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Original Article

Relation between the expression of aminopeptidase N(APN)/CD13 and the clinical significance in osteosarcomas

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Abstract: Aminopeptidase N(APN)/CD13 is highly expressed in many malignant cells. This study aimed to examine CD13 expression and its clinical significance in osteosarcoma patients. CD13 expression was examined with immunohistochemistry (IHC) in 108 osteosarcomas. Correlations between CD13 expression and clinic pathological characteristics were analyzed with Chi-squared test and survival curves were generated with Kaplan-Meier method. IHC results showed that 76.85% (83/108) of the osteosarcomas were positive in CD13. Statistical analysis revealed that CD13 expression was correlated with age, Enneking stages and tumor volume in osteosarcomas ($P < 0.05$). In the multivariate stepwise Cox regression, Enneking staging and CD13 immunostaining was found to be a statistically significant factor on survival ($P < 0.05$) and the survival analysis showed that the survival probability was significantly higher in patients with reduced CD13 expression compared with patients with elevated expression ($P < 0.05$). These findings suggest that negative CD13 expression may implies a good prognosis in patients with osteosarcomas.

Keywords: Aminopeptidase N(APN)/CD13, immunohistochemistry, osteosarcoma, prognosis

Introduction

Osteosarcomas are the most common primary musculoskeletal malignant tumors in children, adolescents and young adults and account for about 5% of all pediatric tumors [1, 2]. It is associated with a high mortality rate. Chemotherapy is the conventional treatment, but in recent years improved chemotherapy cannot improve survival in patients with osteosarcoma. So, searching for new treatments becomes the trend of treating osteosarcoma. Usually, chemotherapy has been utilized to improve patient survival, but this therapeutic approach has reached a plateau phase during the last two decades. In the past decade the target therapy began and archived great success in various tumors such as leukemia, melanoma, and lung and breast cancers. Unfortunately, because of the fact that the genetic etiology of osteosarcomas is very complex and remains unclear, with no specific therapeutic targets yet identified, patients with osteosarcomas have not yet benefited from any targeted therapy.

Aminopeptidase N(APN), also called CD13, is a zine-binding type 2 transmembrane ectopeptidase of 150 kDa that forms a noncovalently bound homodimer on the cellular membrane [3]. The main functions are strengthening the invasive tumor cell, promoting the blood vessels formation, promoting the tumor cell proliferation and inhibition of the cell apoptosis. It highly expressed in many malignant cells, for example leukemia cells, liver cells and cervical cancer cells. But the test of expression in osteosarcomas is rare. So we use the immunohistochemical method to test the expression of CD13 in human osteosarcoma tissues, and analysis the differences of patients' clinical

Table 1. Expression of CD13 according to clinicopathologic features in osteosarcoma

Features	Expression of CD13			
	Number of cases	Negative	Positive	*P*-value
Gender				0.2955
Male	74	15	59	
Female	34	10	24	
Age				0.0489
Age < 18	67	21	46	
Age ≥ 18	41	4	37	
Location				0.7622
Femur	70	16	54	
Tibia and fibula	26	5	21	
Humerus	8	3	5	
Others※	4	1	3	
Histologic subtype				0.9220
Osteoblastic	82	18	64	
Chondroblastic	18	5	13	
Small cell	5	1	4	
Fibroblastic	3	1	2	
Enneking stages				0.0415
II	72	22	50	
III	36	3	33	
Tumor volume (mean, cm^3)		195.85	295.24	0.013

※Others include 2 patient in shoulder blade, 1 patient in rib and manubrium.

appearances and prognosis, to hope to find out the novel targeted therapy.

Materials and methods

Patients and tissue specimens

The study group was composed of 108 patients, aged 16-72 years (mean age of 48) from China. This study was approved by the Internal Review Board of the First Affiliated Hospital of PLA General Hospital, Beijing, China, from 2006-2014, with all pathological specimens in osteosarcomas obtained from the hospital pathology department database. 108 osteosarcoma samples (74 male, 34 female) were collected and all specimens were reviewed by two pathologists. All samples originated from definitive surgeries, except for two osteosarcoma samples derived from biopsies. In osteosarcoma patients, the tumor sites included the femur (70), tibia and fibula (26), humerus (8), shoulder blade (2), rib (1), and manubrium (1). Specimen histological subtypes included osteoblastic (82), chondroblastic (18), fibroblastic (3) and small cell (5). All patients were diagnosed according to histological analysis and radiographic features, with the histological features of osteosarcomas including spindle, roundness and polygon cell proliferation, cytologic anaplasia distinctly. Human kidney tissue served as a control CD13 expression was evaluated via immunohistochemistry.

Immunohistochemistry

Rabbit monoclonal directed against human CD13 and biotin-labeled second antibodies was bought from Abcam and Santa Cruz Biotechnology, USA. Specimens were formalin-fixed, decalcified in 5% nitric acid for 12 h, paraffin-embedded and sectioned (2 μm). Sections were dewaxed and hydrated, with a heat-antigen retrieval method utilized prior to incubation with 0.01 M sodium citrate buffer solution (pH 6.0, 10 min, 90°C). Next, sections were incubated in 3% hydrogen peroxide (H_2O_2) to inactivate endogenous peroxidases (10 min, 37°C), followed by incubation with a drop of goat serum (1:10 with PBS) for 1 h at 37°C. Each section was incubated with primary antibody (1:100) at 4°C overnight. After treatment with a biotinylated secondary antibody for 1 h at room temperature, the sections were colorimetrically detected with HRP-labeled Avidin-Biotin complex and DAB at 37°C for 7 min. Sections were rinsed 3-5 times with PBS between steps, except for the last step. The cerebellar tissue which stained in hematoxylin showed a positive result, with positive control of kidney cells, which also showing CD13 presence in cerebellar (4).

Immunostaining results were evaluated independently by two pathologists who were both blinded to clinical data (Min Zhao, Yiduo Jin). The discrepancies resolved by consensus. The percentage of positively stained cells with only cytoplasmic CD13 staining was calculated as a result. Specimen staining based on “positivity” and “negative” expression values of the CD13. The staining proportion of tumor cells over 10% was defined as immunostain positivity, and the staining proportion below 10% we defined as

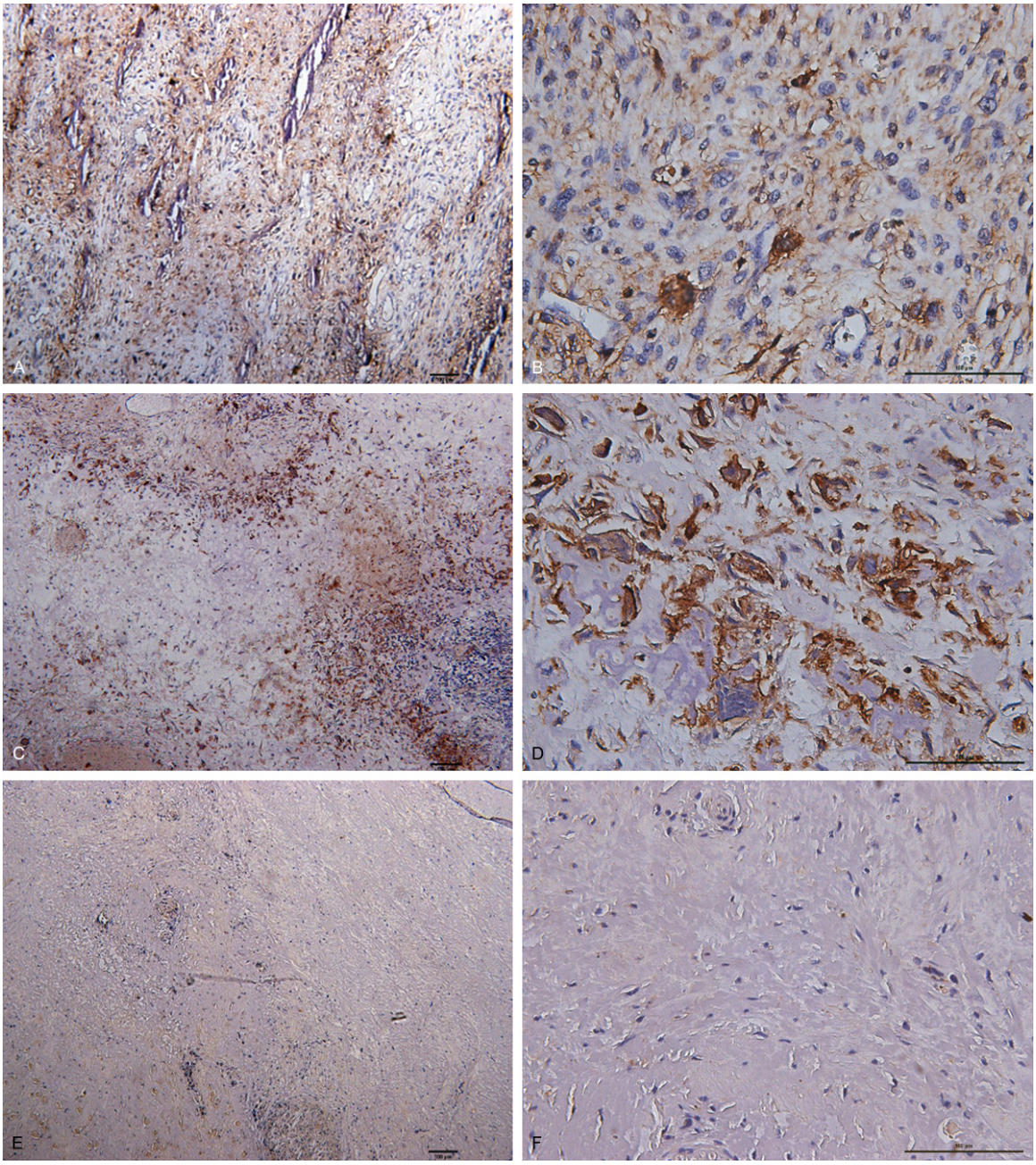

Figure 1. Immunohistochemical staining of CD13 in osteosarcoma. Positive staining of mGluR4 appeared as yellow to brown color in the cytoplasmic area of osteosarcoma cells, representative images from two patients (A × 100 and B × 400; C × 100 and D × 400) and a negative staining control (E × 100 and F × 400). Original magnifications were noted at the lower right corner of each microphotograph. Scale bar = 100 μm.

immunostain negative. CD13 expression as detected by immunohistochemical staining was examined via imaging with a BX 50-32 scanner (Olympus, Union City, CA, USA) and analyzed with NIS - Elements D software (Nikon).

Staining intensity has to be strong to be counted. Otherwise stained were counted as negative.

Statistical analysis

The patient characteristics: age, follow-up, gender, histological subtype, tumor location, Tumor volume,metastasis and Enneking stage were determined according to median and the statistic differences were analyzed using Chi-squared test, Cox and Kruskal-Wallis Test. Survival rate were estimated using the Kaplan-

Table 2. Multivariate Cox regression analyses

Factor	B	SE	Wald	df	Sig.	Exp (B)	95.0% CI
Gender	0.362	0.277	1.711	1	0.191	1.436	0.835-2.469
Age	-0.003	0.016	0.043	1	0.835	0.997	0.966-1.028
Tumor volume	0.000	0.001	0.013	1	0.909	1.000	0.998-1.002
Enneking stages	-1.051	0.471	4.979	1	0.026	0.349	0.139-0.880
CD13 Immunostaining	-0.649	0.279	5.428	1	0.020	0.523	0.303-0.902

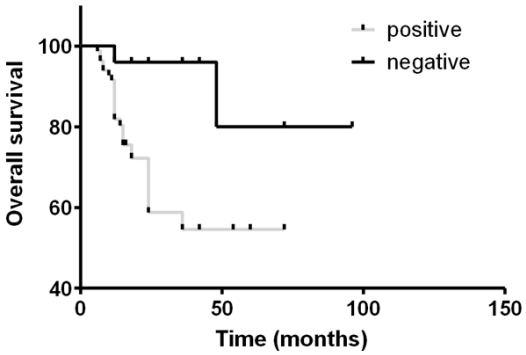

Figure 2. Survival analysis of osteosarcoma patients in 12-96 month. Log-rank analysis showed a higher survival rate in osteosarcoma patients with positivity CD13 expression relative to those with negative expression (P = 0.0019).

Meier method and survival curves were compared using the Log-rank Test. The overall survival analysis was performed to assess the death risk within 12-96 months (mean of 25.1 months). All results with $P < 0.05$ indicated statistical significance. For these analyses, we used the SPSS statistical software package 19.0.

Results

The relation between expression of CD13 and the clinical characteristics from 108 patients in osteosarcoma

The expression of CD13 is mainly on the cytomembrane. The expression of CD13 from 108 patients with osteosarcoma was evaluated by immunohistochemistry for CD13 (**Table 1**), with 76.85% immunostaining positivity (83/108, **Table 1**), while the expression in para-carcinoma tissue is lower. The positive expression and negative expression in osteosarcoma are showed in **Figure 1**.

The correlations between the clinical characteristics, such as gender, age, location, tumor volume and Enneking stages, and the expression of CD13 in osteosarcoma are summarized in **Table 1**. We can see that the proportion of immunostaining positivity has great statistical significance to immunostaining negativity in age (P = 0.0489), Enneking stages (P = 0.0415) and tumor volume (P = 0.013). But there are no statistical significance in gender (P = 0.2955), location (P = 0.7622), and histologic subtype (P = 0.9220). In the multivariate Cox regression, enneking stages and CD13 Immunostaining had a statistically significant influence on survival (P = 0.026 and P = 0.020, **Table 2**).

The relation between expression of CD13 and the prognosis

To investigate whether there is a relation between expression of CD13 and prognosis, Survival rate were estimated using the Kaplan-Meier method and overall survival curves were compared using the Log-rank Test. Of the 108 patients, 30 of 83 patients with positive CD13 expression died, while the 2 of 25 patients with negative CD13 expression died. The differences in the mortality rate (36.1% vs 8%) between the two group was statistically significant. Furthermore, according to the log-rank analysis, compared to the patients with positive CD13 expression, those with negative CD13 expression has a higher survival rate in osteosarcoma (P = 0.0019, **Figure 2**). These findings suggest that negative CD13 expression may implies a good prognosis in patients with osteosarcomas.

Discussion

Osteosarcoma is the most common primary bone cancer, which is characterized by frequent distant metastasis, especially to the lung [5]. Although the overall survival rate for osteosarcoma has increased to 70%, < 30% of patients presenting with metastases survive 5 years after the initial diagnosis [6]. For the primary osteosarcoma, the main therapy is surgery and adjuvant chemotherapy. But for the progress or refractory osteosarcoma, there is no effective therapy. However, in recent years, some study

shows that the occurrence, development and metastasis of osteosarcoma may be characterized by many genetic changes, so searching for the genetic changes for the targeted therapy may be the trend.

CD13 participates in the development of many tumors, confirmed to express in leukemia, liver cancer, lung cancer and ovarian cancer [4, 7, 8]. The main mechanism in the occurrence and development of tumor: 1. Promoting proliferation and inhibiting apoptosis. Studies have shown that, in vitro, using CD13 McAB or CD13 inhibitor can reduce the proliferation rate of mononuclear cells. It implies that CD13 can increase the proliferation rate of mononuclear cells [9]. In addition, Mishima [10] improved that co-culturing the cell lines expressing CD13 and the endothelial cells can resist the apoptosis that endothelium-derived IL-8 causes. But the effect disappears if CD13 inhibitors are used. Also, Van [11] discovered that the activity of CD13 is positive correlation with the tumor load. In some solid tumors, like osteosarcoma, lung cancer, colorectal cancer, it shows an obviously positive correlation between the tumor volume and the activity of CD13. This is the same as the results our study shows. 2. Promote tumor angiogenesis: As we all knows, tumor in situ must have new blood vessels,if the volume is over 1-2 mm^3. Or it cannot be grow. In some studies, the endothelial cells of new blood vessels have shown the increase of CD13, but not in mature blood vessels. It means CD13 may be a regulator of new tumor angiogenesis [12-14]. 3. Increase the virulence of tumor cells and promote tumor metastasis: Saiki [15] found that CD13 is overexpression on the surface of the tumor cells which have the ability to transfer, when they were studying for the chemotherapeutic drugs. They transfected the DNA of CD13 to the non-metastatic carcinoma strain model rats to find out the tumor cells of the rats gain the ability of metastasis. After that, some studies proved that CD13 can accelerate the metastasis because CD13 can degrade of extracellular matrix proteins and increase the secretion of cancer-related adhesion factor or the secretion of growth factors (1). Kidio [16] found that CD13 can mediates the transfer of melanoma, because it can promote the adhesion of tumor cells and the degradation of extracellular matrix proteins. 4. Reduce the immune function to create a microenvironment good for tumor cells survival: CD13 can reduce the expression of IL-8 and MHC II to reduce T cells' recognition of tumor cells [17].

However, the relationship between the expression of CD13 and the prognosis of patients is still confused, and there is little reports all of the world. According to our study, CD13 shows a strong positive staining in human kidney tissue, with a positive rate of 76.85% (83/108) in osteosarcomas. This is similar to Liang W's dates published in 2014 (18). And osteosarcomas with positive CD13 expression tended to be a high mortality, implying a bad prognosis. This is the same as CD13 in other tumors [4, 7, 8]. Statistical analysis showed that CD13 expression is related to age ($P = 0.0489$), Enneking stages ($P = 0.0415$) and tumor volume ($P = 0.013$) in osteosarcomas. The survival curves revealed that there is a significantly higher survival rate in patients with negative CD13 expression compared to positive one ($P = 0.0019$). This results accord with our expectations. On the basis of the Multivariate Cox regression analysis, Enneking stages had a statistically significant influence on survival ($P < 0.01$) and CD13 is a risk factor of prognosis in osteosarcomas. It means that Enneking stages is the most important factors to survival and the expression of CD13 also can be a marker to estimating the prognosis of the patients in osteosarcomas.

CD13 is a potential genetics target to osteosarcomas with potential application prospect to diagnosis and prognosis. There is study [18] showed that in vitro human osteosarcoma cells, treated with APN inhibitor Bestatin, reduced levels of mitogen activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K) pathway proteins, reduced phosphorylation of p38, ERK1/2 and JNK and decreased levels of NF-κB. It implies CD13 participate in the development of osteosarcoma through MAPK and PI3K pathway, and may be associated with the expression or the degree of phosphorylation of p38, ERK1/2, JNK and NF-κB. But the study stop at the experiment in vitro. Also, Marion [19], found that CD13 inhibitor can induce the apoptosis of U937 (acute myeloid leukemia cell line) and K562 (chronic myeloid leukemia cell line) through caspase-3 pathway and phosphatidylinositol 3-glycogen

synthase (PI3K-GSK) pathway. APN/CD13 inhibitor Bestatin's ability to induce the apoptosis of liver cancer calls is also found by Christ [20]. However, almost all the experiments are in vitro, in fact, CD13 is widely distributed in the body tissues, which is involved in various physiological pathology process. Only highly specific and targeted strong drugs can have more anti-tumor effects, with prevention of the complications.

Disclosure of conflict of interest

None.

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