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Comparison of quantitative ground substance analysis in biopsy and resected tumour in osteosarcomas*

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Summary. Osteosarcomas may show variable differentiation. They are divided into osteoblastic, chondroblastic and fibroblastic subgroups depending on their dominant histological differentiation. The pattern of histological differentiation of osteosarcoma is supposed to have an influence on the response to chemotherapy and in this study the relationship between the differentiation of the tumours and response to chemotherapy was examined in 22 osteosarcomas. For this purpose the nuclear size of tumour cells was determined on imprints and the area of the different ground substances on tumour sections after undecalcified preparation. Tumours with little nuclear polymorphism and those with an area of chondroblastic ground substance of more than 20% in the biopsy showed a poor response to chemotherapy. We conclude from our results that lack of nuclear polymorphism and in particular a large area of chondroblastic ground substances in the biopsy, can be regarded as an unfavourable prognostic factor in the response to primary chemotherapy. A comparison of the area of chondroblastic ground substance in biopsy and resection material proves that the biopsy delivers a representative view of the total area of chondroblastic ground substance for the groups of responder and non-responder. Patients with a large amount of chondroblastic ground substance in the biopsy probably require more aggressive chemotherapy.

Key words: Osteosarcoma – Neoadjuvant chemotherapy – Morphometry – Nuclear size – Nuclear polymorphism – Chondroblastic ground substance – Osteoblastic ground substance

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Osteosarcoma is the most frequent primary cancer of bone, uncommon in the first decade of life and reaching its highest frequency at the age between 15 and 19. A slight male preponderance is observed. There are central, periosteal and parosteal forms, with the central type constituting about 90% of the total. Osteosarcoma occurs overwhelmingly in the metaphyses of long bones but the tumour may originate in the diaphyses of long bones (Delling 1984; Weinfeld and Dudley 1962; McKenna et al. 1966; Dominok and Knoch 1971; Spint et al. 1971; Dahlin 1973; Ohno et al. 1975; Unni et al. 1976; Konjetzny 1933; Schajowicz et al. 1972). Therapy of osteosarcoma has changed in the last years, and neoadjuvant chemotherapy is now carried out before surgery. This therapeutic concept has produced a remarkable improvement of prognosis (Rosen et al. 1982; Salzer-Kuntschick et al. 1983; Winkler et al. 1984). The results of the therapy studies Coss 80 and Coss 82 prove that patients with complete necrosis of the tumour have a better prognosis than those with a poor response to primary chemotherapy (Winkler et al. 1984; 1986). About 50% of the osteosarcomas are minimally affected by cytostatic therapy so that there must exist factors which influence the different responses. It is evident that morphological differentiation of osteosarcoma is at partly responsible for the different response to therapy and consequently for the prognosis.

The histological appearance of osteosarcomas is not homogeneous; They are divisible into several different histological types. They may be described as osteoblastic, chondroblastic or fibroblastic, depending on whether osteoid, chondroid or fibromatoid elements predominate (Dahlin et al. 1967; Delling 1984; Dahlin 1975; Kragh et al. 1958; Dahlin 1973). The importance of this histological

Table 1. Age, sex, localisation and regression grade after chemotherapy of 22 examined osteosarcomas

Case	Hist. No.	Age	Sex	Localisation	Regression grade
1	80/1292	24	Male	Prox. tibia	I
2	80/1604	9	Female	Prox. tibia	I
3	81/1747	15	Female	Dist. femur	I
4	81/2330	12	Male	Femur diaphysis	I
5	82/0286	13	Male	Prox. tibia	I
6	84/2386	7	Female	Dist. femur	I
7	81/1598	17	Male	Prox. tibia	II
8	82/0826	18	Male	Prox. tibia	II
9	82/1469	17	Male	Dist. femur	II
10	83/0563	14	Male	Prox. tibia	II
11	83/2128	10	Female	Prox. tibia	II
12	80/2085	16	Female	Femur diaphysis	III
13	82/2121	17	Male	Dist. femur	III
14	81/2281	15	Male	Dist. femur	V
15	81/2283	13	Female	Dist. femur	V
16	82/1117	15	Male	Dist. femur	V
17	83/3214	16	Male	Femur diaphysis	V
18	84/1303	19	Female	Prox. femur	V
19	85/0263	18	Male	Prox. tibia	V
20	85/0331	15	Female	Prox. tibia	V
21	85/1434	21	Male	Dist. femur	V
22	85/1858	16	Male	Prox. tibia	V

classification for prognosis or in the biological behaviour of osteosarcomas is given different emphases by different authors (Garrington et al. 1967; Dahlin 1973; Goidanich et al. 1966; McKenna et al. 1966; Spjut et al. 1971; Larsson et al. 1978).

The desire to match primary chemotherapy to the individual case caused us to concentrate on that information in the biopsy which might be important for prognosis. In future reliable giudes to prognosis in the biopsy might be the basis of specific chemotherapy.

This study deals with the question whether the determination of ground substance formation and nuclear polymorphism in primary biopsy permits the prediction of the therapeutic response of tumours to pre-operative chemotherapy. Does the extent of ground substance formation in the biopsy give a representative view of ground substance formation in the tumour?

Material and methods

This study is based on biopsy and resection material of 22 patients (14 male, 8 female) with osteosarcoma, which were treated according to the therapy protocols of COSS 80 and COSS 82. The patients ranged in age at diagnosis between 9 and 24 years. The localisation of the tumours was the proximal femur (1×), the diaphysis of the femur (3×), the distal femur (8×), and the proximal tibia (10×). Table 1 shows age, sex and localisation of the tumour for these 22 patients.

Imprint preparations were made from the primary biopsies of the 22 tumours and stained by Pappenheim. The biopsy material was embedded in methyl-metacrylate (Delling 1972) after fixation in formalin and stained by Goldner-stain, Kossa-stain and toluidine blue reactions.

From the imprints of each patient the area of at least 100 unselected and undamaged tumour cell nuclei was measured with an interactive image analysis system (IBAS, Kontron).

Determination of the special morphological structures was done in 5 µm thick sections (toluidine blue staining) with the help of a point counting method. The number of fields of view was chosen according to the size of biopsy. As a minimum 100 mm^2 were measured in the biopsies. The following structures were differentiated: tumour cells, chondroid metachromatic ground substance, fully developed tumour cartilage, mineralized tumour cartilage, osteoid ground substance, mineralized tumour bone, fibroblastic ground substance and vessels (Figs. 1–5).

10 repeated measurements of the nuclear size in imprint cytologies and the different components of tumour in the sections in identical areas by the same observer of the same case showed no significant differences. The deviation from the mean value came to 5% for nuclear sizes and tumour components in the biopsy. The tumours of all 22 patients were examined both after preoperative chemotherapy and amputation. At least one complete cross-sectional area of the tumours was reconstructed by large-area sections in each case. The size of the tumour as well as the percentage of necrotic zones and viable tumour were measured by the electronic interactive image analysis system. All 22 osteosarcomas were staged according to the histological grades of regression in bone tumors after chemotherapy by Salzer-Kuntschik et al. (1983) (Table 2).

The analysis mentioned above and quantitative determination of tumour-components in the biopsies was also carried out in the resected specimens of the same cases (detailed description of the method see Delling et al. 1985). As a minimum, 40 unselected regions with 25 mm^2 in size were evaluated. 10 repeated measurements of the identical tumour region of the same case by the same observer showed a variation of not more than 4%.

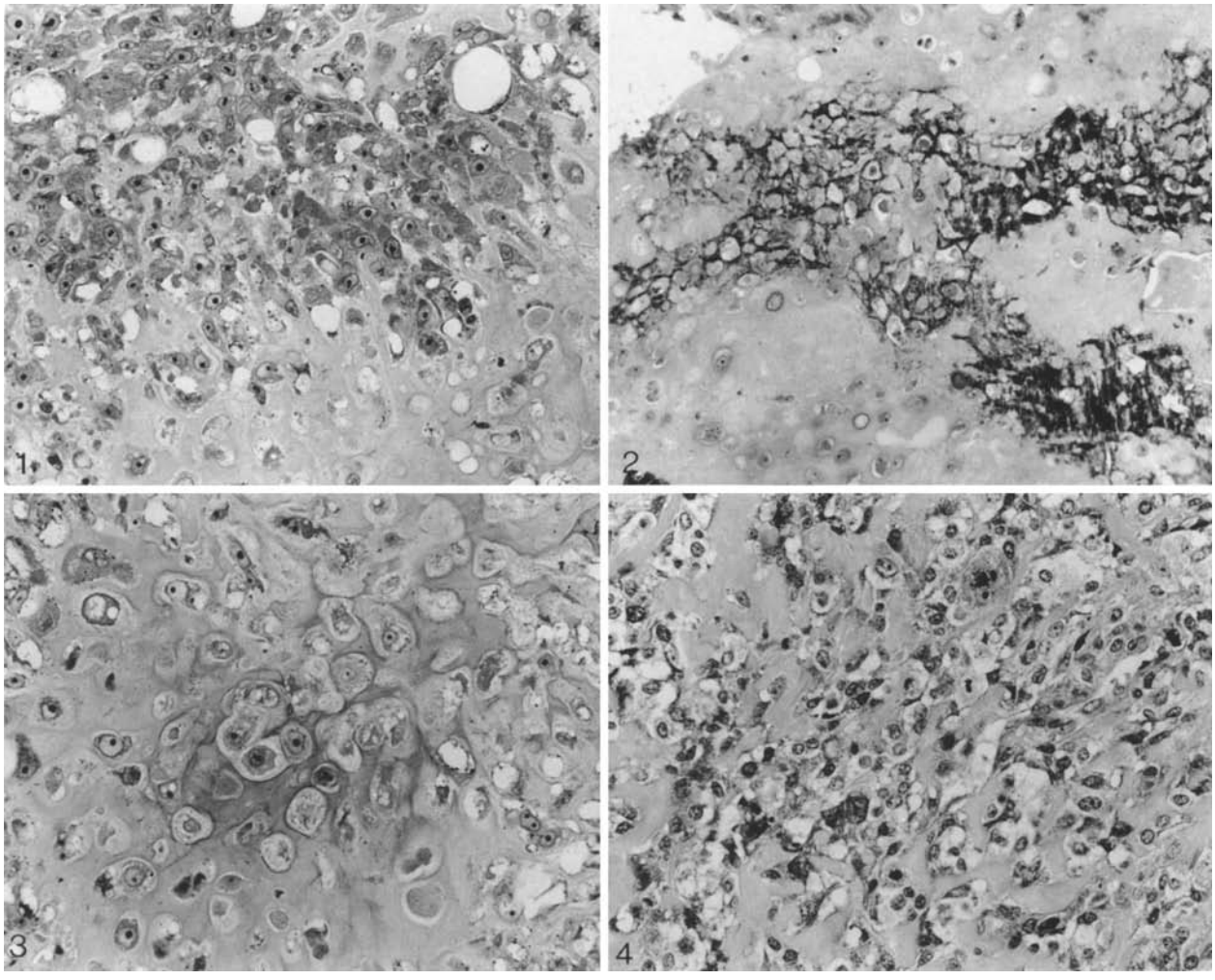

Fig. 1. Tumour-chondroblasts with prominent nuclei. Chondroid ground substance in the lower part of the picture. Toluidine blue reaction, undecalcified, ×264

Fig. 2. Mineralized chondroid matrix. Latticed mineralisation pattern in direct connection to the chondrocyte holes. Toluidine blue reaction, undecalcified, ×160

Fig. 3. Mature tumour cartilage. Large chondrocytes within the cartilage formed by the tumor. Toluidine blue reaction, undecalcified, ×264

Fig. 4. Tumour osteoblasts with formation of relatively wide latticed osteoid. Toluidine blue reaction, undecalcified, ×160

Table 2. Histological regression grades of bone tumours after chemotherapy (Salzer-Kuntschik, Vienna)

I	no viable tumour cells
II	some viable tumour cells or a viable tumour isle of less than 0,5 cm
III	less than 10% viable tumour area
IV	10–50% viable tumour area
V	50–80% viable tumour area
VI	no effect of chemotherapy

Results

Altogether 13 tumours (13/22) showed viable tumour cells in less than 10% of total tumour area after chemotherapy (responder); non-responders had viable tumour cells in more than 10% (9/22). In the group of responders the primary tumour was located in the proximal tibia (7 patients), in the distal femur (4 patients) and in the diaphysis of the femur (2 patients). In the group of non-responders the localisation of the tumours was as follows: distal femur (4 patients), diaphysis of the femur (1 patient), proximal femur (1 patient), and proximal tibia (3 patients).

The mean values of nuclear sizes in imprint cytology of the 22 tumours ranged from 84,5 μm^2 to 225,4 μm^2 (Table 3). In the group of responders (13) the mean value of nuclear size was 152,8 $\mu m^2 \pm 38{,}1\ \mu m^2$, whereas that of the non-re-

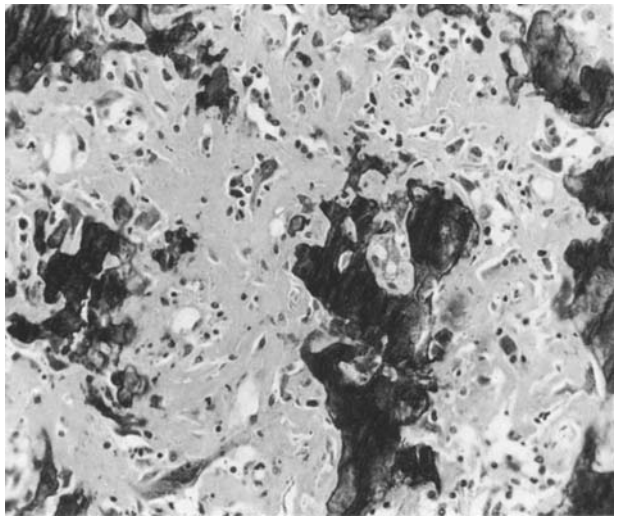

Fig. 5. Mineralized tumour bone and broad areas of osteoid with included tumor osteocytes. Toluidine blue reaction, undecalcified, × 64

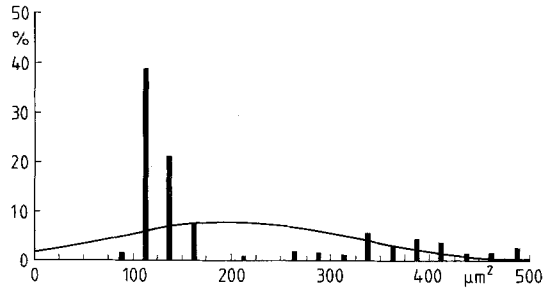

Fig. 6. Example of nuclear size distribution for a tumour with good response to pre-operative chemotherapy (responder)

sponders amounted to 131,3 $\mu m^2 \pm 29,5\ \mu m^2$ (Figs. 6 and 7). The difference of mean nuclear size of responders and non-responders was not significant according to Mann-Whitney U-test ($p = 0.13$).

The amount of chondroid ground substance in the biopsies of the 22 tumours ranged from 0% to 49%. For the responder group the mean value of chondroid ground substance was $4,4\% \pm 11,5\%$. However the non-responder group showed a mean chondroid ground substance of $21,2\% \pm 19,5\%$. This result was significant according to Mann-Whitney U-test ($p = 0.03$). Relating the response of tumours to preoperative chemotherapy, the extent of osteoid ground substance was very similar for responders (21,2% of complete tumour area) and non-responders (23,4%) of complete tumour area). Figure 8 shows the comparison of the extent of chondroid ground substance in biopsy and resection of the 22 osteosarcomas. There is a high degree of correlation (Pearson correlation coeffi-

Table 3. Mean values of nuclear sizes (μm^2) and amount of osteoid ground substance (%) in biopsies (imprint cytologies and histological sections) respectively the amount of chondroid ground substance in biopsies and resections of 22 examined osteosarcomas with evaluation of post-operative regression grade

Case	Hist. No.	RG	Biopsy			Resection
			NS (μm^2)	Osteoid GS (%)	Chondroid GS (%)	Chondroid GS (%)
1	80/1292	I	129,0	0	0	0
2	80/1604	I	142,5	41	0	0
3	81/1747	I	137,1	35	0	0
4	81/2330	I	197,1	2	0	0
5	82/0286	I	165,4	71	0	0
6	84/2386	I	84,5	16	0	0
7	81/1598	II	138,6	9	42	11
8	82/0826	II	199,9	15	0	0
9	82/1469	II	118,3	51	8	0
10	83/0563	II	155,7	8	5	4
11	83/2128	II	165,5	13	0	0
12	80/2085	III	225,4	1	0	0
13	82/2121	III	126,9	13	2	1
14	81/2281	V	103,3	22	19	1
15	81/2283	V	127,0	14	48	6
16	82/1117	V	150,1	11	49	65
17	83/3214	V	185,6	18	0	0
18	84/1303	V	122,1	36	34	52
19	85/0263	V	159,1	13	0	0
20	85/0331	V	128,7	52	19	1
21	85/1434	V	115,7	26	21	63
22	85/1858	V	89,7	19	0	0

Abbreviations: RG: Regression grade; NS: Nuclear size; GS: Ground substance

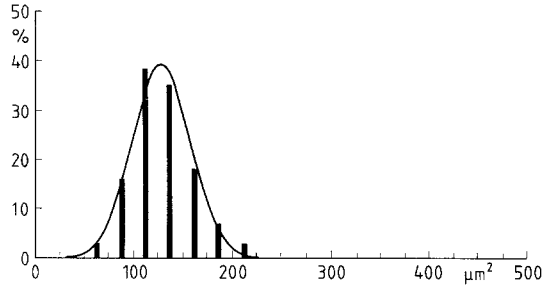

Fig. 7. Example of nuclear size distribution for a tumour with poor response to pre-operative chemotherapy (non-responder)

cient: $r = 0.64$, $p = 0.001$). 12 tumours (9 responders and 3 non-responders) showed neither in the biopsy nor in the resection chondroid differentiated tumour areas.

Discussion

The distribution of mean nuclear sizes of imprints show that tumours with large tumour cell nuclei and a big standard deviation of mean values show a good response to pre-operative chemotherapy more often than those with small nuclei and a small standard deviation. This concurs with the statement of Rosen et al. (1982) based on qualitative observations only. However the marked difference of mean nuclear sizes of responders and non-responders in section material, which was reported by Apel et al. (1985) was not be seen in imprint cytology. An explanation for these distinct results is that the nuclear sizes in imprints and in histological sections have two oppositing artefacts – expansion in imprints and shrinking in sections. The determination of nuclear size in sections seems to be more suitable for the estimation of prognosis of the efficacy of pre-operative chemotherapy.

Apart from the many possible influences on the prognosis of osteosarcoma including localisation, size and vascularisation of the tumour, the age and sex of the patient, stage of metabolism and resistance to certain chemotherapeutic drugs; the amount of chondroid ground substance is obviously important in the response of the tumour to chemotherapy. Large areas of chondroid tumour must be regarded as an unfavourable factor in prognosis of pre-operative chemotherapy. The mean value for the groups (responder/non-responder) is about 20% of chondroid substance in the biopsy. In 3 cases (1 responder, 2 non-responder) we found small amounts of chondroid in the area investigated in the resected tumour despite the presence of 8 and 19% of chondroid in the biopsies. These results show the problem of ground substance in the individual case, because of the variation in the distribution of chondroid within the osteosarcoma. Looking at the group these variations are less pronounced. However the extent of osteoid ground substance in the biopsy has no predictive value in the therapy response of osteosarcoma undergoing chemotherapy. The lack of chondroid substance in 2 out of 9 cases in the non-responders clearly shows the existence of additional but unknown factors determining the response of osteosarcoma cells on chemotherapy.

The reasons for these various therapy responses might be caused by different proliferation kinetics of tumour osteoblasts and chondroblasts. Another explanation is that the chondroid ground substance might create a mechanically or metabolically unfavourable environment so that the applied chemotherapeutic drugs produce little effect.

It is astonishing that a 2 cm^2 biopsy gives a representative image of ground substance formation in such a heterogeneous tumour as osteosarcoma (see Fig. 1). This result is in accordance with the report of Delling et al. (1985) concerning the distribution of single tumour components in extra

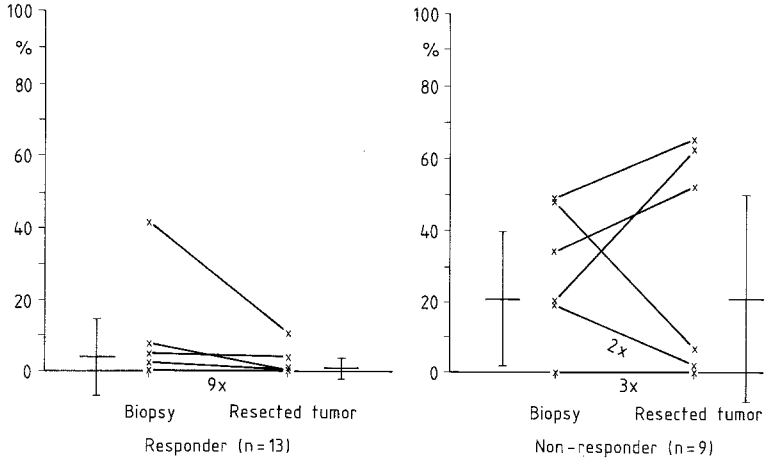

Fig. 8. Comparison of the percentage of chondroid ground substance in tumour biopsy and resection of responder group ($n = 13$) and non-responder group ($n = 9$) with mean value and standard deviation (Pearson correlation coefficient: $r = 0{,}64$; $p = 0{,}001$)

and intraosseus tumour areas. In resected material from 53 osteosarcomas they pointed out that the distribution of single tumour components and especially of chondroid ground substance does not differ in extra- and intraosseus tumour areas.

To simplify and standardize methodical proceeding a staining with special colours for the different ground substances is desirable, so that such an investigation could be done by an automatic colour image analysis. From these results the extent of chondroid ground substance in biopsies has predictive importance for response to chemotherapy. It correlates well with the extent of chondroid ground substance found in resection material. Equally the degree of nuclear anaplasia is – at least tendentiously – important for the therapeutic response of the tumour. It remains uncertain whether there exist further factors which are indicative of the prognosis of osteosarcomas undergoing chemotherapy. A combined analysis of the various factors affecting prognosis might give more information affecting further treatment, directly after confirmation of osteosarcoma in the biopsy. Investigation of DNA-profiles and the proliferation behaviour of osteosarcoma cells are presently being carried out in our laboratory.

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