Clinicopathologic study and Ki-67 proliferative marker evaluation in human osteosarcomas

Primeira submissão em **03/05/05**
Última submissão em **13/11/05**
Aceito para publicação em **18/11/05**
Publicado em **20/12/05**

Estudo clinicopatológico com avaliação do índice de proliferação celular avaliado pelo Ki-67 em osteossarcomas humanos

Larissa Cardoso Marinho[1]; Francy Reis da Silva Patrício[2]; Reynaldo Jesus-Garcia Filho[3]; Antônio Sérgio Petrilli[4]; Luciana Nakao Odashiro[5]; Carla Macedo[6]; Maria Teresa Seixas Alves[7]

key words

Osteosarcoma

Ki-67

Immunohistochemistry

Prognosis

abstract

Background: Osteosarcoma is a relatively uncommon malignant neoplasm and little information has been reported on its cell proliferation using Ki-67. Objectives: Evaluate histological, clinical and immunohistochemical parameters using Ki-67 labeling index (LI), correlate one to another and with follow-up. Material and method: Fifty-seven patients with available clinicopathological data submitted to the study of cell proliferation as determined by Ki-67 expression measured by immunohistochemistry (IHC) staining using formalin-fixed paraffin embedded sections. In each sample, positive cells were quantified on at least a thousand nuclei and expressed as Ki-67 LI according to median value. Results and discussion: Non-significant correlations were observed in metastatic and non-metastatic cases when variables as surgery, tumor size, death and relapse were compared with Ki-67 LI values (cut-off of 45%). In the group of non-metastatic cases there was a direct correlation between higher values of Ki-67 index and better overall survival. Metastatic patients overall survival curve and LI high and low Ki-67 did not show significant differences. Conclusion: Based on our results the Ki-67 LI could be useful as a prognostic marker in patients without metastasis at diagnosis.

unitermos

Osteossarcoma

Ki-67

Imuno-histoquímica

Prognóstico

resumo

Introdução: Osteossarcoma é uma neoplasia rara do tecido ósseo, tendo sido publicados poucos trabalhos na literatura que avaliaram o índice de proliferação celular Ki-67 (IPC-Ki-67) nesse tipo de sarcoma. Objetivo: Avaliação e comparação dos parâmetros clínicos, histológicos e de imunoexpressão do Ki-67 com a evolução clínica dos pacientes. Material e método: Blocos de parafina de 57 casos de osteossarcomas, cujos pacientes apresentavam dados clinicopatológicos fidedignos, foram submetidos a estudo da proliferação celular através da técnica de imuno-histoquímica. Em cada amostra o IPC foi determinado pela contagem de núcleos marcados em pelo menos mil células neoplásicas. Em cada grupo de pacientes o IPC-Ki-67 foi considerado alto ou baixo, tendo como referência a mediana dos valores do Ki-67. Resultados e discussão: Não houve correlação estatística significativa entre os valores do IPC-Ki-67 (valor de corte = 45%) dos osteossarcomas dos pacientes com ou sem metástases ao diagnóstico, em relação às variáveis óbito, recaída tumoral, tamanho do tumor e tipo de cirurgia. No grupo de pacientes sem metástases houve correlação direta entre o tempo de sobrevida e o índice de proliferação celular (isto é, o IPC-Ki-67 > 45% correlacionou-se com maior tempo de sobrevida). Nos casos de pacientes com metástases ao diagnóstico, os valores do IPC-Ki-67 não apresentaram correlação significativa com a sobrevida. Conclusão: Com base nos nossos resultados, o IPC-Ki-67 pode ser utilizado para avaliação prognóstica em pacientes com osteossarcoma sem metástases ao diagnóstico.

1. Mestre em Medicina pela Universidade Federal de São Paulo (UNIFESP); médica patologista da Universidade de Brasília (UnB).
2. Professora-doutora do Departamento de Patologia da UNIFESP.
3. Professor livre-docente do Departamento de Oncologia Ortopédica da UNIFESP.
4. Professor livre-docente do Departamento de Oncologia Pediátrica da UNIFESP.
5. Mestre em Medicina pela UNIFESP.
6. Médica oncologista da UNIFESP.
7. Professora-doutora do Departamento de Patologia da UNIFESP.
Work developed at UNIFESP.

Master dissertation-based article entitled *Estudo clínico-anatomo-patológico e imuno-histoquímico do marcador de proliferação celular Ki-67 em osteossarcomas humanos* (2003), presented at the post-graduation program in pathology of Universidade Federal de São Paulo (UNIFESP).
Oral presentation at the 24th International Congress of the International Academy of Pathology, Amsterdam, Netherlands, October, 2002.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in children and adolescents[21, 30, 31, 37]. Many published reports attempt to identify factors that may provide prognostic information on these tumors[17, 18, 22, 30, 36]. Studies were conducted both before and after the advent of adjuvant chemotherapy. All of them had methodologic variations that make them difficult to interpret and compare[20, 25, 29, 32, 35].

Factors that affect the prognosis of patients with osteosarcoma are age[5, 28], gender[3, 23], location of the lesion[9], size[2, 3, 22, 23, 24, 28, 30], histologic subtype[1, 10], grade and stage of the tumor[17, 18, 23].

Previous studies on cell kinetics in different human neoplasm have shown that cell proliferation can be associated with prognosis[16, 27, 34]. The proliferative characteristics of tumor can be analyzed by a variety of methods, including mitosis count and the evaluation of the synthetic phase of the cell cycle by DNA analysis, thymidine incorporation or bromodeoxyuridine[15].

Ki-67 monoclonal antibody, specific for a nuclear antigen that is expressed throughout the cell cycle (late G1, S, G2 and M), but absent in quiescent cells (G0)[7, 8], is proposed as an invaluable alternative. Ki-67 immunostaining allows an easy and rapid evaluation of tumor samples without the need for preliminary incubation with DNA precursors[14]. Although the nature of this antigen is still incompletely defined, the reliability of Ki-67 immunostaining as a proliferation marker has been investigated in different tumors[6, 12, 13].

In bone tumors, estimation of biologic behavior is an important clinical problem that is only partially solved by the morphologic parameters. For bone tumors only a few data have been reported about cell proliferation and prognosis[11, 19, 20, 25, 29, 32, 35].

Patients and methods

Between 1991 and 2000, we studied 57 patients newly diagnosed high-grade osteosarcoma of extremities at the Department of Pathology of Universidade Federal de São Paulo and treated at the Pediatric Oncology Institute (POI) of this institution. The characteristics of these patients are listed on **Table 1**.

The demographic and clinical variables examined were sex, tumor size, surgery and relapse. The pathologic size was determined at the time of operation based upon the greatest dimension of the macroscopic specimen. The follow-up period began at the date of diagnosis. Patients were followed until death or censored from this analysis at the time of their last visit to hospital. Follow-up ranged from 1 to 113,97 months (median: 29,4 months). Survival was recorded from time of diagnosis to death.

Table 1 Clinical data of patients

Clinical features	Patients (*n*)
Number of patients	57
Median age at diagnosis (years)	14,61
Sex (*n* = 57)	
Male	37
Female	20
Size of tumor (*n* = 50)*	
≥ 12cm	28
< 12cm	22
Surgery (*n* = 56)**	
Limb salvage	32
Radical	24
Relapse (*n* = 56)**	
No	51
Yes	5
Metastases at diagnosis (*n* = 57)	
No	44
Yes	13

**Seven without information; **one refused surgery.*

We have used the event metastases as a point to judge aggressiveness[17, 18, 22] and two groups were separated for the study of Ki-67 immunoexpression: patients with and those without metastases at diagnosis.

In 17 patients we could not retrieve blocks from files and two had only post-chemotherapy specimens. In an effort to avoid potential treatment-related effects on proliferation index, only pre-chemotherapy specimens were selected. In all cases, tissue blocks were taken from original biopsy prior to neoadjuvant chemotherapy. We studied 38 pediatric patients with high-grade osteosarcoma of extremities for Ki-67 expression.

Immunohistochemistry

The expression of Ki-67 (clone Ki-S5 Dako) was determined via standard immunohistochemical techniques on serial paraffin sections. All identifiable archival material (pre-chemotherapy specimens) from patients were retrieved from the Department of Pathology. The blocks were selected

from the primary site in all cases. Bone specimens were decalcified with nitric acid (7.5%) and then washed in water for 30 minutes and immersed in sodium bicarbonate (5%) for 24 hours. Briefly 3-4µm formalin-fixed paraffin-embedded tissue sections mounted on silane-coated slides were deparaffinized with xylene, passed through alcohol solutions and rinsed in deionized water. The sections were submitted to pressure cooker for five minutes after a treatment with 10mM citrate buffer. After that, the slides were rinsed in distilled water and endogenous peroxidase was inhibited with a 6% hydrogen peroxidase solution, in six incubation steps of five minutes each.

Diluted primary antibody was applied to sections, for 18 hours at 4°C. After washing in phosphate-buffered saline (PBS) to remove antibody excess, the amplification step was carried out according to Dako Duet System protocol. The sections were incubated for 20 minutes at 37°C with biotinylated goat antimouse/rabbit immunoglobulins diluted 1:200 and then washed in PBS. The next incubation step employed 1:200 diluted streptavidin-biotin-peroxidase complex and color development was obtained with 60mg% diaminobenzidine plus 0.1% hydrogen peroxidase in PBS. After light counterstaining with Harry's hematoxylin, slides were dehydrated in alcohol solutions and xylene, and permanently mounted.

The positive immunoreactions were counted through use of software (Imagem-Pro plus 3.0 version for Windows). In each sample, positive cells were quantified on at least a thousand cells and expressed as Ki-67 LI (labeling index). In those cases with multiple samples from the same lesion, the highest value ki-67 LI was considered the most representative for tumor proliferation and was used as the parameter for the analysis in this paper.

For those patients without metastases at diagnosis, high Ki-67 LI was defined as greater than 46% of tumor cells showing nuclear immunoreactivity and low Ki-67 LI were those tumors with LI lower than 46%. For those with metastases at diagnosis we considered high Ki-67 LI higher than 45% and low Ki-67 those LI lower than 45% (**Figure 1**).

Statistical analysis

Kaplan-Meier method and long-rank test were used to compare overall survival rates between patients with high and low Ki-67 index value.

The Fisher's exact test and chi-square were used for clinical and IHC parameters. Cox regression and Kruskal-Walles test were used to compare index of Ki-67 and mortality.

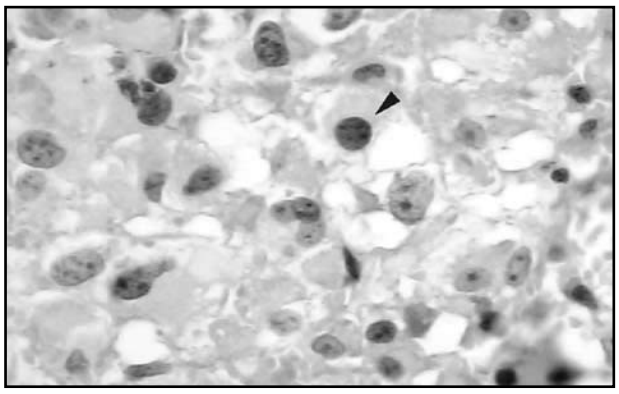

Figure 1 – *Nuclear Ki-67 immunoreactivity in osteosarcomas patients (arrowhead) (x 400)*

Results

General aspects

Table 1 presents the clinical and pathological characteristics of 57 primary osteosarcoma patients included in the current study. Most of them were male.

From these patients, 44 (77.19%) were non-metastatic at diagnosis and 13 (22.81%) were metastatic at diagnosis.

In order to determine the significance of Ki-67 as an independent prognostic factor, patients were separated by Ki-67 expression considered high or low according to the index label expressed by the tumor cells and than correlated with clinical variables (sex, size of the tumor, surgery, relapse, and overall survival).

The median value of Ki-67 to patients without metastasis at diagnosis was 46% and those with metastases at diagnosis were 45%. This difference was not significant ($p = 1$) (**Table 2**). Distribution of results of immunostainings for Ki-67 considering the LI obtained for metastatic and non-metastatic patients is presented in **Figure 2**.

For those 44 grouped as non-metastatic at diagnosis, the Ki-67 LI was available in 26 (Table 2). The variables sex, surgery, tumor size and relapse were compared with Ki-67 high and low index label with non-significant correlation (**Table 3**). In the same group of non-metastatic patients there was significant difference between overall survival curves of those patients with Ki-67 LI high and low ($p = 0.063$) (**Figure 3**).

Among 13 metastatic patients, Ki-67 index label was available in 11. There were no significant correlation between Ki-67 LI high or low and variables sex, size of the tumor, surgery and relapse. The overall survival curve for those with high and low Ki-67 LI did not show significant difference ($p = 0.43$) (**Figure 4**).

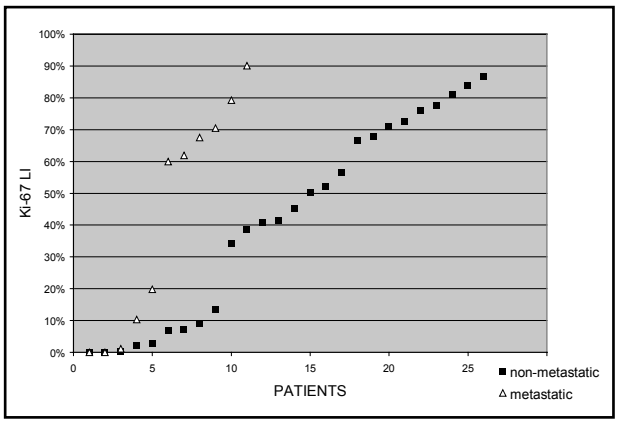

Figure 2 – *Distribution of ki-67 LI for metastatic and non-metastatic osteosarcomas*

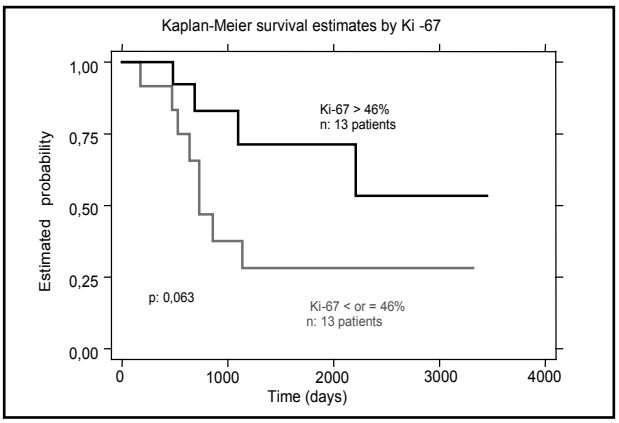

Figure 3 – *Overall survival: non-metastatic osteosarcomas*

Table 2 **Expression of Ki-67 in metastatic and non-metastatic osteosarcomas in pediatric patients**

Event metastases at diagnosis	Low Ki-67 LI		High Ki-67 LI		*p* value
	n	%	*n*	%	
No (*n* = 26)	13	50	13	50	1
Yes (*n* = 11)	6	54.5	5	45.45	

Table 3 **Expression of Ki-67 in non-metastatic osteosarcoma pre-chemotherapy patients**

Variable	Low Ki-67 LI		High Ki-67 LI		*p* value
	n	%	*n*	%	
Sex (n = 44)					
Male (*n* = 24)	11	50	13	59.1	0.381
Female (*n* = 20)	11	50	9	40.9	
Surgery (n = 26)					
Limb salvage (*n* = 16)	6	37.5	10	62.5	0.226
Radical (*n* = 10)	7	70	3	30	
Size of tumor (n =21)					
< 12cm (*n* = 11)	4	36.36	7	63.64	0.198
≥ 12cm (*n* = 10)	7	70	3	30	
Recurrence (n = 26)					
No (*n* = 22)	11	50	11	50	1
Yes (*n* = 4)	2	50	2	50	

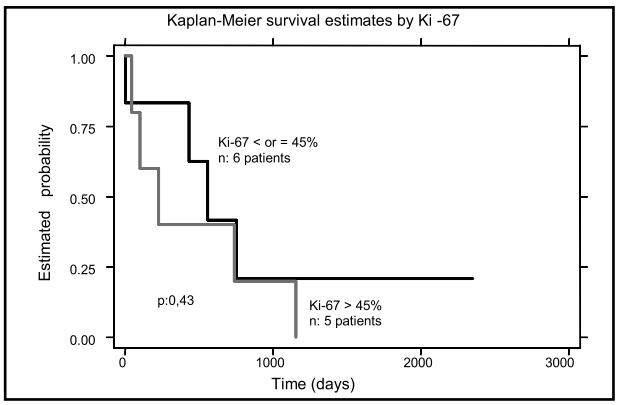

Figure 4 – *Overall survival: metastatic osteosarcomas*

Discussion

The monoclonal antibody Ki-67 exclusively reacts with a protein (Ki-67) expressed only during the proliferative phase of the cell cycle (late G1, G2, S and mitosis)[8]. It was first described in 1983, by Gerdes *et al.*[7], to be used on fresh tissue. With the introduction of the MIB-1 antibody, a true Ki-67 equivalent, and a new antigen retrieval technique with the use of microwaves[26], immunostaining of formalin-fixed paraffin-embedded material was possible with a high reproducibility, equaling results obtained in fresh

material[14]. In a number of publications Ki-67 has proven to be a reliable marker for measuring cell growth in human neoplasms [4, 27, 34].

In previous results regarding prognostic levels of Ki-67 or MIB-1 in osteosarcomas, the mean values of Ki-67 LI vary because of different methodology. Vollmer *et al*.[33], in 1989, studied high malignant intramedullary osteosarcomas using the double indirect peroxidase method in frozen sections, and the count was made in ten fields, each holding about a hundred cells. The mean proliferative rate was 16.8%. Scotland *et al.*[25], in 1995, evaluated the Ki-67 expression by indirect immunofluorescence on cytologic specimens (single-cell suspensions). The percentage of positive cells was calculated for at least 5 hundred neoplastic cells and the mean index was 11.6%, calculated in primary, recurrent and metastatic osteosarcomas. After 1995, we had in literature studies of Ki-67 LI obtained in paraffin-embedded tissue blocks. Oda *et al.*[19], Park and Park[20], Stenzel *et al.*[29], Weiming *et al.*[35] and Hernandez-Rodriguez *et al.*[11] obtained in paraffin-embedded tissue blocks, using different methodologies in cell counting, different mean values of Ki-67 LI in osteosarcomas. In none of them the authors grouped metastatic and non-metastatic patients for analysis, and in none of them we have information about decalcification of bone tissues for obtaining slides sections for the study of Ki-67.

In our study the median proliferative rate was 46% for non-metastatic patients and 45% for those metastatic at diagnosis. This difference was not significant ($p = 1$) (Table 2).

For those patients without metastasis at diagnosis there was statistic correlation between Ki-67 index and overall survival. Patients without metastasis at diagnosis with higher proliferative rate (> 46%) had better overall survival and those with lower proliferative index (≤ 46%) had worse overall survival (Figure 2). Our results suggest the existence of a positive correlation between tumor cell kinetics and effectiveness of preoperative chemotherapy. Scotland *et al.*[25] showed in a group of patients submitted to the same chemotherapy regimen a higher mean Ki-67 LI in good responders than in poor responders to chemotherapy. Hernandez-Rodriguez *et al.*[11] demonstrate that nuclear accumulation of Ki-67 in more than 50% of tumor cells in primary osteosarcoma is associated with increased pulmonary metastases and tumor mortality. Weiming *et al.*[35] further show that nm23 expression parallels with the proliferative ability reflected by Ki-67 labeling is associated with early metastases. The isolated Ki-67 index showed no significant difference in metastases incidence in the same paper.

In our study of metastatic patients there were non-significant correlation of values of Ki-67 and overall survival as showed by Weiming *et al.*[35]. Hernandez-Rodrigues *et al.*[11] observed overexpression of Ki-67 in those patients who developed pulmonary metastases.

In relation to the clinicopathological variables, we found no difference between size of the tumor, sex, surgery, relapse, and death and Ki-67 LI high or low in patients with or without metastasis. Hernandez-Rodriguez found no difference between age, sex, tumor size and histologic grade when comparing patients with high and low Ki-67 index, but when Ki-67 index was associated with tumor size > 10cm patients had poor outcome. Weiming *et al.*[35] showed correlation of Ki-67 index and tumor size and degree of differentiation.

With clinical data, radiologic size, grade and Ki-67 index available, rational preoperative treatment planning, including entry into better follow-up for detection of pulmonary metastasis, could lead to post-operative observation and close examination. Detection of Ki-67 may then improve overall survival of patients together with new treatments.

Conclusion

Based on our results, the Ki-67 LI could be used as a prognostic marker in patients with osteosarcoma without metastasis at diagnosis.

References

1. BACCI, G. et al. Histologic response of high-grade nonmetastatic osteosarcoma of the extremity to chemotherapy. *Clin Orthop Relat Res*, v. 386, p. 186-96, 2001.

2. BIELING, P. et al. Tumor size and prognosis in aggressively treated osteosarcoma. *J Clin Oncol*, v. 14, p. 848-58, 1996.

3. BIELACK, S. S. et al. Prognostic factors in high-grade osteosarcoma of extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol*, v. 20, p. 776-90, 2002.

4. BROWN, D.C.; GATTER, K.C. Monoclonal antibody Ki-67: its use in histopathology. *Histopathology*, v. 17, p. 489-503, 1990.

5. CARSI, B.; ROCK, M.G. Primary osteosarcoma in adults older than 40 years. *Clin Orthop Relat Res*, v. 397, p. 53-61, 2002.

6. CHOONG, P. F. M. et al. Prognostic value of Ki-67 expression in 182 soft tissue sarcomas. Proliferation: a marker of metastasis? *APMIS*, v. 102, p. 915-24, 1994.

7. GERDES, J. et al. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer*, v. 31, p. 13-20, 1983.

8. GERDES, J. et al. Cell cycle analysis of a cell proliferation-associated human nuclear defined by the monoclonal antibody Ki-67. *J Immunol*, v. 133, p. 1710-15, 1984.

9. GLASSER, D. B. et al. Survival, prognosis, and therapeutic response in osteogenic sarcoma. *Cancer*, v. 69, p. 698-708, 1992.

10. HAUBEN, E. I. et al. Does the histological subtype of high-grade central osteosarcoma influence the response to treatment with chemotherapy and does it affect overall survival? A study on 570 patients of two consecutive trials of European Osteosarcoma Intergroup. *Eur J Cancer*, v. 38, p. 1218-25, 2002.

11. HERNANDEZ-RODRIGUEZ, N. A. et al. Ki-67: a proliferative marker that may predict pulmonary metastases and mortality of primary osteosarcoma. *Cancer Detect Prev*, v. 25, n. 2, p. 210-15, 2001.

12. HESLIN, M. J. et al. Ki-67 detected by MIB-1 predicts distant metastasis and tumor mortality in primary, high-grade extremity soft tissue sarcoma. *Cancer*, v. 83, p. 490-7, 1998.

13. HOOS, A. et al. High Ki-67 proliferative index predicts disease specific survival in patients with high-risk soft tissue sarcomas. *Cancer*, v. 92, p. 869-74, 2001.

14. KEY, G. et al. New Ki-67-equivalent murine monoclonal antibodies (MIB-1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. *Lab Invest*, 68, n. 6, p. 629-36, 1993.

15. KROESE, M. C. et al. The relevance of the DNA index proliferation rate in grading of benign and malignant soft tissue tumors. *Cancer*, v. 65, p. 1782-88,1990.

16. LEERS, M. P. G. et al. Multi-parameter flow cytometric analyses with detection of the Ki-67 Ag in paraffin-embedded mammary carcinomas. *Cytometry*, v. 27, p. 289-99, 1997.

17. MARINA, N. M. et al. Improved prognosis of children with osteosarcoma metastatic to the lung(s) at the time of diagnosis. *Cancer*, v. 70, p. 2722-7, 1992.

18. MEYERS, P.A. et al. Osteogenic sarcoma with clinically detectable metastasis at initial presentation. *J Clin Oncol*, v. 11, p. 449-53, 1993.

19. ODA, Y. et al. Expression of growth factors and their receptors in human osteosarcomas. *Gen Diagn Pathol*, v. 141, p. 97-103,1995.

20. PARK, H. R.; PARK, Y. K. Expression of p53 protein, PCNA, and Ki-67 in osteosarcomas of bone. *J Korean Med Sci*, v. 10, n. 5, p. 360-7, 1995.

21. PARKIN, D. M. et al. The international incidence of childhood cancer. *Int J Cancer*, v. 42, p. 511-20, 1988.

22. PEREZ, C. et al. Prognosis after metastases in osteosarcoma. *Cancer Clin Trials*, v. 1, p. 315-20, 1978.

23. PETRILLI, S. et al. IIB Osteosarcoma. Current management, local control, and survival statistics – São Paulo, Brazil. *Clin Orthop Relat Res*, v. 270, p. 60-6, 1991a.

24. PETRILLI, A S. et al. Increased survival, limb preservation, and prognostic factors for osteosarcoma. *Cancer*, v. 68, p. 733-7, 1991b.

25. SCOTLANDI, K. et al. Clinical relevance of ki-67 expression in bone tumors. *Cancer*, v. 75, n. 3, p. 806-14, 1994.

26. SHI, S. R. et al. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem*, v. 39, n. 6, p. 741-8, 1991.

27. SKYTTING, B. T. et al. Ki-67 is strongly prognostic in synovial sarcoma: analysis based on 86 patients from the Scandinavian Sarcoma Group Register. *Br J Cancer*, v. 80, n. 11, p. 1809-14, 1999.

28. SPANIER, S. S. et al. The effect of local extent of tumor on prognosis of osteosarcoma. *J Bone Joint Surg*, v. 72A, p. 643-53, 1990.

29. STENZEL, I. et al. Cell proliferation in bone tumors. Immunohistologic study of Ki-67 protein expression. *Patholog*e, v. 17, n. 1 p. 56-62, 1996.

30. TAYLOR, W. F. et al. Prognostic variables in osteosarcomas: a multi-institutional study. *J Natl Cancer Inst*, v. 81, p. 21-30, 1989.

31. UNNI, K. K. Osteosarcoma. In: DAHLIN, D. C.; UNNI K. K. *Dahlin's bone tumors – general aspects and data on 11,087 cases*. 5 ed. Philadelphia: Lippincott-Raven Publishers, 1996. p. 143-83.

32. VOLLMER, E. et al. Improved grading of bone tumors with the monoclonal antibody Ki-67. *J Cancer Res Clin Oncol*, v. 112, p. 281-2, 1986.

33. VOLLMER, E. et al. *Current topics in pathology*, v. 80, p. 91-114, 1989.

34. WATANABE, T. et al. Malignant peripheral nerve sheath tumors: high Ki-67 labeling index is the significant prognostic indicator. *Histopathology*, v. 39, p. 187-97, 2001.

35. WEIMING, L. et al. Preliminary observation on the correlation between nm23 expression and Ki-67 antigen with early metastasis of human osteosarcoma. *Chin Med J*, v. 111, n. 9, p. 813-7, 1998.

36. YONEMOTO, T. et al. Prognosis of osteosarcoma with pulmonary metastases at initial presentation is not dismal. *Clin Orthop Relat Res*, v. 349, p. 194-9, 1998.

37. YOUNG, J. L. et al. Cancer incidence, survival and mortality for children younger than age 15 years. *Cancer*, v. 58, p. 598-602, 1986.

Mailing adress

Larissa Cardoso Marinho
QRSW 01 Bloco B-14 – Apto. 202 – Sudoeste
CEP 70675-134 – Brasília-DF
e-mail: larissacm@unb.br