

# Elevated Expression of Thymosin $\beta$ 4, Vascular Endothelial Growth Factor (VEGF), and Hypoxia Inducible Factor (HIF)-1 $\alpha$ in Early-Stage Cervical Cancers

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**Abstract** Recent studies have shown that thymosin  $\beta$ 4 (TB-4) is highly related with tumor metastasis and angiogenesis. In addition, TB-4 induced the expression of VEGF in melanoma cells. We investigated the expression patterns of TB-4 and related angiogenic proteins, VEGF, and HIF-1 $\alpha$ , at various stages of cervical cancers and also identified the expression pattern of these proteins in metastatic cervical cancers. Expression patterns of TB-4, VEGF, and HIF-1 $\alpha$  were studied with tissue microarray containing 42 samples of cervical cancers. In addition, 15 cervical cancers and metastatic tumors in lymph nodes from patients who have metastatic tumors were also analyzed to confirm the role of TB-4, VEGF, and HIF-1 $\alpha$  in cervical

cancer metastasis. The expression levels of TB-4, VEGF, and HIF-1 $\alpha$  were very weak at early cancer stages (stages 0 to 1A) but significantly increased at stage 1B. The numbers of blood vessels in tumors were also increased at stage 1B. The expression patterns of TB-4, VEGF, and HIF-1 $\alpha$  were compared in tumors without lymph node metastasis, primary tumors with lymph node metastasis, and metastatic tumors in lymph nodes. The expression levels of TB-4, VEGF, and HIF-1 $\alpha$  in primary tumors with lymph node metastasis and their metastatic tumors in lymph node were less than in tumors without lymph node metastasis. These data suggest that TB-4, VEGF, and HIF-1 $\alpha$  triggered angiogenesis and tumor invasiveness to surrounding tissues at early stage of cervical carcinoma but have a negative or no effect on the metastatic potential.

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## Introduction

Thymosin  $\beta$ 4 (TB-4), a 43 residue acidic peptide, was first isolated from calf thymus [1] as one of a family of closely-related peptides [2]. Although originally postulated to play a role in thymic immune system development [1], TB-4 and its mRNA are now known to be expressed at high levels in many tissues and in cell lines [3]. TB-4 is the major G-actin sequestering protein in motile and in proliferating cells [4, 5]. Many studies have implicated TB-4 in a number of cellular events, such as angiogenesis, wound healing, hair growth, apoptosis, and inflammatory responses [6–11].

TB-4 is involved in tumor growth and metastasis [12–17]. TB-4 regulates malignant mouse fibrosarcoma cell

motility and metastasis and stimulates B16 melanoma cell metastasis and angiogenesis [12, 13]. Overexpression of the TB-4 gene increased invasion of SW480 colon carcinoma cells and the distant metastasis of human colorectal carcinoma. TB-4 triggers an epithelial-mesenchymal transition in colorectal carcinoma by upregulating integrin-linked kinase [14, 15]. The expression of TB-4 was also detected in human breast cancer cells [17]. Although TB-4 has been reported to have important roles in angiogenesis and metastasis, only several studies have showed the expression pattern of TB-4 in metastatic tumors, including small cell lung carcinoma and breast carcinoma [16–18].

Cervical cancer is a common malignant disease responsible for the deaths of a large number of women in the developing world. Angiogenesis and tumor metastasis increase the lethality of cervical cancer and many clinical approaches are aimed at preventing angiogenesis and metastasis [19, 20]. Altered angiogenesis is an important phenotype of high-grade cervical lesions and invasive cervical carcinomas. Oncoproteins of human papillomavirus enhance the expression of vascular endothelial growth factor (VEGF) and the expression of both VEGF and hypoxia-inducible factor (HIF)-1 $\alpha$  is increased during carcinogenesis and progression of cervical cancer [24–27]. Although VEGF and HIF-1 $\alpha$  are important in carcinogenesis and TB-4 is involved in angiogenesis, tumor growth, and metastasis, the expression patterns of TB-4 in cervical cancer have never been studied yet.

Here, the expression patterns of TB-4 and functionally-related proteins, including VEGF and HIF-1 $\alpha$ , were analyzed in 42 specimens of cervical cancers, 4 metastatic tumors in lymph nodes, and 4 samples of normal cervix using tissue microarray. In addition, 15 primary cervical cancer tumors and their lymph node-derived metastatic tumors were analyzed from patients who have lymph nodes metastasis to confirm the role of TB-4 in the cervical cancer metastasis.

## Materials and Methods

### Tissue Microarray

Tissue microarray slides of cervical cancer were purchased from Super Bio Chip (SuperBioChips Laboratories, Seoul, KOREA). No clinical information except the age, gender of each patient, and stage of cancer was available for the tissue on these arrays.

### Patient Population

To analyze the role of TB-4, HIF-1 $\alpha$ , and VEGF expression in metastatic cervical carcinoma, the tissues of 15 patients

with cervical carcinoma and lymph node metastasis were examined by immunohistochemical and Hematoxylin and Eosin (H&E) staining. The detail patient population was described in Tables 1 and 2.

### Immunohistochemistry

For immunohistochemistry, tissue microarray slides were deparaffinized and hydrated. For antigen retrieval, slides were immersed in citrate buffer (0.01 M, pH 6.0) and heated twice in a microwave (700 W or high) for 5 min. Then, slides were quenched with endogenous peroxidase by incubation with 3% hydrogen peroxide solution for 5 min and washed three times in PBS for 5 min. Slides were immunostained with rabbit polyclonal antibody to TB-4 (1:1,000 dilution; ALPCO Diagnostics, Windham, NH, USA) or VEGF (1:2,000 dilution, Abcam Inc., Cambridge, MA, USA) at 4°C for overnight. After primary antibody incubation, slides were washed three times in PBS for 5 min and incubated with secondary antibody (DAKO REAL EnVision, HRP RABBIT/MOUSE, CA, USA) for 1 h. After secondary antibody incubation, slides were washed four times in PBS for 5 min each and the color reaction was developed with Dako's EnVision™ System (DAKO, Carpinteria, CA, USA). TB-4 was stained with DAB (diaminobenzidine) (DAKO, Carpinteria, CA, USA) and VEGF was stained with permanent red (DAKO, Carpinteria, CA, USA). Slides were counterstained Meyer's hematoxylin (DAKO, Carpinteria, CA, USA) for 10 s, dehydrated, and mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA).

### Semiquantitative Assessment

For protein expression assessment, staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Focal intensity of expression was scored and the score was added to the general score (0.5: focally weak, 1.5: focally moderate, 2.5: focally strong). Three well-trained and blinded observers read the slides and scored the expression of TB-4, VEGF and HIF-1 $\alpha$ . The detail score of expressions were described in Tables 1 and 2.

### Quantitation of Blood Vessels in Solid Tumors

We determined the number of blood vessels in cervical tumors by staining with a rat polyclonal anti-CD31/platelet-endothelial cell adhesion molecule-1 (PECAM-1) antibody that recognizes PECAM-1 on endothelial cells (1:100 dilution; Abcam Inc., Cambridge, MA, USA). Antibody binding was detected with the use of an

**Table 1** Analysis of expression patterns of TB-4, VEGF and HIF-1 $\alpha$  in tissue arrays of cervical cancers

| No | Age | Organ  | Diagnosis                              | Mean Expression |      |                |       | No. blood vessels/area (mm <sup>2</sup> ) |
|----|-----|--------|--|-----------------|------|----------------|-------|---|
|    |     |        |  | TB-4            | VEGF | HIF-1 $\alpha$ | Stage |   |
| 3  | 42  | Cervix | squamous cell carcinoma, microinvasive | 0.83            | 1    | 1              | 1A1   | 0.00                                      |
| 4  | 57  | Cervix | squamous cell carcinoma in situ        | 0.33            | 1    | 0.67           | 0     | 0.00                                      |
| 5  | 33  | Cervix | squamous cell carcinoma, microinvasive | 0.33            | 1    | 0.67           | 1A1   | 0.00                                      |
| 6  | 35  | Cervix | squamous cell carcinoma in situ        | 0.33            | 1    | 0.33           | 0     | 0.00                                      |
| 7  | 48  | Cervix | squamous cell carcinoma, microinvasive | 0.50            | 1    | 0.5            | 1A1   | 13.29                                     |
| 8  | 39  | Cervix | squamous cell carcinoma, microinvasive | 0.67            | 1    | 0.17           | 1A1   | 18.10                                     |
| 9  | 58  | Cervix | squamous cell carcinoma, microinvasive | 0.33            | 1    | 0.17           | 1A1   | 0.00                                      |
| 11 | 53  | Cervix | squamous cell carcinoma                | 0.83            | 1    | 0.33           | 1b2   | 14.84                                     |
| 12 | 53  | Cervix | squamous cell carcinoma                | 2.50            | 1.17 | 1              | 3B    | 17.96                                     |
| 13 | 50  | Cervix | adenocarcinoma                         | 0.33            | 1.17 | 0              | 3B    | 3.80                                      |
| 14 | 45  | Cervix | squamous cell carcinoma                | 1.83            | 2.17 | 2.67           | 3B    | 23.24                                     |
| 15 | 49  | Cervix | squamous cell carcinoma                | 0.67            | 2.17 | 2.5            | 3B    | 12.83                                     |
| 16 | 35  | Cervix | squamous cell carcinoma                | 0.83            | 2.17 | 2.5            | 1B1   | 10.57                                     |
| 18 | 39  | Cervix | squamous cell carcinoma                | 2.00            | 2.17 | 1.33           | 1B1   | 4.66                                      |
| 19 | 66  | Cervix | squamous cell carcinoma                | 0.33            | 2    | 2.67           | 3B    | 2.06                                      |
| 20 | 55  | Cervix | squamous cell carcinoma                | 0.67            | 2    | 3              | 1B2   | 0.00                                      |
| 21 | 64  | Cervix | squamous cell carcinoma                | 1.00            | 1    | 0              | 3B    | 12.76                                     |
| 22 | 50  | Cervix | squamous cell carcinoma                | 1.50            | 1    | 0              | 1B1   | 25.15                                     |
| 23 | 48  | Cervix | squamous cell carcinoma                | 3.00            | 2.17 | 1.5            | 3B    | 8.82                                      |
| 24 | 51  | Cervix | squamous cell carcinoma                | 2.67            | 1    | 3              | 3B    | 0.00                                      |
| 25 | 54  | Cervix | squamous cell carcinoma                | 1.50            | 1.83 | 0.33           | 1B1   | 0.00                                      |
| 26 | 39  | Cervix | squamous cell carcinoma                | 2.67            | 3    | 2.83           | 1B1   | 2.54                                      |
| 27 | 58  | Cervix | squamous cell carcinoma                | 2.67            | 2.17 | 0.17           | 1B1   | 17.55                                     |
| 28 | 57  | Cervix | squamous cell carcinoma                | 1.00            | 3    | 0.67           | 1B1   | 0.00                                      |
| 29 | 55  | Cervix | squamous cell carcinoma                | 2.00            | 2.17 | 2.67           | 1B1   | 0.00                                      |
| 30 | 35  | Cervix | squamous cell carcinoma                | 0.50            | 1    | 1.5            | 3B    | 102.70                                    |
| 31 | 62  | Cervix | squamous cell carcinoma                | 1.33            | 1    | 0.83           | 1B1   | 13.54                                     |
| 32 | 64  | Cervix | squamous cell carcinoma                | 1.00            | 1    | 1.5            | 3B    | 5.69                                      |
| 33 | 60  | Cervix | adenosquamous carcinoma                | 1.00            | 1.67 | 1              | 1B1   | 8.94                                      |
| 34 | 43  | Cervix | squamous cell carcinoma                | 1.83            | 1.17 | 0.5            | 3B    | 7.16                                      |
| 35 | 68  | Cervix | squamous cell carcinoma                | 1.17            | 2.33 | 1              | 1B1   | 0.00                                      |
| 37 | 40  | Cervix | squamous cell carcinoma                | 2.50            | 1.17 | 0.67           | 1B1   | 22.92                                     |
| 38 | 58  | Cervix | adenosquamous carcinoma                | 0.33            | 1    | 0              | 1B1   | 33.83                                     |
| 41 | 62  | Cervix | squamous cell carcinoma                | 1.00            | 1    | 0.67           | 3B    | 0.00                                      |
| 42 | 32  | Cervix | squamous cell carcinoma                | 0.33            | 1    | 1.17           | 3B    | 0.00                                      |
| 43 | 55  | Cervix | squamous cell carcinoma                | 0.50            | 1    | 0.67           | 3B    | 0.00                                      |
| 44 | 61  | Cervix | squamous cell carcinoma                | 0.83            | 1    | 0.83           | 3B    | 17.58                                     |
| 45 | 34  | Cervix | adenocarcinoma                         | 0.33            | 1    | 0.67           | 3B    | N/A                                       |
| 46 | 53  | Cervix | squamous cell carcinoma                | 2.50            | 2    | 1              | 1B1   | 19.04                                     |
| 47 | 45  | Cervix | squamous cell carcinoma                | 3.00            | 2.17 | 1              | 1B1   | 4.73                                      |
| 49 | 47  | Cervix | squamous cell carcinoma                | 0.33            | 1.67 | 0.83           | 3B    | 17.90                                     |
| 51 | 62  | Lymph  | node metastatic carcinoma of No.41     | 1.00            | 1    | 1              |       | 0.00                                      |
| 52 | 32  | Lymph  | node metastatic carcinoma of No.42     | 0.67            | 1    | 1              |       | 0.00                                      |
| 53 | 55  | Lymph  | node metastatic carcinoma of No.43     | 0.67            | 1    | 1              |       | 11.20                                     |
| 54 | 61  | Lymph  | node metastatic carcinoma of No.44     | 0.83            | 1    | 0.5            |       | 0.00                                      |
| 56 | 53  | Cervix | normal of No.46                        | 1.50            | 1    | 1.5            |       |   |
| 57 | 45  | Cervix | normal of No.47                        | 1.50            | 1    | 1.17           |       |   |

**Table 1** (continued)

| No | Age | Organ  | Diagnosis       | Mean Expression |      |                |       | No. blood vessels/area (mm <sup>2</sup> ) |
|----|-----|--------|-----------------|-----------------|------|----------------|-------|---|
|    |     |        |                 | TB-4            | VEGF | HIF-1 $\alpha$ | Stage |   |
| 58 | 65  | Cervix | normal of No.48 | 1.50            | 1.33 | 1.5            |       |   |
| 59 | 47  | Cervix | normal of No.49 | 1.50            | 1.17 | 1.5            |       |   |

\*LN meta: Lymph node metastasis

EnVision peroxidase system (DAKO Carpinteria, CA, USA), and the number of blood vessels was counted in a measured tumor area by image analysis software (NIS-Elements D 2.3, Laboratory Imaging, Praha, Czech Republic).

#### Statistical Analysis

The protein expression levels, and number of blood vessels per area were measured for calculation of mean values and 95% confidence intervals. Statistical significance of differ-

**Table 2** Analysis of expression patterns of TB-4, VEGF and HIF-1 $\alpha$  in metastatic cervical cancers (patients)

| No   | Age | Organ  | Diagnosis                     | Mean Expression |      |                |       | No. blood vessels/area (mm <sup>2</sup> ) |
|------|-----|--------|-------------------------------|-----------------|------|----------------|-------|---|
|      |     |        |                               | TB-4            | VEGF | HIF-1 $\alpha$ | Stage |   |
| 01-1 | 73  | Cervix | squamous cell carcinoma       | 2.17            | 2.17 | 2.167          | 1B    | 28.47174                                  |
| 01-2 |     | LN     | Lymph node metastasis of 01-1 | 1.5             | 1.5  | 1.5            |       |   |
| 02-1 | 72  | Cervix | squamous cell carcinoma       | 0.5             | 2.17 | 0.5            | 2B    | 14.58267                                  |
| 02-2 |     | LN     | Lymph node metastasis of 02-1 | 0.5             | 1.17 | 0              |       |   |
| 03-1 | 40  | Cervix | squamous cell carcinoma       | 0.33            | 0.5  | 0.333          | 2A    | 17.27899                                  |
| 03-2 |     | LN     | Lymph node metastasis of 03-1 | 0               | 0    | 0              |       |   |
| 04-1 | 34  | Cervix | squamous cell carcinoma       | 2.83            | 1.83 | 0.833          | 2B    | 26.51738                                  |
| 04-2 |     | LN     | Lymph node metastasis of 04-1 | 1.33            | 1.67 | 0              |       |   |
| 05-1 | 51  | Cervix | squamous cell carcinoma       | 1.17            | 1.5  | 0.167          | 3B    | 0   |
| 05-2 |     | LN     | Lymph node metastasis of 05-1 | 2.5             | 1.83 | 0.333          |       |   |
| 06-1 | 39  | Cervix | squamous cell carcinoma       | 1.67            | 2.33 | 0              | 2B    | 0   |
| 06-2 |     | LN     | Lymph node metastasis of 06-1 | 0.5             | 0.5  | 0.333          |       |   |
| 07-1 | 47  | Cervix | squamous cell carcinoma       | 1.5             | 1.17 | 0.667          | 2B    | 6.930184                                  |
| 07-2 |     | LN     | Lymph node metastasis of 07-1 | 0.83            | 1    | 0.5            |       |   |
| 08-1 | 56  | Cervix | squamous cell carcinoma       | 0.67            | 0.5  | 1              | 2A    | 15.28255                                  |
| 08-2 |     | LN     | Lymph node metastasis of 08-1 | 0.83            | 0.5  | 1.333          |       |   |
| 09-1 | 48  | Cervix | squamous cell carcinoma       | 1               | 1.67 | 1              | 2B    | 23.97947                                  |
| 09-2 |     | LN     | Lymph node metastasis of 09-1 | 0.5             | 0.33 | 0.333          |       |   |
| 10-1 | 60  | Cervix | squamous cell carcinoma       | 0.5             | 0.5  | 0.333          | 2B    | 5.517349                                  |
| 10-2 |     | LN     | Lymph node metastasis of 10-1 | 1               | 1    | 0.667          |       |   |
| 11-1 | 44  | Cervix | squamous cell carcinoma       | 0.83            | 0.5  | 1.5            | 2B    | 0   |
| 11-2 |     | LN     | Lymph node metastasis of 11-1 | 0.67            | 0.83 | 0.833          |       |   |
| 12-1 | 49  | Cervix | squamous cell carcinoma       | 1.67            | 2.17 | 2.5            | 4B    | 1.293939                                  |
| 12-2 |     | LN     | Lymph node metastasis of 12-1 | 0.33            | 0.83 | 0.333          |       |   |
| 13-1 | 51  | Cervix | squamous cell carcinoma       | 3               | 2.67 | 1.167          | 2B    | 7.780872                                  |
| 13-2 |     | LN     | Lymph node metastasis of 13-1 | 1.17            | 1    | 1.667          |       |   |
| 14-1 | 63  | Cervix | squamous cell carcinoma       | 1.33            | 2.17 | 1.5            | 2A    | 0   |
| 14-2 |     | LN     | Lymph node metastasis of 14-1 | 0               | 0.33 | 1.333          |       |   |
| 15-1 | 58  | Cervix | squamous cell carcinoma       | 0.33            | 0.33 | 2              | 2B    | 14.19544                                  |
| 15-2 |     | LN     | Lymph node metastasis of 15-1 | 0               | 0.33 | 0.333          |       |   |

LN: lymph node

ences among the groups was determined using a two-tailed Student's *T*-test. *P* values less than .05 were considered statistically significant.

## Results

### Expression of TB-4, VEGF and HIF-1 $\alpha$ at Various Stages of Cervical Cancers

The expression patterns of TB-4 at various stages of cervical cancers were analyzed (Tables 1 and 2). From stage 0 to 1A, TB-4 expression was low (expression scores are less than 1.5) in all seven patients and the number of patients expressing more than a moderate level of TB-4 (expression scores are more than 1.5) was significantly increased at stage 1B (8 out of 19 show more than a moderate level and 3 out of 19 show strong expression of TB-4). This strong expression was reduced at advanced stages. Three patients out of 12 showed more than moderate expression of TB-4 at stage 2 and six patients out of 20 showed more than moderate expression of TB-4 at stages 3 and 4. Two patients in both stages 2 (out of 12) and stages 3 and 4 (out of 20) showed high expression of TB-4 (Table 3). Examples of the weak, moderate, and strong TB-4 expression patterns and their expression scores are shown in Fig. 1a.

The expression pattern of VEGF was similar with that of TB-4. As shown in Table 3, VEGF expression was low (expression scores are less than 1.5) in all seven patients at stage 0 to 1A but the number of patients expressing more than a moderate level or a high level of VEGF was significantly increased at stage 1B (13 out of 19 show more than a moderate level and 2 out of 19 show strong expression of VEGF). The expression of VEGF was also reduced at advanced stages, similar to TB-4. Seven patients out of 12 showed more than a moderate expression of TB-4 at stage 2 and six patients out of 20 showed more than a moderate expression of TB-4 at stages 3 and 4. Two patients at stage 2 (out of 12) showed strong expression of VEGF while no patient showed strong expression of VEGF at stages 3 and 4. The VEGF expression patterns in weak,

moderate, and strong expression groups and their expression scores are shown in Fig. 1b.

The expression of HIF-1 $\alpha$  was low at stage 0 to 1A (all seven patients showed low expression) but increased at stage 1B (5 out of 19 showed more than a moderate level and 3 out of 19 showed strong expression). The expression was reduced at stage 2 (1 out of 12 showed more than a moderate level and no patient showed strong expression). The expression increased again at stage 3 (5 out of 20 showed more than a moderate level and 3 out of 20 showed strong expression) (Table 3). The HIF-1 $\alpha$  expression patterns at each of these levels and their expression scores are shown in Fig. 1c.

The expression patterns of TB-4, VEGF, and HIF-1 $\alpha$  were compared. As shown in Fig. 2, the expression of TB-4 was increased at stage 1B and reduced after stage 2 with statistical significance determined using a two-tailed Student's *t* test. The *P*-value of TB-4 expression between stages 0-1A and 1B was 0.0017 demonstrating that the TB-4 level was significantly increased at stage 1B. The expression of TB-4 was down-regulated relative to stage 1B at stages 3 and 4 (*P*=0.0814). The expression of VEGF showed the same pattern of expression as that of TB-4. VEGF levels were significantly increased at stage 1B (*P* value is 0.0025) and reduced after stage 2 (*P* value between stage 1B and 3 and 4 was 0.0137). The expression of HIF-1 $\alpha$  was significantly increased at stage 1B (*P* value is 0.0726) and reduced at stage 2 (*P* value between stages 1B and 2 was 0.3402). However, the expression of HIF-1 $\alpha$  was increased at stages 3 and 4. These data suggest that TB-4, VEGF, and HIF-1 $\alpha$  were induced at early stages of tumor growth and spread (from stage 1B) to stimulate tumor growth and angiogenesis and reduced in larger sized tumors (from stage 2). The increased expression of HIF-1 $\alpha$  at stage 3 and higher grade suggested that the hypoxic condition of the larger tumor size re-stimulated the expression of HIF-1 $\alpha$ .

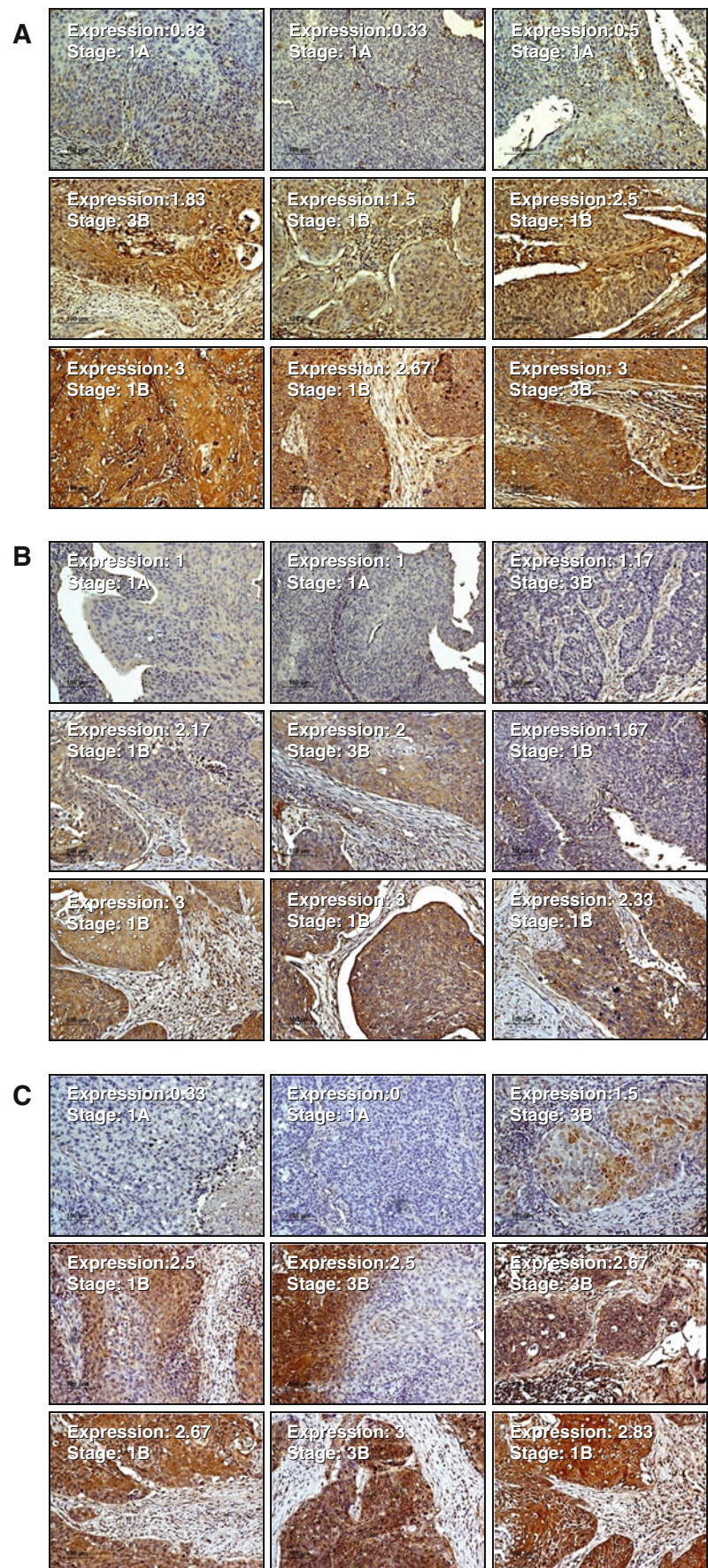
### Angiogenic Activity of Cervical Cancers at Various Stages

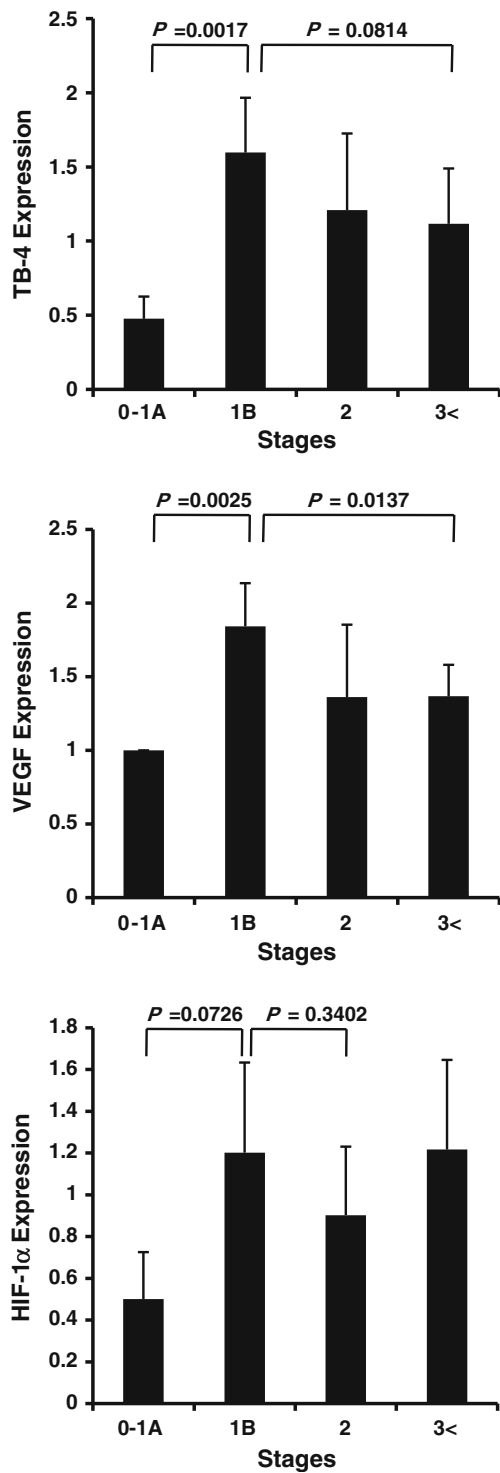
In order to analyze the relationship between elevated levels of TB-4, VEGF, and HIF-1 $\alpha$  at stage 1B and

**Table 3** Expression of TB-4, VEGF and HIF-1 $\alpha$  in cervical cancer patients

| Stage | No of patients | TB-4 expression |         |      | VEGF expression |         |      | HIF-1 $\alpha$ expression |         |      |
|-------|----------------|-----------------|---------|------|-----------------|---------|------|---------------------------|---------|------|
|       |                | 1.5>            | 1.5–2.5 | 2.5< | 1.5>            | 1.5–2.5 | 2.5< | 1.5>                      | 1.5–2.5 | 2.5< |
| 0-1A  | 7              | 7               | 0       | 0    | 7               | 0       | 0    | 7                         | 0       | 0    |
| 1B    | 19             | 9               | 7       | 3    | 6               | 11      | 2    | 14                        | 3       | 2    |
| 2     | 12             | 8               | 2       | 2    | 6               | 5       | 1    | 9                         | 3       | 0    |
| 3<    | 19             | 13              | 4       | 2    | 12              | 7       | 0    | 11                        | 5       | 3    |

**Fig. 1** Expression patterns of the TB-4, VEGF, and HIF-1 $\alpha$  at various stages of cervical cancer. **a** Tissue microarray slides were immunostained with rabbit polyclonal antibody to thymosin  $\beta$ 4 (1:2,000) and stained with DAB. **b** Tissue microarray slides were immunostained with rabbit polyclonal antibody to VEGF (1:2,000) and stained with DAB. **c** Tissue microarray slides were immunostained with rabbit polyclonal antibody to HIF-1 $\alpha$  (1:100) and stained with DAB. Top row, middle row, and bottom row show low expression, moderate expression, and high expression in triplicate, respectively. Slides were counterstained Meyer's hematoxylin. Original magnification X 200. Expression is the mean score of expression read by three well-trained and blinded observers. Stage means the cancer stage of cervical cancer



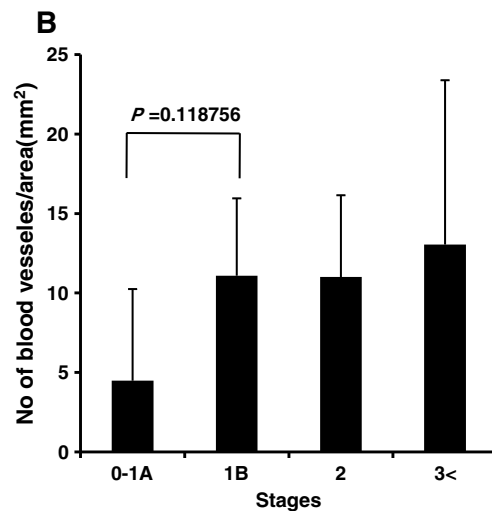
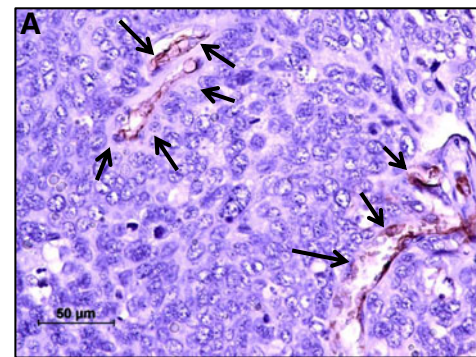


**Fig. 2** Comparison of the TB-4, VEGF, and HIF-1α protein expression at various stages of cervical cancers. For protein expression assessment, staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Focal intensity of expression was scored and added to the total score. Three investigators read the slides and scored the expression of TB-4, VEGF, and HIF-1α. The mean expression scores were calculated and compared

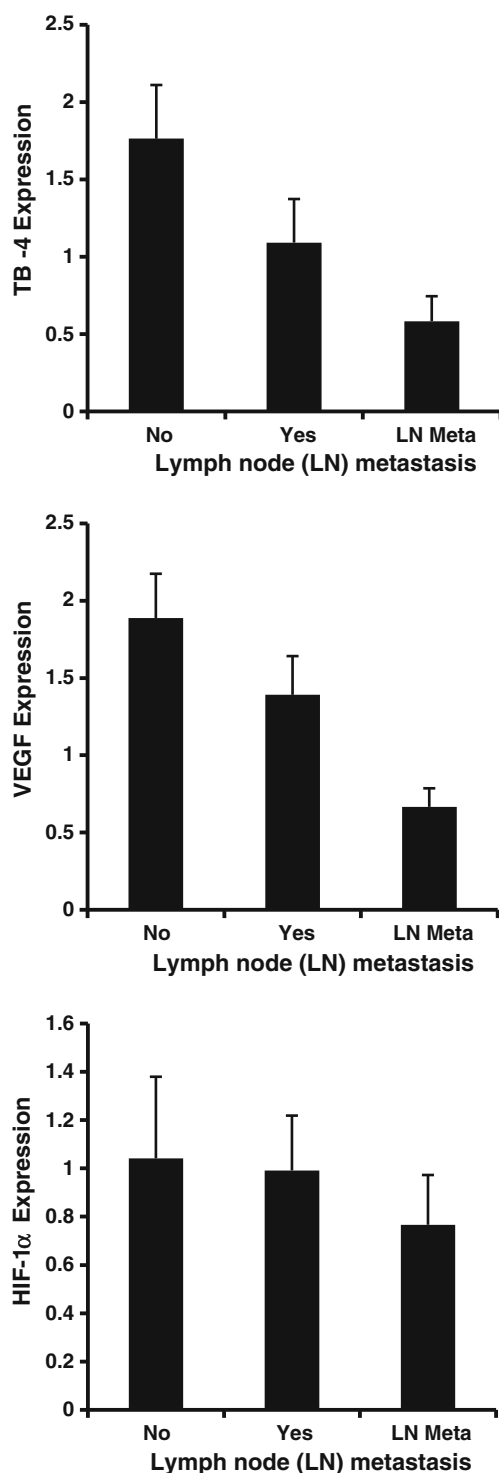
angiogenic activity, the number of blood vessels was counted after immunostaining of blood vessels with anti-CD31/platelet-endothelial cell adhesion molecule-1 (PECAM-1) antibody (Fig. 3a). The density of blood vessels was significantly increased at stage 1B and remained constant at advanced stages (Fig. 3b). These data were coincident with the expression patterns of TB-4, VEGF, and HIF-1α.

Expression of TB-4, VEGF, and HIF-1α in Cervical Cancers that Metastasized to Lymph Nodes

The expression patterns of TB-4, VEGF, and HIF-1α in metastasized cancers were analyzed. Metastatic tumors containing lymph node metastasis showed low expression of TB-4, VEGF, and HIF-1α in both primary and lymph node metastatic tumors in comparison with tumors containing no lymph node metastasis (Fig. 4). We



**Fig. 3** Angiogenic properties in various stages of cervical cancers. The number of blood vessels per area of solid tumor was counted in various stages of cervical cancers after immunostaining of blood vessels with anti-CD31/platelet-endothelial cell adhesion molecule-1 (PECAM-1) antibody (1/100). **a** Immunohistochemical staining of blood vessels with anti PECAM-1 Antibody. **b** Number of blood vessels per area (mm<sup>2</sup>) in various stages of cervical cancers



**Fig. 4** Expression patterns of the TB-4, VEGF, and HIF-1 $\alpha$  in metastatic cervical cancers. The expression patterns of TB-4, VEGF, and HIF-1 $\alpha$  were compared in tumors without lymph node metastasis (No), primary tumor with lymph node metastasis (Yes), and metastatic tumors in lymph node (LN meta). For protein expression assessment, staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Focal intensity of expression was scored and added to the total score. Three investigators read the slides and scored the expression of TB-4, VEGF, and HIF-1 $\alpha$ . The mean expression scores were calculated and compared

conclude that TB-4, VEGF, and HIF-1 $\alpha$  are involved at early stages of cervical cancer as the stimulators of angiogenesis or invasiveness into surrounding tumors but these proteins are not associated with the metastasis of cervical cancers.

## Discussion

Thymosins  $\beta$  were originally isolated from the thymus and constitute a highly conserved family of polypeptides [1, 2]. In humans, thymosins  $\beta$ 4,  $\beta$ 10, and  $\beta$ 15 have been identified in both normal tissue and in tumors. TB-4 is the most abundant of these molecules and has been linked to a number of important biological actions, including actin polymerization, angiogenesis, wound healing, inflammation, and signaling through the Akt pathway [4–7]. The role of TB-4 in actin polymerization is complex and appears to involve both sequestration of monomeric G-actin as well as binding to polymerized F-actin [4, 5]. Recent studies have shown that TB-4 is frequently overexpressed in malignant tumors [12, 13, 21, 22]. In addition, induced overexpression of TB-4 in melanoma cells is associated with increased metastatic capability, angiogenesis, and production of VEGF [12]. Wang *et al.* [14] also present data showing that cultured colon carcinoma cells progressed toward a more malignant phenotype after overexpression of TB-4. In contrast, Yamamoto *et al.* [23] described downregulation of TB-4 in metastatic cells of colorectal carcinomas. Our results also showed that the level of TB-4 was first increased at the early cancer stages but decreased at advanced stages and in metastatic cervical tumors.

Among the many functions of TB-4, angiogenesis is closely related with tumor formation and metastasis. TB-4 induced angiogenesis by stimulating endothelial cell differentiation and migration [6]. TB-4 also increased VEGF expression which is a key angiogenic protein [12]. Several studies have shown that angiogenesis and carcinogenesis and grade of cervical cancer are closely related and that VEGF and HIF-1 $\alpha$  expression are very important in cervical neoplasia [24–26]. TB-4 was also reported to have anti-inflammation effect to reduce swelling, pain, tissue damage, and death due to septic shock [11, 27–29]. These studies suggest the TB-4 elevation during the tumor progression can be related with anti-inflammation effect against tumor formation.

This study determined the expression patterns of TB-4 and related proteins involved in angiogenesis, including VEGF and HIF-1 $\alpha$ , in tissue microarray. The expression of TB-4, VEGF, and HIF-1 $\alpha$  were very weak at early stages (stages 0 to 1A) but significantly increased at stage 1B. These data suggest that TB-4, VEGF, and HIF-1 $\alpha$  triggered

early stage tumor invasion and angiogenesis. The transition of stage from 1A to 1B in cervical cancer is very critical point for clinical treatment. Only a very small amount of cancer can be seen with a microscope in the tissues of the cervix at stage 1A but some cancers can be seen without a microscope and are more than 5 mm deep or more than 7 mm wide at stage 1B. Surgical treatment should be initiated whether the stage is 1A or 1B. Therefore, the induction of TB-4, VEGF, and HIF-1 $\alpha$  expression may be early biomarkers for early stage of cervical cancer [30].

We found that the expression of TB-4, VEGF, and HIF-1 $\alpha$  was decreased at stage 2 and the angiogenic properties of the tumor did not change after stage 2. These results show that angiogenic stimulation by TB-4, HIF-1 $\alpha$ , and VEGF is reduced after stage 2. In contrast with TB-4 and VEGF, the expression of HIF-1 $\alpha$  was increased again after stage 3 suggesting that HIF-1 $\alpha$  was up-regulated by the hypoxic condition in the larger tumors.

In order to clarify the relationship between the expressions of TB-4, VEGF, and HIF-1 $\alpha$  and metastasis, the expression patterns of TB-4, VEGF, and HIF-1 $\alpha$  were compared in tumors without lymph node metastasis, primary tumors with lymph node metastasis, and metastatic tumors in lymph nodes. The expression levels of TB-4, VEGF, and HIF-1 $\alpha$  in primary tumors which have lymph node metastasis and in metastatic tumors in lymph nodes were less than tumors not in lymph nodes. These data are in accord with Yamamoto *et al.* [23] and but opposite to Wang *et al.* [14]. We conclude that the level of TB-4 can be regulated and is different at various stages of tumors even if the tumors are metastatic. In addition, induced overexpression of TB-4 can stimulate the metastatic potential of certain tumors but the natural TB-4 expression can be downregulated in metastatic tumor. To identify the role and balance of TB-4 expression in metastatic tumors, further studies are needed.

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