EXPRESSION OF BASIC FIBROBLAST GROWTH FACTOR, TRANSFORMING GROWTH FACTOR β_1 AND THEIR RECEPTORS IN OSTEOSARCOMA AND ITS RELATIONSHIP TO ANGIOGENESIS

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ABSTRACT

Objective: To investigate the expression of angiogenic factors, basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-β_1 in osteosarcoma, its association with neovascularization and prognosis. Methods: The expression of bFGF, TGF-β_1 and their receptors, as well as intratumoral microvessel count (MVD) were studied in 80 osteosarcomas by immunohistochemical staining and morphometry. The relationship between the angiogenic factors expression and prognosis was evaluated by a multivariate analysis using Cox proportion hazard model. Results: Among 80 cases of osteosarcoma, 46 cases were positive for bFGF/bFGFr (57.5%), and 31 cases for TGF-β_1/ TGF-β (RI)(38.8%) respectively. The MVD and bFGF, TGF-β_1 were important indicators to predict the prognosis of patients with osteosarcoma by the Cox proportion hazard model analysis. Conclusion: The angiogenic factors bFGF and TGF-β_1 are involved in the angiogenesis of osteosarcoma, and the angiogenesis influences the prognosis. Also they may be useful in the evaluation of the prognosis of patients with osteosarcoma.

Key words: Osteosarcoma, Angiogenic factors, Prognosis

The angiogenesis is a critical step in tumor growth and metastasis.[1] Recent studies have indicated that intratumoral microvascular density (MVD) was an important factor to influence the prognosis in many tumors, including osteosarcoma.[2,3] The angiogenesis is regulated by many angiogeneic factors, in which the basic fibroblast growth factor (bFGF) and transforming growth factor-β_1(TGF-β_1) were confirmed playing an important role in the angiogenic process.

In this study, we investigated the correlation between bFGF, TGF-β_1 expression and prognosis in osteosarcomas by detecting the expression of bFGF and TGF-β_1 immunohistochemically, as well as evaluating the MVD quantitatively.

MATERIALS AND METHODS

Clinical Materials

Eighty patients with intramedullary osteosarcoma in the long bones of the extremities were treated in hospital from 1968 to 1993. From them, 75 surgical specimens and 5 biopsies were obtained. Of the patients, 52 were male and 28 female with an age ranging from 11 to 68 (mean=23). 54 cases (62.5%) were in the second decade of their lives. The tumor invaded the soft tissue in most of the patients (92.5%). The tumor size varied from 5 to 25 cm (mean=10cm) in diameter. The histological gradings and types were determined according to Price's method[4] and Dahlin's classification,[5] as well as WHO's classification.[6]

Out of the 80 patients, 35 were treated with amputation and chemotherapy, 24 with amputation only, 17 with excision of the tumoral segment followed by inactivation and replantation or end-to-end connection of the amputated ends and 4 without any treatment. All the patients were followed up. The 2-year and 5-year survival rates were 33.8% and 18.3% respectively.

Immunohistochemical Staining

All specimens were fixed with 10% formalin, embedded in paraffin and then sectioned. The

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characteristics of the used antibodies were listed in Table 1. LSAB kit is the product of Dako Company; the immunohistochemical staining was performed according to the manufacturer's manual. The tissue sections were digested with trypsin for twenty min before the incubating with primary antibodies against F-VIIIRA and CD31. PBS was substituted for the primary antibody as the negative control.

Table 1. The type and source of antibodies

Antibodies	Type	Source	Dilation
FVIII-Rag	MC	Dako. Inc	1: 20
CD31	MC	Dako. Inc	1: 20
bFGF	PC	Santa Cruz	1: 20
FGFr	PC	Santa Cruz	1: 20
TGFβ-1	PC	Santa Cruz	1: 20
TGFβ (RI)	MC	Santa Cruz	1: 20

MC: monoclonal, PC: polyclonal

Counting of the Intratumoral MVD[7]

The MVD was determined with CMIAS image analysis system. The hemorrhagic and necrotic part and the peritumoral area of every section were excluded. The sections containing regular tissues were screened with a lens of 40-fold magnification to identify the areas of the highest vascular density in the tumor (so named spots). Then the MVD of three such areas in each tumor was counted under a microscope of 200-fold magnification (× 20 objective and × 10 ocular, 0.785 mm^2 per field, Olympus BX-50 microscope). The average of three counting was the MVD of the sarcoma.

Statistical Analysis

The Chi-square test was used to estimate the association between the MVD, bFGF, TGF-β_1 and the various clinicopathologic factors. Spearman's rank correlation co-efficient was calculated for a comparison of bFGF and TGF-β_1 with MVD. Cox proportional hazards model was applied to estimate the influence of MVD, bFGF and TGF-β_1 to the prognosis. These analyses were performed using a statistical computer package of SAS.

RESULTS

Expression of bFGF/bFGFR in Osteosarcoma

bFGF expression was consistent with bFGFR expression, together positive or together negative. Their product was localized in the cytoplasm of tumor cells, also was be observed in some epithelium of new capillaries. Forty-six out of 80 cases exhibited positive expression of (57.5%)(Figure 1).

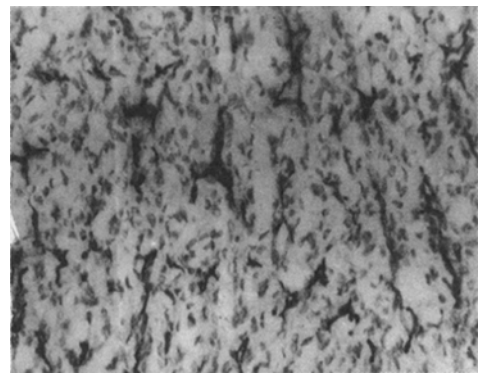

Fig. 1. The intratumoral microvessel density by immunohistochemical staining for endothelial cells with CD31 in osteosarcoma. LSAB × 200

Expression of TGF-β_1/TGF-β (RI) in Osteosarcoma

TGF-β_1 and it's receptor TGF-β (RI) positive staining mainly appeared in the epithelium of new capillaries in a cluster pattern and there was little in the tumor cells. Also TGF-β_1 expression was in agreement with TGF-β (RI), positively or negatively. Thirty-one out of 80 cases showed positive expression of them (38.8%)(Figure 2).

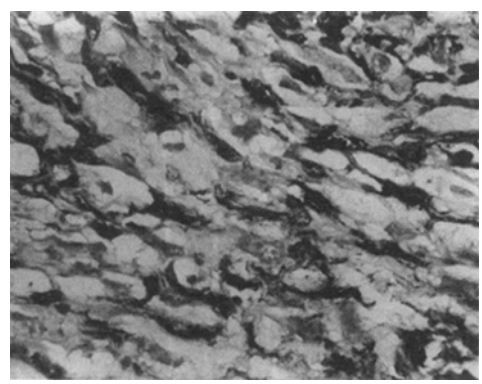

Fig. 2. bFGF positive product was localized in cytoplasm of tumor cell by immunohistochemical staining. LSAB × 200

Correlation of MVD bFGF and TGF-β_1 Expression with Clinicopathological Factors

As shown in Table 2, there was no correlation between MVD as well as bFGF, TGF-β_1 expression and tumor size, Price's grade and Dahlin's classification. But a significant association was found

between MVD, as well as bFGF, TGF-β_1 expression and WHO's classification. The MVD, bFGF and TGF-β_1 expressions were very rare in well-differentiated types than in other types (Figure 3).

Table 2. Correlation of MVD bFGF and TGF-β_1 expression with clinicopathological factors

Clinico-pathological data	N	MVD X (S)	VEGF +	VEGF −	bFGF/bFGFr +	bFGF/bFGFr −	TGFβ$_1$/TGFβ(RI) +	TGFβ$_1$/TGFβ(RI) −	*P* value
Tumor size									
<10 cm	40	33.1(19.6)	24	16	18	22	13	27	>0.05
≥10 cm	40	36.6(21.8)	18	22	28	12	18	22	
Price's grade									
Grade I	7	25.3(22.9)	3	4	4	3	3	4	
Grade II	38	30.5(15.7)	18	20	18	20	14	24	>0.05
Grade III	35	43.3(21.9)	21	14	24	11	14	21	
Dahlin's type									
Osteoblastic	33	35.5(19.8)	19	14	20	13	11	22	
Chondroblastic	16	33.2(24.1)	8	8	10	6	7	9	>0.05
Fibroblastic	24	33.9(20.1)	11	13	14	10	11	13	
Others	7	37.4(18.1)	4	3	2	3	2	4	
WHO classification									
Conventional	59	45.2 (19.8)	36	23	38	18	25	34	
Telangiectatic	4	34.7(11.4)	2	2	2	2	2	2	
Well differentiate	14	25.3(22.7)*	2	12*	4	10	2	12*	
Round cell	3	39(16.3)	2	1	2	1	2	1	

*$P<0.05$

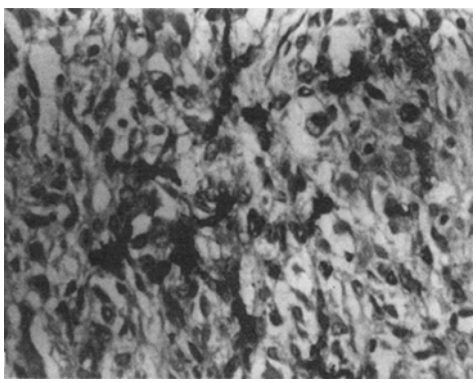

Fig. 3. The endothelial cells were mainly positive for TGF-β_1 in a cluster pattern by immunohistochemical staining. LSAB × 200

Correlation of bFGF, TGF-β_1 Expression with MVD

It was found that there is a significant correlation between bFGF, TGF-β expression and MVD by Spearman's analysis (Table 3).

Correlation of MVD, bFGF and TGF-β_1 Expression with the Prognosis

The results of the multivariate analysis for prognosis are summarized in Table 4. The MVD and bFGF, TGF-β_1, were important indicators to predict the prognosis of patients with osteosarcoma by Cox proportion hazard model analysis, as a sequencing of bFGF> TGF-β_1>MVD according their ratio risk value.

Table 3. Correlation of bFGF, TGF-β_1 Expressions with MVD

	MVD	bFGF	TGF-β_1
MVD	1.0000	0.40195	0.19615
P value	0.0	0.0002	0.0412
bFGF	0.40195	1.00000	0.42431
P value	0.0002	0.0	0.0001
TGF-β_1	0.19615	0.42431	1.00000
P value	0.0412	0.0001	0.0

Table 4. Correlation of MVD, bFGF and TGF-β_1 expression with the prognosis

Variable	DF	Parameter Estimate	Standard Error	Wald Chi-Square	Pr > chi-square	Risk Ratio
MVD	1	0.020568	0.00676	9.24803	0.0024	1.021
bFGF	1	0.603786	0.23147	6.80431	0.0097	1.829
TGF-β_1	1	0.520184	0.23689	4.82199	0.0281	1.682

DISCUSSION

bFGF is one of the most important angiogenic factors. It can stimulate the endothelial cells to proliferate, produce the proteases, and increase the expression of integrin on endothelial cells, and promote the endothelial cell to migrate.[8] Relf, et al. have found that the mRNA level and protein expression of bFGF were markedly higher in breast cancer tissues than in normal breast tissues.[3] Also our results showed that 57.5 percent of osteosarcomas showed high expression of bFGF, the bFGF expression was in agreement with the FGFr expression, and was significantly associated with the MVD count. The results confirmed that bFGF is a major inducer of angiogenesis in osteosarcoma.

TGF-β_1 is also expressed in endothelial cells during wound healing. *In vitro* TGF-β_1 may stimulate angiogenesis indirectly by inducing macrophage cells to produce some cytokines, but *in vivo* the bFGF have a similar role of VEGF by a different mechanism. bFGF can increase integrin expression of α 2, α 5, β_1 and β_3, while TGF-β_1 can increase integrin expression of α 2, α 5 and β_1 in endothelial cells.[9] These results revealed that TGF-β_1/TGFβ(RI) were highly specifically in endothelial cell, but rare or lowly expressed in tumor cells. Moreover, there was a significant correlation between the TGF-β_1/TGFβ(RI) expression and MVD count (P<0.05). It suggests that TGF-β_1 may contribute to angiogenesis by a self-secreted role.

It is known that the vesicular endothelial growth factor (VEGF) is an angiogenic factor of endothelial cell-specific,[10] while both the bFGF and TGF-β_1 are pleiotropic regulatory factors of tissue remodeling. In this paper, the expressions of bFGF and TGF-β_1, as well as their relationships were studied immunohistochemically. The results showed: (1) The expression of bFGF and TGF-β_1 were consistent with their respective receptors, which suggests a para-secreted and self-secreted mechanism of the above factors may play a major role in the angiogenic process; (2) bFGF expression was correlated with TGF-β_1 expression, and (3) The expression of bFGF and TGF-β_1 were correlated significantly with the MVD. The results indicate that bFGF and TGF-β_1 may act synergistically in promoting the neovasculization, furthermore, contribute to the prognosis of patients with osteosarcoma.

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