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Expression of neurotrophins and their receptors in primary osteosarcoma.

Expressão de neurotrofinas e de seus receptores no osteossarcoma primário.

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A B S T R A C T

Objective: to determine the expression of neurotrophins and their tyrosine-kinase receptors in patients with osteosarcoma (OS) and their correlation with clinical outcomes. **Methods:** we applied immunohistochemistry to biopsy specimens of patients consecutively treated for primary OS at a single institution between 2002 and 2015, analyzing them for expression receptors of tyrosine kinase A and B (TrKA and TrKB), neural growth factor (NGF) and brain derived neurotrophic factor (BDNF). Independently, two pathologists classified the immunohistochemical markers as negative (negative or weak focal) or positive (moderate focal/diffuse or strong focal/diffuse). **Results:** we analyzed data from 19 patients (10 females and 9 males), with median age of 12 years (5 to 17.3). Tumors' location were 83.3% in the lower limbs, and 63.2% of patients had metastases at diagnosis. Five-year overall survival was 55.3%. BDNF was positive in 16 patients (84%) and NGF in 14 (73%). TrKA and TrKB presented positive staining in four (21,1%) and eight (42,1%) patients, respectively. Survival analysis showed no significant difference between TrK receptors and neurotrophins. **Conclusion:** primary OS samples express neurotrophins and TrK receptors by immunohistochemistry. Future studies should explore their role in OS pathogenesis and determine their prognostic significance in larger cohorts.

Keywords: Osteosarcoma. Nerve Growth Factors. Brain-Derived Neurotrophic Factor. Receptor, trkA. Receptor, trkB.

INTRODUCTION

Osteosarcoma (OS) is a malignant bone tumor found preferentially in individuals between ten and 25 years old. At diagnosis, up to 30% of patients have metastases, considered the main prognostic factor. The development of chemotherapy at high doses increased survival considerably. However, since the early 2000s, this therapeutic option has reached its plateau. Overall, there was no significant progress in OS treatment during this period[1-6]. Consequently, the search for therapies based on the molecular profile of the tumor has grown considerably[7].

Neurotrophins and their tyrosine kinase receptors (Trk) are responsible for synaptic modulation of the central nervous system. Recent studies with sarcoma samples have demonstrated the expression of the TrkA receptor and its ligand, nerve growth factor (NGF), seen as a potential marker of prognosis and treatment. Increased NGF expression may possibly be associated with the tumor stage and risk of metastases in certain neoplasms. TrkA signaling has also been described as a promoter of mitotic and anti-apoptotic activity in osteoblasts of different cell lines[8-10].

The Trk B receptor (TrkB) has affinity for the brain-derived neurotrophic factor (BDNF).

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Increased BDNF expression is closely related to tumor viability, migration and invasion of healthy tissues in various malignancies. Different samples of human uterine leiomyosarcoma have demonstrated expression of BDNF and TrkB, and increased expression of TrkB and its ligands in this mesenchymal neoplasm has been associated with resistance to multiple chemotherapeutic agents. Increased expression of TrkB and its ligands has also been associated with unfavorable clinical outcomes in some tumors of neuroectodermal origin[11-18].

OS is a bone tumor of mesenchymal origin that may share some features with other sarcomas and tumors that metastasize to the bones. Studies correlating OS with TrkA, TrkB and neurotrophins (NGF and BDNF) are rare[7] and not include humans. In the present study, we analyzed the expression of TrkA, TrkB and its ligands (NGF and BNDF) in OS tumor samples from.

METHODS

Patients and histological samples

The study was approved and registered by the institutional Review Board of the Hospital de Clínicas de Porto Alegre through the Office of Research and Graduate Studies (IRB nº 00000921) under reference number 15-0499.

The eligible participants were all OS patients treated in a single institution in accordance with the Guidelines of the Brazilian Osteosarcoma Treatment Group (BOTG) and the Latin American Osteosarcoma Treatment Group (GLATO) in two consecutive treatment protocols (BOTG V, GCBTO 2006), between 2002 and 2015. All patients signed an informed consent form prior to their inclusion in the 2006 BOTG V and GCBTO protocols[3,19].

We included patients without previous treatment, whether metastatic or not, who had their biopsies analyzed in the Department of Pathology of Hospital de Clínicas of Porto Alegre. Details of the chemotherapeutic regimens were previously published[3,19]. Exclusion criteria were insufficient material for immunohistochemical tests and not undergoing the aforementioned treatment protocol.

All included patients had their biopsies submitted to immunohistochemistry for the following tumor markers: BNDF, NGF, TrkA and TrkB. We extracted clinical and surgical information from the patients' medical records. We graded tumor necrosis according to the Huvos-Ayala classification[20].

Immunohistochemistry

All samples were fixed in paraffin, cut into 4-µm thick slices, incubated and rehydrated in alcohol. Antigen retrieval was performed in a microwave oven. Endogenous peroxidase activity was blocked by incubation of the slides in hydrogen peroxide, and non-specific binding sites were blocked with normal serum. The slides were incubated with the primary antibody at a dilution of 1:50 for 12 hours at 4°C, then immunoblotted with the streptavidin-biotin-peroxidase complex (LSAB, Dako) and grown with diaminobenzidine tetrachloride (DAB Kit, Dako). The primary antibodies were rabbit polyclonal anti-NGF (sc-33603; Biotecnologia Santa Cruz), anti-BDNF (sc-20981, Biotecnologia Santa Cruz), mouse polyclonal TrkB (sc-377218, Biotecnologia Santa Cruz) and goat polyclonal anti-TrkA (sc-20539, Biotecnologia Santa Cruz).

Two specialists in surgical pathology and immunohistochemistry independently evaluated the slides for the expression of NGF, BDNF, TrkB and TrkA.

In case of disagreement among the pathologists, they reviewed the slides together until reaching consensus. They scored the immunohistochemical staining according to the intensity on a scale of 0 to 3, where 0 indicates absence of staining (negative); 1, weak staining; 2, moderate staining; and 3, strong staining. Regarding the percentage of immunoreactive cells, 1 indicated less than 10% of stained cells (focal) and 2 indicated more than 10% of stained (diffuse) cells. We divided the patients into two groups: negative and focal weak (negative); moderate focal/diffuse and strong focal/diffuse (positive).

Statistics

We expressed the variables in absolute and relative frequencies, with the exception of age, expressed as mean and standard deviation. We evaluated differences between groups concerning age with the Student's t test, and used the Fisher's exact test for all other variables. We used the log-rank test to compare the overall survival and disease-free survival curves, estimated by the Kaplan-Meier method. We considered P=0.05 as significant. We analyzed the data with SPSS, version 18.0, and Epi Info.

RESULTS

Patients' characteristics and markers

Out of 28 patients treated for OS in the Service, we excluded four due to insufficient or unavailable sample to perform the immunohistochemical study, and five that did not undergo the BOTG V and GCBTO 2006 protocol (by choice of the oncologist, of the patient, or due to clinical conditions that prevented the treatment). We did not exclude patients who presented poor response to chemotherapy and required treatment change during the study. We therefore analyzed data from 19 patients (9 male and 10 female, with mean age 12 years), from a single institution, who underwent the BOTG V and GCBTO 2006 protocols. The characteristics of the sample were highlighted in table 1.

Table 1. *Sample characteristics.*

Variables	N=19
Age (years), mean (±SD*) [min.-max.]	12.0 (±3.6) [5-17.3]
Gender, N (%)	
Male	9 (47.4)
Female	10 (52.6)
Metastasis, N (%)	
No	12 (63.2)
Yes	7 (36.8)
Huvos-Ayala, N (%)	
<90%	5/11 (45.5)
=90%	6/11 (54.5)
Recurrence, N (%)	
No	15 (78.9)
Yes	4 (21.1)

*Source: author database. * SD= standard deviation.*

The median age was 12 years (range 5 to 17.3), and the median follow-up time, 2.7 years (range 0.6 to 14). Fifteen tumors (78.9%) were located in the lower extremities, two (10.5%) in the upper ones, one (5.3%) in the pelvis and one (5.3 %) had no record of location. Twelve (63.2%) patients were metastatic and seven (36.8%) had localized tumor. The main metastatic site were the lungs (83%), followed by bones (17%). The response to chemotherapy was poor in five patients, good in six and not recorded in eight patients.

Four (21.1%) patients presented disease progression, with two local and two systemic recurrences. Of the 19 patients, 17 were operated on, of whom nine (52.9%) underwent procedures that allowed preservation of the limb, and eight (47.1%), amputation.

BDNF was positive in 16 (84.2%) patients, while NGF was positive in 14 (73.7%) (Figure 1). On the other hand, TrkA and TrkB were more often negative than positive, presenting four (21,1%) and eight (42,1%) cases of positive staining, respectively, (Figure 2). There was a significant difference between the positive and negative markers of the sample (P<0.001) (Table 2).

Table 2. *Immunohistochemical markers data.*

Variables	N=19
BDNF*, N (%)	
Negative and weak focal	3 (15.8)
Positive, weak and strong	16 (84.2)
NGF**, N (%)	
Negative and weak focal	5 (26.3)
Positive, weak and strong	14 (73.7)
TrkA***, N (%)	
Negative and weak focal	15 (78.9)
Positive, weak and strong	4 (21.1)
TrkB****, N (%)	
Negative and weak focal	11 (57.9)
Positive, weak and strong	8 (42.1)

*Source: author database. * BDNF= brain-derived neurotrophic factor; ** NGF= nerve growth factor; *** TrkA= tyrosine kinase A receptor; **** TrkB= tyrosine kinase B receptor. There was a significant difference in positivity between the markers (P<0.001).*

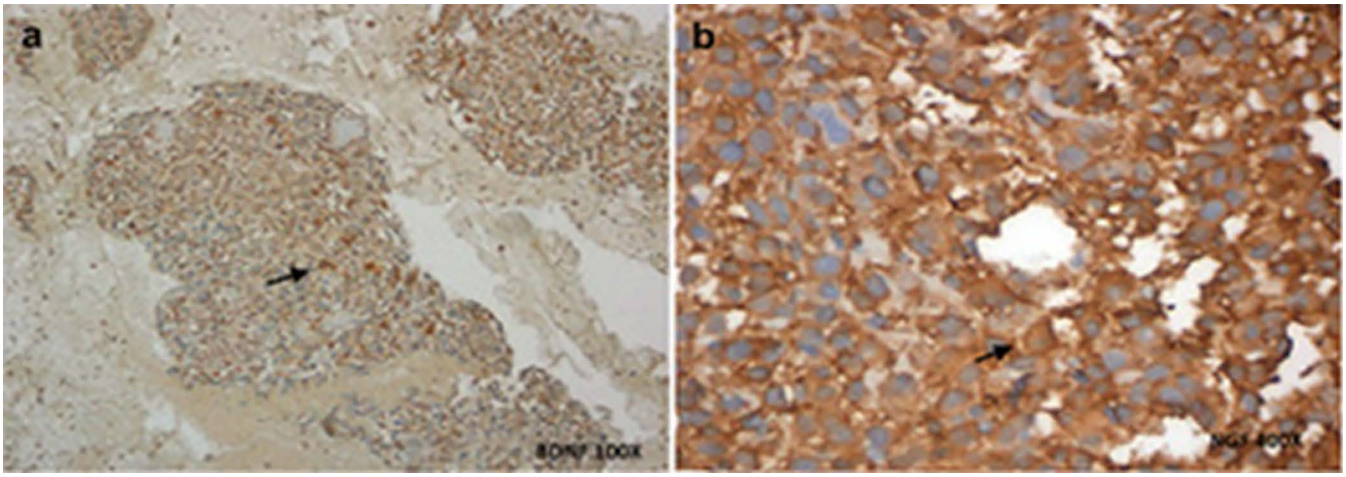

Figure 1. *Expression of BDNF (A, 100x) and NGF (B, 400x) in osteosarcoma cells. Arrows indicate stained cells.*

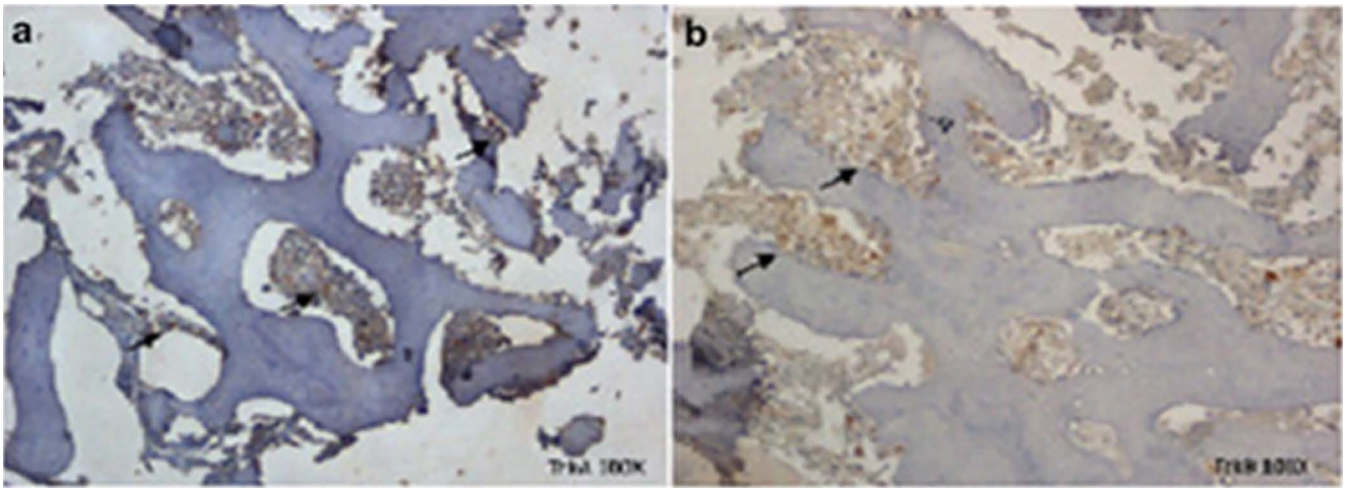

Figure 2. *Expression of TrkA (A) and TrkB (B) in osteosarcoma cells (100x). Arrows indicate stained cells.*

Global analysis

Overall five-year survival was 55.3%. The five-year survival rate was 40% for metastatic disease and 60 % for localized disease (Relative Risk [RR]=2.04, P=0.39). Although not significant, the five-year survival rate was only 25% (*hazard ratio* [HR]=3.02) for patients with disease recurrence, being 72.5% for patients without relapse (P=0.17).

Immunohistochemical analysis

Five-year cumulative survival was greater in patients who presented positive staining for BDNF and NGF than for those with negative staining (61.3 vs. 33.3%, 66.7 vs. 33.3, respectively). The RR for patients with positive staining for BDNF and NGF was 0.51 (95% CI: 0.09-2.8), 0.69 (95% CI: 0.13-3.81), respectively. However, survival was higher in patients with negative staining for TrkA and TrkB than in those with positive staining (58.2 vs. 37.5%; 56.3% vs. 54.7, respectively). There was no significant difference between neurotrophin and Trk receptors in the survival analysis. The RR for patients with positive staining for TrkA and TrkB was 3.48 (95% CI: 0.62-19.70) and 1.84 (95% CI: 0.37-9.20), respectively. We submitted both the TrkA receptor and the recurrence characteristics to risk-adjusted analysis, since the P-value was lower than 20%; however, none of them presented statistically significant differences associated with the risk of death (Table 3).

Despite the known affinity between TrkA and NGF, tumors stained positively for both markers only in three (15.7%) cases, and negative for both markers in four (21,1%). Regarding the TrkB-BDNF relation, tumors stained positively for both markers in eight (42.1%) cases and negative for both in three (15.7%). Combinations of TrkA-NGF-positive, TrkA-NGF negative, TrkB-BDNF-positive and TrkB-BDNF-negative were not significantly associated with survival.

DISCUSSION

This is the first study evaluating the expression of neurotrophins and their TrK receptors in primary OS tumors in humans. We found positive staining by immunohistochemistry for BDNF, NGF, TrkA and TrkB in samples from 19 OS patients treated at a single institution within the BOTG V and GCBTO 2006 trials conducted by Petrilli *et al*.[3,19]. In addition, the studied group presented characteristics similar to those reported in the BOTG V and GCBTO 2006 studies, with a high prevalence of metastasis and a survival rate of approximately 55%[19]. Survival rates for metastases, recurrence, and gender (male) followed the pattern and approximate values found in other studies, although they were not statistically significant (probably due to the small sample size)[3,19]. Likewise, the expression of the markers evaluated by immunohistochemistry was not significantly associated with survival, presence of metastases, recurrence or response to chemotherapy. However, there was a trend of association between survival, recurrence and presence of positive TrkA (P<0.20).

Although a TrkA-NGF relation is frequently present in the repair and proliferation of bone tissue, studies investigating these proteins in OS cells are rare. A study in canine OS cells has shown that in vitro blocking of TrkA-NGF binding can induce apoptosis and inhibit cell proliferation.

Table 3. *Univariate analysis.*

Variables	Death		
	5 years	RR*(95% CI**)	P
All patients (N)	55.3%	-	-
Age years)		0.98 (0.74-1.30)	0.889
Gender			
Male	30.0%	1.45 (0.29-7.24)	0.648
Female	68.6%	1.00	
Metastasis			
No	66.0%	1.00	
Yes	40.0%	2.04 (0.40-105)	0.392
Huvos-Ayala			
<90%	40.0%	¥	0.427
=90%	75.0%	1.00	
Recurrence			
No	72.2%	1.00	
Yes	25.0%	3.02 (0.60-15.1)	0.178
BDNF***			
Negative and weak focal	33.3%	1.00	
Positive, weak and strong	61.3%	0.51 (0.09-2.80)	0.436
NGF****			
Negative and weak focal	33.3%	1.00	
Positive, weak and strong	66.7%	0.69 (0.13-3.81)	0.669
TrkA#			
Negative and weak focal	58.2%	1.00	
Positive, weak and strong	37.5%	3.48 (0.62-19.70)	0.158
TrkB##			
Negative and weak focal	56.3%	1.00	
Positive, weak and strong	54.7%	1.84 (0.37-9.20)	0.457

*Source: author database. * RR= relative risk; ** 95% CI= 95% confidence interval; *** BDNF= brain-derived neurotrophic factor; **** NGF= nerve growth factor; # TrkA= tyrosine kinase A receptor; ## TrkB= tyrosine kinase B receptor. ¥ The risk could not be estimated due to the small sample size.*

In addition, TrkA receptors were identified in primary tumors and in pulmonary metastases[7]. We found positive staining for TrkA in 21% of cases and for its NGF ligand in 73%. Our study suggests a lower survival with the positive expression of TrkA when compared with its negative expression (37% vs. 58% in five years, respectively). When analyzing the NGF samples, we observed greater survival in patients without its expression (66% *versus* 33% at five years, respectively). Although the results are not statistically significant, perhaps due to sample size, the TrkA-NGF binding may play a potential role in OS prognosis.

Signaling of TrkB, through its ligand BDNF, has been linked to the prognosis of certain malignancies.

In neuroblastoma, the presence of BDNF and TrkB demonstrated greater resistance to chemotherapy and aggressiveness of the local tumor. Similarly, in cases of uterine leiomyosarcoma, *in vitro* assays indicate that endogenous signaling of the TrkB pathway is associated with tumor growth. Consequently, TrkB and BDNF were investigated in greater detail because of their potential relevance as targets for antineoplastic therapy[17,18,21-28].

In the present study, TrkB and BDNF were present in 42% and 84% of the OS samples, respectively. Among TrkB-positive patients, 80% had a low necrosis rate and 25% had tumor recurrence. Among TrkB-negative patients, only 16% had a low necrosis rate and 18% had OS recurrence. Patients positive for TrkB tended to show lower survival than patients with negative staining, although the difference was not statistically significant (P=0.44). On the other hand, patients with BDNF-expressing cells had a higher necrosis rate, a lower frequency of metastases and a higher survival rate than those with negative staining (P=0.42). Further studies are needed to investigate whether BDNF-TrkB play a role in the pathogenesis of OS.

Data in the literature on the expression of neurotrophin and Trk receptors are very limited and, to the best of our knowledge, we are the first to report the expression of these receptors on human OS samples. Our findings showed a considerable prevalence of these markers. We believe that studies with larger samples and associated molecular essays, as well as the stratification of patients by morbidity criteria, may contribute to a better use of these markers in patients with OS.

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R E S U M O

Objetivo: *determinar a expressão de neurotrofinas e seus receptores tirosina quinases em pacientes com osteossarcoma (OS) e sua correlação com desfechos clínicos.* **Métodos:** *biópsias de tumores primários de pacientes com OS tratados em uma única instituição, consecutivamente, entre 2002 e 2015, foram analisados através de imuno-histoquímica para expressão de receptores de tirosina quinase A e B (TrKA e TrKB), fator de crescimento neural (NGF) e fator neurotrófico derivado do cérebro (BDNF). De forma independente, dois patologistas classificaram os marcadores de imuno-histoquímica como negativos (negativos e focais fracos) ou positivos (moderado focal/difuso ou forte focal/difuso).* **Resultados:** *foram analisados dados de 19 pacientes (10 do sexo feminino e 9 do masculino) com mediana de idade de 12 anos (5 a 17,3 anos). Dos tumores, 83,3% estavam localizados em membros inferiores e 63,2% dos pacientes eram metastáticos ao diagnóstico. A sobrevida global em cinco anos foi de 55,3%. BDNF foi positivo em 16 pacientes (84%) e NGF em 14 pacientes (73%). TrKA e TrKB apresentaram coloração positiva em quatro (21,1%) e oito (42,1%) pacientes, respectivamente. A análise de sobrevida não demonstrou diferença significativa entre receptores TrK e neurotrofinas.* **Conclusão:** *amostras de OS primário expressam neurotrofinas e receptores TrK através de imuno-histoquímica. Estudos futuros podem auxiliar na identificação do papel das mesmas na patogênese do OS e determinar se há possível correlação prognóstica.*

Descritores: *Osteossarcoma. Fatores de Crescimento Neural. Fator Neurotrófico Derivado do Encéfalo. Receptor trkA. Receptor trkB.*

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