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miR-211 regulates the expression of *RRM2* in tumoral metastasis and recurrence in colorectal cancer patients with a *k-ras* gene mutation

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Abstract. Colorectal cancer (CRC) ranks as the third-leading cause of cancer-associated mortalities in Taiwan. The expression of ribonucleotide reductase M2 (*RRM2)* and *p53R2* is associated with tumoral malignancy and progression in several types of cancer. The aim of the present study was to determine the association of *p53R2*/*RRM2* with the upstream expression of microRNA (*miR)-211* and the association of expression levels of *p53*, *APC* and *k-ras* with clinical outcomes in patients with CRC. The study consisted of 192 tumor tissue samples obtained from patients with CRC. Immunohistochemistry and direct sequencing of DNA were performed to analyze *p53R2*/*RRM2* protein expression and *p53*/*APC*/*k-ras* gene mutations in these samples. The expression level of *miR-211* was detected by reverse transcription-quantitative polymerase chain reaction. The results showed that the expression of *p53R2* was lower and that of *RRM2* was higher in patients with lymph node metastasis, distant metastasis, and late-stage CRC compared with patients without lymph node metastasis, distant metastasis and early-stage CRC. A high expression of *RRM2* in patients had a negative effect on overall survival (OS) and disease-free survival (DFS) in CRC. Positive expression of *RRM2* was detected in tumor tissues, and expression associated with the presence of *k-ras* gene mutation. Furthermore, it was detected that the upstream *miR-211* expression was negatively associated with *RRM2* expression in tumor tissues of patients with CRC. *miR-211* expression was associated with survival and tumoral recurrence in patients with *k-ras* mutations. The present authors suggest that the downregulation of *miR-211* and overexpression of *RRM2* in tumor tissues of patients with CRC could be used to predict metastases and disease prognosis, particularly in patients with *k-ras* gene mutations.

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Introduction

Colorectal cancer (CRC) ranks as the third-leading cause of cancer-associated mortalities in Taiwan (1). The majority of Taiwanese patients with CRC (60-70%) present with stage II-IV disease at initial diagnosis (2,3). Among newly diagnosed cases, ~20-25% of patients have advanced disease with distant metastases (2,3). Recurrence occurs in ~30% of patients with advanced CRC, even following curative resection (2). Patients with stage I and II CRC have an 86-95% five-year survival rate, whereas those with stage III and IV metastatic diseases have five-year survival rates of ~67 and ~12%, respectively (3). In a previous study by the present authors, it was reported that

the genetic background of Taiwanese patients with CRC was different from that of Caucasian patients with CRC (4). In the study, ~33.8% of tumor tissues in Taiwanese patients with CRC contained Adenomatous polyposis coli *(APC)* mutations. This figure is close to that reported in Asia but significantly lower compared with the values reported in western countries (70-80%) (5). Therefore, studies of the dynamic nature of molecular signatures in CRC are required to determine the prognosis of patients.

Ribonucleotide reductase (RR) is a highly regulated rate-limiting enzyme that is used in the conversion of ribonucleoside diphosphate to 2'-deoxyribonucleoside diphosphate (6), and it is essential for DNA synthesis (6,7). In humans, one large subunit (M1) and two small subunits (RRM2 and p53R2) of RR have been identified (6,7). Although the protein sequence of the two small RR subunits, p53R2 and RRM2, show 80% similarity, their biological function is markedly different (7). Previous reports demonstrated that the regulation of *RRM2* and *p53R2* might play a critical role in the invasion potential of cancer cells and the establishment of the metastatic phenotype (8,9). Research also suggested that *RRM2* might potentially serve as a biomarker for predicting aggressive CRC, with poor survivability and progression-free survival (10). Inhibition of *RR* activity has been tested as a potential therapy in anticancer settings (11). Therefore, the overexpression of *RRM2* has an important role in the pathogenesis of CRC.

Research has suggested that microRNAs (miRNAs), which are small, noncoding RNAs, modulate gene expression by degrading mRNA and/or inhibiting protein translation (12). The dysregulation of miRNA has been implicated in numerous processes during tumoral progression (12). In previous reports by the present authors, it was demonstrated that different miRNAs were involved in the pathogenesis in CRC, depending on the presence or absence of an *APC* gene mutation (5,13-15). For example, *miR-224* suppressed the migration of CRC cells by targeting cell division cycle 42 in patients with CRC and an *APC* gene mutation (13). It was also demonstrated by the present authors that the downregulation of *let-7a-5p* in sera and tumor tissues of patients with CRC could be used to predict lymph node metastasis and disease prognosis (14). In addition, the present authors demonstrated that *let-7a* appeared to regulate the expression of miR-21 (15). *miR-21* has oncogene-like activity and is highly expressed in several types of cancer (16). In a recent study, it was also found that patients with *APC* mutations and high *miR-21* expression had lower *APC* gene expression and exhibited poorer overall survival (OS) rates compared with patients with *APC* mutations and low *miR-21* expression, *APC* wild-type and high *miR-21* expression, and *APC* wild-type and high *miR-21* expression (5). The same study demonstrated that the downregulation of *APC* gene expression was associated not only with expression of the *APC* gene mutation but also with upregulation of *miR-21* (5). Based on these findings, the present authors speculated that the expression of the *p53*, *APC* and *k-ras* genes may be associated with miRNA expression in patients with CRC.

The *k-ras* gene is a member of the ras gene family (H-, K- and N-ras) (17). Oncogenic *k-ras* mutations have been detected in several types of cancer (e.g., lung, colon and pancreatic) (17). Additionally, ~20-50% of primary colorectal tumors contain oncogenic *k-ras* mutations (18). Recent clinical trials verified that the *k-ras* gene mutation was associated with cetuximab resistance in patients with metastatic CRC (19). However, whether mutant *k-ras* genes affect survival rates, tumoral recurrence and drug resistance in patients with CRC and who receive adjuvant chemotherapy is unclear. Previous research identified a positive association between *RRM2* and *k-ras* genes, showing that re-expressed *k-ras* genes in a HKe3 colon cancer cell line induced *RRM2* expression (20). Based on these findings, we suggest that the interaction of *k-ras* with *RRM2* may play a role in survival rates and drug resistance in CRC patients.

In the present study, it was hypothesized that the downregulation of *miR-211* would induce *RRM2* expression and promote tumorigenesis in CRC and that the expression levels of *miR-211, p53R2* and *RRM2* may be used as biomarkers to predict clinical outcomes and tumoral recurrence in CRC. It was also hypothesized that *p53/APC/k-ras* gene mutations would result in overexpression of *RRM2* and have a role in disease progression and clinical outcomes of patients with CRC. Therefore, the associations between *miR-211, p53R2* and *RRM2* gene expression and clinical outcomes in CRC patients with and without *p53/APC/k-ras* mutations were analyzed.

Patients and methods

Study population. CRC tumor tissue samples were collected from 192 non-selected patients who underwent surgical resection for CRC at the Department of Surgery, Taipei Medical University Hospital (Taipei, Taiwan) between December 2011 and December 2013 (14). The acquisition of the samples and their subsequent examination were approved by the Institutional Review Board of Taipei Medical University (Taipei, Taiwan). Informed written consent was obtained from all the patients and/or guardians prior to the use of the resected specimens.

None of the participants had a previous history of cancer. The clinical stages and pathological features of primary tumors were defined according to the criteria of the American Joint Commission on Cancer (https://cancerstaging.org/references-tools/Pages/What-is-Cancer-Staging.aspx). A total of 33 of the 192 patients with stage IV disease and distant metastasis were enrolled in the present study, of which 5 had lung metastasis, 16 had liver metastasis, 9 had peritoneum metastasis and 3 had para-aortic lymph nodes metastasis. All of the patients had oligometastatic disease and had undergone surgery but not chemotherapy. Postoperative follow-up visits were scheduled every three months thereafter during the first two years, then every six months thereafter, or more frequently if needed. Survival and recurrence were followed up in all the patients in the present study.

The CRC tissues and paired non-tumor tissues from the aforementioned patients were obtained from the Tissue Bank of Taipei Medical University (Taipei. Taiwan) between December 2011 and December 2013. In the present study, a total 192 patients were enrolled, including 90 females and 102 males, age ranging from 40 to 90 years old. A pathologist confirmed that >95% of the cells were tumor cells based on H&E-stained frozen sections. The normal tissues were used as a control. These tissue samples were obtained from the same patient and were checked by a pathologist.

Table I. Association of *p53R2* and *RRM2* expression and clinical parameters in tumor tissues of patients with colorectal cancer.

A, *p53R2* expression

Parameters	Low (n=99)	High (n=93)
Age, years		
≤65	44	50
>65	55	43
P-value	0.248	
Sex		
Female	43	47
Male	56	46
P-value	0.386	
T factor		
1	5	2
2	12	16
3	57	53
4	25	22
P-value	0.562	
N factor		
0	43	40
1+2	56	53
P-value	1.000	
M factor		
0	74	85
1	25	8
P-value	0.002	
Stage		
I	13	10
II	24	29
III	36	46
IV	26	8
P-value	0.008	

B, *RRM2* expression

Parameters	Low (n=96)	High (n=96)
Age, years		
≤65	47	47
>65	49	49
P-value	1.000	
Sex		
Female	40	50
Male	56	46
P-value	0.193	
T factor		
1	4	3
2	19	9
3	51	59
4	22	25
P-value	0.206	
N factor		
0	53	30
1+2	43	66
P-value	0.001	
M factor		
0	82	77
1	14	19
P-value	0.445	
Stage		
I	18	5
II	33	20
III	30	52
IV	15	19
P-value	0.001	

RRM2, ribonucleotide reductase M2; p53R2, ribonucleotide reductase regulatory TP53 inducible subunit M2B; T, tumor, M, metastasis; N, node.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) -based detection of miR-211. Total RNA was extracted from the tumor tissue samples or sera of the patients with CRC using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The expression of mature miRNA was detected by a TaqMan miRNA assay (Applied Biosystems, Thermo Fisher Scientific, Inc; catalog no. 4427975; sequence; UUCCCUUUGUCAUCCUUCGCCU) and normalized relative to U6B using the $2^{-\Delta\Delta Cq}$ method (21). All TaqMan PCRs were performed in triplicate. The PCR reaction was conducted with the following conditions: starting with 95˚C for 10 min, followed by 40 cycles of amplification (95˚C for 15 sec and 60˚C for 1 min). The definition of high and low expression of *miR-211* was dependent on the mean value of the expression of these genes in the normal tissues of the patients. High expression was defined as expression levels higher than the mean expression in non-tumor tissues. Low expression was defined as expression levels lower than he mean expression in normal tissues. The mean expression level of *miR-211* in normal colon tissue samples was 20.21±10.81. The PCR-based detection of *miR-211* was conducted as described in a previous report by the present authors (14).

Detection of p53R2 and RRM2 protein expression by immunohistochemistry. Formalin-fixed and paraffin-embedded specimens were sectioned at a thickness of 3 μm. All the sections were deparaffinized in xylene, sequentially rehydrated through serial dilutions of alcohol, and washed in phosphate-buffered saline. The sections used for *p53R2* and *RRM2* detection were immersed in a citrate buffer (pH 6.0) and heated in a microwave oven twice for 5 min. Mouse anti-p53R2 (catalog no. SC-137174) and RRM2

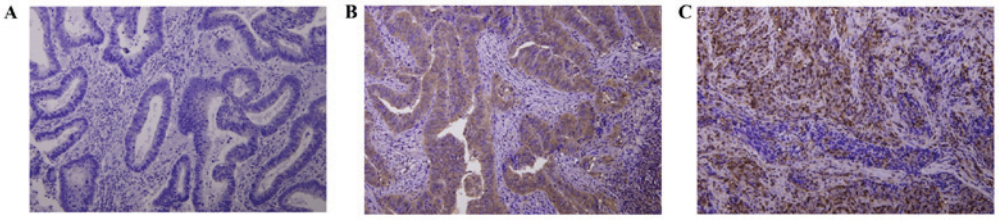

Figure 1. Immunohistochemical analysis of RRM2 and p53R2 proteins in colorectal cancer tissues. (A) Negative immunostaining (x200 magnification). Expression of (B) RRM2 (x200 magnification) and (C) p53R2 proteins (x400 magnification) in tumors. p53R2, ribonucleotide reductase regulatory TP53 inducible subunit M2B; RRM2, ribonucleotide reductase regulatory subunit M2.

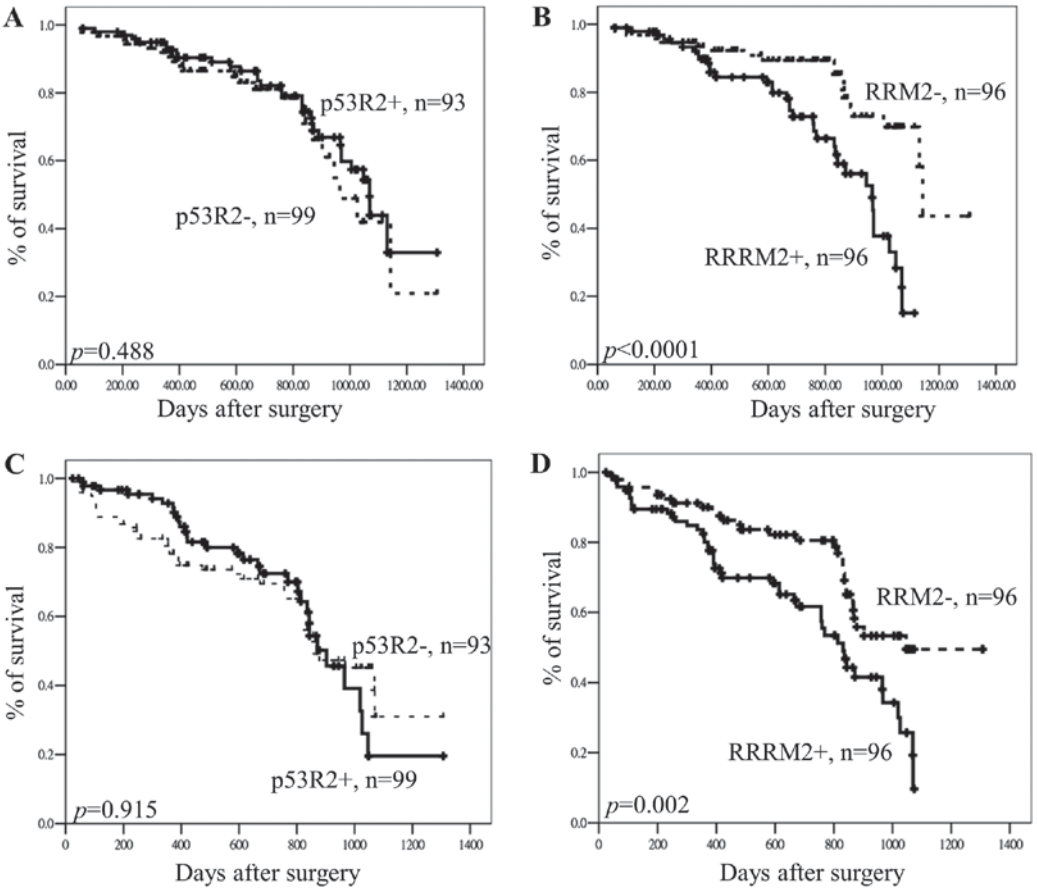

Figure 2. Overall survival curves of all patients with (A) p53R2 and (B) RRM2 expression. Disease-free survival curves of all patients with (C) p53R2 and (D) RRM2 expression. p53R2, ribonucleotide reductase regulatory TP53 inducible subunit M2B; RRM2, ribonucleotide reductase regulatory subunit M2.

(catalog no. SC-81850) monoclonal antibodies (all 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used as the primary antibodies. The conventional streptavidin peroxidase method (DAKO; Agilent Technologies, Inc., Santa Clara, CA, USA; LSAB Kit K675) was performed to develop signals according to the manufacturer's protocol and the cells were counter-stained with hematoxylin. The details of the protocol used have been described previously (22). Negative controls that did not include the primary antibodies were also prepared.

A total of three observers independently evaluated the results and scored the percentage of positive expression in the samples. In each specimen, the cells that positively stained for anti-p53R2 and RRM2 antibodies were recorded as a percentage (%) using a labeling index, and the measurements were calculated. The scores were as follows: 0, no positive staining; +, from 1 to 10% positive cells; ++, from 11 to 50% positive cells; and +++, >50% positive cells. Among the 192 CRC patients, 99 samples were negative for p53R2 protein expression. None of the patients were scored +, 57 were scored ++ and 36 were scored +++. For RRM2 protein expression, 96 patients were negative. None of the patients were scored +, 35 were scored ++ and 61 were +++. The scores of ++ and +++ were considered to represent high immunostaining, and scores of 0 and + were classified as low immunostaining.

Mutation analysis of APC, k-ras and p53 genes. Genomic DNA was prepared from 192 frozen CRC tissues using standard proteinase K digestion and phenol/chloroform extraction following homogenization. Mutations in the *APC*, *p53* and *k-ras* genes were determined by direct sequencing of PCR

products. The detailed protocol used has been described previously (23). The target sequences were amplified in a 50 μl reaction mixture containing 20 pmol of each primer, 2.5 U Taq polymerase (Takara Bio, Inc., Otsu, Japan), 0.5 mmol/l dNTPs, 5 μl PCR reaction buffer and 1 μl genomic DNA as the template. Oligonucleotide primers were used to amplify the mutation cluster region of the *APC*, *p53* and *k-ras* genes. A total of four sets of oligonucleotide primers were used for *APC*: Forward, 5'-CAGACTTATTGTGTAGAAGA-3' and reverse, 5'-CTCCTGAAGAAAATTCAACA-3'; forward, 5'-AGGGTTCTAGTTTATCTTCA-3' and reverse, 5'-TCTGCTTGGTGGCATGGTTT-3'; forward, 5'-GGCATTATAAGCCCCAGTGA-3' and reverse, 5'-AAATGGCTCATCGAGGCTCA-3'; forward, 5'-ACTCCAGATGGATTTTCTTG-3' and reverse, 5'-GGCTGGCTTTTTTGCTTTAC-3'. A total of three sets of oligonucleotide primers were used for *p53*: Forward, 5'-TGCCCTGACTTTCAACTCTG-3' and reverse: 5'-AGTTGCAAACCAGACCTCAGG-3'; forward, 5'-CCTGTGTTATCTCCTAGGTTG-3' and reverse, 5'-TCTCCTCCACCGCTTCTTGT-3'; forward, 5'-AAGGCGCACTGGCCTCATCTT-3' and reverse, 5'-GAATCTGAGGCATAACTGCAC-3'. One set of oligonucleotide primers was used for *k-ras*: Forward, 5'-AGGCCTGCTGAAAATGACTGAA-3' and reverse, 5'-AAAGAATGGTCCTGCACCAG-3'.

Ingenuity Pathways Analysis (IPA). The Ingenuity Pathways Analysis (IPA) platform was used (http://www.ingenuity.com/) to investigate associations between RRM2, p53R2 (RRM2B), KRAS, and miR-211. The platform can reveal molecular interactions according to records contained in its Ingenuity Knowledge Base. The Path Explore tool in the platform was used to identify interactions between the molecules. In total, 13 interacting proteins were identified, including 4 transcription regulators, 3 enzymes, 1 transporter, and 5 proteins with other functions. Among the proteins, BCL2, an apoptosis regulator, serves a central role by interacting with RRM2, KRAS, and miR-211.

Statistical analysis. All data were analyzed using the Statistical Package for the Social Sciences, software (version 13.0; SPSS, Inc., Chicago, IL, USA). A chi-square test (χ^2 test) was used to compare the association of *p53R2* and *RRM2* gene expression with clinical parameters in CRC. A total of two independent tests and k-independent nonparametric tests were used to compare the expression level of *miR-211* in the presence of different clinical parameters in CRC. A probability value of P<0.05 was considered statistically significant. Kaplan-Meier survival curves were constructed for overall survival (OS) and disease-free survival (DFS), and the log-rank test was used to evaluate the differences in survival curves of patients with and without *p53R2* and *RRM2* expression. In the present study, survival was defined as the time from the date of the surgical intervention until 31st July 2016.

Results

RRM2/p53R2 expression is associated with tumoral metastasis in CRC. The *p53R2* gene was expressed in 93 of 192 (48.4%) patients, and *hRRM2* was expressed in 96 of 192 (50.0%) patients. Both proteins were expressed in CRC tumor cells (Fig. 1). As shown in Table I, the expression level of *p53R2* was significantly lower in patients with distant metastasis and late-stage CRC compared with patients with no metastasis and early-stage CRC (M factor, distant metastasis, P=0.002; stage, P=0.008; Table I). No additional associations were identified between *p53R2* expression and other clinical parameters (Table I). In addition, *RRM2* expression was significantly higher in patients with lymph node metastasis and late-stage CRC compared with patients with no metastasis and early-stage CRC (N factor, lymph node metastasis, P=0.001; stage, P=0.001). No additional associations were detected between *RRM2* and other clinical parameters (Table I).

Effect of RRM2 and p53R2 expression on OS and DFS in CRC. We hypothesized that the expression levels of *p53R2*

Table II. Association between *p53R2* and *RRM2* expression and *APC*, *p53*, and *k-ras* gene mutations in patients with colorectal cancer.

A, *p53R2*		
APC, n	- (n=99)	+ (n=93)
Wild-type	63	49
Mutant	36	44
P-value	0.144	
P53, n		
Wild-type	73	73
Mutant	26	19
P-value	0.397	
K-ras, n		
Wild-type	74	69
Mutant	25	24
P-value	1.000	

B, *RRM2*		
APC, n	- (n=96)	+ (n=96)
Wild-type	55	57
Mutant	41	39
P-value	0.884	
P53, n		
Wild-type	70	76
Mutant	25	20
P-value	0.398	
K-ras, n		
Wild-type	80	63
Mutant	16	33
P-value	0.008	

APC, adenomatous polyposis coli; RRM2, ribonucleotide reductase M2; p53R2, ribonucleotide reductase regulatory TP53 inducible subunit M2B.

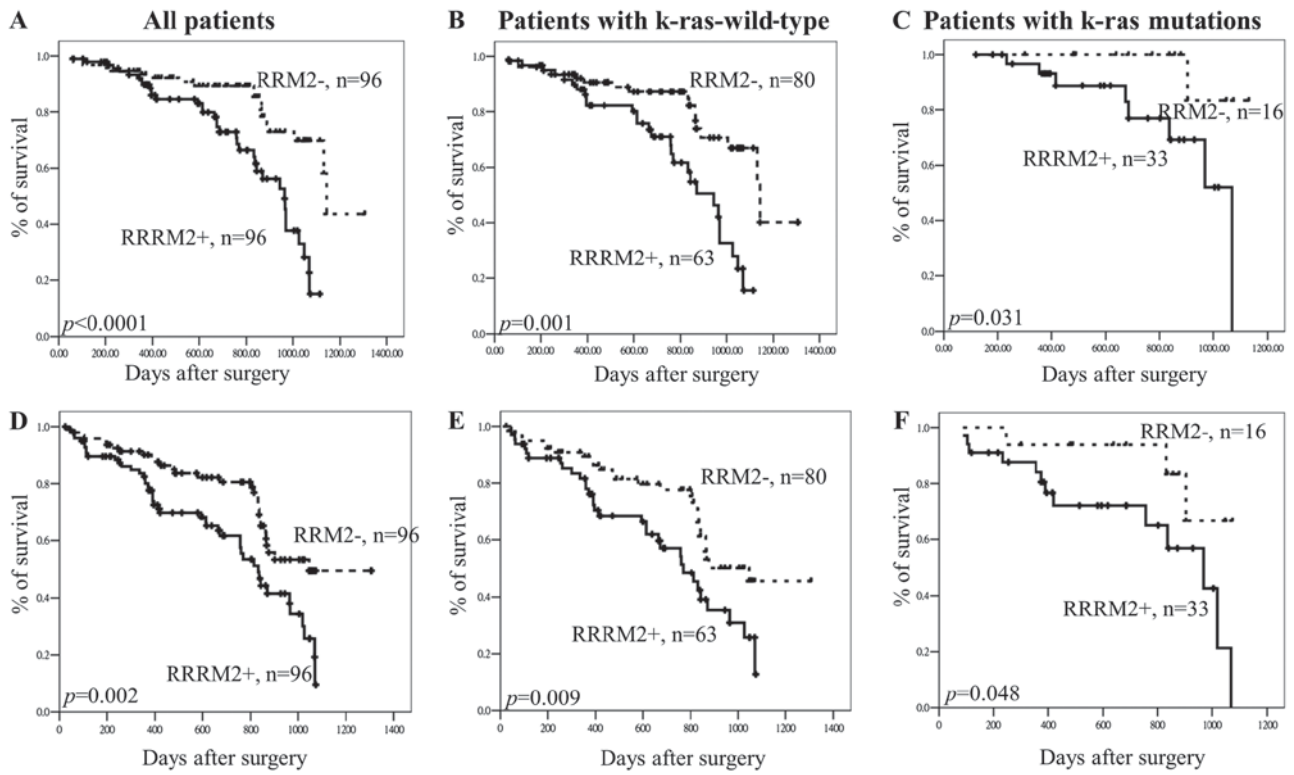

Figure 3. Kaplan-Meier analysis of the effect of *RRM2* on the overall survival curves of (A) all patients, and patients with (B) wild-type *k-ras* gene and (C) mutant *k-ras* gene. The effect of *RRM2* on the disease-free survival curves of (D) all patients, and patients with (E) wild-type *k-ras* gene and (F) mutant *k-ras* gene. RRM2, ribonucleotide reductase regulatory subunit M2.

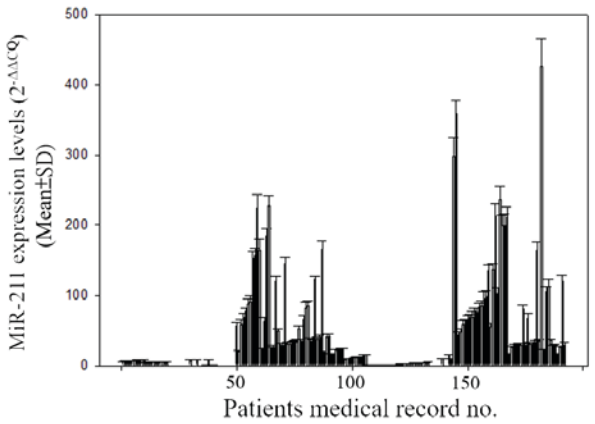

Figure 4. Expression levels of *miR-211* in tumor tissues of patients with colorectal cancer as detected by a TaqMan-based miRNA assay and normalized relative to *U6B* using the $2^{-\Delta\Delta Cq}$ method. miR, miRNA; SD, standard deviation.

and *RRM2* would contribute to tumoral progression and metastasis in CRC and that the expression of *p53R2* and *RRM2* would be associated with OS and DFS in CRC. As indicated by the results of the Kaplan-Meier analysis, *p53R2* protein expression levels exhibited no association with OS or DFS in CRC (OS, P=0.488; DFS, P=0.915 for; Fig. 2). However, the results of the Kaplan-Meier analysis demonstrated that *RRM2* protein expression was associated with OS and DFS in patients with CRC (OS, P<0.0001; DFS, P=0.002; Fig. 2). Patients with low *RRM2* expression had longer OS and DFS compared patients with high *RRM2* expression. Therefore, it is suggested that *RRM2* expression has potential to be a prognostic and tumoral recurrence biomarker in patients with CRC.

RRM2 expression is associated with the k-ras gene mutation but not with p53/APC gene mutations in patients with CRC. The associations between *p53R2* and *RRM2* expression and gene mutations (*APC, p53* and *k-ras*) in patients with CRC were further analyzed. As indicated in Table II, the expression of *p53R2* and *RRM2* was not associated with *APC* or *P53* gene mutations in tumors of patients with CRC. A positive association was observed between *RRM2* protein expression and the *k-ras* gene mutation. However, there was no association between *p53R2* expression and the *k-ras* mutation. In addition, the expression of *RRM2* was higher in patients with the *k-ras* gene mutation compared with patients with wild-type *k-ras* (P=0.008; Table II).

Effect of RRM2 expression on OS and DFS of patients with CRC according to k-ras status. The results indicated that *RRM2* expression was associated with the *k-ras* gene mutation. Therefore, it was hypothesized that the effects of *RRM2* expression on OS or DFS in CRC would differ among patients, depending on the presence or absence of *k-ras* mutations. The results of the Kaplan-Meier analysis indicated that *RRM2* expression was associated with clinical outcomes in patients with wild-type *k-ras* (P=0.001) and those with *k-ras* gene mutations (P=0.031) (all patients, P<0.0001; Fig. 3A-C). The association between *RRM2* expression and DFS in patients with and without *k-ras* mutations was also analyzed. The

results revealed that *hRRM2* expression was associated with overall DFS in patients with wild-type *k-ras* (P=0.009) and in those with the *k-ras* gene mutation (P=0.048) (all patients, P=0.002; Fig. 3D-F). Therefore, it is suggested that *RRM2* is not associated with the *k-ras* gene status but that *RRM2* expression may have potential as a prognostic and recurrence marker in patients with CRC.

miR-211 expression negatively regulates RRM2 expression in patients with CRC and k-ras gene mutations. The association of *miR-211* with *RRM2* expression in tumor tissue samples of patients with CRC was analyzed. The levels of *miR-211* expression in tumor tissue samples from patients with CRC are illustrated in Fig. 4. As indicated in Table III, the level of *miR-211* was significantly negatively associated with *RRM2* protein expression in patients with CRC and *k-ras* gene mutation (P<0.0001) but not in patients with CRC and wild-type *k-ras* gene (P=0.634). In addition, *miR-211* expression was negatively associated with lymph node metastasis, distant metastasis, and cancer stage (all P<0.0001; Table III). Patients with lymph node metastasis, distant metastasis, and late-stage disease had lower *miR-211* expression compared with those with early-stage disease and without metastasis (Table III).

Effect of miR-211 expression on OS and DFS in CRC. It was hypothesized that *miR-211* expression would be associated with clinical outcomes in patients with CRC, depending on the *k-ras* gene status of the patient. As indicated by the results of the Kaplan-Meier analysis, there was no association between OS and *miR-211* expression in overall patients (P=0.488; Fig. 5A) and patients with the wild-type *k-ras* gene (P=0.400; Fig. 5B), but had effects in patients with k-ras gene mutations (P=0.003, Fig. 5C). In addition, there was no association between *miR-211* and DFS in all patients (P=0.255; Fig. 5D) and patients with the wild-type *k-ras* gene (P=0.744; Fig. 5E). However, *miR-211* expression had effects on OS and DFS in patients with CRC and *k-ras* gene mutations (Fig. 5C and F), patients with high *miR-211* expression having longer OS and DFS compared with patients with low *miR-211* expression (OS, P=0.003; Fig. 5C; DFS, P=0.004; Fig. 5F).

The effects of miR-211 and RRM2 expression on OS and DFS were analyzed using the Kaplan-Meier method is presented in Fig. 6. The results indicated that patients with CRC and high *RRM2*/low *miR-211* expression and high *RRM2*/high *miR-211* expression had significantly poorer OS (P<0.0001; Fig. 6A) and DFS (P=0.015; Fig. 6B) compared with those with low *RRM2*/high *miR-211* expression and low *RRM2*/low *miR-211* expression. A shown by the results of the Cox regression analysis, patients with late-stage disease and high *RRM2* expression had a higher hazard ratio compared with patients with early-stage disease and low *RRM2* expression (Table IV). *miR-211* expression and the presence of the *k-ras* mutation showed no significant different with overall survival of patients with CRC. These observations demonstrated that the expression of *RRM2* in tumor tissues rather than the expression of *miR-211* could be used as an independent biomarker to predict OS in CRC (Table IV). It is suggested that a combination of *miR-211* and *RRM2* expression could be used as a prognostic and tumoral recurrence marker only in patients with CRC and the *k-ras* gene mutation.

Table III. Association of *miR-211* expression levels and clinical parameters in tumor tissues of patients with colorectal cancer.

Parameters	*miR-211* expression (Mean ± SD)	P-value
Age, years		
≤65	54.18±68.41	0.501
>65	48.23±71.53	
Sex		
Female	53.63±77.48	0.769
Male	48.94±62.76	
T factor		
1	52.89±65.55	0.055
2	84.07±93.65	
3	44.29±62.90	
4	47.29±66.46	
N factor		
0	70.58±77.04	<0.0001
1+2	36.34±60.20	
M factor		
0	58.26±73.51	<0.0001
1	16.83±31.86	
Stage		
I	95.22±102.20	<0.0001
II	75.37±76.91	
III	37.55±53.40	
IV	16.33±31.50	
RRM2 expression		
Overall		
-	56.39±69.67	0.087
+	45.90±70.10	
K-ras wild-type		
-	43.45±60.84	0.634
+	52.91±74.54	
K-ras mutation		
-	121.08±76.77	<0.0001
+	32.50±59.50	

miR, miRNA; RRM2, ribonucleotide reductase M2; SD, standard deviation.

Discussion

Previous reports demonstrated that patients with high *RRM2* expression had poor prognoses and tumoral recurrence in several types of cancer, including hepatocellular cancer, prostate cancer, pancreatic cancer, CRC and lung cancer (9,22,24-26). In the present study, it was detected that *RRM2* expression in tumor tissues of patients with CRC and lymph node metastasis was significantly higher compared with patients without lymph

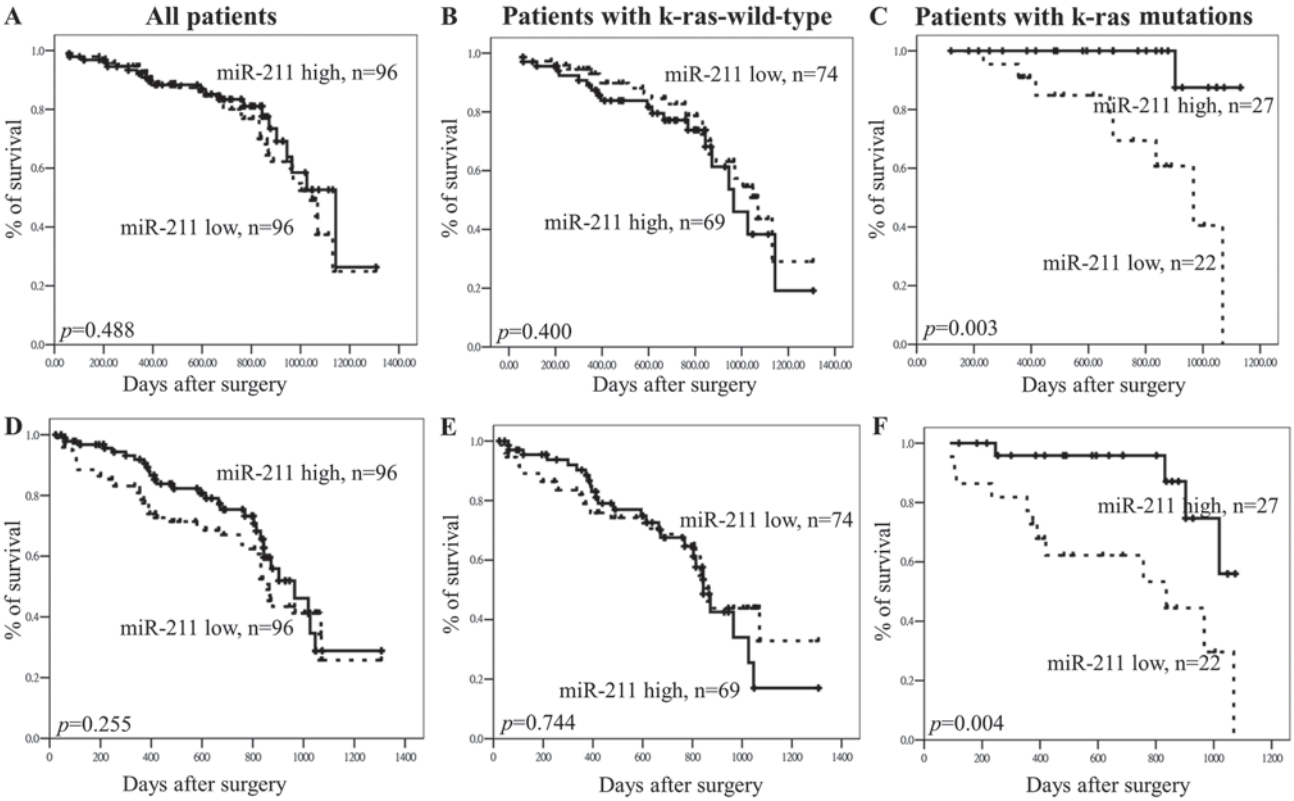

Figure 5. Kaplan-Meier analysis of the effect of *miR-211* on overall survival of (A) all patients, and patients with (B) wild-type *k-ras* gene and (C) mutant *k-ras* gene. The effect of *miR-211* on the disease-free survival curves of (D) all patients, and patients with (E) wild-type *k-ras* gene and (F) mutant *k-ras* gene.

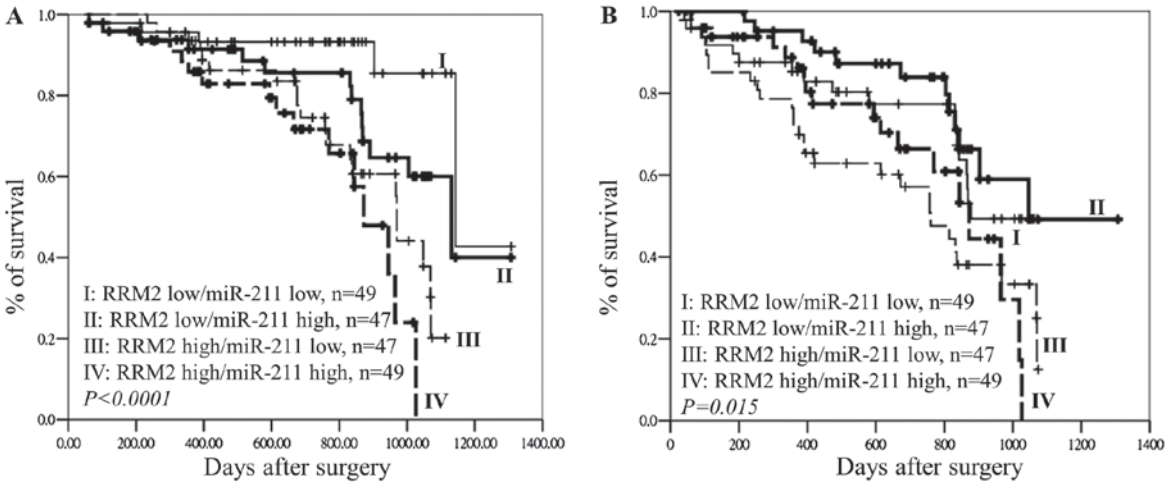

Figure 6. Kaplan-Meier analysis of the effects of a combination of *RRM2* and *miR-211* expression on the overall survival (A) and disease free survival (B) of patients with colorectal cancer. miR, miRNA; p53R2, ribonucleotide reductase regulatory TP53 inducible subunit M2B; RRM2, ribonucleotide reductase regulatory subunit M2.

node metastasis (Table I). In addition, patients with high *RRM2* expression in tumors had poor DFS (Fig. 2). A previous study by Maftouh *et al* (27) on SUIT2-007 and SUIT2-028, subclones of a human pancreatic adenocarcinoma cell line (SUIT-2), reported that *miR-211* targeted *RRM2* and modulated its sensitivity to gemcitabine. In the present study, the expression of *miR-211* was negatively associated with *RRM2* expression in tumor tissues of patients with CRC and *k-ras* gene mutation (Table III). Tumoral recurrence was lower in patients with CRC and *k-ras* mutation and high *miR-211* expression compared with patients with the *k-ras* mutation and low *miR-211* expression. Therefore, it was proposed that the level of *RRM2* expression and upstream expression of *miR-211* in tumor tissues of patients with CRC may be useful biomarkers to predict tumoral metastasis and tumoral recurrence, particularly in patients with the *k-ras* gene mutation.

A previous study reported that *RRM2* cooperated with a variety of oncogenes to promote cell transformation and tumorigenesis in cell model experiments (28). In addition, human and mouse cell models demonstrated that *RRM2* played a critical role in enhancing the invasive potential of

Table IV. Multivariate Cox regression analysis of the combined effects of *RRM2*, *miR-211*, distant metastasis, tumor stage and *k-ras* gene mutation on the overall survival of patients with colorectal cancer.

Parameters	HR	95% CI	P-value
Tumor stage			
Early/late	3.008	1.358-6.662	0.007
Distant metastasis			
No/yes	1.233	0.669-2.273	0.501
K-ras mutation			
No/yes	0.503	0.242-1.043	0.065
RRM2			
Low/high	2.175	1.186-3.987	0.012
miR-211			
High/low	1.063	0.607-1.864	0.830

CI, confidence interval; HR, hazard ratio; miR, miRNA; RRM2, ribonucleotide reductase M2.

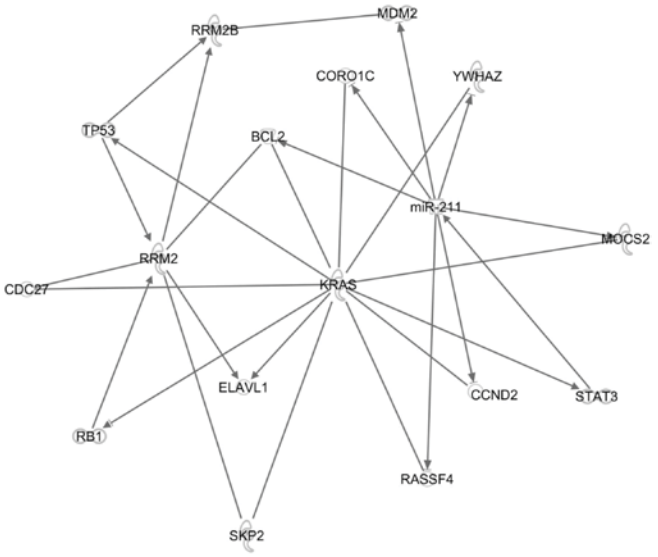

Figure 7. Potential associations between *p53R2*, *RRM2*, *miR-211*, *k-ras* and associated target genes as detected using the Ingenuity Pathways Analysis platform. miR, miRNA; p53R2, ribonucleotide reductase regulatory TP53 inducible subunit M2B; RRM2, ribonucleotide reductase regulatory subunit M2.

tumor cells (28-31). In the present study, *RRM2* expression was higher in lymph nodes invasion groups compared with the group without invasion (Table I). Therefore, it was suggested that *RRM2* expression in patients with CRC may be associated with increased metastasis of tumor cells.

As shown in a previous study, *miR-211* modulated gemcitabine activity and inhibited the invasive ability of cancer cells by negatively regulating the expression of *RRM2* (27). The downregulation of *let-7a* and *miR-211* was associated with the overexpression of *k-ras* in 9, 10-dimethyl-1,2-benz [a]anthracene-induced mouse skin tumorigenesis (32). In the present study, the expression of *miR-211* was negatively associated with *RRM2* expression in tumor tissues of patients with CRC and *k-ras* mutation. Patients with low *miR-211* expression and high *RRM2* expression had poor DFS, particularly those with the *k-ras* mutation. Therefore, it was suggested that the *k-ras* gene mutation may be associated with downregulation *of miR-211* and that this results in the overexpression of *RRM2* and the induction of CRC tumorigenesis.

A meta-analysis indicated that the *k-ras* mutation was present in 1,364 of 4,687 (29.10%) patients with CRC (33). This result is similar to the results found in the present study (25.5%; Table II). Previous studies reported an association between *k-ras* gene mutation and clinicopathological characteristics (34-37). However, the association remains unclear, where some reports demonstrated that the *k-ras* gene mutation appeared to be associated with clinical outcomes, while other studies found no evidence to suggest that it could be used to predict clinical outcomes (34-37). The present study demonstrated that patients with low *miR-211* expression had poor DFS, as did patients with the *k-ras* gene mutation. By contrast, the wild-type *k-ras* gene was not associated with poor DFS. A negative association was detected between *RRM2* and *miR-211* expression in patients with CRC and *k-ras* gene mutation (Table III). These findings indicate that a combination of *k-ras* gene mutation and *miR-211* and *RRM2* expression, may be a useful biomarker to monitor tumoral recurrence in CRC. However, *k-ras* alone cannot be used as a biomarker.

In the present study, the IPA platform was used to investigate the associations between *RRM2, p53R2,* (*RRM2B*), *k-ras* and *miR-211*. The platform can reveal molecular interactions between these genes based on data recorded in the Ingenuity Knowledge Base. The Path Explore tool in IPA was used to identify interactions between the molecules (Fig. 7). The following 13 interacting proteins were identified: Transcription regulators (n=4), enzymes (n=3), transporters (n=1) and proteins (n=5) with other functions. One of the proteins identified, *Bcl2* is an apoptosis regulator, and it plays a central role by interacting with *RRM2*, *k-ras* and *miR-211*. The associations of these 13 target genes (Fig. 7) and the clinical application of these genes need to be examined in future studies.

In conclusion, it was detected that *RRM2* and *p53R2* protein expression was associated with lymph node and distant metastasis in CRC. Additionally, the expression level of *RRM2* was regulated by *miR-211*. The protein expression of *miR-211* and *RRM2* but not that of *p53R2* could be used to monitor metastasis, OS, and DFS in CRC, particularly in patients with CRC and *k-ras* gene mutation. The present authors propose that the activation of the *k-ras* gene may downregulate the expression of *mir-211*, resulting in *RRM2* overexpression and the induction of tumoral recurrence in patients with CRC and *k-ras* gene mutation.

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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

YWC designed the study and wrote the paper. YWC, CCC, KTY, CCH and NYH designed the experiments, wrote the paper, and prepared the figures. CCL, CHW, KTY, PLW and CCH collected the colorectal tumor samples and clinical data. TWK evaluated the immunohistochemistry results. KCH analyzed the molecular pathway using the Ingenuity Pathways Analysis platform. All the authors gave their approval for the manuscript to be submitted for publication.

Ethics approval and consent to participate

The acquisition of the samples and their subsequent examination were approved by the Institutional Review Board of Taipei Medical University (Taipei, Taiwan). Informed written consent was obtained from all the patients and/or guardians prior to the use of the resected specimens.

Consent for publication

All identifying information is removed.

Competing interests

The authors declare that they have no competing interests.

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