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The prognostic significance of DNA cytophotometry and proliferation index (Ki-67) in giant cell tumors of bone

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Abstract We studied DNA ploidy by smear cytophotometry and proliferation activity by Ki-67 MIB immunohistochemistry in 69 primary and recurrent giant cell tumors (GCT) from 50 randomly selected patients. The obtained results were evaluated with comparisons made to the available clinical data. From the 46 primary tumors 63% showed diploidy and 37% aneuploidy. A significantly ($P=0.026$) higher recurrence rate (64%) was observed in aneuploid than in diploid tumors (31%). In the course of the recurrences, both the ratio of aneuploid tumors as well as the proliferation index of the tumors increased, though the degree of the latter was non-significant. Aneuploidy did not mean an unambiguous tendency towards malignant transformation; however, a close follow-up of recurrent aneuploid tumors, and wide excision of the recurrence instead of intralesional curettage are the recommended procedures. The DNA cytophotometry and proliferation index of GCTs – as compared to other histologic examinations – are of prognostic value in the evaluation of the recurrence potential of the GCTs.

Resume Les auteurs ont examine l’anomalie chromosomique de DNA a l’aide de cytophotometrie de frottis; et l’activite de proliferation par le moyen de l’immunohistochimie Ki-67 MIB dans 69 tumeurs de cellules geantes de 50 patients choisis accidentellement. Ils ont value les resultats en les comparant aux donnees cliniques disponibles. Parmi les 46 tumeurs primaires 63% ont manifeste un caractere diploide et 37% un caractere aneuploide. On a observe une proportion de recidive significativement plus levee ($P=0.026$) dans les tumeurs aneuploides (64%) que dans les tumeurs diploides (31%).

Au cours des recidives on a remarque une augmentation tant dans la proportion des tumeurs aneuploides que dans l’indice de proliferation mais en ce dernier l’augmentation n’etait pas significative. Le caractere aneuploide ne signifie pas forcement une tendance univoque a la transformation maligne cependant dans les tumeurs aneuploides recidivantes il est necessaire de suivre la maladie avec beaucoup de soin et comme intervention chirurgicale on conseille une excision large au lieu du curettage intralesionnel.

Introduction

Giant cell tumor (GCT) of bone is a rare neoplasm and accounts for approximately 8% of bone tumors. In general, the epi- or metaphysis of the long tubular bones are involved. GCT exhibits local aggressive growth, having a high tendency for recurrence, between 10–40% [25]. Statistically, 4% of GCT are of primary malignant nature, 80% show benign behavior, 5–10% undergo malignant transformation, while another 8–10% present pulmonary metastases besides a benign histological appearance [4,6].

There have been several attempts to predict the biological behavior of GCT. Jaffe et al. [18], Dahlin et al. [8], and Huvos [17] classified GCT into 3 grades by its histological appearance, but the clinical and prognostic value of this grading has been disputed because these criteria are, in part, subject to personal bias [9,26].

On the basis of the clinical-radiomorphologic picture, Enneking [10] and Campanacci [6] defined latent, active and aggressive groups, with reference to biological behavior. Eckhart [9] observed the relationship between the number of mitoses and recurrences during histological examinations. Fukunaga et al. [11] studied the DNA content of GCT of bone by flow cytometry and failed to find a method suitable for drawing conclusions related to prognosis, while the results of others seem to verify this [1,2,13,31].

During the course of our retrospective study, Ki-67 analysis and smear cytophotometry of GCT were evalu-

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ated and correlated with the clinical data, to find out whether the proliferation activity and DNA-ploidy of the GCT of bones are suitable prognostic factors for evaluating the biological behavior of the GCT.

Patients and methods

Fifty patients with 69 GCTs were retrospectively evaluated. To avoid the influence of the different surgical procedures on the recurrence rate, only cases treated with curettage were included. Cases of primary malignant GCT were excluded from the survey.

Case histories, surgical reports, radiograms and where applicable, CT images, histological material were all known and at our disposal. The proportion of males and females was 21:29. The mean age was 34 (14–69) years. Mean follow-up period was 6.7 (2–18) years. The localisation of GCTs showed the typical distribution, 50% occurred in the knee region, followed by distal radial and proximal humeral involvement. From the 69 studied tumors 46 were primary treated GCTs and 23 recurrences. From the recurrences 11 were first, 7 were second, while 5 were third occurrences. Histological grading was done according to criteria of Dahlin [8].

Staging of the GCTs of bone was performed according to Enneking [10]. Hence, latent or surgical stage 1 indicates: preserved bone shape and cortical thinning; active stage 2; cortical thinning and bulging, involvement of the subchondral bone; the aggressive stage 3; the tumor has broken through the cortex with an associated soft tissue component. Distribution of our patients according to these stages showed 4 cases belonging to stage 1; 13 cases to stage 2; and 28 cases to stage 3.

DNA-smear cytophotometry was performed on 69 GCTs. For histological examination, paraffin-embedded, 4–6 μm thick sections were prepared from both primary and recurrent tumors and stained with hematoxylin-eosin, PAS and Gömöri for reticulin fibres.

Three 50 μm sections were cut from each paraffin block in order to prepare a nuclear extract for image cytometry. Sections (5 μm) were cut immediately from the thick sections for histological confirmation of the tumor. Suitable tumor details, including appropriate quantity and quality of tumor cells and lymphocytes, from non-necrotic areas were selected by light microscopy. Extraction of nuclei from the 50 μm sections for the image studies was accomplished using the method of Hedley et al. [14], with minor modification.

Briefly, tissue sections were deparaffinized and rehydrated in a series of decreasing concentrations of ethanol using a tissue processor (Technicon, Tarrytown, N.Y., USA). The tissue was incubated for 60 min at 37°C in 1 ml of 0.5% pepsin (pH=1.5; activity 2660 U/mg) in 1.1% NaCl and 3.5% polyethylene glycol, with vortexing every 5 min. Following pepsin digestion, the resulting nuclear extract was washed with phosphate buffered saline and filtered through a 44 μm nylon mesh (Small Parts, Miami, FL, USA). The concentration of the nuclear extract was adjusted to 1.10^6 nuclei/ml with phosphate-buffered saline.

For the nuclear extract 100 μl (approximately 1×10^5 cells) was deposited into the etched circle of a poly L-lysine coated glass slide and incubated in a humidified chamber for 15 min. A single drop of cold (–20°C) 95% ethanol was carefully added to the drop containing the nuclear suspension. After 15 s, the slide was placed into a staining jar containing cold 95% ethanol for 1 min and allowed to air dry.

The chromatin substance of the nuclei on the smears was made suitable for quantitative determination by Feulgen staining (hydrolysis 1 N HCl, 60°C, 9 min). Measurements were done with the help of an image analyzer with our own software, which corresponded to the criteria of DNA cytophotometry as defined by Carey [7].

The visual fields were photographed with a video camera built into the light microscope. The signs were digitized by means of a computer system performing the analysis (so-called “grey-level”

Fig. 1 Diploid DNA histogram of a 25-year-old male patient with a GCT located in the distal part of the radius

Fig. 2 Aneuploid DNA histogram of the recurrent GCT of the same patient 8 years later. Normal histology revealed only later a malignant transformation. The patient recently died of pulmonary metastases

determination), during which the parts not to be studied were excluded. The DNA content of the cell nuclei was quantitatively determined on the basis of the so-called integrated optical density (IOD) of the image points. The obtained results were graphically drawn by the system, in the form of a DNA histogram. Calculation of DNA-ploidy values was done with consideration of the observed standard reference population (usually lymphocytes). A count of 20–30 lymphocytes and 100–120 tumor cells, respectively, was measured from each slide during cytophotometry.

All histograms were reviewed by two observers (A.I. and S.Z.) who gave a consensus interpretation. Image cytometry histograms obtained from paraffin-extracted nuclei were classified based on the DNA-index was according to the so-called Boecking-algorithm. [The tumor is regarded as diploid if the DNA-index of the tumor cells falls between 0.9 and 1.2, and less than 15% of the nuclei is manifest in the tetraploid field (Fig. 1). The tumor is aneuploid if the DNA-index is between 1.21 and 1.79, and two segre-

gated peaks appear in the tumor cell population (Fig. 2). The tumor is considered tetraploid if the DNA-index is between 1.8 and 2.2.]

The Ki-67 antigen expression was demonstrated on decalcified, paraffin-embedded tissue samples of 56 GCTs. This required a special procedure, with the use of antigen-exploiting solution (of high pH value, containing NaOH) recommended for decalcified tissues. The method is described in detail elsewhere [20,24].

The Ki-67 antigen expression was evaluated by light microscopy. All stained tumor cell nuclei – irrespective of the intensity of the staining – were regarded as being positive. The Ki-67 representing proliferative activity was expressed as a percentage, referring to 100 counted cells.

The chi-square probe, and Fischer exact test were used for the statistical analysis. Identical populations showing a probability of $P < 0.05$ were regarded as populations indicating significant differences.

Results

Out of the 46 primary tumors 30 proved to be diploid and 16 to be aneuploid (Table 1). From the tumors showing diploidy, 9 (i.e. 31%) later revealed recurrences, while from the aneuploid ones there were 10 such cases (i.e. 64%) ($P=0.026$). Dividing the GCTs into groups according to Enneking [10] the proliferation index (ratio of Ki-67 positive cells) displayed an average of 7.5% in stage 1 tumors, 13% in stage 2 tumors, and 14% in stage 3 GCT. Considering the small number of patients, the great degree of dispersion, the differences found between the various groups cannot be regarded as significant. At the same time, studies on the diploidy of the tumors belonging to stages 1–3 revealed that while the tumors belonging to stage 1 all proved to be diploid, the tumors belonging to stage 2 were found to be aneuploid in 22%, while those of stage 3 in 48% of the cases. Statistical analyses gave no significant deviations between stages 2 and 3 ($P=0.084$). Notable differences for the stages 1–3 were not observed considering the frequency of recurrences.

A total of 23 recurrent GCTs were studied in respect to diploidy and proliferation index. The investigations revealed diploidy in 5 cases (22%), while aneuploidy in 18 cases (78%).

Table 2 shows the changes regarding first, second and third recurrences and the diploidy-aneuploidy ratios. First recurrences displayed diploidy in around one third of the cases while without exception, third recurrences all proved to be aneuploid.

By comparing ploidy and proliferation index of the primary tumors and the recurrent tumors in relation to the frequency of recurrences, a steady decrease in diploidy was detectable, while Ki-67 positivity showed a moderate but not noteworthy percentile increase in the various groups (Fig. 3). In about half of the cases curettage of the GCT was completed with adjuvant therapies (use of phenol and/or bone cement), which can also influence the frequency of recurrences (Table 3). Our cases were therefore examined separately according to the applied adjunctive therapy, scrutinizing the frequency of recurrences, ploidy as well as proliferation index.

Considerably fewer recurrences (38%) were observable in the diploid group as compared to the aneuploid

Table 1 Distribution of primary GCTs according to their stage (Enneking), ploidy and proliferation index

Stage	Diploid	Aneuploid	Ki-67 (%)	Recurrence
I: latent	3	0	7.50%	2
II: active	14	4	13%	6
III: aggressive	13	12	14%	7
Total	30	16		

Table 2 Alteration of the ploidy in recurrent GCTs

	Diploid	Aneuploid
First recurrence	3	8
Second recurrences	2	5
Third recurrences	0	5
Total	5	18

Table 3 Evaluation of ploidy and recurrences in GCTs treated by different surgical methods

$P=0.04357$	Surgical method	Total number	Recurrence (%)
Diploid (29)	Curettage	18	8
	Curettage + Adjunctive therapy	11	3 (38%)
Aneuploid (20)	Curettage	8	6
	Curettage + Adjunctive therapy	12	9 (75%)

Fig. 3 Comparison of cytophotometric DNA analysis and proliferation index (Ki-67) values in recurrent and non-recurrent GCTs

group where the ratio of recurrences amounted to 75%, irrespective of whether curettage, or curettage + adjunctive therapy was the given treatment ($P=0.0106$). Adjunctive therapy seemed to decrease the ratio of recurrences in the group comprising diploid tumors.

Discussion

Giant cell tumors belong to the group of tumors of intermediary dignity [21,22,27]. Based on data in the litera-

ture, 84–86% of GCTs are benign despite the fact that a part of them contain tumor emboli in the surrounding arterial lumens [26]. This, however, serves as an explanation to the finding that a certain percentage of histologically benign GCT (Goldenberg et al. 4% [12], Katz et al. 2% [19], Bertoni et al. 7% [3] give pulmonary metastases, the histological appearance of which corresponds in full to that of the primary tumor.

Secondary malignant transformation of GCTs is not a rare occurrence either, observed mainly following previous radiotherapy, but on occasions spontaneously [9]. Although without any results, numerous attempts have been made to predict the biological behavior of GCT, including their aggressiveness, tendency to metastasize, develop recurrences, and become malignant.

Certain authors have found that the site of the tumor is of prognostic significance, e.g. GCTs in the region of the wrist behave more aggressively in comparison to other locations [25]. Histological grading is, however, found to be of little value in the prognosis of recurrences, because of the lack of histological criteria and the subjectivity of the pathologist. McDonald [23] and Sanerkin [26], respectively found no correlation between the Enneking's [10] clinical staging system, the recurrences, as well as histological appearance of GCTs [23,26]. Mitosis count per square millimeter was held by these authors as a more important prognostic factor, even though they found significant over-lapping between the borderline aggressive and malignant forms.

The role of flow cytometry in GCTs was evaluated differently by different authors. Fukunaga et al. [11] as well as Heliö et al. [15,16] found no relationship between the aggressiveness, histological grading and tumor diploidy/aneuploidy. Other authors [13,31,32]; however, considered DNA-cytophotometry a useful addition to histological studies since the technique is suitable for detecting aneuploidy [2] even before the histological signs of malignancy become manifest, not only in the case of GCTs but also in cartilaginous borderline lesions (e.g. central chondroma) [1,5].

In our opinion, the varying conclusions drawn in respect to DNA cytophotometry are due to the different methods used [1,15,16,31]. In GCTs, where the cell population is complex, the objectivity of flow cytometry is doubtful. The technique of smear cytophotometry used by us, however enabled us to investigate the different cell populations of GCTs separately.

The biological behavior of tumors is greatly influenced by their proliferation activity [28,30,33]. In case of bone tumors – according to the experiences of certain authors [28,33] – the degree of Ki-67 gene expression shows correlation with the degree of malignancy; thus, such analysis is of diagnostic and prognostic value. In our material, the Ki-67 expression was higher in the primary tumors showing late recurrences and in the recurrent tumors, as compared to the non-recurring osteoclastomas (Table 3). In our study, the primary GCTs belonging to Enneking's stages 1–3 showed some increase in respect to either proliferation index or recur-

ring ability (Table 1) but without statistical significance.

The surgical staging according to Enneking, as well as the radiomorphological grouping defined by Campanacci refer therefore to the actual growth stage of the tumor, which has no strict correlation to the biological behavior of the tumor.

Our DNA cytomorphometric analyses revealed that two thirds of the primary GCT were diploid, with a considerably lower tendency to recur (31%) as compared to the aneuploid tumors (63%). This was also verified when the different surgical interventions (curettage versus curettage and adjuvant therapy) were evaluated separately. Aneuploidy was found to show an increase directly proportional to the increase in the number of recurrences of the certain tumors.

Accordingly, one third of recurrences were always found to be aneuploid, which would explain the finding of some authors that malignant transformation takes place following multiple recurrences [29].

We have found no data in the literature on parallel studies related to DNA-smear-cytophotometric study results and proliferation activity evaluations using the Ki-67 MIB method in GCTs. Neither have we encountered any comparisons of such results with prognostic purposes – though the ploidy values seem to be a quite sensitive prognostic marker, showing tight relationship with the biological behavior of osteoclastomas.

Based on our results, it is suggested to perform smear cytophotometric DNA analysis supplemented with proliferation activity determination in primary and recurrent GCTs. Aneuploidy as well as higher Ki-67 expression values (being above 12% in our material) call attention to the greater risk of recurrences, warranting closer follow-ups of these patients. Aneuploidy does not unambiguously mean malignant transformation. When a recurring GCT becomes aneuploid or an increase is manifest in its aneuploidy and proliferation index, resection is the recommended procedure rather than curettage of the tumor.

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