrosis and/or vasculopathy; 'grade 2' - fibrosis with scattered cancer cells; 'grade 3' - only few scattered cancer cells with fibrosis background; 'grade 4' - no visible cancer cells.

UBE2B immunohistochemistry and scoring. Tissue sections from Pre-Tx specimens were cut from paraffin-embedded tissue blocks at 3-mm thickness and placed on precoated slides. UBE2B expression was scored by two pathologists who were blinded to the information using a multiheaded microscope to reach a consensus for each case. The percentage of tumor cells showing UBE2B immunoexpression in each specimen was classified into five groups of various expression levels from 0 to 4, denoting none, 1-24%, 25-49%, 50-74%, and 75-100% of tumor cells with moderate to strong nuclear reactivity, respectively.

Statistical analysis. The SPSS 14 software package was used for statistical analysis (SPSS, Inc., Chicago, IL, USA). Scores of 3+ or 4+ were defined as having high expression of UBE2B for statistical analysis. The correlations between UBE2B expression and clinicopathological parameters were analyzed by Chi-square test. Survival curves were analyzed by using the Kaplan–Meier method and log-rank tests were performed to evaluate prognostic differences between different groups. Cox proportional hazards model was prepared by multivariate analysis. For all analyses, two-sided tests with $p<0.05$ were considered as significant.

Results

Cells surviving from ionizing radiation display high levels of UBE2B. To identify novel histone ubiquitination-modifying enzymes for predicting the cell response to IR exposure, we established radio-resistant cells. The HCT-116 cell line, which is substantially more sensitive to IR *in vivo* (20), was used as an *in vitro* cell-based model. HCT-116-R cells, which survived exposure to 8 Gy IR, were evaluated. As shown in Figure 1A, HCT-116-R cells showed a radioresistant phenotype compared to parental HCT-116 cells. Next, we performed a qPCR assay to evaluate the levels of histone ubiquitination-modifying enzymes in HCT-116 and HCT116-R cells. We found that *UBE2B* and *UBE2A* expression was markedly increased in HCT-116-R cells compared to other histone ubiquitination-modifying enzymes including *RNF2, RNF20, USP16*, and *USP22* (Figure 1B). Similarly, the levels of UBE2B protein were also increased in HCT-116-R cells (Figure 1C). These results indicate that UBE2B is involved in the development of the IR-induced radioresistant phenotype.

Depletion of UBE2B increases radiosensitivity in CRC cells. To verify the role of UBE2B in radioresistance, we performed a clonogenic cell survival assay to assess the effects of UBE2B depletion on the cellular response to IR. As shown in Figure 2A, UBE2B knockdown in HCT-116-R cells enhanced the cellular response to IR. TZ9 has been reported to be a selective UBE2B inhibitor. Consistent with UBE2B silencing in HCT-116-R cells, treatment with TZ9 enhanced the radiosensitivity of HCT-116-R cells (Figure 2B).

UBE2B is required for the recruitment of DDR proteins. Recent evidence has indicated that UBE2A/2B cooperates with RNF168 in recruiting DNA damage repair proteins, such as BRCA1 and 53BP1, to the damaged site after IR (21). Therefore, we sought to dissect the functional involvement of UBE2B in HCT-116-R. γ-H2AX is well known as a biomarker for DNA damage. As shown in Figure 3A, we found that UBE2B was inhibited in cells treated with TZ-9, and the foci of γ-H2AX were significantly increased by UBE2B inhibition, even without IR exposure. Furthermore, the impairment of UBE2B consequently prolonged γ-H2AX foci formation. These results indicated that endogenous DNA damage and impaired DNA repair characterized UBE2B-deficient cells (Figure 3A). To gain mechanistic insights into how UBE2B might regulate the radioresistant phenotype of HCT-116-R, we examined whether inhibiting UBE2B could affect the recruitment of 53BP1 and Rad51, which are required for DNA repair. Compared with control cells, the recruitment of 53BP1 and Rad51 was remarkably prolonged in cells after pre-treatment with TZ9 for 24 h, suggesting that UBE2B inhibition resulted in defective DNA repair pathway (Figure 3B).

UBE2B regulates radioresistance in CRC cells. To further verify that UBE2B regulates the radioresistant phenotype, we compared the basal levels of expression of UBE2B in a series of colorectal cancer cell lines (Caco-2, WIDR, SW48, SW480, SW620, HCT-116). As shown in Figure 4A, we found that radioresistant cells (Caco-2 and SW480) displayed high levels of UBE2B compared to radiosensitive cells (SW48 and HCT-116). Notably, we found that ecto-expression of UBE2B in SW48 cells increased their survival after irradiation (Figure 4B). UBE2B depletion caused hypersensitivity to IR in Caco-2 cells, suggesting that UBE2B plays a vital role in modulating the genotoxicity of IR exposure (Figure 4C).

High expression of UBE2B is associated with several clinicopathologic parameters in rectal cancer following

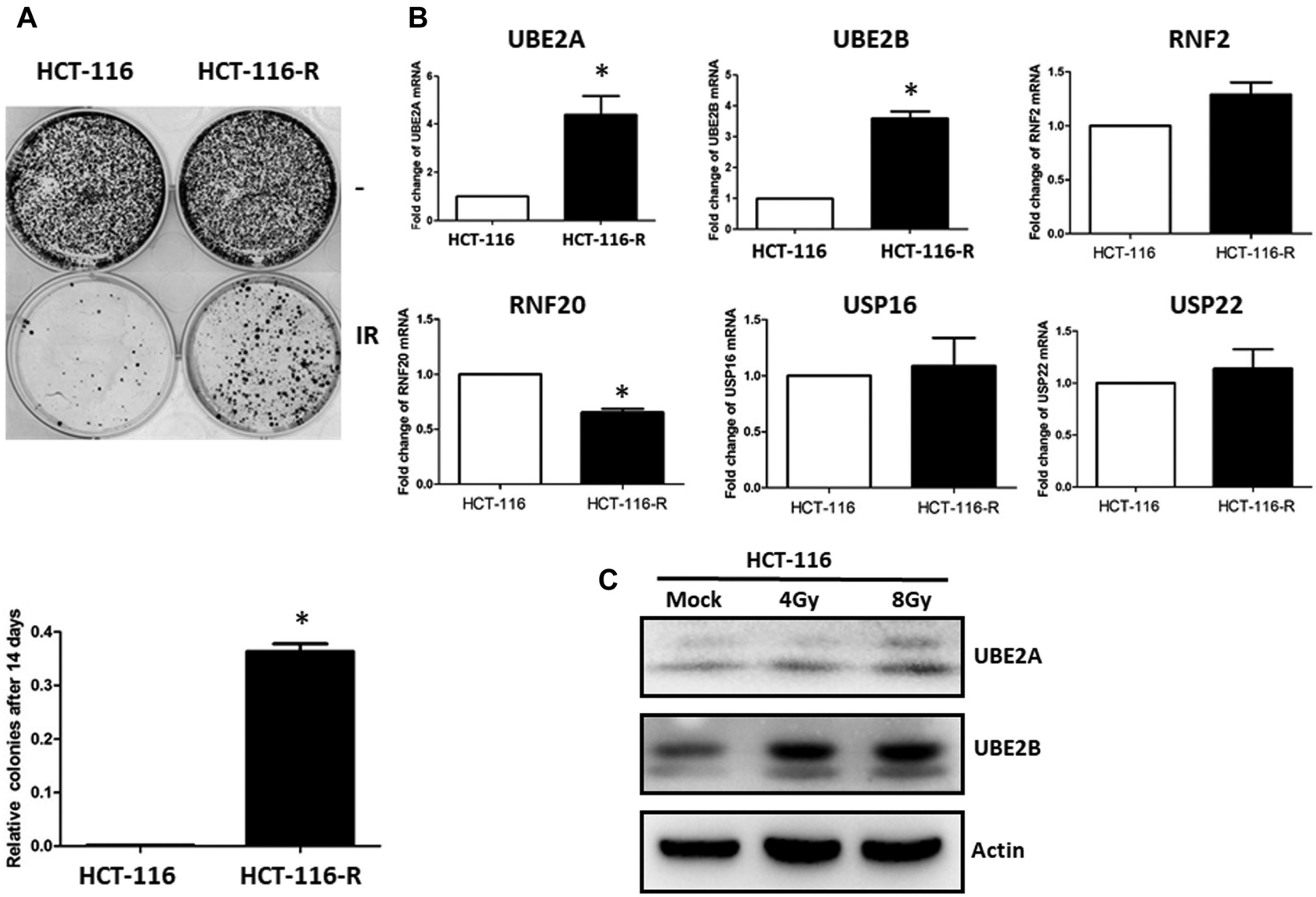

Figure 1. *Cells surviving from ionizing radiation express high levels of UBE2B. (A) HCT-116-R cells were those that survived exposure of HCT-116 cells to 8 Gy IR. 1×103 cells of the parental HCT-116 and HCT-116-R cells were seeded into 6 cm dishes and irradiated with 4 Gy for 14 days to study the clonogenic activity. Relative survival was defined as the number of colonies after IR divided by the number of colonies without IR. Data were statistically analyzed using paired t-test. *indicates p<0.05. (B) The levels of UBE2A, UBE2B, RNF2, RNF20, USP16, and USP22 in parental HCT-116 and HCT-116-R cells were determined by RT-qPCR analysis. Data were statistically analyzed using paired t-test. *p<0.05. (C) The levels of UBE2A and UBE2BB in parental HCT-116 and HCT-116-R cells were analyzed by immunoblots.*

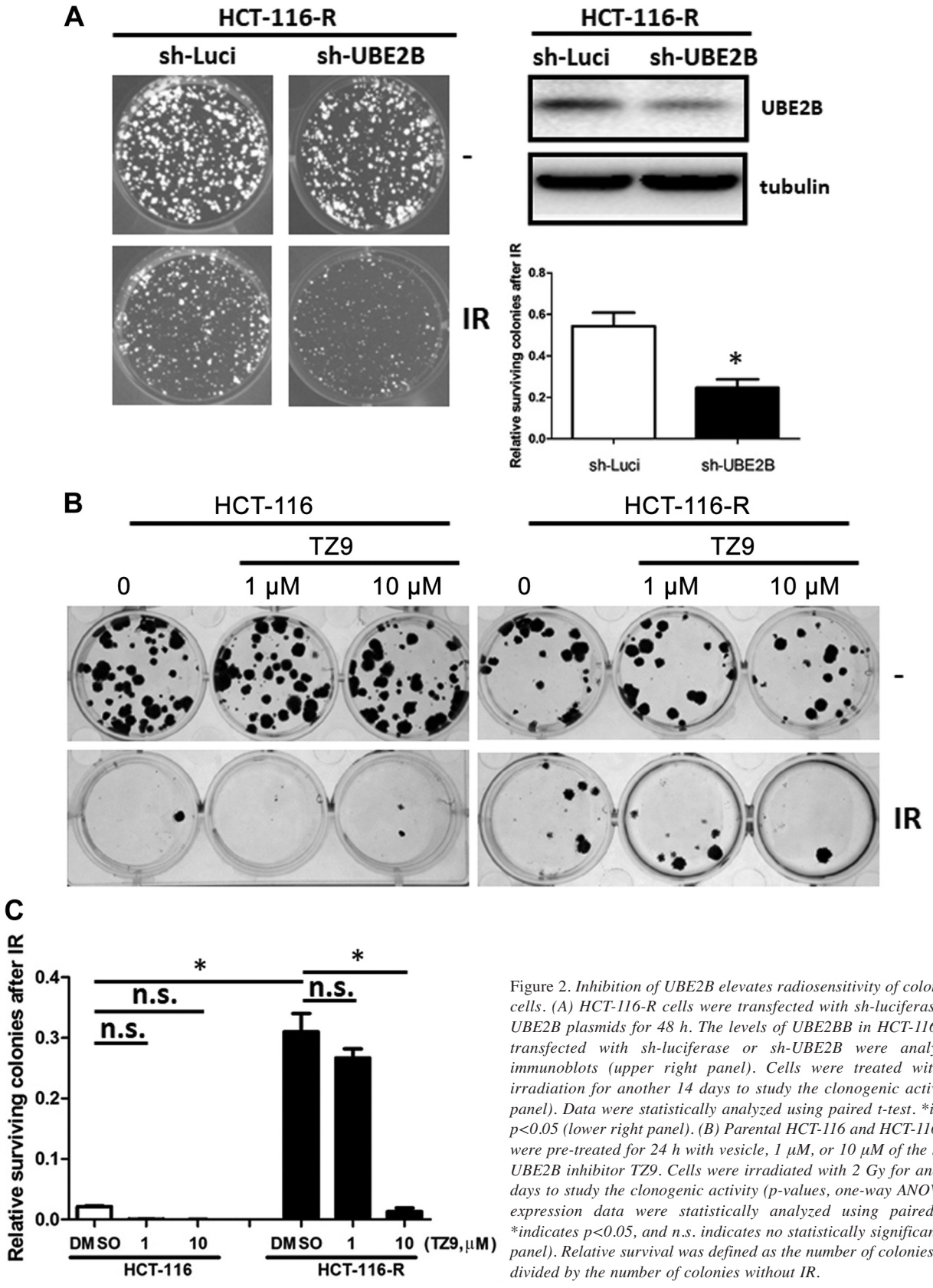

Figure 2. *Inhibition of UBE2B elevates radiosensitivity of colon cancer cells. (A) HCT-116-R cells were transfected with sh-luciferase or sh-UBE2B plasmids for 48 h. The levels of UBE2BB in HCT-116-R cells transfected with sh-luciferase or sh-UBE2B were analyzed by immunoblots (upper right panel). Cells were treated with 2 Gy irradiation for another 14 days to study the clonogenic activity (left panel). Data were statistically analyzed using paired t-test. *indicates p<0.05 (lower right panel). (B) Parental HCT-116 and HCT-116-R cells were pre-treated for 24 h with vesicle, 1 μM, or 10 μM of the selective UBE2B inhibitor TZ9. Cells were irradiated with 2 Gy for another 14 days to study the clonogenic activity (p-values, one-way ANOVA, gene expression data were statistically analyzed using paired t-test). *indicates p<0.05, and n.s. indicates no statistically significant (lower panel). Relative survival was defined as the number of colonies after IR divided by the number of colonies without IR.*

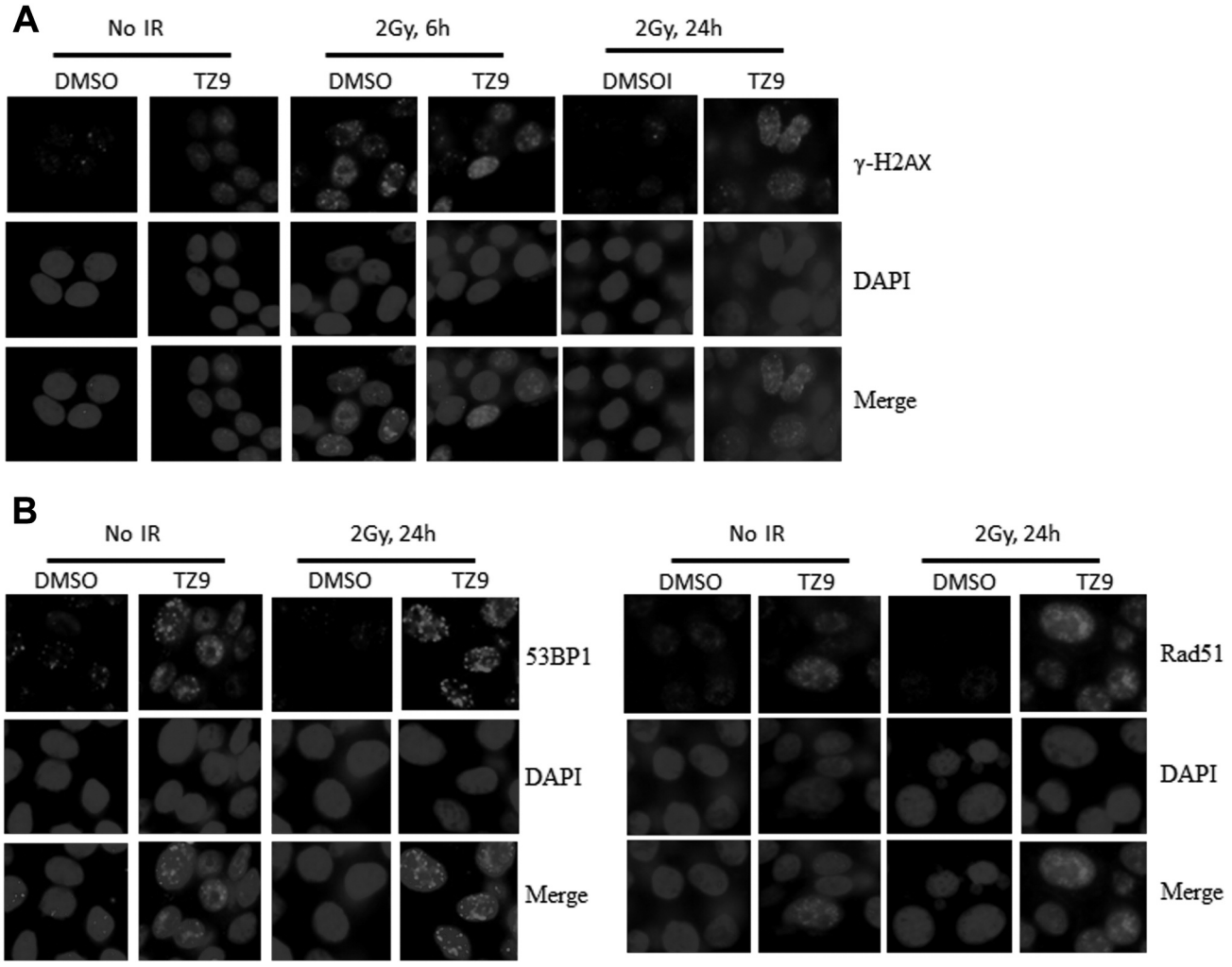

Figure 3. *UBE2B is involved in regulating DNA damage response. (A) HCT-116-R cells were pre-treated with 10 μM TZ9 for 24 h. Analysis of γH2AX phosphorylation and DAPI was performed by immunofluorescence at the indicated time points after exposure to IR (2 Gy). (B) HCT-116-R cells were pre-treated with 10 μM TZ9 for 24 h. Then, cells were exposed to IR (2 Gy) for another 2 h. Foci formation of 53BP1 and Rad51 and DAPI were analyzed by immunofluorescence at the indicated time points after exposure to IR (2 Gy).*

neoadjuvant CCRT. Given the ability of UBE2B to modulate the cellular response to IR, we examined the clinical relevance of UBE2B in 172 patients with rectal cancer administered neoadjuvant CCRT. The immunohistochemistry results for UBE2B in normal and tumor cells are illustrated in Figure 5A. As shown in Table I, UBE2B expression was significantly correlated with the post-treatment tumor status, post-treatment nodal status, vascular invasion, and tumor regression grade. There was no significant correlation between UBE2B expression and gender, age, pre-treatment tumor status, pre-treatment nodal status, and perineurial invasion.

Prognostic impact of UBE2B expression for rectal cancer patients treated with neoadjuvant CCRT. The association between UBE2B expression and the prognosis of patients with rectal cancer who were administered neoadjuvant CCRT was examined by Kaplan–Meier analysis. According to univariate analysis in Table II, clinico-pathological parameters including the post-treatment tumor status, vascular invasion, tumor regression grade and UBE2B expression were predictive of DSS. The pre-treatment nodal status, post-treatment tumor status, vascular invasion, tumor regression grade, and UBE2B expression were correlated with local recurrence-free survival (LRFS). Meanwhile, post-treatment tumor status, tumor regression grade, and UBE2B were significantly associated with metastasis-free survival (MeFS). It is worth noting that high expression of UBE2B was associated with all three endpoints of our study (Figure

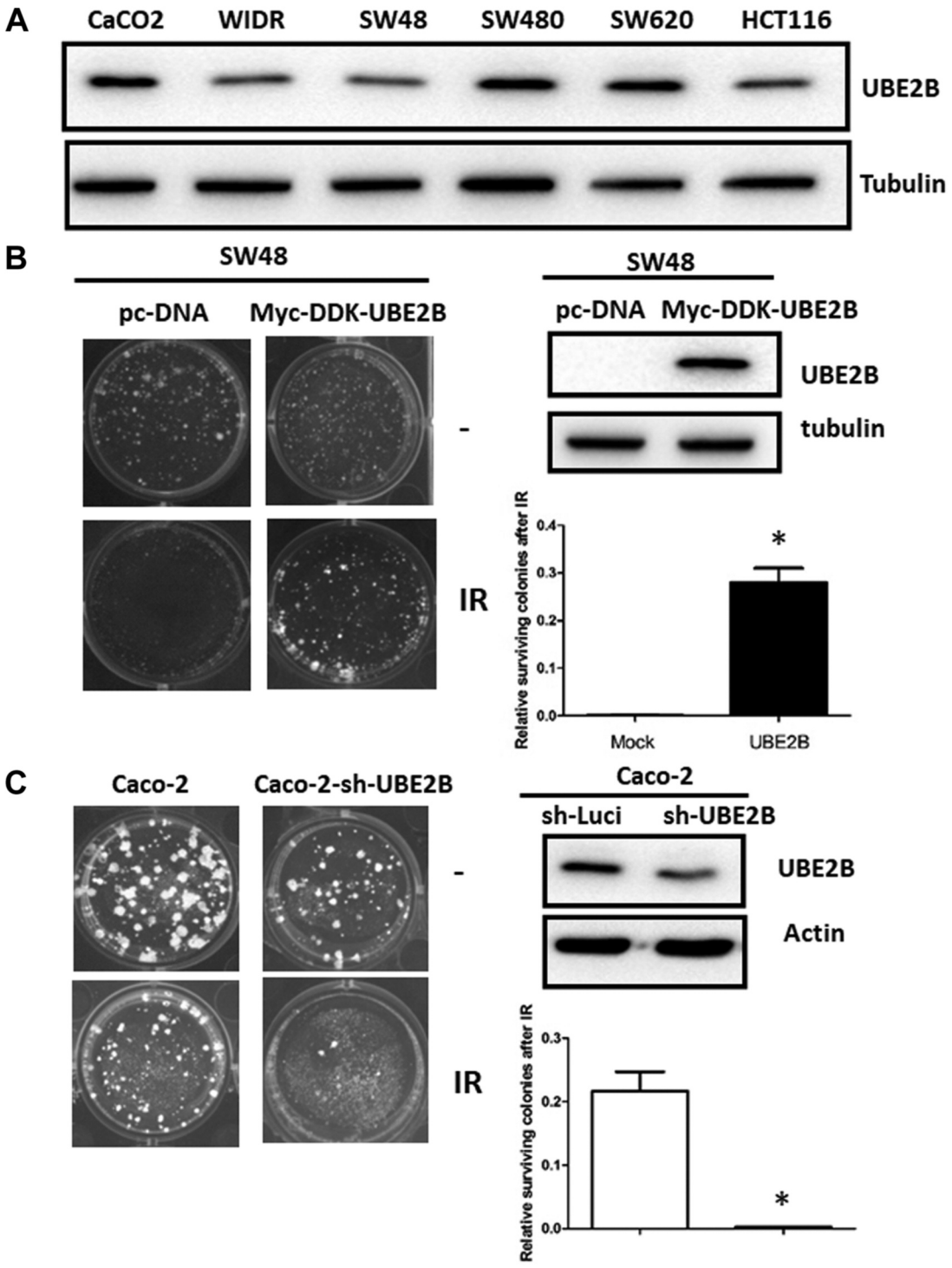

Figure 4. *UBE2B levels in CRC cell lines. (A) The expression levels of UBE2B in a series of colorectal cancer cells (Caco-2, SW480, and SW620, WIDR, SW48, and HCT-116) were analyzed by immunoblotting. Tubulin was used as an internal loading control. (B) SW48 cells were transfected with UBE2B expressing plasmid for 48 h. The protein levels of UBE2B were examined by immunoblot analysis (upper right panel). Parental SW48 and UBE2B over-expressing SW48 cells were treated with 4 Gy irradiation for another 14 days to study the clonogenic activity (left panel). Data were statistically analyzed using paired t-test. *indicates p<0.05 (lower right panel). (C) Caco-2 cells were transfected with sh-Luci and sh-UBE2B plasmids for 48 h. The protein levels of UBE2B were examined by immunoblot analysis (upper right panel). Cells were treated with irradiation for another 14 days to study the clonogenic activity (left panel). Data were statistically analyzed using paired t-test. *indicates p<0.05 (lower right panel). Relative survival was defined as the number of colonies after IR divided by the number of colonies without IR.*

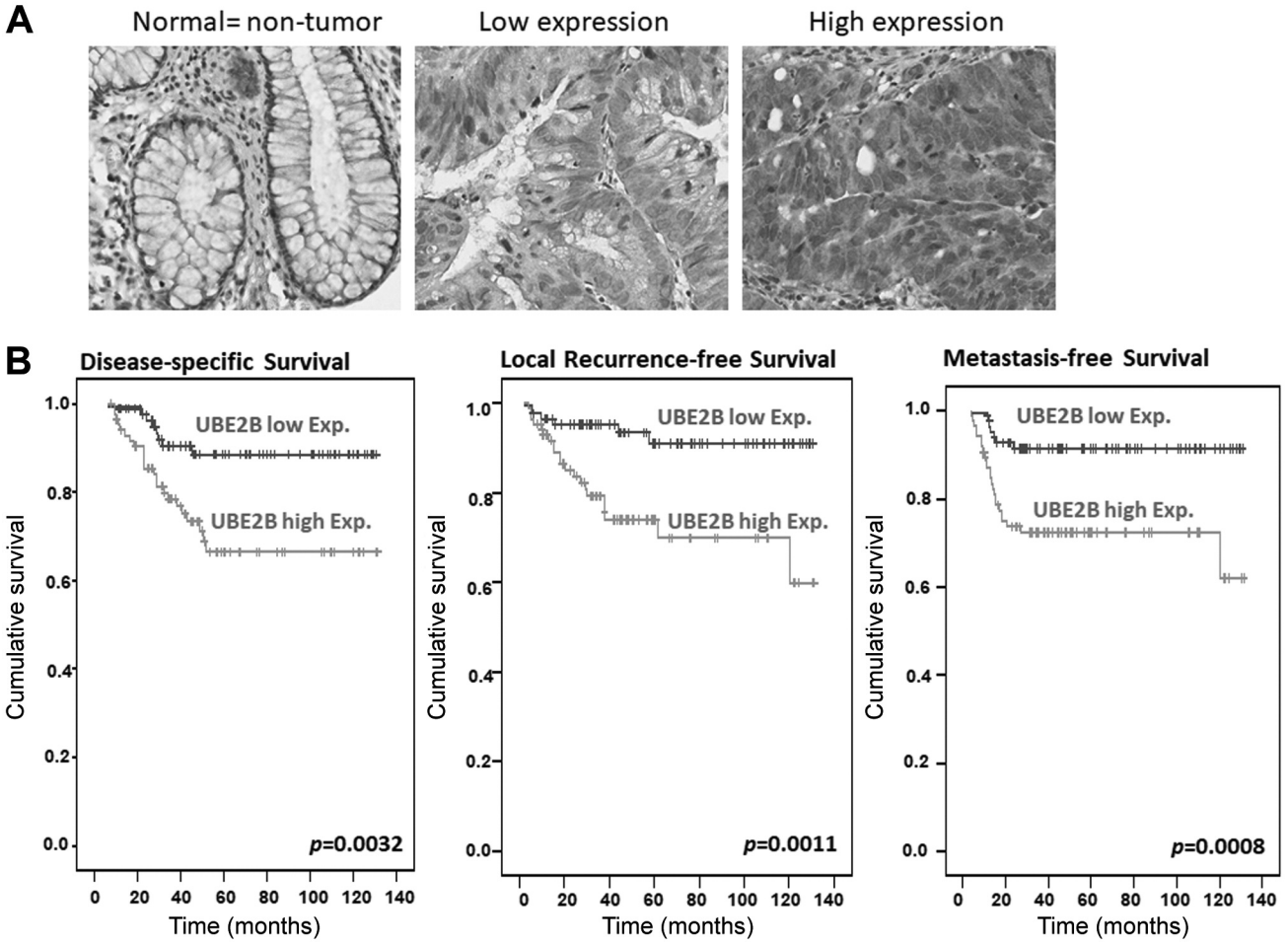

Figure 5. *Kaplan–Meier survival curves plotted to predict survival. (A) The non-neoplastic mucosa reveals no expression of UBE2B as compared with rectal cancers with low expression and high expression of UBE2B in patients with no treatment. (B) Using the log-rank test, high expression of UBE2B predicts inferior disease specific survival (p=0.0032), as well as local recurrence free survival (p=0.0011). It also significantly predicts metastasis-free survival (p=0.0008).*

5B). In addition, high expression of UBE2B remained significant for DSS (p=0.03), LRFS (p=0.001) and MeFS (p=0.008) in the multivariate analysis in Table III.

Discussion

UBE2B is a ubiquitin-conjugating enzyme of 17-kDa molecular weight. The protein is highly conserved in all eukaryotes. The protein was originally identified as a DNA repair protein. Previous studies have demonstrated that both UBE2B and UBE2A regulate DNA damage tolerance and DNA repair pathways by forming a ubiquitination complex with the E3 ubiquitin-protein ligase RAD18 (11, 22). UBE2A and UBE2B have similar ubiquitin conjugating activities and share 95% sequence identity with each other in humans (23). However, in contrast to UBE2A UBE2B has been demonstrated to have a relationship with histone modification and carcinogenesis. UBE2B-mediated histone modification is crucial for DNA repair and damage tolerance (24). Furthermore, UBE2B regulates gene transcription via histone 2B ubiquitination-mediated chromatin modifications and leads to changes in DNA damage tolerance (12, 13, 25). Besides its role as a DDR protein, UBE2B has been reported to play a role in the malignant phenotype of cancers. Gajan *et al*. has identified UBE2B splice variants that represent truncated UBE2B or modified functional versions of the parent UBE2B to be selectively increased in melanoma cell lines and patient-derived melanoma brain metastases (26). The over-expression of UBE2B has also been observed in breast cancer and ovarian cancer and is associated with tumorigenesis and platinum resistance (25, 27, 28). In breast cancer, UBE2B stabilizes β-catenin by adding a K63-linked

Table I. *The correlations between UBE2B expression and clinicopathological factors in 172 rectal cancer patients receiving neoadjuvant CCRT.*

Parameter	No.	UBE2B Expression		*p*-Value
		Low Exp.	High Exp.	
Gender				
Male	108 (62.8)	58 (33.7)	50 (29.1)	0.207
Female	64 (37.2)	28 (16.3)	36 (20.9)	
Age				
<70	106 (61.6)	52 (30.2)	54 (31.4)	0.754
≥70	66 (38.4)	34 (19.8)	32 (18.6)	
Pre-Tx tumor status (Pre-T)				
T1-T2	81 (47.1)	43 (25.0)	38 (22.1)	0.445
T3-T4	91 (52.9)	43 (25.0)	48 (27.9)	
Pre-Tx nodal status (Pre-N)				
N0	125 (72.7)	66 (38.3)	59 (34.3)	0.231
N1-N2	47 (27.3)	20 (11.6)	27 (15.7)	
Post-Tx tumor status (Post-T)				
T1-T2	86 (50.0)	51 (29.7)	35 (20.3)	0.015*
T3-T4	86 (50.0)	35 (20.3)	51 (29.7)	
Post-Tx nodal status (Post-N)				
N0	123 (71.5)	68 (39.5)	55 (32.0)	0.028*
N1-N2	49 (28.5)	18 (10.5)	31 (18.0)	
Vascular invasion				
Absent	157 (91.3)	83 (48.3)	74 (43.0)	0.015*
Present	15 (8.7)	3 (1.7)	12 (7.0)	
Perineurial invasion				
Absent	167 (97.1)	85 (49.4)	82 (47.6)	0.173
Present	5 (2.9)	1 (0.6)	4 (23.3)	
Tumor regression grade				
Grade 0-1	37 (21.5)	16 (9.3)	21 (12.2)	0.004*
Grade 2~3	118 (68.6)	55 (32.0)	63 (36.6)	
Grade 4	17 (9.9)	15 (8.8	2 (1.2)	

polyubiquitin chain to Lys394 of β-catenin (29, 30). However, the detailed mechanism of carcinogenesis and the chemoradiotherapy response controlled by UBE2B remains largely unknown.

UBE2B has been reported to mainly play a role in controlling the error-prone and the error-free DNA damage repair pathway by regulating the ubiquitination of the PCNA protein (31). UBE2B regulates the ubiquitination of the PCNA protein by co-operating with the E3 ligase RAD18, which recruits UBE2B to chromatin to regulate the mono-ubiquitination of PCNA at Lys164 (11, 32). The mono-ubiquitinated PCNA is crucial for error-prone DNA damage repair. Furthermore, UBE2B and the heterodimeric ubiquitin-conjugating enzyme, formed by UBC13 and MMS2, are recruited to chromatin through the interaction with the RING-finger-containing RAD18-RAD5 complex. This large complex is required for error-free DNA damage repair (11, 22). UBE2B initiates different DNA damage repair pathways to repair damaged DNA in both error-prone and error-free manners. However, it is noteworthy that our data indicated that UBE2B expression was significantly correlated with tumor regression grade (Table I). UBE2B deficiency elevated radiosensitivity (Figure 2). These results indicated that other DDRs are involved in the UBE2B-mediated regulation of DNA damage repair in CRC cells. It has recently been shown that UBE2A/2B work together with RNF168 in recruiting DNA damage repair proteins, such as BRCA1 and 53BP1, to the damaged site after ionizing radiation (IR) of U2OS cells (21). To address the role of UBE2B-mediated DNA repair in CRC cells, we quantified the formation of γ-H2AX, 53BP1, and Rad51 foci after IR exposure. As shown in Figure 3, we found that loss of UBE2B triggered the endogenous DNA damage and elongation of DNA repair ability. These results suggested that UBE2B is required for genomic stability and the IR-mediated DNA repair pathway.

A few clinical studies have evaluated microsatellite instability (MSI) status of cancers as a marker of radiation response (33-35). MSI has been shown to predict radiation outcome in early-stage endometrial cancer (35), but its relationship with radiation response in rectal cancer has not been demonstrated (33, 34). This discrepancy might be explained by fewer MSI-related rectal cancers. MSI constitutes about 2-9.3% of rectal cancer (36, 37), while MSI is present in 20%-30% of endometrial cancer (38). Accumulating evidence suggests that DNA mismatch repair (MMR) proteins may directly and indirectly influence DDR after radiation. MSI tumors have increased mutation rates of not only the DSB recognition proteins ATM and MRE11 (38, 39), but also of DNA PKcs, which is necessary for non-homologous end joining repair of DSBs (40). The radiation sensitivity of MSI tumors may also result from a loss of MMR proteins, which play a crucial direct role in the pathways affecting cell cycle arrest, subsequent DSB repair and apoptosis after IR (41-45). As shown in Figure 4A, we examined the UBE2B levels in microsatellite stable (Caco2, SW480, and SW620) and microsatellite unstable (WIDR, SW48, and HCT-116) cell lines by immunoblotting. These results suggested that a positive correlation between UBE2B and MSI might provide another potential mechanism for maintaining genome stability. However, the hypothesis still needs to be further clarified.

Clinical data related to the role of UBE2B in patients administered neoadjuvant CCRT are limited. This is the first study to use immunohistochemistry and surgical samples from patients administered neoadjuvant CCRT and subjected to radical surgery to investigate the CCRT response and prognostic significance of UBE2B in human CRC. We demonstrated that the expression of UBE2B in CRC tissues

Table II. *Univariate log-rank analysis for UBE2B expression.*

Parameter	No. of case	DSS		LRFS		MeFS	
		No. of event	*p*-Value	No. of event	*p*-Value	No. of event	*p*-Value
UBE2B expression							
Low	86	8	0.0032*	6	0.0011*	7	0.008*
High	86	23		21		24	

*Indicates statistically significant values.

Table III. *Multivariate analysis.*

Parameter	DSS			LRFS			MeFS		
	HR	95%CI	*p*-Value	HR	95% CI	*p*-Value	HR	95%CI	*p*-Value
Tumor regression grade	2.849	1.447-5.618	0.002*	2.571	1.142-5.780	0.023*	2.506	1.227-5.102	0.012
UBE2B expression	2.467	1.089-5.589	0.030*	2.807	1.095-7.195	0.032*	3.148	1.350-7.339	0.008
Vascular invasion	2.183	0.851-5.601	0.105	1.950	0.680-5.592	0.214	-	-	-
Post-Tx tumor status (Post-T)	1.661	0.761-3.623	0.202	1.789	0.704-4.549	0.222	1.956	0.839-4.559	0.120
Pre-Tx nodal status (Pre-N)	-	-	-	1.887	0.739-4.492	0.151	-	-	-
Perineurial invasion	-	-	-	1.083	0.219-5.348	0.922	-	-	-

*Indicates statistically significant values.

after IR was closely correlated with several clinicopathologic parameters, including vascular invasion, post-CCRT status of size, spread of the tumor, and tumor regression grade (Table I). In addition, high UBE2B expression was significantly associated with all three endpoints of our study. We highlight that high UBE2B expression was significantly associated with MeFS even in the multivariate analysis. Consistent with the *in vivo* data, our results indicated that UBE2B plays a role in modulating the cell response to IR exposure. Knockdown of UBE2B significantly enhanced the genotoxicity effect of IR in radioresistant cells (HCT-116-R and Caco-2 cells). In contrast, UBE2B over-expression elevated the sensitivity of SW48 cells to IR exposure (Figure 2). Because the treatment response to IR stems from the DNA damage directly caused by IR and indirectly from the formation of free radicals (46), high expression of UBE2B, which regulates DNA damage tolerance and initiates the DNA repair pathway, may result in worse outcomes after radiotherapy, including reduced tumor regression and poor survival. The development of radiation resistance is highly unpredictable, and our clinical and *in vitro* results suggest that the expression levels of UBE2B are highly correlated with radioresistance and lower survival. However, our *in vitro* results cannot explain how UBE2B expression influences MeFS. Published evidence reveals that while cancer cells with high UBE2B expression comprise cells with epithelial–mesenchymal transition (EMT) phenotype, cancer cells with low UBE2B expression lack this phenotype (30). The initiation of metastasis requires EMT (47, 48). In conclusion, we hypothesize that UBE2B increases metastasis by upregulating the EMT phenotype. As a result, patients with low UBE2B expression levels have better MeFS than patients with high UBE2B expression levels.

In recent decades, numerous studies have evaluated the use of neoadjuvant therapy for locally advanced rectal cancer. Adding the radiosensitizer oxaliplatin to current fluoropyrimidine-based CRT failed to increase the pCR rate or downstage, but dramatically increased adverse effects (49, 50). Preclinical and clinical data indicate that the VEGF-specific antibody bevacizumab has anti-vascular effects in rectal cancer (51). However, attempts to administer bevacizumab concomitantly with fluoropyrimidine-based CRT have only shown mild to no benefits in terms of pCR, with a slight increase in peri-operative complications (52, 53). Preclinical evidence suggests that the EGFR inhibitor cetuximab is a potent radiosensitizer. However, pCR was not improved by the addition of cetuximab (54, 55). In contrast to the disappointing results of combining radiation with platinum-based antineoplastic drugs, VEGF-specific antibody, and EGFR inhibitor, our findings indicate that adding the UBE2B inhibitor enhanced the radiation response and reversed the acquired radioresistance in rectal cancer cells. Recent studies have

reported that high levels of UBE2B increased stemness-related gene expression and led to the acquisition of stem cell phenotype, contributing to the recurrence and metastasis of ovarian cancer (56). Inhibition of UBE2B induced G_2-M arrest and apoptosis in breast cancer. Moreover, the depletion of UBE2B has been shown to sensitize nasopharyngeal carcinoma cells to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) by regulating ubiquitination of O^6-methylguanine-DNA methyltransferase (57). Treatment with a UBE2B inhibitor has been shown to sensitize CRC cells to platinum drug-based treatment (58). Thus, UBE2B may regulate the sensitivity of cancer cells to various chemotherapeutic drugs through DNA damage tolerance and repair efficiency. Herein, we provide more evidence showing that UBE2B might be a potential target for developing agents to improve the efficacy of CCRT for rectal cancer.

Based on our results, we conclude that UBE2B is an appropriate biomarker to predict the outcome of CCRT and guide the development of agents to improve the efficacy of CCRT for rectal cancer. Future studies need to identify the E3 ligase downstream of UBE2B and the mechanistic association with MSI, as well as evaluate the efficacy of UBE2B inhibitors *in vivo*. Our findings may further the clinical use of CCRT for rectal cancer treatment.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

WLH, CWL and MRP designed and conducted experiments, acquired and analyzed the data, wrote the article. CLC, CCY collected patient tissues. TJC and CFL conducted experiments analyzed the data of immunohistochemistry. MRP reviewed the manuscript and provided administrative support.

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