ORIGINAL RESEARCH PAPER

Surgery

PROLIFERATIVE ACTIVITY EVALUATION OF OSTEOSARCOMAS IN CHILDREN

KEY WORDS:

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ABSTRACT

The purpose of this study is to quantify the proliferative activity of high degree osteosarcomas and to determine if the proliferation is an independent or associated prognostic factor. In order to evaluate the proliferative activity, we used two methods: PCNA stain (proliferating cell nuclear antigen) and computerized quantitative assessment using the PRODIT interactive digital program. The intensity of the PCNA expression varied from slight positive (20% marked nuclei) to strong positive (75% or more marked nuclei). The imunopositivity for PCNA was present on all the analysed tumour categories, progressively increasing from grade 1 to 4. The quantification of proliferative activity using the three parameters proves the same mode of alterations, the values are increasing suddenly at 2-3 stage, reaching the peak in grade 4. The highest mitosis density was found at grade 4 but also in grade 3 the values are very high. The lowest MAI value is found on stage 2, while in the third and fourth stages the values were similar. Any of these objective assessment methods of the proliferative activity might be used with the same degree of accuracy, including in the case of the high risk grade of osteosarcoma. Any of the quantitative assessment methods of the proliferative activity might be used with the same degree of accuracy, including in the case of the high risk grade of osteosarcoma.

Introduction

Osteosarcomas are malignant bone tumours, which are characterised by osteoid producing, atypical cells. In the last two decades, the treatment of the osteosarcomas patients undergone revisions, shifting from an exclusive surgical approach to a multimodal complex therapy, in which the essential change is the preoperative chemotherapy being the essential change[1,2].

With this purpose in view, the investigations were focused on diagnosis and prognosis of the patients based on characteristics of the primary biopsy. A special place was given to the evaluation of the proliferative activity[3].

The purpose of this study is to quantify the proliferative activity of high degree osteosarcomas and to determine if the proliferation is an independent or associated prognostic factor.

Materials and methods

In order to evaluate the proliferative activity, we used two methods:

1. PCNA stain (proliferating cell nuclear antigen),
2. Computerized quantitative assessment using an interactive digital program.

As for the ***immunohistochemically study*** we used the method based on immunoenzimatic soluble complexes, namely LSAB/HRP (labelled streptavidin biotin), the one named ABC method (avidin-biotin complex) and the kit used was DAKA LSAB 2 system HRP (Universal DAKO Labbeled Streptavidin Biotin 2 System Horseradish Peroxidase). For each antibody used we have done the positive external control as well as the negative external control, using the same working procedure. Also, on the diagnostic slides we checked/ followed up the presence of the positive internal control used. On this study we used serial sections of 3-4 microns wide and DAKA Cytomation antibodies (Denmark), with proper dilution and pre-treatment. In order to assess the proliferative activity with PCNA, we used the PC10 clone, 1:100 dilution, 5 cycles MW in citrate buffer pre-treatment and a 30 minutes TA incubation period.

The quantitative evaluation of the proliferative activity was done on 10 or 30 consecutive fields, as it is required in the PRODIT program, using am ocular with a magnification power of 10x, objective lenses of 40x with a digital aperture of 0.75, and a circular field of 450 microns diameter and 0.159043 mm2 area. Quantification was done for:

- *Mitotic activity index* (MAI) which represents the total number of mitotic figures counted on these fields,
- *Mitotic rate* which evaluate the percentage value of mitosis on the quantifying fields,
- *Mitotic density* which represents the number of mitosis per mm2.

Results

The imunopositivity for PCNA ***(proliferating cell nuclear antigen)*** was present on all the analysed tumour categories, but with different values for each of these, progressively increasing from grade 1 to 4.

PCNA expression have been studied on 10 selected cases from the total blocks. For these selected cases the intensity of the PCNA expression varied from slight positive (20% marked nuclei) to strong positive (75% or more marked nuclei).

The PCNA intensity was correlated for osteosarcomas with the degree of differentiation and with the newly formed quantity of osteoid. In the poorly differentiated osteosarcomas, the PCNA expression was positive in the tumoral cells nuclei in 75% percentage or more. (Fig. 1)

In these cases the osteoid formation was absent or barely visible (Fig. 2)

When the osteoid formation was becoming obvious, the positivity of PCNA decreases to 50% (Fig.3).

Quantitative study of the proliferative activity for the classical osteosarcomas (Fig. 7) was done by correlating the clinical evolution of the cases with microscopic features. I used the selection of the cases in batches according to the histological grading. We do mention we haven't any tumor stage 1.

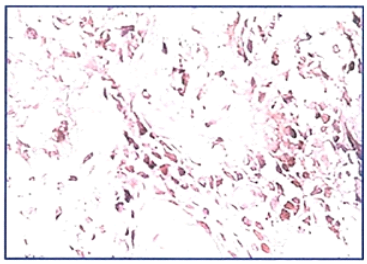

Fig. 1. The positivity of PCNA expression of 20% in the tumoral cells nuclei which surrounds the pre-existing bone trabecula (imunohystochemical for PCNA x400).

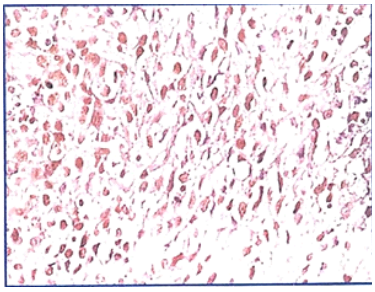

Fig. 2. The positivity of PCNA expression of 75% in the tumoral cells nuclei (imunohystochemical stain for PCNA x400).

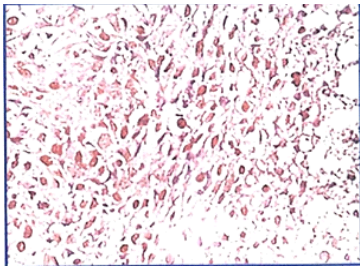

Fig. 3. The positivity of PCNA expression of 75% in the tumoral cells nuclei (imunohystochemical stain for PCNA x400).

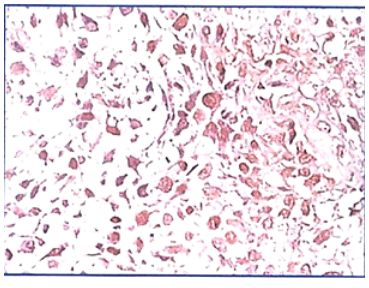

Fig. 4. Osteosarcoma with a sketch of osteoid tissue. The positivity of PCNA expression of 70% in the tumoral cells nuclei (imunohystochemical stain for PCNA x400).

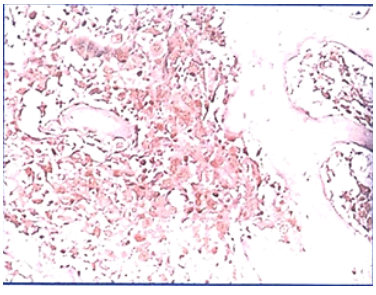

Fig. 5. Osteosarcoma with obvious training of osteoid tissue. The positivity of PCNA expression of 50% in the tumoral cells nuclei (imunohystochemical stain for PCNA x400).

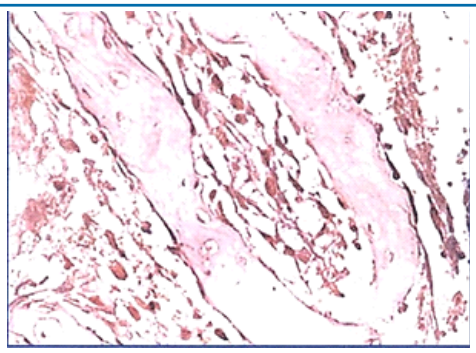

Fig. 6. Osteosarcoma with the presence of osteoid tissue lamella. PCNA expression of 50% in the tumoral cells nuclei (imunohystochemical stain for PCNA x 400).

1. *Mitosis density* (mitosis per mm2) was quantified in all grades of osteosarcomas (Fig. 7). It is noticed the progressive increment of mitosis on the standard area (1mm2), the sudden increase was from grade 2 (9.6 mitosis per mm2) to grade 2-3 (16.6 mitosis per mm2). The highest proliferative activity was found at grade 4 (25.3 mitosis per mm2) but also in grade 3 the values are very high (20.7 mitosis/mm2).
2. The quantification report of *mitotic activity index* (MAI) shows the average number of mitosis per 10 fields (hpt = high power field) (Fig. 7). The lowest value is found on stage 2 (7 mitosis per 10 fields), while in the third and fourth stages the values were similar (17.19 respectively mitosis per 10 fields.)
3. *Mitosis rate* was assessed according to the statistic report of quantification for each individual grade expressed by percentage with the difference being 2 percent for the last three grades (Fig. 7).

Tumor grade	*Mitosis density*	*Mitotic activity index*	*Mitosis rate*
2	9.6	7	8 %
2-3	16.6	12	12 %
3	20.7	17	14 %
4	25.3	19	16 %

Fig. 7. Centralizing the results of quantifying proliferative activity.

Discussions

PCNA, proliferating cell nuclear antigen, known as cyclin also, is an intranuclear polypeptide found in (both) normal and tumoral cell, identified as an auxiliary protein of delta polynucleosis of ADN, related to the replication sites of the ADN. There is a good correlation between the PCNA level and the degree of cellular proliferation.

PCNA is acting as a cofactor for the delta polynucleosis ADN in the S phase of the cellular cycle as well as in the ADN synthesis associated with its repair process[5, 6]. PCNA levels are increasing in the nucleus during the G1 phase and are decreasing in the G2 and M phases of the cellular cycle.

PCNA is a 36 KD molecule, very strongly preserved during the evolution of species. The complexity of the PCNA molecule was presented in details by Mc Cornick and Hall[5].

In the rapidly multiplying tumours the marking is do for the great majority of the nuclei. The intensity of the marking is heterogeneous, varying from very fade to very intense, probably reflecting the stages of the evolution cycle. The method can be used on paraffin included material, but also on preserved material if it was properly fixed[7].

Because the PCNA has a very long half-time, the cells presents detectable levels of PCNA for extended periods, starting from the exit of the M phase of the cellular cycle. Additional, very low levels of PCNA may also be detected even in the uncycling cells, adjacent

to the tumours. The same authors considers that this aspect proves the tumour aggressivity and it might be important to establish the prognosis.

We had noticed that in all non-osteoblastic areas there is a high value of PCNA index by comparison to osteoblastic areas. There are differences regarding the proliferative ability in the various intratumoral arias of the same osteoblastic osteosarcoma, similar features being pointed out by Sakayama Kenshi in 2003[8].

Tumour proliferation doesn't seems to be prognostic for the high degree osteosarcomas. Subsequent studies might show if the evaluation of this characteristic in conjunction with other features of the tumour might have a prognostic value[3].

The percentage of the proliferated tumour cells from the investigated series of osteosarcomas varied between 5% and 90%. This represents a wider range than that obtained in the same studies[3], but are similar to the figures obtained by German scientists[4].

The studies done in the last two decades[9] considers the tumoral necrosis as the important factor in the osteosarcomas of the bone extremities. There is no consensus regarding any variable which can be used for prognostic value and order the patients before starting the therapy. Immunochemical PCNA stain shows positivity between 3-91% nuclear stain in all cases. For 12 tumors the PCNA grade was higher (40%). Between histological subtypes, for the fibroblastic osteosarcomas the positivity was the highest (50.5 %), followed by 24.42 % (as next value recorded). According to this study, the PCNA survivors group was less (34.4 %) than those who died (45.26 %), but the difference was not statistically significant[9].

Conclusions

1. The PCNA intensity was correlated for osteosarcomas with the degree of differentiation and with the newly formed quantity of osteoid. The imunopositivity for PCNA was present on all the analysed tumour categories, but with different values for each of these, progressively increasing from grade 1 to 4. In the poorly differentiated osteosarcomas, the PCNA expression was positive in the tumoral cells nuclei in 75% percentage or more.
2. The quantification of proliferative activity using the three parameters proves the same mode of alterations, the values are increasing suddenly at 2-3 stage, reaching the peak in grade 4.
3. The highest mitosis density was found at grade 4, but also in grade 3 the values are very high. The lowest MAI value is found on stage 2, while in the third and fourth stages the values were similar. Any of these objective assessment methods of the proliferative activity might be used with the same degree of accuracy, including in the case of the high risk grade of osteosarcoma.

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