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Overexpression of RalBP1 in colorectal cancer is an independent predictor of poor survival and early tumor relapse

Nathan M. Mollberg,[1,2,†,*] Gunnar Steinert,[2,†] Maximillian Aigner,[2] Alexander Hamm,[2] Fang-Ju Lin,[3] Heike Elbers,[2] Christoph Reissfelder,[2] Jürgen Weitz,[2] Markus W. Büchler[2] and Moritz Koch[2]

[1]Department of Surgery; University of Illinois at Mount Sinai Hospital; Chicago, IL USA; [2]Department of General, Visceral and Transplantation Surgery; University of Heidelberg; Heidelberg, Germany; [3]Department of Biostatistics; University of Illinois at Chicago; Chicago, IL USA

[†]These authors contributed equally to this work.

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The non-ABC transport protein RalBP1 has been shown to be overexpressed in various cancer cell lines and implicated in the process of metastasis formation, but its expression in tissue samples and prognostic significance has not been shown. In this study, matched tumor-mucosa tissue samples from 78 CRC patients were investigated. The RalBP1 mRNA and protein levels were quantified by real-time quantitative PCR (qPCR) and ELISA. RalBP1 was found to be overexpressed in tumor at the mRNA level both overall ($p = 0.027$), and for stages I ($p = 0.024$), II ($p = 0.038$) and IV ($p = 0.004$). At the protein level, RalBP1 was only significantly overexpressed in stage IV patients ($p = 0.018$). Expression of RalBP1 mRNA and protein were inversely correlated ($r = 0.4173$; $p = 0.0004$). Multivariate Cox regression analysis including sex, age, stage, grade and nodal status as covariates showed that overexpression of RalBP1 protein, but not mRNA, was an independent predictor of both decreased disease free survival ($p = 0.016$, RR = 6.892) and overall survival ($p = 0.039$, RR = 5.986). These results suggest that RalBP1 protein is an independent predictor of poor survival and early relapse for CRC patients. Owing to its multifunctional intermediary role in cell survival, chemotherapeutic resistance and metastasis formation, RalBP1 represents a promising novel therapeutic target.

Introduction

RalA and RalB are nearly identical proteins (85% amino acid identity) within the Ras family of monomeric G proteins which, in addition to normal cellular functions, contribute to cancer cell migration, chemotherapy resistance, invasion and metastasis.[1-6] The best-characterized Ral effector is Ral-binding protein (RalBP1), whose association with Ral-mediated tumorigenesis has previously been suggested.[7] RalBP1 is a multifunctional membrane protein that has been implicated in cancer cell proliferation,[8] radiation and chemoresistance,[9-13] and ligand dependent receptor internalization.[14-16] Recent studies have shown that overexpression of RalBP1 enhanced migration and invasion of fibrosarcoma cells,[17] whereas depletion inhibited tumor growth and metastasis formation in prostate and bladder metastasis models.[8] Depletion or inhibition of RalBP1 increased apoptosis in cultured cells from various malignancies, such as small cell lung cancer, non-small cell lung cancer, melanoma, ovarian cancer, prostate cancer, lymphoma and myeloid leukemia.[18] Xenograft models in which RalBP1 was depleted by antisense caused regression of commercial cell lines of lung and colon cancer.[21] In summary, RalBP1 may be a promising therapeutic target for cancer therapy owing to its intermediary signaling role in affecting cellular migration, metastasis formation and chemotherapeutic resistance. The objective of the present study is to establish the relative baseline RalBP1 expression levels in matched human CRC and normal mucosa tissue.

Results

Quantitative RT-PCR analysis for RalBP1 mRNA expression. Quantitative RT-PCR (qPCR) analysis showed mRNA expression for RalBP1 in all tumor and mucosa samples; however, marked differences in mRNA levels were seen between normal mucosa and tumor samples for RalBP1 (**Fig. 1**). Pairwise comparison demonstrated that 70.0% (53/76) overall, 82.4% (14/17) in stage I, 63.6% (14/22) in stage II, 54.5% (12/22) in stage III and 86.7% (13/15) of patients in stage IV overexpressed RalBP1 in tumor as compared with mucosa. Significantly higher RalBP1 mRNA levels were observed in tumor than in normal mucosa for patients in UICC stage I ($p = 0.024$), stage II ($p = 0.038$) and stage IV ($p = 0.004$). When grouping all stages, RalBP1 mRNA was significantly overexpressed in tumor when compared with normal mucosa ($p = 0.027$).

*Correspondence to: Nathan Mollberg; Email: nathan.mollberg@gmail.com
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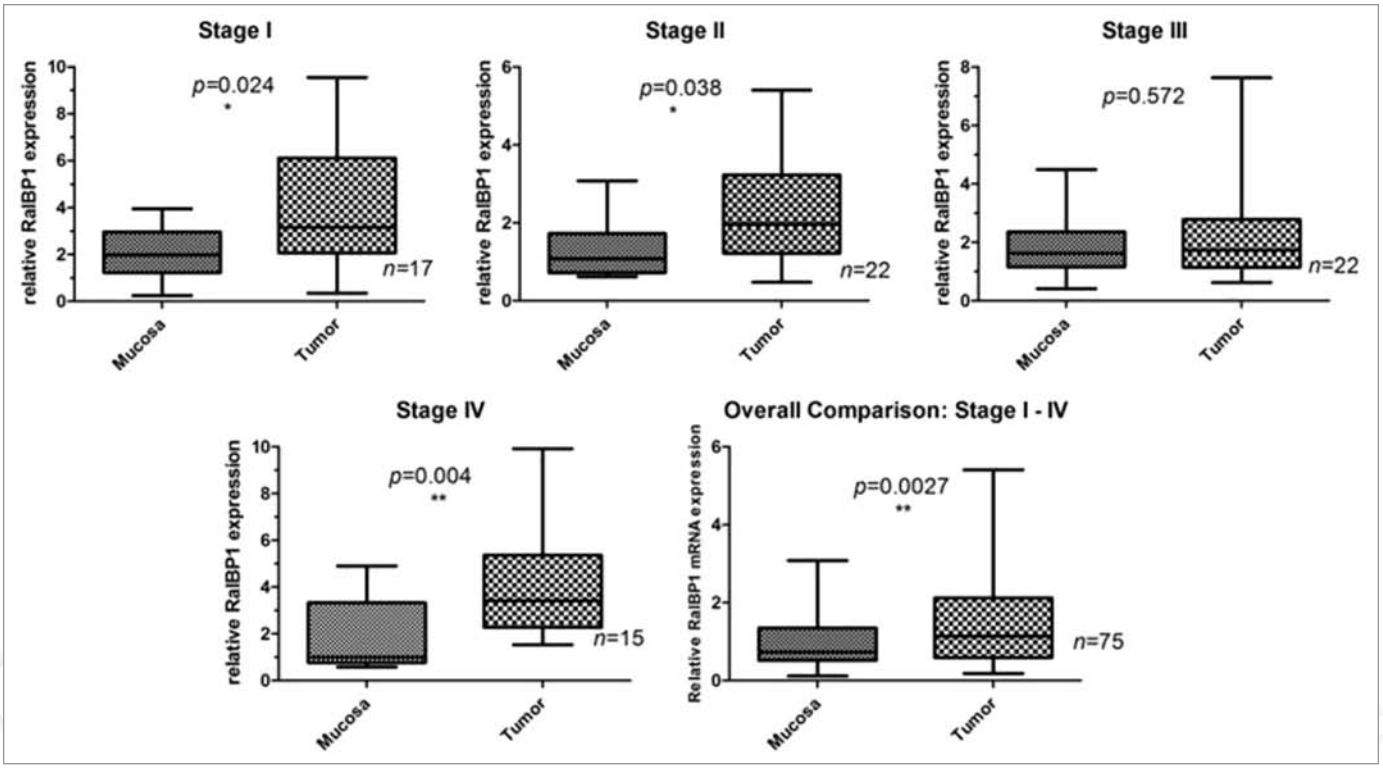

Figure 1. Summary of RalBP1 mRNA expression in normal mucosa and tumor by both stage and overall. mRNA levels are expressed as a relative ratio according to the Equation $2^{-\Delta\Delta CT}$.

ELISA analysis for RalBP1 protein expression. All of the tumor and mucosa samples were found to be positive for RalBP1 measured by ELISA. Pairwise comparison demonstrated that 50.0% (37/74) overall, 17.6% (3/17) in stage I, 37.5% (9/24) in stage II, 54.5% (12/22) in stage III and 100% (13/13) of patients in stage IV overexpressed RalBP1 in tumor as compared with mucosa. Significantly higher RalBP1 protein levels were observed in tumor than in normal mucosa only for stage IV patients ($p = 0.018$, **Fig. 2**).

RalBP1 expression relationship. By crosstable calculation (χ^2-test), we found RalBP1 protein overexpression to have a significant correlation with nodal status ($p = 0.044$), UICC stage ($p < 0.001$), recurrence ($p < 0.001$) and death ($p = 0.001$, **Table 1**). There were no significant correlations between RalBP1 mRNA overexpression and any of the clinicopathologic parameters.

Spearman correlation coefficient analysis showed a statistically significant direct correlation between mRNA Cp (crossing point) values and tissue protein levels for both tumor ($r = 0.4173$; $p = 0.0004$) and mucosa ($r = 0.2933$; $p = 0.0160$).

Kaplan-Meier survival curves. Survival curves were compared via the log rank test, and the corresponding p values are printed on each graph (**Figs. 3 and 4**). Survival probabilities are comparable for patients with and without mRNA overexpression (DFS: $p = 0.595$; OS: $p = 0.490$). Patients with protein overexpression have lower survival probabilities at each time-point (DFS: $p < 0.001$; OS: $p = 0.002$). As expected, more advanced colorectal cancer stages have lower survival rates (DFS: $p < 0.001$; OS: $p < 0.001$). When analyzing prognostic factors for disease-free and OS, univariate analysis using the log-rank test confirmed well-known prognostic parameters such as lymph node status ($p < 0.001$), UICC tumor stage ($p < 0.001$) and grade ($p = 0.026$, **Table 2**) to be of prognostic relevance in our patient cohort. Regarding RalBP1 expression, median DFS and OS were significantly reduced in tumors with protein overexpression. In a multivariate analysis based on the Cox proportional hazards regression model, we tested the independent predictive value for all relevant clinical and pathological parameters and RalBP1 expression. Lymph node status was excluded for its linear depending covariance with tumor stage. The group of patients who overexpressed RalBP1 protein expression had significantly worse DFS ($p = 0.016$, HR = 6.892) and OS ($p = 0.039$, HR = 5.986) at each time point, whereas mRNA expression had no effect on DFS or OS (**Table 3**).

Discussion

Multivariate analysis found that overexpression of RalBP1 protein was an independent predictor of both decreased DFS and OS in CRC patients. To our knowledge, our results are the first to show that RalBP1 protein expression provides prognostic information for CRC patients. From a functional point of view, these results could be explained by the intermediary signaling role that RalBP1 plays in metastasis formation, and its protective role against oxidative stress metabolites and xenobiotics.

A number of studies have demonstrated the role of RalBP1 in cellular migration and metastasis. Wu et al. showed that RalBP1

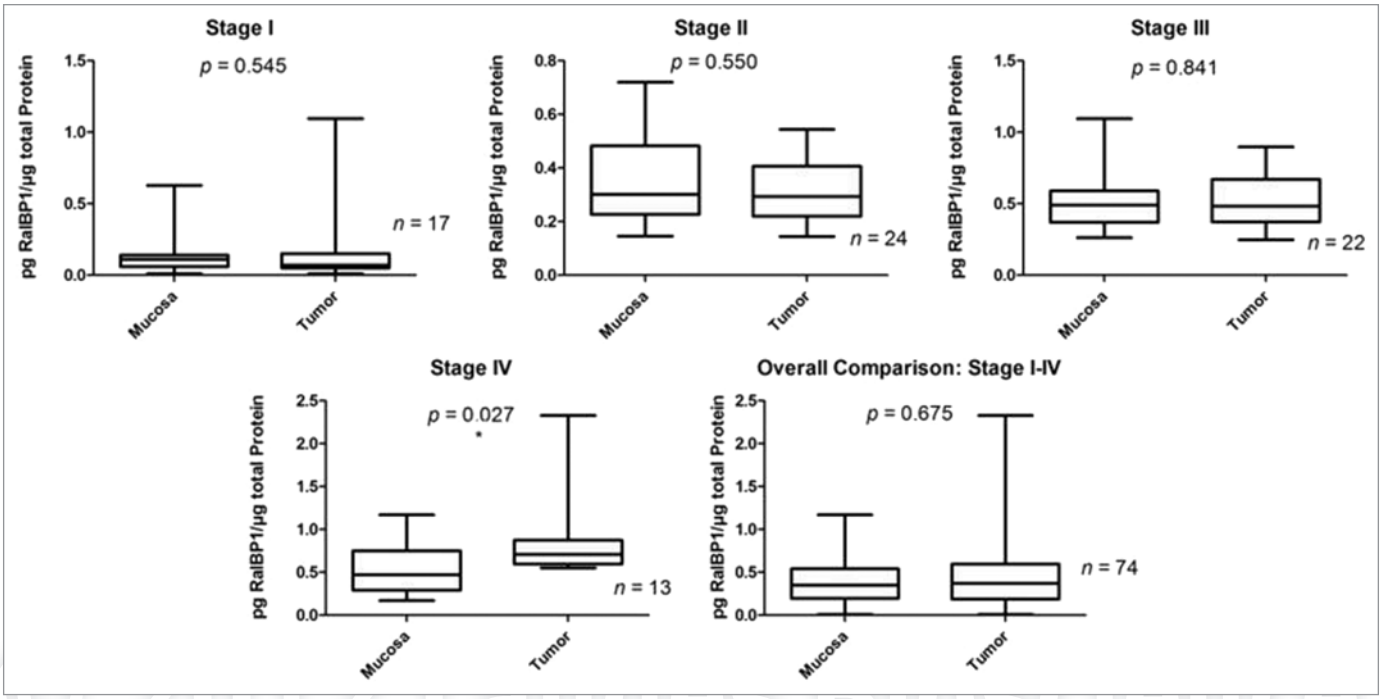

Figure 2. Summary of RalBP1 protein expression in normal mucosa and tumor by both stage and overall by ELISA method.

depletion not only inhibited cell migration, but also inhibited metastasis formation in an experimental metastasis model of bladder cancer.[8] RalBP1 has also been shown to act upon CDK1 (cdc42), a protein known to be involved in regulating cell cycle progression and migration.[24] After binding to CDK1, RalBP1 dissociates from the cell membrane to act as a motor for spindle movement at the mitotic spindle.[25,26] Our data demonstrated a significant correlation with RalBP1 protein overexpression, increasing stage and nodal positivity; providing further evidence for the role of RalBP1 in invasion and metastasis formation.

RalBP1 is an ATP-dependent non-ABC transporter which actively transports chemotherapeutic agents, in addition to anionic metabolites like glutathione-conjugates of electrophiles (GS-E).[32,33] Studies performed in RalBP1$^{-/-}$ mice showed a significant increase in the concentration of aldehydes, lipid hydroperoxides and alkenals in tissues as a consequence of RalBP1 loss.[33] This loss translates into greater sensitivity to xenobiotic toxins including traditional chemotherapeutic agents, which are substrates of RalBP1, as well as other alkylating agents and platinum-coordinates that are metabolized to GS-E. Induction of RalBP1 thus results in cellular resistance to apoptosis by metabolizing and excluding stress metabolites at a higher rate.[37]

Although RalBP1 mRNA was overexpressed in tumor vs. mucosa, it was not a significant predictor of either disease-free or overall survival. Indeed, there was an inverse correlation demonstrated between RalBP1 mRNA and protein expression. Furthermore, as RalBP1 mRNA transcript amounts decreased with increasing stage (data not shown), RalBP1 protein amounts increased as stage increased. Oncogenes have been demonstrated to co-opt the translational process during transformation, and interestingly RalBP1 has been linked to translational regulation of some mRNAs.[35] Panner et al. demonstrated that RalA binding of RalBP1 induced suppression of S6 kinase and the translation of the antiapoptotic FLICE-like inhibitory protein. Further research will be needed to determine the mechanism by which the translation of RalBP1 begins to occur at an accelerated rate during tumorigenesis.

In conclusion, overexpression of the RalBP1 protein is an independent predictor of poor survival and early relapse for CRC patients. Owing to its multifunctional intermediary role in cell survival, chemotherapeutic resistance and metastasis formation, RalBP1 represents a promising novel therapeutic target.

Materials and Methods

Patients and samples. Matched tissue samples (histologically proven) of normal colon mucosa and tumor were obtained from the surgical specimens of 78 patients with curatively resected (R0) primary sporadic colon adenocarcinomas treated between February 2004 and April 2007 at the Department of Surgery, University of Heidelberg in accordance with the ethics committee. Tissue samples were frozen in liquid nitrogen immediately after surgical removal and maintained at 80°C until RNA extraction. Clinical and pathological data were documented prospectively, and entered into a specific tumor registry at the time of surgery and at each follow-up. The patients' ages ranged from 39 to 90 y (mean age, 65 y). Median follow-up time of patients alive at last follow-up was 35 mo.The tumors were staged according to the Unio Internationalis Contra Cancrum (UICC) system with the following distribution: 21.8% stage I (17/78), 30.8% stage II (24/78), 28.2% stage III (22/78) and 19.2% stage IV (15/78) patients.

Table 1. Correlation between RalBP1 expression and clinical and pathologic parameters (χ^2-test)

Characteristics	RalBP1 mRNA			RalBP1 Protein		
	Overexpressed	Not overexpressed	p value	Overexpressed	Not overexpressed	p value
Gender			0.512			0.876
Male	31	16		22	23	
Female	22	7		14	12	
Age			0.454			0.701
> Mean	28	15		20	22	
≤ Mean	25	8		16	13	
Lymph node status			0.355			**0.044**
N0	31	11		15	26	
N1	12	4		11	5	
N2	9	8		9	4	
N3	1	0		1	0	
Differentiation status			0.791			0.305
Well	1	1		0	2	
Moderate	36	16		23	23	
Poor	16	6		13	10	
UICC Stage			0.207			**< 0.001**
I	14	3		3	12	
II	14	8		9	14	
III	12	10		12	9	
IV	13	2		12	0	
Recurrence[a]			0.633			**< 0.001**
Yes	16	5		18	2	
No	37	18		18	33	
Death[a]			0.536			**0.001**
Yes	42	16		21	33	
No	11	7		15	2	

UICC, Unio Internationalis Contra Cancrum; statistically significant p values are highlighted in bold; [a]recurrence or death at any time.

RNA extraction and qPCR. Fresh tumor and mucosa tissue were frozen in RNAlater Reagent (Invitrogen) and stored at -80°C. Approximately 20 mg of tissue was used for each RNA extraction. SV total RNA isolation (Promega) was performed, followed by ImProm-II reverse transcription (Promega) for cDNA synthesis. The cDNA was amplified with real-time quantitative polymerase chain reaction (qPCR) and was performed with the LightCycler 2.0 Real-Time PCR system (Roche Applied Science). qPCR conditions for RalBP1 were as follows: initial denaturation for 5 min at 95°C, 35 cycles (95°C for 30 sec, annealing at 55°C for 45 sec, 30 sec at 72°C), followed by 15 min at 72°C; incubated with appropriate forward (5'-TCT ATA GTG CTC AGC CCA AC-3') and reverse (5'-ATC GCA GAG GTT TCA TCA C-3') primers (Invitrogen). cDNA was also amplified with β-actin primers (*ACTB*) forward (5'-ATG TGG CCG AGG ACT TTG ATT-3') and reverse (5'-AGT GGG GTG GTT TTA GGA TG-3') (Invitrogen), serving as an internal control. Threshold cycle (Ct) and melting curves were acquired. Only genes with clear and single melting peaks were taken for further data analysis. Samples with irregular melting peaks were excluded from the calculation. The threshold was set manually, using identical threshold levels for one gene in all analyzed samples. Reaction efficiency was established for each set of primers, after quantification of four different dilutions of a reference cDNA.

Analysis of RalBP1 expression using the $2^{-\Delta\Delta Ct}$ method. Details of the $2^{-\Delta\Delta Ct}$ method have been previously described in reference 23 and 24. Briefly, the mean target gene mRNA expression level for the three mRNA measurements was calculated. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. In the present study, the data are presented as the fold change in target genes RalBP1 expression in tumors normalized to the internal control gene (*ACTB*) and relative to the normal control (matched normal as calibrator). Results of the qPCR data were represented as threshold cycle (Ct) values, where Ct was defined as the threshold cycle number of PCR at which amplified product was first detected. There is an inverse correlation between Ct and amount of target: lower amounts of target correspond to a higher Ct value. The average Ct was calculated for both the target genes and *ACTB* and the ΔCt was determined as (the mean of the triplicate Ct

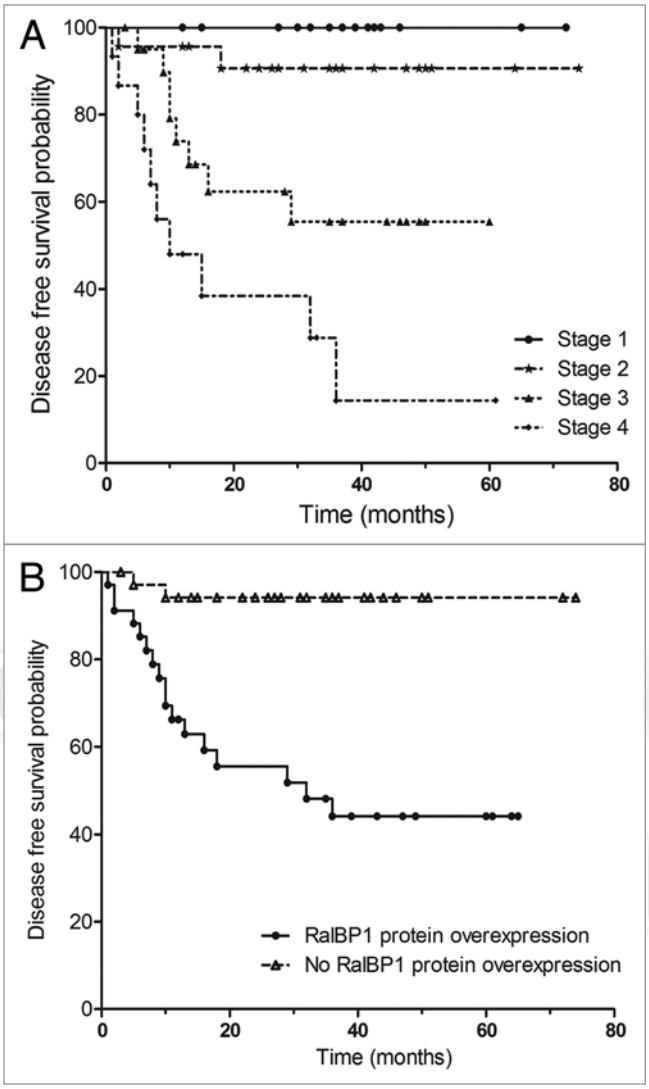

Figure 3. Univariate analysis (log-rank test, Kaplan-Meier curves) of statistically significant prognostic parameters for disease free survival in colorectal cancer. (A) Stage; (B) RalBP1 protein overexpression.

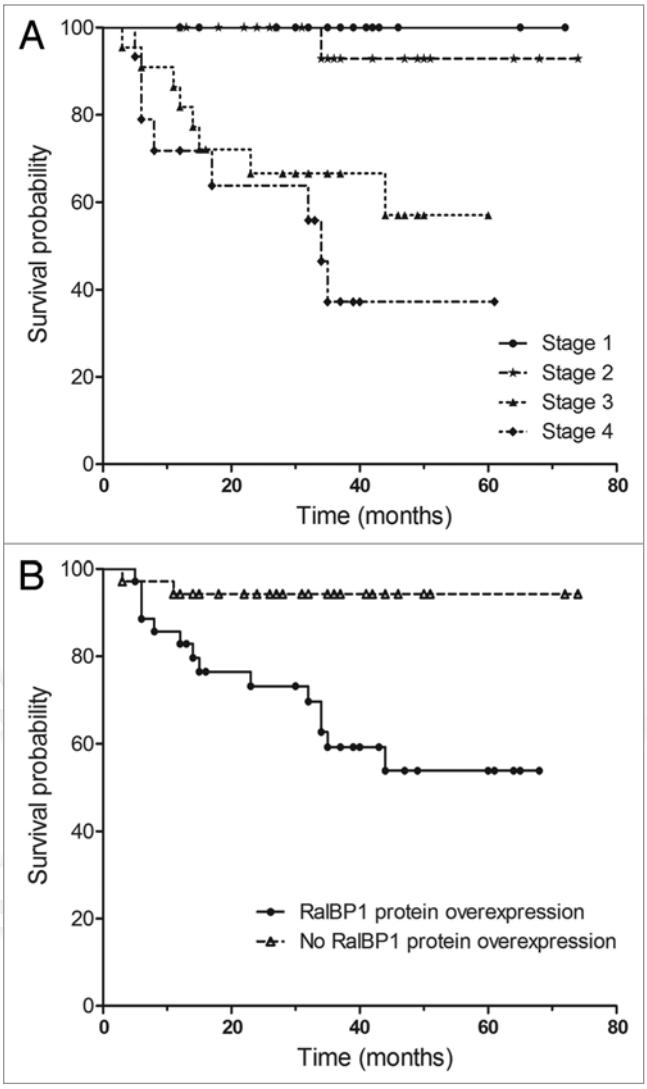

Figure 4. Univariate analysis (log-rank test, Kaplan-Meier curves) of statistically significant prognostic parameters for overall survival in colorectal cancer. (A) Stage; (B) RalBP1 protein overexpression.

values for the target gene) minus (the mean of the triplicate Ct values for *ACTB*). The ΔΔCt represented the difference between the paired tissue samples, as calculated by the formula ΔΔCt = (ΔCt of tumor - ΔCT of normal). The differential expression in the target gene of a tumor sample compared with the normal counterpart was expressed as $2^{-\Delta\Delta Ct}$.[20,21]

Protein extraction and ELISA. Tissue extracts were prepared from frozen tissues by a standard extraction protocol.[22] The protein content of cell lysates and tissue extracts was determined using the Lowry protein assay (Sigma). The enzyme-linked immunosorbent assay (ELISA)Kit for human RalBP1 was purchased from USCN Life Science, Inc. (E97265Hu), and performed as indicated by the manual. The standard was reconstituted and a dilution series was made with the standard serving as a blank. The blank and samples were loaded and incubated for 2 h at 37°C. Every item of the standard dilution series, the blank and samples were loaded as duplicates. The liquid of each well was removed and a detection reagent was added and incubated for 1 h at 37°C. The liquids were removed and washed (Wash Solution and aqua dest) three times after the incubation was finished. A second detection reagent was added to each well and incubated for 30 min at 37°C, after which the samples were washed five times as described above. The detection procedure continued by adding 90 µl Substrate Solution to each well and incubation for 15–20 min at 37°C. The microplate was immediately measured by 450 nm in a standard ELISA microplate reader.

Statistical analysis. All statistical analyses were performed using Statistical Analysis Systems (SAS) (SAS Corp., NC). The t-test was used to compare all continuous parameters from normal vs. tumor samples, and the χ^2 test was used for comparison of discrete parameters. The relationship between RalBP1 protein and mRNA expression levels within the same samples

Table 2. Univariate analysis (log-rank test) of prognostic parameters in colorectal cancer for progression-free survival and overall survival

Characteristics	Cases	Progression free survival		Overall survival	
		Time (months)	p value	Time (months)	p value
Gender			0.921		0.947
Male	48	38.4		29.2	
Female	30	30.0		23.6	
Age			0.062		0.375
> Mean	45	34.2		12.9	
≤ Mean	33	31.2		30.0	
Lymph node status			**< 0.001**		**< 0.001**
Negative	44	33.3		30.3	
Positive	34	31.0		22.0	
Differentiation status			**0.025**		0.120
Well/Moderate	55			27.3	
Poor	23			25.7	
UICC stage[a]			**< 0.001**		**< 0.001**
II	24	34.0		17.3	
III	22	33.4		21.1	
IV	15	25.1		17.9	
RalBP1 mRNA			0.490		0.595
Overexpressed	53	38.0		28.6	
Normal expression	23	26.5		14.4	
RalBP1 Protein			**0.002**		**< 0.001**
Overexpressed	36	10.7		9.9	
Normal expression	35	33.7		22.8	

UICC, Unio Internationalis Contra Cancrum; [a]there were no stage I patients who experienced either recurrence or death at time of last follow-up; statistically significant p values are highlighted in bold.

Table 3. Multivariate analysis (cox proportional hazards regression model) of prognostic parameters in colorectal cancer for progression-free survival and overall survival

	Progression Free Survival			Overall Survival		
Parameter	**Hazard ratio**	**95% CI**	**p value**	**Hazard ratio**	**95% CI**	**p value**
Gender	0.566	0.217–1.476	0.2441	0.491	0.172–1.406	0.1853
Age	0.998	0.957–1.040	0.9207	0.965	0.921–1.011	0.1301
Differentiation status	0.825	0.318–2.138	0.6921	0.582	0.204–1.656	0.3104
UICC stage	8.811	1.847–42.047	**0.0063**	17.348	1.953–33.125	**0.0105**
RalBP1 mRNA expression	0.709	0.233–2.160	0.5449	0.276	0.083–0.920	0.0797
RalBP1 protein expression	6.892	1.429–33.249	**0.0162**	5.986	1.094–32.766	**0.0391**

UICC, Unio Internationalis Contra Cancrum; CI, confidence intervals; statistically significant p values are highlighted in bold.

was examined using the Spearman correlation coefficient analysis. The Kaplan-Meier method was used to estimate the survival probability, and the log-rank test was used to compare the survival curves between groups. Independent predictive factors affecting survival were analyzed by the Cox multivariate proportional hazards regression model with stepwise and enter models, respectively. All p values were two-sided and considered statistically significant if $p < 0.05$. Overall survival (OS) was defined as the time interval between the date of surgery and the date of death or last follow-up. Disease free survival (DFS) was defined as the time interval between the date of surgery and the date of disease recurrence or death from any cause, whichever came first, or date of last follow-up evaluation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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