Decreased expression of sestrin 2 predicts unfavorable outcome in colorectal cancer

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Abstract. Sestrin 2 is a conserved antioxidant protein that is involved in p53-dependent antioxidant defenses and protects cells against oxidative stresses. The present study was conducted to examine the expression of sestrin 2 in colorectal cancer (CRC) and investigate a possible relationship between sestrin 2 expression and prognosis in CRC. The expression of sestrin 2 in human CRC tissues and cell lines was evaluated by immunohistochemical or immunofluorescent staining and western blot analysis. The correlations between sestrin 2 expression in human CRC tissues and clinicopathological variables, including overall survival (OS) and disease-free survival (DFS), were analyzed. Both human CRC tissues and cell lines showed a decreased expression of sestrin 2. Furthermore, a low expression of sestrin 2 was significantly correlated with advanced tumor stage, lymphatic invasion, lymph node metastasis, vascular invasion and liver metastasis. Survival analysis showed that patients with low sestrin 2 staining had a significantly worse DFS and OS. Additionally, early or advanced stage CRC patients with a low expression of sestrin 2 had a shorter survival. In univariate analysis, the patients with low sestrin 2 expression, advanced tumor stage, lymphatic invasion, lymphatic node metastasis, vascular invasion, liver metastasis and peritoneal metastasis had shorter OS and DFS. In multivariate analysis, only low sestrin 2 expression, advanced tumor stage, lymphatic node metastasis, vascular invasion and liver metastasis remained as independent prognostic factors of poor OS and DFS. The findings suggested that a decreased expression of sestrin 2 is associated with an unfavorable prognosis, which suggests that it is a novel and crucial predictor for CRC metastasis.

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Introduction

Colorectal cancer (CRC) remains a major cause of cancer worldwide and accounts for approximately 9% of overall cancer incidence (1-2). Although recent advances in chemotherapy have prolonged the survival of patients with advanced disease, the recurrence rates remain high (3). Thus, improved understanding of CRC development may facilitate the identification of molecular targets for therapeutic intervention and improve prognosis of the disease.

Oxidative stress plays a major role in CRC development and progression (4), and results from an excess production of free radicals or insufficient antioxidant defenses. The tumor-suppressor protein p53 has received attention mainly because the gene is mutated and/or inactivated in the majority of human cancers, including CRC (5-6). p53 protein accumulation and activity are induced by genotoxic, oxidative and oncogenic stresses (7). Many p53 target genes have been thoroughly characterized and are involved in its tumor suppressive functions (8). Among these antioxidant genes activated by p53, sestrins are important for the inhibition of reactive oxygen species (ROS) and protection from oxidative stress, transformation and genomic instability (9-10). Sestrins are members of a family of highly conserved antioxidant proteins. Mammalian cells express three members of this family, including sestrin 1, 2 and 3 (11-12). Sestrin 2, transcriptionally regulated by p53, has a cytoprotective function based on regeneration of the overoxidized peroxiredoxins (10), which are supposed to be involved in CRC (13-14). Sestrin 2 emerges as a novel player in autophagy induction and tumor suppression (6,15). Upregulation of sestrin 2 expression via JNK pathway activation contributes to autophagy induction in cancer cells (16). Wang *et al* found that fangchinoline, a novel anti-tumor agent, induced autophagic cell death via p53/sestrin2/AMPK signalling in human hepatocellular carcinoma cells (17). Analysis of gene expression has shown that sestrin 1 and 2 are downregulated in lung cancers of different origin such as large cell carcinoma, adenocarcinoma, squamous cell carcinoma

and small cell lung carcinoma (18-20). It is also reported that sestrin 2 interacted directly with AMPK and mediated sensitization of breast cancer cells to ionizing radiation (21). These studies suggest that the sestrin 2 may be important in tumorigenesis by regulating oxidative stress. More recently, the upregulation of sestrin 2 was found to induce apoptosis through the AMPK/p38 signaling pathway in HT-29 colon cancer cells, which are p53 mutant, treated with quercetin (22). However, the role of sestrin 2 in CRC has to be elucidated.

In the present study, we reported the expression of sestrin 2 in human CRC tissues and cell lines. The correlation between pathological factors and protein expression of sestrin 2 in human CRC tissues was examined, as well as the correlation between protein expression and disease-free survival (DFS) and overall survival (OS). To the best of our knowledge, this study provides the first evidence that sestrin 2 may be involved in CRC.

Materials and methods

Human subjects and clinical data. The parafin-embedded tissue samples of the CRC patients (130 males and 107 females) who underwent surgery between 2004 and 2008 were obtained from the Department of Gastrointestinal Surgery of the following hospitals: The First Affiliated Hospital of Chongqing Medical University and The Chongqing Three Gorges Central Hospital. The patients did not receive chemo- or radiotherapy prior to sample collection. The histological type was independently determined by two pathologists in the study. The paraffin-embedded tissue specimens from 32 normal mucosa, 22 polyp, 30 adenomas (24 cases with mild dysplasia, 4 cases with moderate dysplasia, 2 cases with severe dysplasia) and 26 borderline tissues were used as controls. Demographics (age and gender) and tumor features (differentiation, TNM stage, lymphatic invasion, lymphatic node metastasis, invasion, liver metastasis, peritoneal metastasis and serum CEA) were obtained from clinical and pathological records (Table I). Surgical staging was determined using criteria based on International Union Against Cancer (UICC). DFS was regarded as the interval between the day that surgery was performed and the day that recurrence was identified. If recurrence was not diagnosed, the date the patient succumbed or that of last follow-up was used. OS was regarded as the interval between the dates of surgery and death. After the initial operation for the primary lesion there was a 5-year period for DFS and OS. .

Forty-two fresh CRC tissues as well as 19 normal mucosa, 20 polyp, 22 adenomas (18 cases with mild dysplasia, 3 cases with moderate dysplasia, and 1 case with severe dysplasia) and 26 borderline tissues collected between 2013 and 2014 were immediately placed in a cryovial and stored in liquid nitrogen until subsequent use for western blot analysis. Clinical features of the CRC patients are shown in Table II.

The study was approved by the Medical Ethics Review Committee of the First Affiliated Hospital of Chongqing Medical University. Informed and written consent was obtained from the patients or their relatives for the use of any data and tissues for this study. The study was performed as per the Declaration of Helsinki of the World Medical Association.

Immunohistochemistry. Tissue sections were deparaffinized in xylene, immersed in graded ethanol series, and then incubated in 3% hydrogen peroxide for 15 min. For the antigen retrieval, the sections were heated in a microwave oven for 10 min at 92-98˚C in 10 mmol/l sodium citrate buffer (pH 6.0). Non-specific binding was blocked by incubating the sections with 10% goat serum (Zhongshan Golden Bridge, Beijing, China) in 0.1 M phosphate-buffered saline (PBS) at room temperature for 30 min as described previously (14). The sections were then incubated with primary sestrin 2 antibody (mouse monoclonal antibody; 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; cat. no. sc-101249) overnight at 4˚C followed by incubation with goat anti-mouse antibody (Zhongshan Golden Bridge, Inc., Beijing, China) for 30 min at 37˚C. Sections were then treated with ABC solution (Zhongshan Golden Bridge, Inc.) at 37˚C for 30 min and washed with PBS. Immunoreactivity was detected with 3,3'-diaminobenzidine (DAB, Zhongshan Golden Bridge, Inc.) for 5 min. Counterstaining was performed using hematoxylin. For the negative controls, the primary antibodies were replaced with PBS. A LEICA DM6000B automatic-microscope (Leica, Solms, Germany) was employed for collecting of images.

The cells with buffer stain in the cytoplasm were considered to be positive. Ten random visual field images for each sample were analyzed. Staining intensity was graded on a 0-3 scale as: 0, absence of staining;, 1, weakly stained; 2, moderately stained; and 3, strongly stained. The percentage of positive tumor cells was scored as: 0, absence of positive cells; 1, <33% positive tumor cells; 2, 33-66% positive tumor cells; and 3; >66% positive tumor cells. The staining score, calculated as the staining intensity score multiplied by the percentage score ranged from 0 to 9 (23). Low and high expression was regarded as a staining score of 0-4 and 5-9, respectively. The staining score was evaluated independently by two experienced pathologists. Concordance was achieved when the two pathologists concurred on the same score for a patient. Discordant patient cases were discussed by all the pathologists from the Department of Pathology in Chongqing Medical University to reach a consensus.

Cell lines and culture conditions. The FHC human normal colorectal mucosa cell line and the human CRC HT-29, SW480, SW620 and LoVo cell lines were purchased from the Shanghai Cell Bank at the Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in Leibovitz L-15 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Shanghai, China) and 2% penicillin/streptomycin (Beyotime, Jiangsu, China). The cells were then maintained at 37˚C in a humidified atmosphere.

Western blot analysis. Total proteins extracted from human tissues and cell lines were prepared in lysis buffer (Keygen Biotech, Nanjing, China) consisting of 50 mM Tris (pH 7.4), 1% Triton X-100, and a protease inhibitor mixture supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF). The insoluble material was centrifuged at 12,000 x g for 10 min at 4˚C, and the supernatant was obtained. The protein concentrations were quantified by bicinchoninic acid (BCA)

Table I. Association of sestrin 2 expression with clinicopathological characteristics in 237 CRC patients.

Clinicopathological factors	No. of patients (n=237)	Sestrin 2 expression		P-value
		Low no. (%)	High no. (%)	
Age (years)		171	660.762	
≥65	104	74 (71.2)	30 (28.8)	
<65	133	97 (72.9)	36 (27.1)	
Gender				0.601
Male	130	92 (70.8)	38 (39.2)	
Female	107	79 (73.8)	28 (26.2)	
Tumor site				0.688
Distal	152	111 (73)	41 (27)	
Proximal	85	60 (70.6)	25 (29.4)	
Histology				0.208
Well	96	65 (67.8)	31 (32.2)	
Moderate/poor (mucinous)	141	106 (75.2)	35 (24.8)	
TNM stage				**<0.001**
I/II	78	43 (55.1)	35 (44.9)	
III/IV	159	128 (80.5)	31 (19.5)	
Lymphatic invasion				**0.004**
Yes	149	117 (78.5)	32 (21.5)	
No	88	54 (61.4)	34 (38.6)	
Lymph node metastasis				**0.006**
Yes	106	86 (81.1)	20 (18.9)	
No	131	85 (64.9)	46 (35.1)	
Vascular invasion				**0.012**
Yes	32	29 (90.6)	3 (9.4)	
No	205	142 (69.3)	63 (30.7)	
Liver metastasis				**0.006**
Yes	35	32 (93.5)	3 (6.5)	
No	202	139 (68.9)	63 (31.1)	
Peritoneal metastasis				0.359
Yes	29	23 (79.3)	6 (20.7)	
No	208	148 (71.2)	60 (28.8)	
Serum CEA level (μg/l)				0.218
≥5	175	130 (74.3)	45 (25.7)	
<5	62	41 (66.1)	21 (33.9)	

Bold, P<0.05.

assay (Pierce, Rockford, IL, USA). Electrophoresis was carried out using a Mini-Protean system (Bio-Rad Laboratories, Hercules, CA, USA). Total proteins (50 μg) were separated on 10% SDS-PAGE gel and transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA) by an electrophoretic transfer system (Bio-Rad Laboratories). The PVDF membranes were blocked with 5% non-fat dry milk in TBS with 0.1% Tween-20 for 1 h at 37˚C, and then incubated with primary antibodies, anti-sestrin 2 antibody (mouse monoclonal antibody, 1:200; Santa Cruz Biotechnology, Inc.; cat. no. sc-101249) and GAPDH antibody (mouse monoclonal antibody, 1:1,000; Abcam Biotechnology, Cambridge, MA, USA; cat. no. ab125247) overnight at 4˚C. After washing, the membranes were incubated with secondary antibodies (1:2,000 dilution, goat anti-mouse IgG-HRP; Santa Cruz Biotechnology, Inc.) for 1 h at 37˚C. Proteins were detected by enhanced chemiluminescence plus detection reagents (Pierce). The membranes were scanned (Bio-Rad Laboratories), and the pixel density of the images was quantified using Quantity One software (Bio-Rad Laboratories). The band intensity ratio of sestrin 2 relative to GAPDH (sestrin 2/GAPDH) was analyzed.

Table II. Association of sestrin 2 protein expression with clinicopathological characteristics in CRC patients.

Clinicopathological factors	No. of patients (n=42)	Sestrin 2 expression protein	P-value
Age (years)			0.700
≥65	18	0.234±0.085	
<65	24	0.223±0.102	
Gender			0.979
Male	23	0.230±0.097	
Female	19	0.229±0.087	
Tumor site			0.815
Distal	25	0.227±0.096	
Proximal	17	0.234±0.089	
Histology			0.340
Well	14	0.249±0.083	
Moderate/poor (mucinous)	28	0.220±0.096	
TNM stage			**0.005**
I/II	13	0.287±0.077	
III/IV	29	0.204±0.087	
Lymphatic invasion			**0.008**
Yes	24	0.198±0.084	
No	18	0.272±0.086	
Lymph node metastasis			**0.012**
Yes	20	0.193±0.088	
No	22	0.263±0.083	
Vascular invasion			**0.037**
Yes	9	0.174±0.072	
No	33	0.245±0.091	
Liver metastasis			**0.001**
Yes	6	0.146±0.043	
No	36	0.244±0.090	
Peritoneal metastasis			0.622
Yes	4	0.208±0.146	
No	38	0.232±0.087	
Serum CEA level (μg/l)			0.413
≥5	28	0.238±0.093	
<5	14	0.213±0.089	

Bold, P<0.05.

Immunofluorescence and confocal microscopy. The cells were seeded and cultured on glass coverslips the day prior to the analysis. Following incubation for 24 h, the cells were fixed with 4% paraformaldehyde at room temperature for 15 min. After fixation, the cells were permeabilized with 0.2 % Triton X-100 (Beyotime, Jiangsu, China) and blocked with 10% normal goat serum for 1 h at room temperature, as previously described (?). The cells were incubated with anti-sestrin 2 antibody (mouse monoclonal antibody, 1:50; Santa Cruz Biotechnology, Inc.; cat. no. sc-101249) overnight at 4˚C. After washing with PBS, the cells were incubated with DyLight 594-conjugated goat anti-mouse IgG (1:500, Zhongshan Golden Bridge, Inc.) for 1 h at 37˚C. The nuclei were counterstained with DAPI (Keygen Biotech) for 10 min, and the images were captured with an Olympus microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis. Continuous data are presented as mean ± standard deviation (SD). Continuous variables were measured using an independent Student's t-test. The associations between sestrin 2 immunohistochemical staining and clinicopathological variables were analyzed by the Mann-Whitney U test. The log-rank test and the Kaplan-Meier analysis were used to the associations between sestrin 2 expression and the OS/DFS. Factors independently associated with OS were identified using the Cox proportional hazards model for univariate and multivariate analyses. Statistical analysis was performed using SPSS Ver. 17.0 for Windows. P<0.05 was considered to indicate a statistically significant result.

Results

Sestrin 2 is decreased in CRC and is correlated with clinicopathological characteristics. In the normal mucosa, polyp, adenomas and borderline tissues, sestrin 2 was strongly and predominantly localized in cytoplasm, whereas faint immunoreactivity for sestrin 2 was observed in CRC patients (Fig. 1A-F). A significantly lower expression of sestrin 2 was detected in the CRC group as compared to the normal mucosa, polyp, adenomas and borderline groups respectively (P<0.05; Fig. 1G). No significant difference was identified among the normal mucosa, polyp, adenomas and borderline groups (P>0.05; Fig. 1G). In the CRC group, 72.2% of cases exhibited a low expression of sestrin 2 and 27.8% a high expression (Fig. 1H). By contrast, sestrin 2 expression was high in the normal mucosa (87.5%), polyp (71.8%), adenomas (80%) and borderline (80.8%) samples (Fig. 1H). The Mann-Whitney U test was used to evaluate whether a low expression of sestrin 2 in CRC samples was associated with specific clinicopathological variables (Table I). A low expression of sestrin 2 was significantly associated with TNM stage (P<0.001), lymphatic invasion (P=0.004), lymph node metastasis (P=0.006), vascular invasion (P=0.012) and liver metastasis (P=0.006). However, no significant associations were found between sestrin 2 expression and age, gender, tumor site, histology, peritoneal metastasis and serum carcinoembryonic antigen (CEA) level, respectively (all P>0.05).

Correlation between the protein level of sestrin 2 and clinicopathological characteristics. Western blot analysis was performed to evaluate the sestrin 2 protein level from frozen tissues of 42 CRC patients and controls including 19 normal mucosa, 20 polyp, 22 adenomas and 26 borderline (Fig. 2). The sestrin 2 expression was strong in the normal mucosa, polyp, adenomas and borderline samples, while it was faint in CRC samples. The protein expression of sestrin 2 in CRC tissues was significantly lower than that in normal mucosa, polyp, adenomas and borderline groups (P<0.05). No statistical significance was found among normal mucosa, polyp,

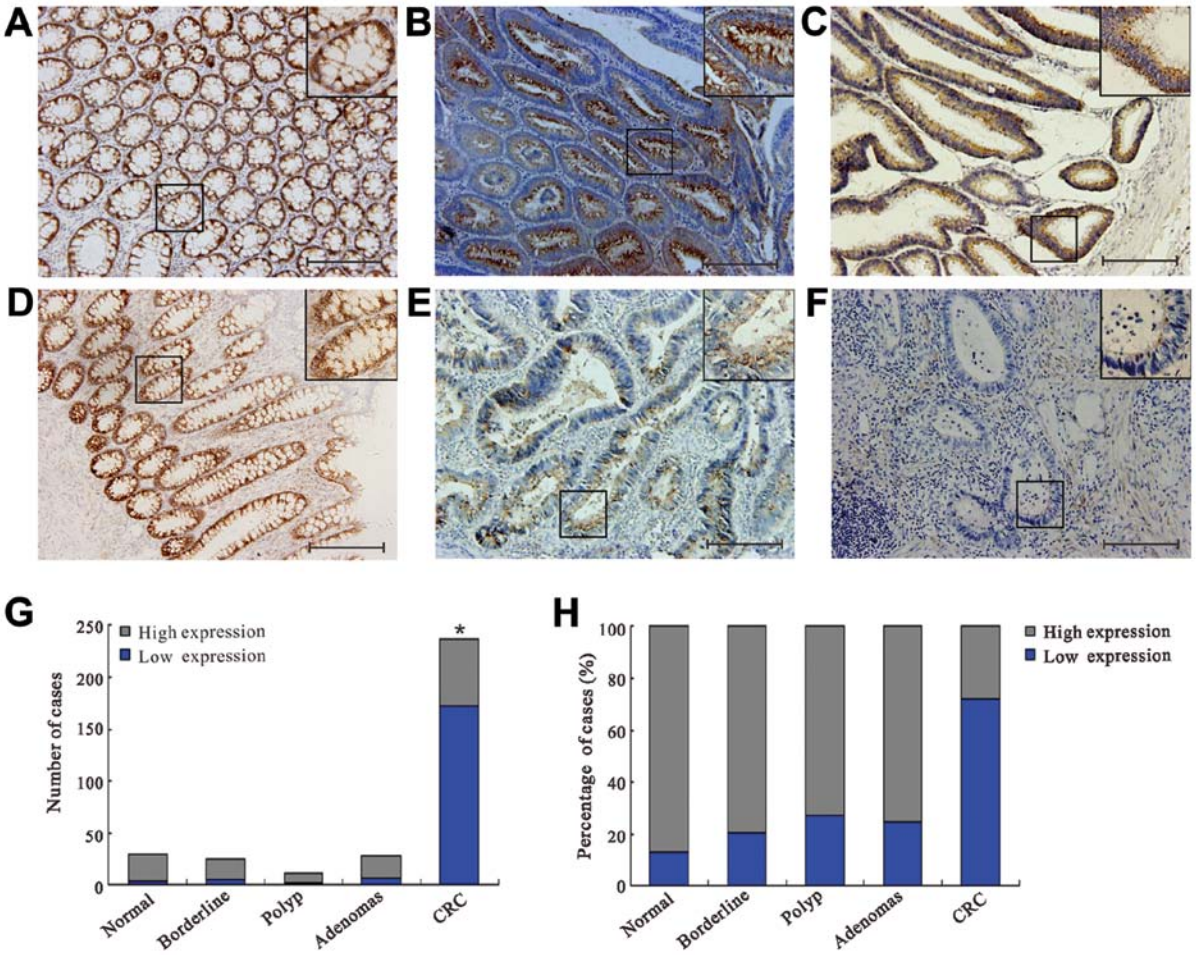

Figure 1. Expression of sestrin 2 in the CRC samples and controls using immunohistochemical staining. Sestrin 2 staining was mainly observed in the cytoplasm of (A) the normal mucosa, (B) polyp, (C) adenomas and (D) borderline and (E and F) CRC tissues. (G) The number of case and (H) the percentage of case showed that the proportion of low expression of sestrin 2 was higher in the CRC group than that in the normal mucosa, adenomas, polyp and borderline groups. *P<0.05, statistically significant differences between different control groups and the CRC group. Scale bar, 100 μm.

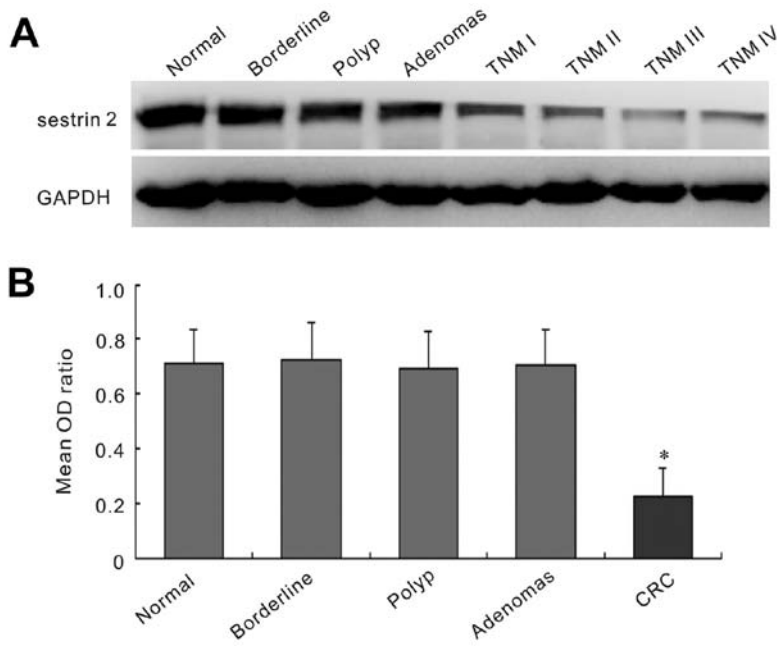

Figure 2. Western blot analysis of sestrin 2 expression in the CRC samples and controls. Representative western blotting showing sestrin 2 expression was strong in the normal mucosa, polyp, adenomas and borderline samples, while it was faint in (A) CRC samples. Histogram of the normalized sestrin 2 mean OD ratio for the (B) normal mucosa, polyp, adenomas, borderline and CRC samples. The mean OD ratio shows the density of sestrin 2 immunoreactivity relative to GAPDH immunoreactivity. *P<0.05, statistically significant differences between different control groups and the CRC group.

Table III. Univariate and multivariate analyses of the associations between prognostic variables and DFS and OS in 237 CRC patients.

Variables	5-year DFS HR (95% CI), P-value		5-year OS HR (95% CI), P-value	
	Univariate	Multivariate	Univariate	Multivariate
Age (≥65 years vs. <65 years)	1.204 (0.830-1.746), P=0.328	NA	1.230 (0.843-1.794), P=0.283	NA
Gender (male vs. female)	1.046 (0.724-1.512), P=0.810	NA	1.107 (0.762-1.606), P=0.594	NA
Tumor site (distal vs. proximal)	0.987 (0.673-1.446), P=0.944	NA	1.032 (0.702-1.517), P=0.873	NA
Histology (poor/moderate vs. well)	1.273 (0.870-1.862), P=0.215	NA	1.378 (0.933-2.035), P=0.107	NA
TNM stage (III/IV vs. I/II)	3.678 (2.219-6.096), **P<0.001**	3.043 (1.143-8.103), **P=0.026**	3.822 (2.276-6.420), **P<0.001**	3.352 (1.249-8.999), **P=0.016**
Lymphatic invasion (yes vs. no)	2.773 (1.777-4.327), **P<0.001**	0.556 (0.218-1.419), P=0.219	2.825 (1.796-4.446), **P<0.001**	0.479 (0.186-1.238), P=0.129
Lymph node metastasis (yes vs. no)	2.821 (1.934-4.114), **P<0.001**	1.776 (1.054-2.993), **P=0.031**	3.035 (2.064-4.462), **P<0.001**	1.992 (1.158-3.425), **P=0.013**
Vascular invasion (yes vs. no)	2.538 (1.616-3.986), **P<0.001**	2.012 (1.218-3.325), **P=0.006**	2.469 (1.559-3.912), **P<0.001**	1.929 (1.156-3.219), **P=0.012**
Liver metastasis (yes vs. no)	3.767 (2.418-5.867), **P<0.001**	2.469 (1.512-4.031), **P<0.001**	3.809 (2.445-5.934), **P<0.001**	2.516 (1.540-4.112), **P<0.001**
Peritoneal metastasis (yes vs. no)	2.351 (1.481-3.733), **P<0.001**	1.311 (0.799-2.152), P=0.284	2.302 (1.436-3.689), **P=0.001**	1.279 (0.772-2.119), P=0.339
CEA level (≥5 μg/l vs. <5 μg/l)	0.972 (0.640-1.474), P=0.892	NA	0.962 (0.630-1.469), P=0.856	NA
Sestrin 2 (high vs. low)	0.412 (0.254-0.668), **P<0.001**	0.553 (0.334-0.915), **P=0.021**	0.398 (0.249-0.654), **P<0.001**	0.537 (0.321-0.899), **P=0.018**

DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; OS, overall survival; NA, not applicable; TNM, tumor node metastasis; bold, P<0.05.

adenomas and borderline groups (P>0.05). Furthermore, we analyzed the correlation between the protein level of sestrin 2 and clinicopathological characteristics. The results positively correlated with the immunohistochemical findings. A significantly lower sestrin 2 protein level was associated with TNM stage (P=0.005), lymphatic invasion (P=0.008), lymph node metastasis (P=0.012), vascular invasion (P=0.037) and liver metastasis (P=0.001) (Table II).

Sestrin 2 expression in colon normal mucosa and CRC cell lines. The control cell line FHC and the HT-29, SW480, SW620 and LoVo human CRC cell lines were selected to analyze the expression of sestrin 2 at the protein level by immunofluorescence and western blot analysis. Immunofluorescence showed the expression of sestrin 2 was localized mainly in cytoplasm (Fig. 3A). Sestrin 2 expression was strong in the FHC cells and moderate in HT-29 and SW480 cells, while it was faint in the SW620 and LoVo cells (Fig. 3A). Western blot analysis also revealed a markedly strong expression of sestrin 2 in FHC group and a moderate expression in the HT-29 and SW480 groups, but a markedly weak expression in the SW620 and LoVo groups (Fig. 3B). Compared with the FHC group, the expression of sestrin 2 was significantly lower in the HT-29, SW480, SW620 and LoVo groups (P<0.05; Fig. 3C). Furthermore, the expression of sestrin 2 in the SW620 and LoVo cells was significantly lower than that in the HT-29 and SW480 cells (P<0.05; Fig. 3C).

Low expression of sestrin 2 in CRC predicts an unfavorable outcome. The correlation of sestrin 2 low expression and clinical outcome was analyzed. Following 5-year follow-up, the mean OS and DFS periods were 39.04±22.46 and 38.70±22.56 months, respectively. To assess sestrin 2 as a predictor of survival, the Kaplan-Meier analysis method was used to investigate the correlation between sestrin 2 expression and survival. The log-rank test showed that patients with low sestrin 2 staining had a significantly worse OS and DFS than patients with high sestrin 2 staining (P<0.001 and P<0.001, respectively; Fig. 4A and B). Additionally, patients with early or advanced stage CRC with low expression of sestrin 2 had a shorter survival than patients with high expression (P=0.029 and P=0.023, respectively; Fig. 4C and Fig. 4D). At the 5-year follow-up, 50.99% of the patients with high sestrin 2 level survived. However, only 37.67% of patients with low sestrin 2 staining survived.

The univariate analysis showed that the patients with low sestrin 2 expression [DFS, hazard ratio (HR) =0.412, P<0.001; OS, HR =0.398, P<0.001], advanced tumor stage (DFS, HR =3.678, P<0.001; OS, HR =3.822, P<0.001), lymphatic invasion (DFS, HR =2.773, P<0.001; OS, HR =2.825, P<0.001), lymph node metastasis (DFS, HR =2.821, P<0.001; OS, HR =3.035, P<0.001), vascular invasion (DFS, HR =2.538, P<0.001; OS, HR =2.469, P<0.001), liver metastasis (DFS, HR =3.767, P<0.001; OS, HR =3.809, P<0.001) and peritoneal metastasis (DFS, HR =2.351, P<0.001; OS, HR =2.302, P=0.001) had shorter OS and DFS (Table III). Furthermore, the multivariate analysis showed that only low sestrin 2 expression (DFS, HR =0.553, P=0.021; OS, HR =0.537, P=0.018), advanced TNM stage (DFS, HR =3.043, P=0.026; OS, HR =3.352, P=0.016), lymphatic node metastasis (DFS,

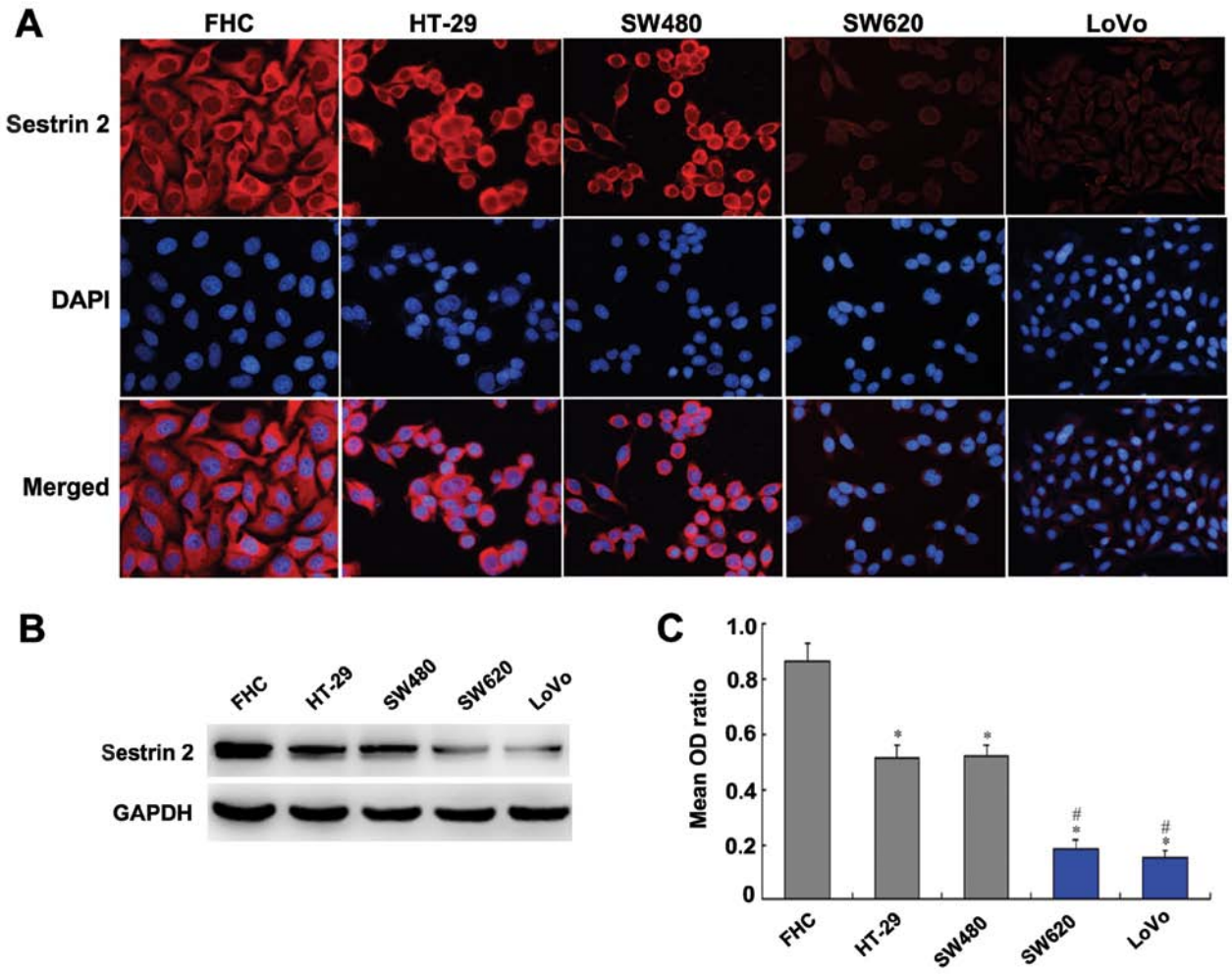

Figure 3. Immunofluorescence and western blot analysis of sestrin 2 expression in colon normal mucosa and CRC cell lines. (A) Immunofluorescence showed sestrin 2 expression was strong in the FHC cells and moderate in HT-29 and SW480 cells, while it was faint in the SW620 and LoVo cells. Sestrin 2 was marked in red, and the nuclei were marked in blue with DAPI. (B) Representative western blotting showing sestrin 2 expression was strong in the FHC group and moderate in HT-29 and SW480 groups, while it was faint in the SW620 and LoVo groups. (C) Histogram of the normalized sestrin 2 mean OD ratio for the FHC, HT-29, SW480, SW620 and LoVo groups. The mean OD ratio shows the density of sestrin 2 immunoreactivity relative to GAPDH immunoreactivity. *P<0.05, statistically significant differences between the FHC group and the different CRC groups. #P<0.05, statistically significant differences when compared with the HT-29 and SW480 groups, respectively.

HR =1.776, P=0.031; OS, HR =1.992, P=0.013), vascular invasion (DFS, HR =2.012, P=0.006; OS, HR =1.929, P=0.012) and liver metastasis (DFS, HR =2.469, P<0.001; OS, HR =2.516, P<0.001) remained independent prognostic factors of poor OS and DFS (Table III). However, no significant correlation was detected between survival and other clinicopathological variables including age, gender, tumor site, histology, lymphatic invasion, peritoneal metastasis and serum CEA level (all P>0.05; Table III).

Discussion

ROS, which are thought to be a major source of endogenous DNA damage, directly contribute to tumor progression and metastasis (24-26). A great deal of evidence support the view that oxidative stress and the accompanying ROS are genotoxic and may contribute to the development of CRC (27). Furthermore, the genetic reduction of mitochondrial oxidative stress reduces tumor grade and inhibits metastasis (28). Therefore, tumors occur when there is an imbalance between overproduction of ROS and a decrease of antioxidant molecules in the body.

In the present study, we showed that the antioxidant protein sestrin 2 was decreased in human CRC tissues. Similarly, sestrin 2 was downregulated in human CRC cell lines. The expression of sestrin 2 in SW620 and LoVo cells, which were derived from the metastatic site of CRC, was significantly lower than that in the HT-29 and SW480 cells, which were derived from the primary lesion of CRC. In subsequent analysis of the association between the sestrin 2 expression and clinicopathological variables, we found that the low expression of sestrin 2 was correlated with lymph node and liver metastasis. The findings reveal that there may be a connection between the decreased expression of sestrin 2 and tumor metastasis. Results of studies have shown that oxidative stress directly contributes to tumor progression and metastasis (28,29). In clinical findings, most current chemotherapy agents and radiation therapy increase oxidative stress, leading to tumor recurrence and metastasis (28). Since sestrin 2 protect cells from oxidative stress, the

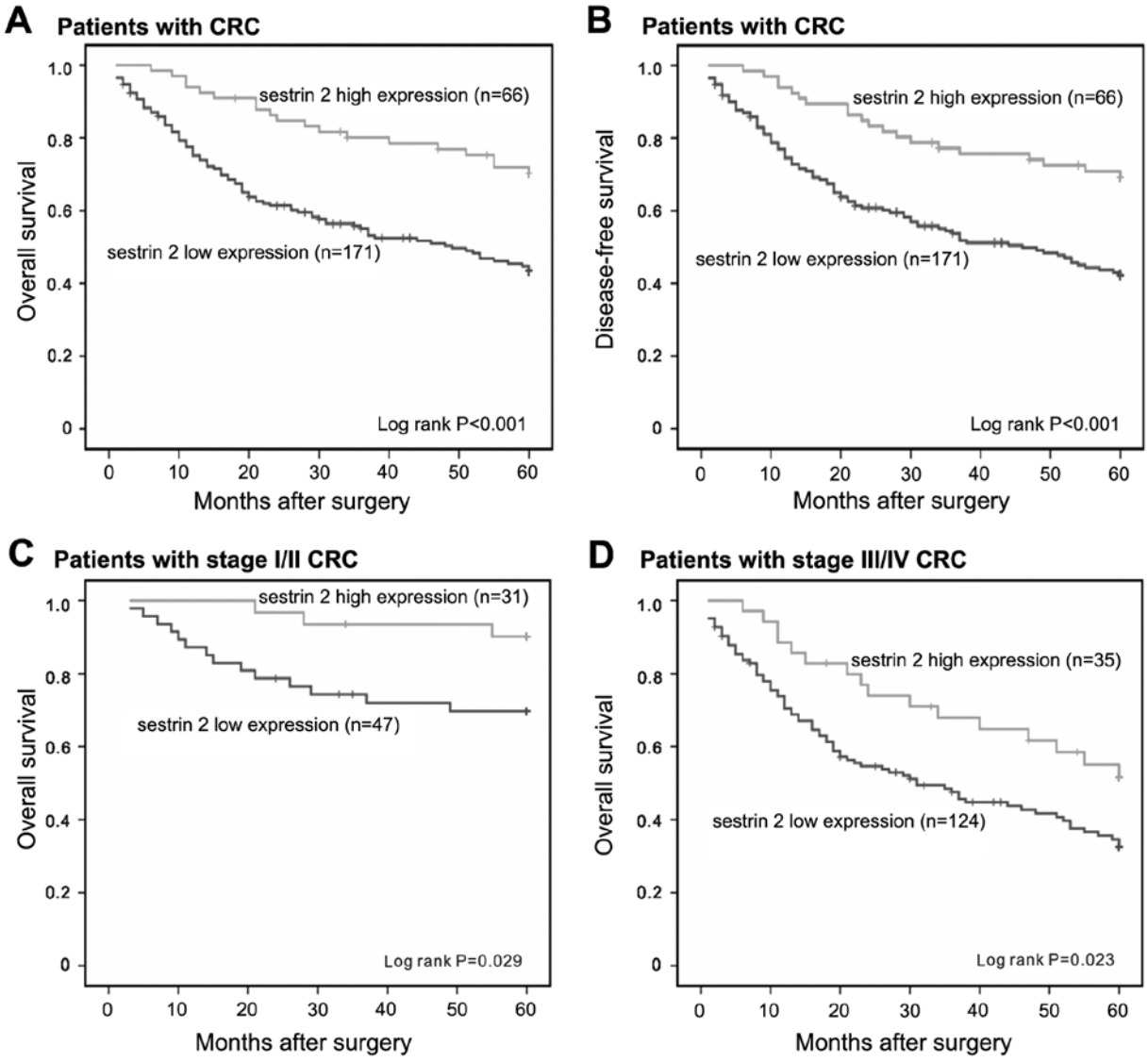

Figure 4. Kaplan-Meier analysis for the correlation between sestrin 2 expression and survival. The (A) OS and (B) DFS of CRC patients with a high and low expression of sestrin 2 are shown, respectively. Log-rank test showed that patients with high sestrin 2 staining had a significantly better OS and DFS versus patients with low sestrin 2 staining. The (C) stage I/II survival and (D) III/IV survival of CRC patients with high and low expression of sestrin 2, respectively are shown. Log-rank test showed that patients with a low expression level of sestrin 2 in stage I/II and stage III/IV had a shorter outcome.

downregulation of sestrin 2 may increase oxidative stress, thus aggravating tumor metastasis. However, the molecular pathway that connects downregulation of sestrin 2 to the acquisition of metastatic capacity during tumor progression remains to be investigated. Besides the lymph node and liver metastasis, we found that low expression of sestrin 2 was significantly correlated with advanced tumor stage, lymphatic invasion and vascular invasion.

Previous studies reported that the abnormalities of sestrin 2-related protein, p53, was associated with CRC patient survival (30-33). However, the association between sestrin 2 and cancer mortality has not been investigated in clinical samples. Our results clearly demonstrate that a decreased expression of sestrin 2 was an independent and significant prognostic factor for 5-year DFS and OS. Additionally, early or advanced stage CRC patients with a low expression of sestrin 2 had a shorter survival than patients with a high expression. To the least of our knowledg, this is the first study to show an association between sestrin 2 expression and CRC patient survival. These findings suggest that sestrin 2 and its associated proteins may be crucial in CRC patient prognosis.

In conclusion, our study of patients with CRC revealed the downregulation of sestrin 2 protein in human CRC tissues compared with the normal mucosa, polyp, adenomas and borderline tissues. The expression of sestrin 2 was decreased in HT-29, SW480, SW620 and LoVo human CRC cell lines when compared with the FHC control cell line. Additionally, decreased sestrin 2 was associated with an unfavorable prognosis and was an independent prognostic factor for CRC, suggesting that sestrin 2 is a crucial predictor for sestrin 2 metastasis. The results thus shed light on the potential of sestrin 2 as a tumor-suppressor gene with a novel antioxidant function in CRC, and that downregulation of sestrin 2 may aggravate tumor metastasis. However, future studies should be conducted to examine the effects of changing sestrin 2 activity and identify the possible mechanisms based on this novel protein involved in CRC.

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