Expression of endothelial cell-specific molecule-1 regulated by hypoxia inducible factor-1α in human colon carcinoma: Impact of ESM-1 on prognosis and its correlation with clinicopathological features

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Abstract. Based on a previous finding that endothelial cell-specific molecule-1 (ESM-1) is a potential serum marker for colorectal cancer (CRC), the aim of this study was to clarify the clinicopathological significance of ESM-1 expression in CRC, and to explore the correlation between ESM-1 and HIF-1α in the tumorigenesis of CRC related to hypoxic conditions. ESM-1 mRNA expression was examined in CRC and corresponding normal mucosal tissues by reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR. This experiment confirmed that ESM-1 levels were high in CRC. We screened the tissue samples of 143 CRC patients. By immunohistochemistry, we determined that the ESM-1 immunoreactivity was significantly correlated with the tumor size, depth of invasion, nodal status, distant metastasis and Dukes' stage, and was an independent prognostic factor for disease recurrence and worse survival outcome (P=0.001). The modulation of ESM-1 under hypoxia was investigated, and it was confirmed that ESM-1 expression was induced by HIF1-α and significantly attenuated by small interfering RNA (siRNA) targeting HIF-1α in CRC cells. These results showed that ESM-1 is significantly overexpressed, which is regulated by HIF-1α in CRC patients, and can be used as a potential biomarker and a therapeutic target for CRC.

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Introduction

Human colon cancer is one of the major causes of morbidity and mortality worldwide. Its tumorigenic mechanism is a multi-step process related to the genetic instability associated with genetic alterations in the invariably less well-oxygenated tumor state (1-6). The unstable hypoxic microenvironment induces the expression of hypoxia inducible factor-1 (HIF-1), a key transcriptional regulator which plays a central role in the regulation of biological processes, including glucose metabolism, cell proliferation, angiogenesis, migration, and survival (7-13). HIF-1α activity is dependent on the localization of HIF-1α protein in the nucleus (14). Upregulation of the HIF pathway has been shown in aggressive phenotypes of colorectal cancer (CRC) (15-18).

Endothelial cell-specific molecule-1 (ESM-1), also known as endocan, is a 50-kDa secretory proteoglycan, which was originally cloned from a human endothelial cell cDNA library by Lassalle and collaborators (19). The structure of ESM-1 is composed of a mature polypeptide of 165 amino acids and a single dermatan sulfate chain covalently linked to the serine residue at position 137 (19-21). ESM-1 expression in endothelial and epithelial cells is upregulated by tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and vascular epidermal growth factor (VEGF), downregulated by IL-4 and interferon (IFN)-γ (19,22-24), and secreted by vascular endothelial cells, epithelial cells lining distal tubules, bronchi and lung submucosal glands (19,22,25). In addition to ESM-1 expression in normal human tissue, differential expression of ESM-1 has been reported in the vascular endothelium of renal carcinoma (26), breast carcinoma (27,28), glioblastoma (29), non-small cell lung cancer (24), and liver cancer (30). In colon cancer, we reported that ESM-1 expression was increased in the tissue and serum samples of CRC patients, and it can be used as a potential serum marker for early detection of CRC (31). ESM-1 has previously been described to be upregulated by VEGF *in vitro* and *in vivo* (20). VEGF is a major target gene of HIF-1α regulatory genes (32). In addition, Maurage *et al* showed that

ESM-1 expression was increased under hypoxic condition (1% O_2) in a glioblastoma cell line (29). Although there are several reports that ESM-1 overexpression was closely related to the process of angiogenesis in the endothelial cells from tumor tissues (26,33-35), whether ESM-1 modulation is directly regulated by HIF-1α or whether the overexpression of these two proteins in the tissue samples of CRC patients significantly correlate with each other has not yet been elucidated.

In this study, we investigated the overexpression of ESM-1 and HIF-1α in the tissue samples of 143 CRC patients using RT-PCR and immunohistochemistry. Next, the clinicopathological significance of ESM-1 immunoreactivity and its correlation with poor prognosis of CRC was analyzed, and we confirmed the functional inter-correlation of ESM-1 with HIF-1α in colon cancer cells and tissues.

Materials and methods

Cell lines and transfection. The human colon cancer cell line, HT29, was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum (FBS; Hyclon, Logan, UT, USA), and kept at 37˚C in a humidified incubator, which was maintained with 5% CO_2. siRNA directed against human HIF-1α (5'-CUGAU GACCAGCAACUUGATT-3' and 5'-UCAAGUUGCUGGUC AUCAGTT-3') (siGENOME SMARTpool, catalog no. M-013858-00) was purchased from Bioneer (Daejeon, Korea) with its control non-targeting siRNA (catalog no. 1068432), and treated according to the manufacturer's instructions. As an example, 12 pmol of HIF-1α siRNA was premixed with 15 μl of Lipofectamine RNAiMAX reagent (Invitrogen) following the manufacturer's instructions, incubated for 20 min at room temperature (RT), and used to treat colon cancer cells plated on a 60-mm dish with 40% confluency. The plasmid-containing wild type HIF-1α-coding region was transfected into the HT29 cell lines using Lipofectamine Plus reagent (Invitrogen), according to the manufacturer's instructions.

Antibodies and western blotting. Cells were washed with phosphate-buffered saline (PBS) and lysed with cell lysis buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM NaF, and Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA)] on ice for 30 min. SDS-PAGE was used to resolve 30-50 μg of the lysate by using 10 or 12% gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies followed by incubation with peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (Calbiochem, EMD Chemicals Inc., San Diego, CA, USA) and ECL reagent (Amersham Biosciences Inc., Piscataway, NJ, USA) for band visualization. To verify equal loading and adequate transfer, the membranes were probed with anti-γ-tubulin antibody (Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA). The primary antibodies were anti-ESM-1 (Abnova, Taipei, Taiwan) and anti-HIF-1α (Novus Biologicals, Littleton, CO, USA).

RT-PCR analysis. A 2-step RT–PCR reaction was performed using reverse transcriptase with oligo-dT primer, and *Taq* polymerase (Takara, Shiga, Japan), with specific primer pairs. Total RNA was isolated by a standard protocol (36), and cDNA was synthesized using the AccuScript High Fidelity First Strand cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. One microliter of the synthesized cDNA was used per 20 μl of PCR reaction mixture, which comprised 0.2 U ExTaq DNA polymerase, 1X buffer, and 1 mM dNTP mix (Takara), with specific primer pairs, and amplified as follows: 1 cycle of 94˚C for 5 min; then 30 cycles of 94˚C for 45 sec, 56˚C for 45 sec and 72˚C for 1 min; followed by a final extension of 7 min at 72˚C, using the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). PCR primers were designed using the Primer3 program purchased from Bioneer. ESM-1 gene-specific primers used for PCR were 5'-GCCCTTCCTTGGTAGG TAGC-3' (sense) and 5'-TGTTTCCTATGCCCCAGAAC-3' (antisense). The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, visualized by Gel Doc 2000 UV transilluminator (Bio-Rad Laboratories, Hercules, CA, USA), and analyzed using Quantity One software (Bio-Rad Laboratories). Each sample was tested more than 2 times, and the representative data are shown. The primers used for ESM-1 real-time RT-PCR were 5'-AAGGC TGCTGATGTAGTTC-3' (sense), 5'-GCTATTTATGGAAGT GTATGTGTTT-3' (antisense), gapdh; 5'-AGTCAGCCGCAT CTTCTT-3' (sense), 5'-GCCCAATACGACCAAATCC-3' (antisense). Optimized PCR was carried out as follows: 1 cycle of 95˚C for 10 sec; 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec, and 95˚C for 15 sec; and a final extension at 60˚C for 15 sec. The relative levels of gene expression were normalized to GAPDH expression.

Patient samples and immunohistochemistry. Human colorectal carcinoma samples were obtained from patients who underwent routine surgery for colorectal cancer at the Department of Surgery, Eulji University Hospital, between January 2000 and June 2005. Our study protocol (Protocol No. 10-24) was approved by our Institutional Review Board, Eulji University Hospital. For immunohistochemical study, 143 colorectal carcinoma tissue samples and paired normal mucosal tissue samples taken from a site distant from the tumor lesion were fixed in 10% neutralized-buffered formalin solution for 24 h and embedded in paraffin wax. Serial sections (4-μm) were cut and mounted on charged glass slides (Superfrost Plus; Fisher Scientific, Rochester, NY, USA). IHC conditions for ESM-1 and HIF-1α were optimized and evaluated by 2 independent pathologists. In brief, tissue sections were microwaved twice for 10 min in citrate buffer (pH 6.0) for antigen retrieval. The sections were then treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with 1% BSA. Mouse monoclonal antibodies against ESM-1 (Abnova, Taipei, Taiwan) and HIF-1α (Novus Biologicals) were used at dilutions of 1:200 and 1:50, respectively. The tissue sections were incubated with antibody overnight at 4˚C in a wet chamber. The sections were stained using a standard EnVision-HRP kit (Dako, Glostrup, Denmark) and developed with diaminobenzidine as a substrate. An irrelevant mouse IgG of the same isotype or antibody dilution solution served as a negative control.

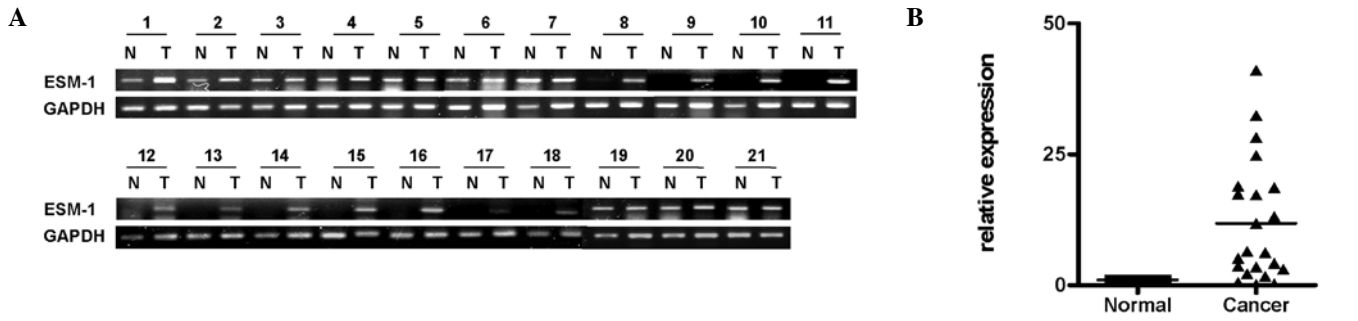

Figure 1. Expression of the ESM-1 gene in human colorectal tissues. (A) In the RT-PCR analysis, comparative expression levels of ESM-1 are shown from 21 normal colon mucosa (N) and colon carcinoma tissue (T) samples. Expression of the ESM-1 gene was higher in the colon carcinoma tissue than that in the corresponding normal mucosa. (B) Real-time RT-PCR analysis was used to determine the level of relative expression of ESM-1 by using the ratio of ESM-1 to GAPDH. The value of tumor-originated ESM-1 was relative to the ESM-1/GAPDH of matched normal controls.

Assessment of immunostaining and statistical analysis. Each slide was evaluated for ESM-1 and HIF-1α immunoreactivity by using a semi-quantitative scoring system for both the intensity of the stain and the percentage of positive neoplastic cells. In the colorectal and mucosal cells, ESM-1 immunoreactivity corresponded to the cytoplasm and HIF-1α to the nuclei. The intensity of membrane staining was coded as follows: 0, weaker than that in the adjacent normal-appearing mucosal epithelium; 1, similar to that in the adjacent mucosal epithelium; and 2, stronger than that in the adjacent mucosal epithelium. The percentage of cells displaying a stronger staining intensity than that in the adjacent mucosal epithelium was scored as either 1 (0-24% tumor cells stained), 2 (25-49% tumor cells stained), 3 (50-74% tumor cells stained), or 4 (75-100% tumor cells stained). For the purpose of statistical analysis, the median of this series (25% of malignant cells showing a stronger intensity than adjacent colonic epithelium) was used as a cut-off value to distinguish tumors with low (<25%) or high (>25%) levels of ESM-1 and HIF-1α expression. The relationship between the results of the immunohistochemical study and the clinicopathological parameters was determined using the SPSS software package (version 14.0; SPSS Inc., Chicago, IL, USA). The correlation between staining index scores and other categorical factors was analyzed using the Pearson's Chi-square test of independence.

Prognostic parameter for recurrence-free survival and overall survival. Recurrence-free survival was defined as the time from the date of surgery to the first date of recurrence of cancer, or death from any cause. Overall survival was defined as the time from the date of surgery to the date of last follow-up or death from any cause. The median follow-up period for all patients was 54.2 months (inter-quartile range, 23.2-80.7). Survival and median survival curves were estimated using the Kaplan-Meier method. The log-rank test was used to evaluate the statistical significance of differences in survival distribution. Multivariate analysis was carried out using the Cox proportional hazards regression analysis. Results were considered statistically significant if P-values were <0.05.

Results

ESM-1 is differentially expressed in human CRC tissues. To compare the ESM-1 expression levels in the colon cancer tissues, we examined the mRNA level of ESM-1 by performing RT-PCR or real-time RT-PCR analysis on pairs of tissue containing normal and tumor tissue samples from the same donor. GAPDH was used as a reference gene to correct for the variations for mRNA in individual samples. As shown in Fig. 1A and B, 21 cases of colon cancer tissues, randomly selected from clinically diagnosed patients, showed a significant increase in ESM-1 mRNA expression compared to the normal tissue from the same patients.

Hypoxic stress induces ESM-1 expression via HIF-1α in CRC cells. To investigate the effect of hypoxic stress on ESM-1 expression in colon cancer cells, HT29 cells were exposed to 1% hypoxia. Under a hypoxic condition, the protein level of HIF1-α was maximized at 8 and 12 h and was gradually decreased to reach basal level; ESM-1 showed the same trend (Fig. 2A). To test whether ESM-1 induction is correlated with HIF-1α, siRNA targeting HIF-1α was transfected into HT29 cells and the cells were then incubated for indicated times under a hypoxic condition. siRNA targeting HIF-1α attenuated the induction of ESM-1 under a hypoxic condition (Fig. 2B). When HIF-1α was overexpressed in order to confirm whether ESM-1 expression is led by HIF-1α, the expression level of ESM-1 was found to be consistent with that of HIF-1α (Fig. 2C). Taken together, transcription factor HIF-1α, which is induced under hypoxic conditions, induced ESM-1 expression.

Association of ESM-1 and HIF-1α expression levels with the clinicopathological characteristics. ESM-1 and HIF-1α levels were evaluated by immunohistochemical analysis. The elevated expression of ESM-1 and HIF-1α was detectable in 76 (53.1%) and 66 (46.1%) CRC cases, respectively. ESM-1 immunoreactivity was found primarily in the cytosol, but expression of ESM-1 in the cell membrane was occasionally noted in some malignant cells. HIF-1α was noted predominantly in the nucleus. Fig. 3 shows representative expression patterns of ESM-1 (Fig. 3A and B) and HIF-1α (Fig. 3C) in CRC tissues. Clinical and pathological characteristics of the 143 CRC patients who underwent surgical resection are summarized in Table I. The median age at the time of surgical resection was 60.3 years. A high level of ESM-1 expression was observed in 76 (53.1%) of the 143 patients. When we tested for an association between the level of ESM-1 expression and the clinicopathological factors potentially predictive of prognosis,

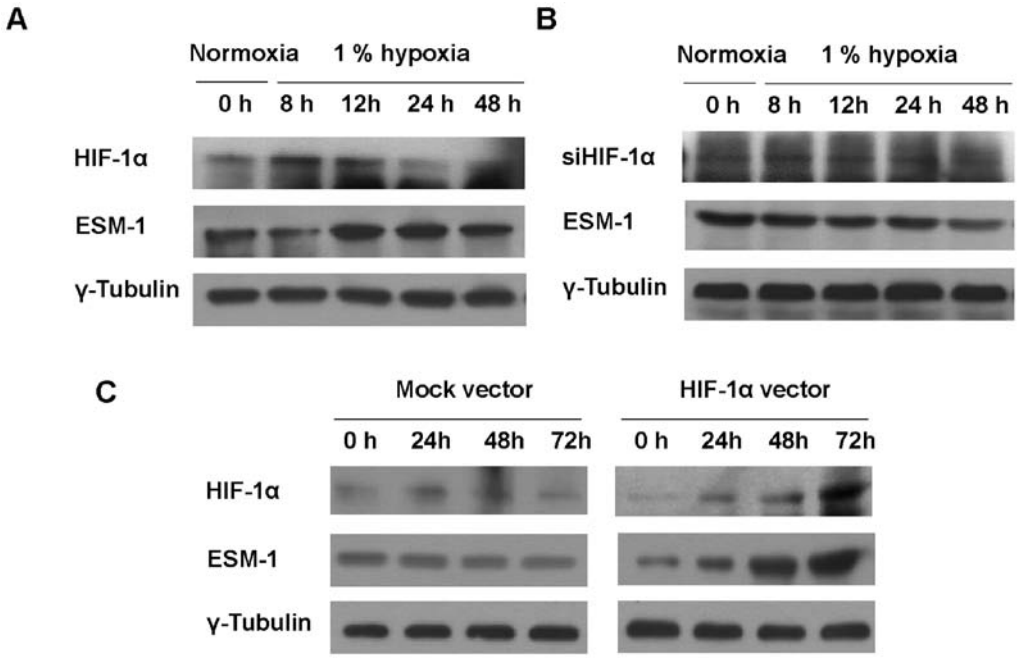

Figure 2. ESM-1 expression is regulated by HIF-1α induced under hypoxia condition. (A) Human colon cancer cell line HT29 was exposed to hypoxia (1% oxygen) or normoxia for 3 days. In western blot analysis of HT29 cells, HIF-1α and ESM-1 overexpression was noted. (B) Downregulation of ESM-1 was induced in the HIF-1α siRNA-transfected HT29 cells. (C) Modulation of HIF-1α expression by transfection with a HIF-1α plasmid vector induced overexpression of ESM-1.

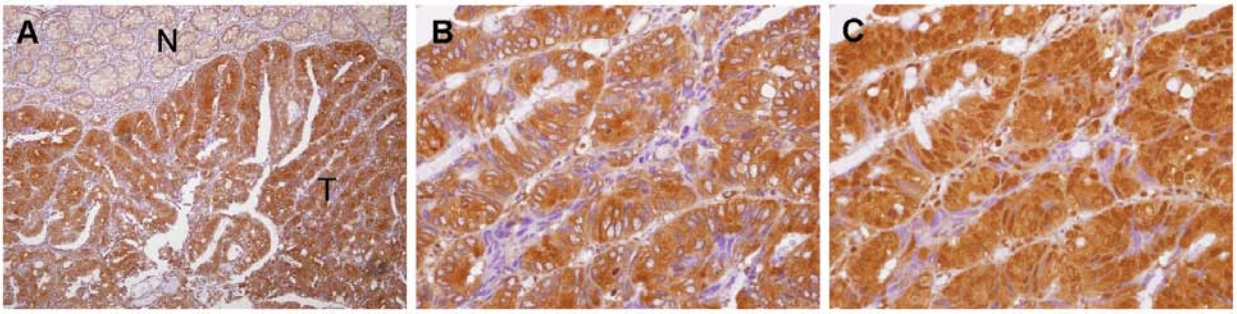

Figure 3. Immunohistochemical expression of ESM-1 and HIF-1α in human CRC. (A) ESM-1 was highly expressed in the colorectal tumor cells, but not or weakly expressed in the normal colonic mucosa. Original magnification, x40. (B) ESM-1 was strongly expressed primarily in the cytosol of tumor cells. Original magnification, x200. (C) HIF-1α was highly expressed in tumor cells of the serial tissue section; it was localized in both the nucleus and the cytosol of tumor cells. Original magnification, x200. N and T represent normal and tumor tissue, respectively.

tumor size, depth of invasion, nodal status, distant metastasis, and Dukes' stage, these clinicopathological variables showed a statistically significant association with ESM-1 expression status. We also tested for an association between HIF-1α status and these variables. A significant correlation between high nuclear HIF-1α and Dukes' stage (P=0.005) was noted (data not shown). A significant correlation between high nuclear HIF-1α and ESM-1 (P<0.001) was also noted (Table II).

A high level of ESM-1 expression is an independent prognostic factor for disease recurrence and a worse survival outcome. We first carried out univariate analyses to examine whether the expression status of ESM-1 correlates with recurrence-free survival. A total of 40 patients (28.0%) presented with recurrence during the follow-up period. At the end of the follow-up, 81 (56.6%) patients were alive, and 62 had died. The analysis showed that a high level of ESM-1 expression was negatively associated with recurrence-free survival (P<0.001), as shown in Fig. 4A. A high level of ESM-1 expression also correlated significantly with negative overall survival (P<0.001). Cumulative overall survival curves of patients were significantly split by ESM-1 expression status (Fig. 4B). The mean overall survival times for patients with high and low levels of ESM-1 expression, and all patients were 53.4, 100.4, and 78.1 months, respectively. We carried out multivariate analyses to assess the predictive value of ESM-1 expression status for recurrence-free survival and overall survival by adjusting for other potentially prognostic factors, including age, gender, tumor site, tumor size, cell differentiation and tumor stage. The results corroborated a worse survival outcome in patients with a high level of ESM-1 expression. In a multivariate Cox regression analysis, the independent prognostic factors significantly associated with overall survival were ESM-1 (P=0.001) and tumor stage (P=0.039). The relative risk (RR) of death was

Table I. Clinicopathological variables and the expression status of ESM-1.

Characteristics	Total	ESM-1 expression level		P-value
		Negative/low	High	
		n (%)	n (%)	
Age (years)				
<50	26	12 (17.9)	14 (18.4)	0.937
≥50	117	55 (82.1)	62 (81.6)	
Gender				
Female	68	35 (52.2)	33 (43.4)	0.292
Male	75	32 (47.8)	43 (56.6)	
Site				
Right/transverse colon	34	14 (20.9)	20 (26.3)	0.447
Left colon and rectum	109	53 (79.1)	56 (73.7)	
Size (cm in diameter)				0.030
<5	61	35 (52.2)	26 (34.2)	
≥5	82	32 (47.8)	50 (65.8)	
Differentiation				
Well	34	20 (29.9)	14 (18.4)	0.266
Moderately	82	36 (53.7)	46 (60.5)	
Poorly	27	11 (16.4)	16 (21.1)	
Depth of invasion				
T1	5	5 (7.5)	0 (0.0)	<0.001
T2	21	16 (23.9)	5 (6.6)	
T3	105	43 (64.2)	62 (81.6)	
T4	12	3 (4.5)	9 (11.8)	
Nodal status				
N0	67	44 (65.7)	23 (30.3)	<0.001
N1	23	10 (14.9)	13 (17.1)	
N2	53	13 (19.4)	40 (52.6)	
Distant metastasis				
M0	121	63 (94.0)	58 (76.3)	0.003
M1	22	4 (6.0)	18 (23.7)	
Dukes' stage				
A	8	7 (10.4)	1 (1.3)	<0.001
B	58	36 (53.7)	22 (28.9)	
C	56	20 (29.9)	36 (47.4)	
D	21	4 (6.0)	17 (22.4)	

more than 3 times higher in patients with high ESM-1 levels (RR, 3.062; 95% CI, 1.630-5.751) than those with low ESM-1 levels. A high level of ESM-1 expression was also predictive of increased disease recurrence with a P-value of <0.001. The RR of disease recurrence for patients with high ESM-1 level was 2.65 (95% CI, 1.543-4.550). The results from Cox proportional hazards analysis are summarized in Table III.

Discussion

Intra-tumoral hypoxia is a major event that occurs in most solid tumors (37,38), and HIF is a key transcription factor activating survival machinery in cancer cells under intra-tumoral hypoxic conditions (39). To date, there has been one report suggesting the modulation of ESM-1 expression by hypoxia

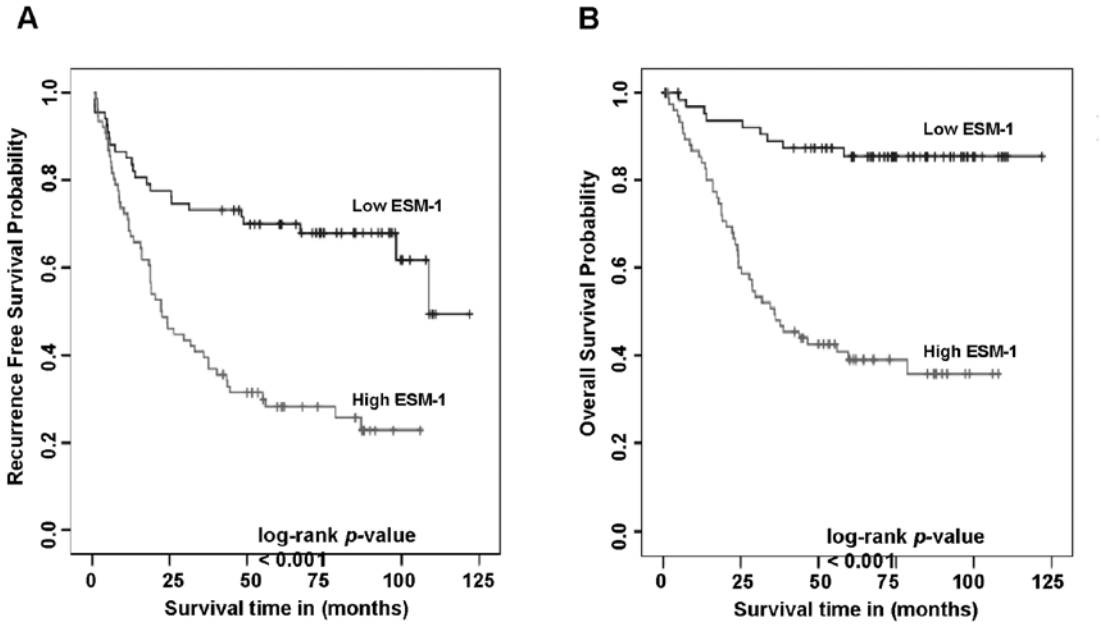

Figure 4. Kaplan-Meier survival analysis by ESM-1 expression status. (A) Cumulative recurrence-free survival differences between patients with high and low ESM-1 expression. (B) Cumulative overall survival differences between patients with high and low ESM-1 expression. The P-value of the difference was obtained using the log-rank test.

Table II. Correlation between ESM-1 and HIF-1α expression status.

		HIF-1α expression	
Frequency	Total	Low/negative, n (%)	High, n (%)
ESM-1 expression			
Low, n (%)	67	51 (76.1)	16 (23.9)
High, n (%)	76	26 (34.2)	50 (65.8)

in human glioblastoma cells in addition to TNF-α, fibroblast growth factor (FGF)-2 and VEGF (29). Although we could anticipate the functional correlation of HIF-1α with ESM-1, there was no *in vitro* or *in vivo* evidence to suggest whether there is a significant direct correlation between ESM-1 and HIF-1α. Therefore, in this study, we screened the tissues of 143 CRC patients, and showed that overexpression of ESM-1 in CRC was closely related to the restricted overexpression of HIF-1α in the nuclei of CRC cells. We also provided the first report that HIF-1α stimulated the induction of ESM-1 in CRC cells.

ESM-1 has been studied in a number of cell lines and human tumor tissues, and has been shown to influence a variety of normal and pathological processes. ESM-1 is a key player in tumor progression as well as in the regulation of inflammatory disorders (23,40,41), wherein it is either downregulated or overexpressed. In this study, we showed that a high level of cytosolic ESM-1 is an independent and clinically significant prognostic indicator for colon cancer patients who underwent surgery. However, in contradiction to our results, Zuo *et al* (42) showed that a lower expression of ESM-1 was detected in CRC tissue compared to that in normal colon and rectal tissue samples, and its expression was positively correlated with tissue differentiation of CRC. Although we could not explain the exact reason why our results differ from those of Zuo *et al*, we are convinced by our results based on many efforts to confirm our data, including those of a previous published report (31). It is noteworthy that ESM-1 was elevated in the colon cancer cells under hypoxic conditions. Since previous studies showed that HIF-1α is one of the important regulators in hypoxia, we explored a possible correlation between ESM-1 and HIF-1α expression status. We showed that ESM-1 was upregulated in the human colon cell line HT29 under 1% hypoxic conditions, inhibited by siRNA of HIF-1α, and overexpressed following HIF-1α plasmid vector transfection, suggesting that ESM-1 may be regulated by HIF-1α in the hypoxic tumor microenvironment during tumor development and progression (27). Indeed, of the 67 tumors containing a high level of nuclear HIF-1α immunoreactivity, 51 displayed a high level of ESM-1 expression. Of the 76 tumors containing a low level of nuclear HIF-1α, 25 showed a correspondingly low level of ESM-1. The likelihood of observing a high level of ESM-1 expression in a tumor containing a high level of nuclear HIF-1α was significantly greater than that in a tumor with a low level of nuclear HIF-1α ($P<0.01$). Ji *et al* recently reported that ESM-1 was secreted in human colon cancer tissue and cells (31). ESM-1 is a novel soluble dermatan sulfate proteoglycan that is secreted from endothelial cells (19,22), and its expression is regulated by tumor cell-derived factors, including vascular endothelial growth factor, in the unstable hypoxic microenvironment (25). This, together with our findings, suggests that ESM-1 may be involved in hypoxia-associated angiogenesis during tumor development and progression.

HIFs are essential mediators in regulating transcription in tumor cells in response to hypoxia (19,39,43). HIF-1α is

Table III. Multivariate Cox proportional hazards analysis for recurrence-free and overall survival.

		Recurrence-free survival		Overall survival	
	n	RR (95% CI)	P-value	Median (95% CI)	P-value
ESM-1 level					
Low/negative	77	1.000	0.010	1.000	0.001
High	76	2.109 (1.196-3.716)		3.531 (1.632-7.644)	
Age (years)					
<50	26	1.000.	0.900	1.000	0.123
≥50	117	0.962 (0.531-1.744)		1.862 (0.845-4.104)	
Gender					
Female	68	1.000	0.131	1.000	0.387
Male	75	1.432 (0.899-2.282)		1.278 (0.733-2.228)	
Site					
Right colon	34	1.000	0.011	1.000	0.096
Left colon	109	2.197 (1.195-4.041)		1.804 (0.901-3.612)	
Size (cm, diameter)					
<5	61	1.000	0.344	1.000.	0.104
≥5	82	1.27 (0.774-2.085)		1.626 (0.905-2.922)	
Differentiation					
Well	34	1.000	0.236	1.000	0.009
Moderately	81	0.771 (0.411-1.446)		0.894 (0.404-1.979)	
Poorly	28	1.299 (0.589-2.864)		2.758 (1.100-6.915)	
Dukes' stage					
A and B	66	1.000	0.020	1.000	0.039
C and D	77	1.942 (0.981-3.027)		2.046 (0.846-3.242)	

P-values were obtained by Cox proportional hazards analysis modeled for the high and low/negative levels of ESM-1 expression.

the most well-characterized, and HIF-2α (44) has emerged as a non-redundant player, but the role of full-length HIF-3α (45,46) is not yet known. It is well known that HIF-1α but not HIF-1β plays an important role in the HIF-1 pathway and regulates HIF target genes (47). HIF-1α is induced by hypoxia in almost all cell types, and is frequently overexpressed in solid tumors (11,48). Furthermore, the upregulation of HIF-1α correlates with cancer progression or aggressiveness in many human tumors, although the prognostic significance of HIF-1α induced by hypoxia in colon cancer has only been restrictively elucidated. Using immunohistochemical screening, we showed that HIF-1α protein was overexpressed in colon cancer tissues, and that this expression is significantly correlated with Dukes' stage (P=0.005) and poor overall survival (data not shown). These results were coincident with observations of others that HIF-1α overexpression is correlated with worse clinical prognosis (14,49).

The present results demonstrated that HIF-1α enhances ESM-1 expression in response to hypoxia in human CRC. We suggest that ESM-1, in addition to serving as a prognostic marker, may also serve as a therapeutic target by using the HIF pathway in human CRC. Further studies into the potential of ESM-1 inhibition as an effective means of enhancing tumor response to treatment and/or delaying tumor progression are warranted.

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