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■ ORIGINAL ARTICLE ■

Increased Expression of Thymosin β_4 Is Independently Correlated with Hypoxia Inducible Factor-1α (HIF-1α) and Worse Clinical Outcome in Human Colorectal Cancer

Seung Yun Lee · Mee Ja Park
Hye Kyung Lee · Hyun Jin Son
Chang Nam Kim[1] · Joo Heon Kim
Dong Wook Kang

Departments of Pathology and [1]Surgery, Eulji University Hospital, Daejeon, Korea

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Corresponding Author
Joo Heon Kim, MD, PhD
Department of Pathology, Eulji University Hospital, 95 Dunsanseo-ro, Seo-gu, Daejeon 35233, Korea
Tel: +82-42-611-3454
Fax: +82-42-611-3459
E-mail: kjh2000@eulji.ac.kr

Dong Wook Kang, MD, PhD
Department of Pathology, Eulji University Hospital, 95 Dunsanseo-ro, Seo-gu, Daejeon 35233, Korea
Tel: +82-42-611-3454
Fax: +82-42-611-3459
E-mail: astrias@eulji.ac.kr

Background: Thymosin β_4 is a multi-functional hormone-like polypeptide, being involved in cell migration, angiogenesis, and tumor metastasis. This study was undertaken to clarify the clinicopathologic implications of thymosin β_4 expression in human colorectal cancers (CRCs). **Methods:** We investigated tissue sections from 143 patients with CRC by immunohistochemistry. In addition, we evaluated the expression patterns and the clinico-pathological significance of thymosin β_4 expression in association with hypoxia inducible factor-1α (HIF-1α) expression in the CRC series. **Results:** High expression of thymosin β_4 was significantly correlated with lymphovascular invasion, invasion depth, regional lymph node metastasis, distant metastasis, and TNM stage. Patients with high expression of thymosin β_4 showed poor recurrence-free survival ($p = .001$) and poor overall survival ($p = .005$) on multivariate analysis. We also found that thymosin β_4 and HIF-1α were overexpressed and that thymosin β_4 expression increased in parallel with HIF-1α expression in CRC. **Conclusions:** A high expression level of thymosin β_4 indicates poor clinical outcomes and may be a useful prognostic factor in CRC. Thymosin β_4 is functionally related with HIF-1α and may be a potentially valuable biomarker and possible therapeutic target for CRC.

Key Words: Thymosin β_4; Hypoxia inducible factor-1α; Hypoxia; Immunohistochemistry; Colorectal cancer

The incidence of colorectal cancer (CRC) has been rapidly increasing and CRC is one of the leading causes of cancer mortality worldwide. Despite earlier detection and advances in molecular pathology-based treatments, we continue to encounter CRC patients with early-stage cancer who present with recurrence. Human CRC is a heterogeneous and complex disease with various genetic and/or epigenetic alterations that result in a biologically aggressive tumor phenotype.[1-3] Various genetic alterations accompanied with genetic instability can be induced in the hypoxic tumor state.[1-7] Interactions between neoplastic cells and the hypoxic microenvironment modify CRC tumor cell phenotype.[8-11]

The β thymosins are a family of hormone-like polypeptides that consist of 40–44 amino acids and are further divided into 15 subfamilies.[12] Of these β thymosins, thymosin β_4 is the most common subtype and the differential expression of thymosin β_4 has been reported in fibrosarcoma,[13] malignant melanoma,[14] breast cancer,[15] gastric cancer,[16] and colon cancer.[17-23] We previously reported that thymosin β_4 expression was increased in breast cancer tissue and correlated this expression with tumor progression and lymph node metastasis via hypoxia inducible factor-1α (HIF-1α) modulation.[24]

In this study, we examined the expression patterns of thymosin β_4 and HIF-1α in 143 CRC patients using immunohistochemical staining. We also evaluated the clinico-pathological significance of thymosin β_4 and HIF-1α expression levels and their correlation with various prognostic factors of CRC.

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MATERIALS AND METHODS

Case selection and immunohistochemistry

CRC cases were selected from patients who underwent surgical treatment at Eulji University Hospital from January 2000 to June 2005. We excluded those specimens obtained from patients who underwent preoperative neoadjuvant chemoradiation. Pertinent clinical and pathological information was obtained from electronic operation records and pathology reports. All cases were histologically confirmed to be primary colorectal adenocarcinoma and hematoxylin and eosin slides were re-evaluated by two independent pathologists. The tumor grade of the adenocarcinoma was classified into low grade (≥50% of tumor glands) and high grade (<50% of tumor glands).[25] For signet ring cell carcinoma and mucinous adenocarcinoma, less than 50% glands were defined as high grade. Tumor budding was defined as a single or group of less than five detached tumor cells and classified into two grades.[26,27] Tumor recurrence was designated as tumor occurring at the anastomosing site, in the regional lymph nodes, and the pelvic cavity diagnosed by radiology, colonoscopy, exploratory surgical, and/or histological examination. In addition, metastasis was defined as the presence of tumor cells outside the area of resection, including the liver, pancreas, lung, and other organs.

All cases of CRC tissue with accompanying normal mucosal tissue were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. Tissue sections of 3–4 μm thickness were cut and mounted on ProbeOn slides (Fisher Scientific, Pittsburgh, PA, USA). Sections that contained both tumor and adjacent uninvolved colonic mucosa were selected for immunohistochemistry (IHC) in most cases. In a few cases, sections were trimmed in order to decrease the surface area for an even distribution of antibodies, so that only the tumor portion was included in the IHC evaluation. IHC conditions for thymosin β_4 and HIF-1α were optimized according to the manufacturers' instructions. Paraffin embedded tissue sections were deparaffinized and rehydrated through a series of xylene and alcohol. Slides were then treated with 10 mM/L sodium citrate buffer (pH 6.1) for 15 minutes and autoclaved at 120°C for antigen retrieval. All slide sections for IHC were incubated in 3% H_2O_2 for 10 minutes to inactivate endogenous peroxidase, washed with 10 mM/L phosphate buffered saline buffer (pH 7.4), and then incubated with normal bovine serum to reduce false-positive staining. Mouse monoclonal antibodies against thymosin β_4 (1:100, Biodesign Int., Saco, ME, USA) and HIF-1α (1:50, Novus Biologicals, Littleton, CO, USA) were used as primary antibodies. Slide sections were incubated with primary antibodies overnight at 4°C in a wet chamber and stained with diaminobenzidine as the substrate using an EnVision-HRP kit (Dako, Glostrup, Denmark). An irrelevant mouse IgG of the same isotype served as a negative control. Sections were counterstained with Mayer's hematoxylin solution and then mounted.

Assessment of IHC staining

To evaluate the expression of thymosin β_4 and HIF-1α in association with various clinico-pathological factors, the immunoreactivity of both thymosin β_4 and HIF-1α were analyzed in a semi-quantitative manner by two independent pathologists who were blinded to outcomes. Immunoreactivity for thymosin β_4 and HIF-1α was observed primarily in the cytoplasm and nuclei of normal mucosal epithelium and tumor cells, respectively. The intensity of immunohistochemical staining was scored as 0 to 2 (0, weaker staining than the normal mucosal epithelium; 1, staining similar to the normal mucosal epithelium; and 2, stronger staining than the normal mucosal epithelium). The percentage of positive cells was scored as 1 (<25% of tumor cells), 2 (25%–49% of tumor cells), 3 (50%–74% of tumor cells), and 4 (≥75% of tumor cells). To evaluate the statistical significance between thymosin β_4 and HIF-1α expression and clinico-pathological factors, the median value (25% of tumor cells showing a strong positive reaction than normal epithelium) of the series was used as the cutoff value to distinguish between tumor cells with low expression (<25% tumor cells) and high expression (≥25% of tumor cells). Cases with conflicting results were re-evaluated and a consensus was reached.

Statistical analysis of prognostic parameters

We performed statistical analysis using the SPSS ver. 18 (SPSS Inc., Chicago, IL, USA). The correlation between thymosin β_4 and the various clinico-pathological parameters were analyzed with the Pearson's chi-square test or Fisher exact test. To evaluate statistical analysis, recurrence-free survival was defined as the duration from the date of surgery to the first date of recurrence or the date of last follow-up. Similarly, overall survival was defined as the duration from the date of surgical therapy to the date of death or date of last follow-up. The mean follow-up duration for all patients was 53.3 months, ranging from 0.6 to 121.9 months. Using the Kaplan-Meier method, the recurrence-free survival curve and the overall survival curve were formulated. To examine the statistical significance of the differences in survival distribution, log-rank test was utilized. Multivariate analysis for overall survival and recurrence-free survival was performed using Cox proportional hazard regression analysis. In all statistical

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analyses, p-values less than .05 were considered statistically significant.

Ethical permission

The Institutional Review Board of Eulji University Hospital approved the study protocol and provided all necessary ethical permission.

RESULTS

Association of clinico-pathological characteristics with thymosin β4 and HIF-1α expression status

The median age of the 143 CRC patients (75 men and 68 women) at surgery was 62.2 years (range, 28 to 86 years) and the median tumor size was 5.2 cm (range, 0.8 to 12.0 cm) in maximum diameter. The majority of CRCs were moderately differentiated adenocarcinoma and 111 cases (77.6%) were classified as low grade and 32 cases (22.4%) as high grade (poorly differentiated 19, signet ring cell carcinoma 3, and mucinous carcinoma 10). One hundred and four cases (72.7%) showed lymphovascular tumor invasion and 106 cases (74.1%) exhibited high-grade tumor budding. According to the seventh edition of the AJCC TNM system,[28] 26 patients (18.2%) were diagnosed with early-stage tumor invasion (five cases of pT1 and 21 cases of pT2) and 117 patients (81.8%) were diagnosed with advanced-stage tumor invasion (105 cases of pT3 and 12 cases of pT4). Seventy-six patients (53.1%) presented with regional lymph node metastasis and 22 patients (15.4%) with distant metastasis. Twenty-one patients (14.7%) were at pTNM stage I, 45 patients (31.5%) were at stage II, 55 patients (38.5%) were at stage III, and 22 patients (15.4%) were at stage IV. Twenty-seven patients (18.9%) were treated with chemoradiation after the first surgery (data not shown). Clinico-pathological characteristics of the 143 CRC patients are summarized in Table 1.

In the normal colonic epithelium, immunoreactivity for thymosin β4 and HIF-1α was mostly none or weak. While thymosin β4 expression was found primarily in the cytoplasm of cancer cells, HIF-1α was stained predominantly in the nuclei of tumor cells (Fig. 1A–H). A high level of thymosin β4 and HIF-1α expression was observed in 66 of the 143 patients (46.2%) and in 67 of the 143 patients (46.9%), respectively. We analyzed whether thymosin β4 expression level was associated with clinico-pathological factors. We found that predictive factors for prognosis, such as lymphovascular invasion, invasion depth (pT), regional lymph node metastasis (pN), distant metastasis, and TNM stage showed statistically significant correlations with thymosin β4 immunoreactivity (Table 1). Patients with high thymosin β4 expression levels showed a significantly greater presence of lymphovascular invasion, more frequent regional lymph node metastasis, deeper invasion depth, and more advanced tumor

Table 1. Clinico-pathological variables and thymosin β4 expression status

Characteristic	Total	Thymosin β4 expression level		p-value
		Negative/Low	High	
Age (yr)				
<50	26	10 (13.0)	16 (24.2)	<.082
≥50	117	67 (87.0)	50 (75.8)	
Gender				
Female	68	35 (45.5)	33 (50.0)	<.587
Male	75	42 (54.5)	33 (50.0)	
Site				
Right/Transverse colon	34	20 (26.0)	14 (21.2)	<.505
Left colon and rectum	109	57 (74.0)	52 (78.8)	
Size				<.814
<5 cm in diameter	60	33 (42.9)	27 (40.9)	
≥5 cm in diameter	83	44 (57.1)	39 (59.1)	
Grade				<.926
Low	111	60 (54.1)	51 (45.9)	
High	32	17 (53.1)	15 (46.9)	
LV invasion				<.001*
Not identified	39	31 (40.3)	8 (12.1)	
Present	104	46 (59.7)	58 (87.9)	
Tumor border				.560
Pushing	13	8 (10.4)	5 (7.6)	
Infiltrating	130	69 (90.6)	61 (92.4)	
Tumor budding				<.118
Low	37	24 (31.2)	13 (19.7)	
High	106	53 (68.6)	53 (80.3)	
Invasion depth				<.001*[a]
pT1	5	5 (6.5)	0	
pT2	21	18 (23.4)	3 (4.5)	
pT3	105	50 (64.9)	55 (83.3)	
pT4	12	4 (5.2)	8 (12.1)	
LN metastasis				<.001*
pN0	67	52 (67.5)	15 (22.7)	
pN1	23	8 (10.4)	15 (22.7)	
pN2	53	17 (22.1)	36 (54.5)	
Distant metastasis				<.001*[a]
M0	121	74 (96.1)	47 (71.2)	
M1	22	3 (3.9)	19 (28.8)	
TNM stage				<.001*[a]
I	21	20 (26.0)	1 (1.5)	
II	45	32 (41.6)	13 (19.7)	
III	55	22 (28.6)	33 (50.0)	
IV	22	3 (3.9)	19 (28.8)	

Values are presented as number (%).
LV, lympho-vascular invasion; LN, lymph node.
*p<.05.
[a]Fisher exact test.

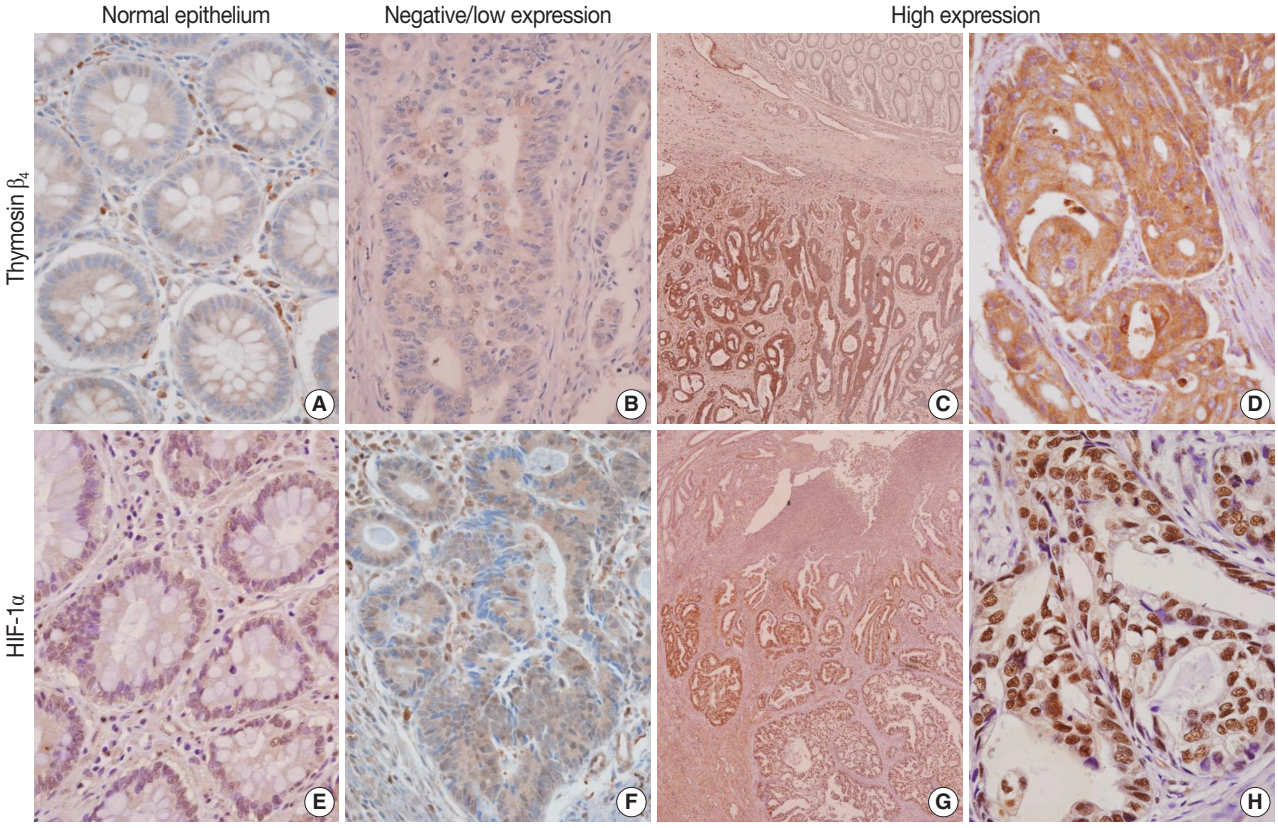

Fig. 1. Immunohistochemical expression of thymosin β_4 (A–D) and hypoxia inducible factor-1α (HIF-1α) (E–H) in human colorectal cancer. (A) No or weak expression of thymosin β_4 in normal colonic epithelium. (B) Low expression of thymosin β_4 in tumor glands. (C) Tumor cells show high thymosin β_4 expression, but no or weak thymosin β_4 expression in normal colonic epithelium. (D) Tumor cells reveal strong thymosin β_4 expression primarily in the cytoplasm of tumor cells. (E) No or weak immunoreactivity of HIF-1α in normal colonic epithelium. (F) Low expression of HIF-1α in tumor cells. (G) Tumor cells show strong HIF-1α expression. (H) HIF-1α is highly expressed predominantly in the nucleus of tumor cells.

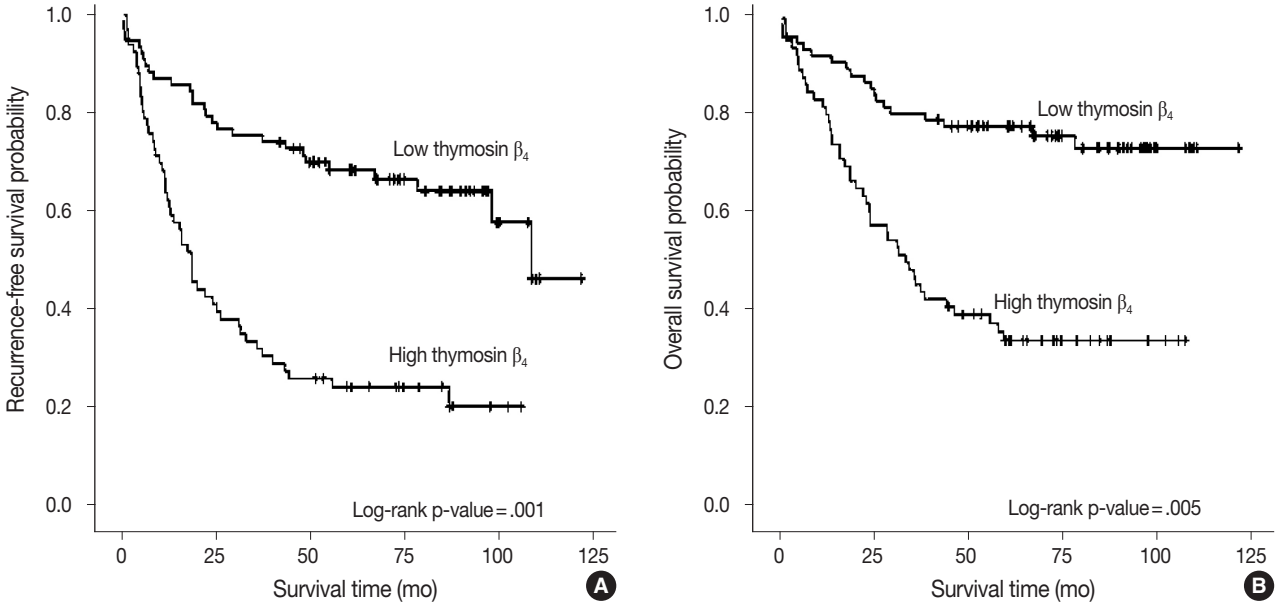

Fig. 2. Kaplan-Meier survival analysis by thymosin β_4 expression status. (A) Cumulative recurrence-free survival differences between patients with high and low thymosin β_4 expression. (B) Cumulative overall survival differences between patients with high and low thymosin β_4 expression. p-values were obtained using the log-rank test of differences.

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stage than in those with low thymosin β_4 expression levels ($p < .001$). We also evaluated the association between HIF-1α expression levels and clinico-pathological variables. We found a statistically significant correlation between high HIF-1α immunohistochemical expression and clinico-pathological factors such as lymphovascular invasion ($p = .006$), invasion depth ($p = .002$), regional lymph node metastasis ($p = .007$), distant metastasis ($p = .029$), and TNM stage ($p = .002$) (data not shown).

High thymosin β_4 expression level correlated with tumor recurrence and overall survival

We performed multivariate analysis to examine the correlation between thymosin β_4 expression levels with recurrence-free survival and overall survival. Forty patients (28.0%) presented with cancer recurrence during follow-up and 55 patients (38.5%) died of CRC with or without metastasis. Seven patients (4.9%) died of unknown causes, 17 patients (11.9%) were alive with local recurrence and/or distant metastasis, and 64 patients (44.8%) remained alive and recurrence-free. Kaplan-Meier analysis showed that high thymosin β_4 expression was significantly correlated with decreased recurrence-free survival ($p < .001$) (Fig. 2A).

Recurrence-free survival was shorter in patients with high expression levels of thymosin β_4, with a mean duration of 37.3 months (95% confidence interval [CI], 27.833 to 46.684), and was longer in patients with low levels of thymosin β_4 expression, with a mean duration of 84.6 months (95% CI, 73.538 to 95.571). We also found that high thymosin β_4 expression significantly correlated with worse overall survival ($p = .001$). Thymosin β_4 expression status also significantly split the cumulative overall survival curves (Fig. 2B). While the overall survival of CRC patients with high thymosin β_4 expression was mean duration of 51.7 months (95% CI, 41.381 to 61.939), the overall survival of CRC patients with low thymosin β_4 expression was longer with mean duration of 96.8 months (95% CI, 86.952 to 106.732). Multivariate analysis was also performed to assess the prognostic value of thymosin β_4 expression for recurrence-free survival and overall survival using various clinico-pathological parameters. Patients with high thymosin β_4 expression were found to have worse survival outcomes. Statistically significant clinico-pathological factors that were correlated with overall survival were high thymo-

Table 2. Multivariate Cox proportional hazard analysis for recurrence-free survival and overall survival

Characteristic	No.	Recurrence-free survival		Overall survival	
		Relative risk (95% CI)	p-value	Relative risk (95% CI)	p-value
Thymosin β_4			.001*		.005*
Low/negative	77	1.000		1.000	
High	66	2.540 (1.479–4.362)		2.457 (1.315–4.592)	
Size (diameter, cm)			.230		.094
<5	60	1.000		1.000	
≥5	83	1.349 (0.828–2.197)		1.636 (0.919–2.913)	
Grade			.048*		.013*
Low	111	1.000		1.000	
High	32	1.785 (1.005–3.171)		2.241 (1.186–4.236)	
LV invasion			.938		.974
Not identified	39	1.000		1.000	
Present	104	1.025 (0.547–1.922)		1.012 (0.485–2.111)	
Budding			.022*		.047*
Low	37	1.000		1.000	
High	106	2.094 (1.112–3.942)		2.068 (1.010–4.235)	
Invasion depth			.022*		.074
pT1+pT2	26	1.000		1.000	
pT3+pT4	117	3.491 (1.194–10.206)		3.849 (0.878–16.868)	
LN metastasis			.833		.437
Not identified	67	1.000		1.000	
Present	76	1.060 (0.617–1.821)		1.290 (0.678–2.455)	
Distant metastasis			.005*		.001*
M0	121	1.000		1.000	
M1	22	2.368 (1.301–4.311)		2.997 (1.586–5.664)	

p-values were obtained by Cox proportional hazards analysis modeled.
CI, confidence interval; LV, lympho-vascular invasion; LN, lymph node.
*$p < .05$.

sin β_4 expression (p = .005), high tumor grade (p = .013), high tumor budding (p = .047), and presence of distant metastasis (p = .001). The relative risk (RR) of death in patients with a high expression level of thymosin β_4 was more than two times greater (RR, 2.457; 95% CI, 1.315 to 4.592) than those with low thymosin β_4 expression levels. High thymosin β_4 expression was also an independent and relevant factor of decreased recurrence-free survival (p = .001). The RR of recurrence for patients with high thymosin β_4 expression level was 2.540 (95% CI, 1.479 to 4.362). Table 2 summarizes the results from the Cox proportional hazards analysis.

A statistically significant correlation between high HIF-1α expression and high thymosin β_4 expression was also found (p < .001). Specifically, of the 66 cases exhibiting high thymosin β_4 expression, 49 cases (74.2%) also showed high nuclear immunoreactivity of HIF-1α. Of the 77 cases expressing low thymosin β_4 expression, 59 cases (76.6%) revealed corresponding low HIF-1α expression (Table 3).

DISCUSSION

Growing tumors require oxygen and nutrient delivery through neovasculature. However, intratumoral hypoxia induced by imbalance between tumor growth and insufficient angiogenesis can lead to expression of HIF-1α, which is a transcriptional factor that activates tumor survival in an unstable hypoxic tumor microenvironment.[29-32] Recent reports have shown that thymosin β_4 stabilizes HIF-1α in human cancer cells[7] and that thymosin β_4 also induces migration and metastasis of colon cancer cells via the ILK/IQGAP1/Rac1 signal transduction pathway.[22,33]

Few studies have evaluated whether overexpression of thymosin β_4 influences clinical prognosis and whether thymosin β_4 is related to HIF-1α in CRCs. To better understand the relationship, we analyzed the clinical significance and expression status of thymosin β_4 and HIF-1α in CRC patients. Our study demonstrates that high thymosin β_4 expression has a significant association with lymphovascular invasion, nodal status, distant metastasis, and tumor progression in CRC patients (p < .001). This finding is consistent with our previous study showing hypoxia-induced high expression of thymosin β_4, which also significantly correlated with regional lymph node metastasis in breast cancer.[24] In a previous study, we found thymosin β_4 to be up-regulated under hypoxic conditions (5% O_2) using an *in vitro* hypoxia-induced model to generate transcription profiles in human CRC. Based on these findings, for this study we examined the association between thymosin β_4 expression and HIF-1α expression in CRC specimens. We then discovered that the overexpression of thymosin β_4 in CRC is closely related to the restricted overexpression of HIF-1α in the CRC cells (p < .001).

Recently, there have been reports regarding the association between thymosin β_4 expression with tumor development and epithelial mesenchymal transition (EMT).[18,34] In particular, Nemolato *et al.*[34] reported high expression of thymosin β_4 at the invasive front in colon cancer and discussed its associated with EMT as well as invasion and metastasis of tumor cells. However, from our study, since we were unable to find an association between thymosin β_4 expression and tumor budding (p = .118) and tumor border (p = .560), we found the direct association of thymosin β_4 expression with EMT to be weak.

The HIF complex, which involves various hypoxia-regulated genes, is a group of critical gene products in the tumor microenvironment of hypoxic adaptation and in angiogenesis.[35] The HIF complex is also an essential mediator in coordinating transcription of various factors in the tumor cells to survive in the hypoxic environment and its overexpression has been associated with increased mortality in various cancer types.[31,35-37] Among HIF complex proteins, HIF-1α is the best-characterized isoform. Whether HIF-2α, HIF-3α, and HIF-1β also play critical roles in the HIF pathway and regulate HIF target genes is not yet clearly known.[38-41] Hypoxic conditions induce HIF-1α expression in normal cells. HIF-1α is frequently upregulated in various cancer cells and the overexpression of HIF-1α correlates with advanced cancer progression or aggressiveness.[42] However, the clinical significance of HIF-1α in CRC has not been extensively studied. In this study, we observed a significant association between thymosin β_4 expression and HIF-1α expression (p < .001). This result coincides with previous studies that found overexpression of HIF-1α to be associated with poor prognosis.[36,37]

Table 3. Correlation between thymosin β_4 and HIF-1α expression status

	Frequency	Total	HIF-1α		p-value
			High	Low/negative	
Thymosin β_4	High	66	49 (74.2)	17 (25.8)	< .001
	Low/negative	77	18 (23.4)	59 (76.6)	

Values are presented as number (%).
HIF-1α, hypoxia inducible factor-1α.

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Thymosin β_4 has various functional roles in normal cell biology and its mechanism of action has recently been studied in various tumors. In this study, we found that high cytoplasmic expression of thymosin β_4 is clinically important and an independent prognostic factor for CRC patients. As our results demonstrate that high thymosin β_4 expression significantly correlates with tumor recurrence and worse overall survival, we suggest that high thymosin β_4 expression may be a useful prognostic factor in CRC.

Our results demonstrate that HIF-1α is correlated with overexpression of thymosin β_4 in human CRC. Although further studies are necessary to further validate our findings, we suggest that thymosin β_4, has potential as a prognostic biomarker and has potential as a HIF pathway target in human CRC.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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