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# DNA cytometry of osteosarcoma

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ACTA ORTHOPAEDICA SCANDINAVICA SUPPLEMENTUM NO. 228, VOL. 59. 1988

MUNKSGAARD · COPENHAGEN

Printed in Sweden
Ortonova
Wallin & Dalholm
1988

# Contents

# Abstract

The relationship between cytochemical features and histomorphology in osteosarcoma, and the clinical significance of DNA content were investigated by microspectrophotometry (MSP) of tissue sections and flow cytophotometry (FCM) of cell suspensions.

MSP of tissue sections entails the methodological error of determining the DNA content of sectioned cell nuclei. By analyzing 184 normal mesenchymal cell populations, an upper limit of diploidy (normal DNA content) was deduced. Applying this upper limit for 42 sarcomas, 6 were diploid and 36 hyperploid. Comparative analysis of the same lesions by MSP of imprint preparations and by FCM disclosed complete agreement in ploidy classification (diploid versus hyperploid).

Retrospective MSP analysis of bone tumors is often impeded by previous demineralization in acid, which destroys DNA. EDTA as an alternative was found to slightly reduce Feulgen DNA stainability of osteosarcomas, but did not affect tumor ploidy determination. Hence, EDTA offers a means of retaining DNA stainability of bone tumors requiring demineralization.

MSP analysis of different histologic areas, and comparative FCM analysis of biopsy and surgical specimens, dislosed that individual osteosarcomas are cytochemically uniform despite morphologic heterogeneity. Hence, a single tumor sample for DNA analysis can be relied upon as representative for the tumor as a whole.

In a consecutive series of 83 osteosarcoma patients treated by surgery and adjuvant Interferon, the 7-year survival rate was 0.44. MSP DNA analysis gave no significant prognostic information. Multivariate analysis identified 3 risk factors for tumor related death, i.e., male sex, proximal tumor location, and histologic grade IV. In a prognostication model, the 7-year survival rates, for patients with 0, 1, 2, or 3 risk factors, were 0.80, 0.59, 0.42, and 0.13, respectively. Hence, it is possible to identify subgroups of high grade osteosarcoma patients with different prognosis.

In a study of 166 primary bone tumors, the applicability of DNA analysis for differential diagnostic purposes was investigated. The series included high grade osteosarcomas, parosteal osteosarcomas and benign bone tumors, which may be mixed up histologically with osteosarcoma. Out of 166 tumors, 149 (90%) were histologically noncontroversial, whereas 17 (10 %) posed diagnostic difficulties. In the diagnostically noncontroversial group, all benign tumors and parosteal osteosarcomas were diploid, whereas 97 of 102 osteosarcomas were hyperploid. Hence, hyperploidy seems to be a characteristic feature of high grade osteosarcoma. In the diagnostically controversial group of 17 cases, uncertainty had prevailed as to benignity or malignancy. All 8 patients, who developed local recurrence or died, had hyperploid lesions. In contrast, none of the diploid lesions recurred.

The clinical significance of DNA analysis in osteosarcoma is mainly diagnostic. In general, it confirms the histopathologic assessment. Occasionally, it questions diagnosis, and provides information of decisive therapeutic implication. Applied routinely, ploidy determination can contribute to increased diagnostic accuracy of primary bone tumors.

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# Introduction

Osteosarcoma is a highly malignant tumor of bone with a peak age incidence in the second and third decade (Broström 1979; Dahlin and Coventry 1967; Mirra 1980; Price 1961). Although a rare entity, it is the most common primary malignant bone tumor. The annual incidence is approximately 2 per million inhabitants (Larsson and Lorentzon 1974; Stark et al. 1988). Males are more often affected than females. The most common location is about the knee. Osteosarcomas predominantly metastasize to the lungs; other sites are uncommon except in late stages of the disease.

Historically, the clinical course in osteosarcoma has been dismal. Survival was less than 20 percent in series reported from the 1950s and 1960s, despite ablative surgery (Broström 1979; Dahlin and Coventry 1967; Lockshin and Higgins 1968; Marcove et al. 1970; Price 1961).

During the last decade, several centres have reported improved survival in osteosarcoma (Bacci et al. 1986; Harvei and Solheim 1981; Rosen and Nirenberg 1985; Simon et al. 1986). The results have mainly been attributed to the introduction of adjuvant chemotherapy (Cortes et al. 1974; Jaffe et al. 1974; Rosen et al. 1974). The rationale for this treatment is to eradicate micrometastases, not evident at presentation (Rosen et al. 1979). However, controversy prevails about the role of adjuvant chemotherapy in osteosarcoma (Bertino 1987; Carter 1984; Holland 1987; Lange and Levine 1982; Souhami 1981). One major reason is the lack of conclusive randomized trials (Edmonson et al. 1984; Eilber et al. 1987; Link et al. 1986). Most important, improved survival has also been reported for patients treated by surgery only (Broström et al. 1980; Gilchrist et al. 1981).

There have been speculations about a change in the natural course of the disease, questioning the validity of historical controls (Fisher 1978; Giuliano et al. 1984; Taylor et al. 1978). Broström et al. (1980), reported that osteosarcoma patients from the 1970s exhibited different clinicopathologic features than those from the 1960s. Moreover, in a recent epidemiological study from Sweden covering the period 1971 to 1983, a continuously increasing age of the patients was noted (Stark et al. 1988).

At present, there seems to be several factors involved, which create difficulties in attaining consensus about treatment and in evaluating different osteosarcoma series. A major concern, in this context, remains the application of generally adopted criteria for diagnosis and patient selection.

## Histopathology

The diagnosis of osteosarcoma rests upon clinical, radiological and histopathological findings. Osteosarcomas, commonly, exhibit characteristic histologic features indicative of high grade malignancy (Dahlin 1975; Mirra 1980). However, there is a great morphologic variability among the tumors, even within individual lesions. It is customary to classify osteosarcoma according to predominant tissue type and degree of differentiation. Histologic subtypes include osteo-, chondro-, fibroblastic and telangiectatic variants (Dahlin 1975). Degree of differentiation is based on a 4 grade scale, Grade IV representing the most extreme anaplasia (Broders et al. 1939). Although useful for diagnostic purposes, the subclassification of high grade osteosarcomas has been of limited prognostic value.

The heterogeneous morphology of osteosarcomas sometimes also causes diagnostic problems (Ackerman 1976; Dahlin 1979; Mirra 1980). Hence, it may be difficult to distinguish high grade osteosarcoma from parosteal osteosarcoma, which commonly is of low grade malignancy. Of greater clinical significance, is that benign bone tumors, such as osteoblastoma and aneurysmal bone cyst, are being mixed up histologically with osteosarcoma, and *vice versa* (Dorfman 1973; Lichtenstein 1950; Mirra et al. 1976; Merryweather et al. 1980).

The diagnostic problems illustrate the limitation of histopathologic assessment. The fact that the diagnosis of high grade osteosarcoma does not rest upon clear-cut criteria, would also seem to be reflected by the difficulties in predicting the clinical course. Hence, there is a need for better characterization of this tumor entity.

## DNA cytophotometry

The normal human nonproliferating cell is characterized by a constant DNA content, corresponding to 46 chromosomes. Malignant transformation of cells involves chromosomal changes often resulting in an abnormal DNA content (Atkin et al. 1966; Hughes 1965; Sandberg and Hossfeld 1970). When sufficiently pronounced, this can be detected by quantitative cytophotometry (Atkin and Richards 1956; Böhm and Sandritter 1975; Caspersson 1979; Lomakka 1965). In several malignant tumor entities, lesions with either a normal (diploid) or an abnormally increased (hyperploid) DNA content have been encountered. Although, hyperploidy is a sign of chromosomal abnormality, it does not necessarily reflect specific malignant properties, such as increased proliferative activity or propensity for metastatic spread. Nevertheless, a relationship between nuclear DNA content and clinical course has been reported for several carcinoma entities. Thus, diploid tumors have been shown to be associated with a better prognosis than hyperploid (Barlogie et al. 1983; Laerum and Farsund 1981; Tribukait 1987). Since this does not apply to all malignant entities, the relationship between DNA content and clinical course has to be investigated for each tumor entity separately.

In sarcoma, the clinical significance of DNA content has only been reported for chondrosarcoma (Kreicbergs et al. 1982). Diploid lesions were associated with a better prognosis than hyperploid. DNA analysis was actually found to provide better prognostic information than clinicopathologic assessment. In soft tissue sarcoma, a relationship has been demonstrated between histologic malignancy grade and DNA content; the higher the grade, the larger the proportion of hyperploid lesions (Kreicbergs et al. 1987). Preliminary data on osteosarcoma indicate that the majority is hyperploid (Heliö et al. 1985; Hiddeman et al. 1987; Kreicbergs et al. 1984; Mankin et al. 1985; Xiang et al. 1987). So far, studies relating DNA content to clinical course in osteosarcoma have not been reported.

## Specific aims

The present work comprised methodological, morphological and clinical investigations. The aims were:

**Methodologically** to (1) establish an upper limit of diploidy for microspectrophotometric DNA measurements in tissue sections; (2) examine the reliability of ploidy determination by microspectrophotometry as compared to flow cytometry; (3) investigate Feulgen DNA staining properties of bone tumors after demineralization in EDTA; (4) test the reliability of ploidy determination in poorly Feulgen stained sections of archival paraffin embedded tumors.

**Morphologically** to (1) determine whether individual osteosarcomas are cytochemically uniform; (2) analyze the relationship between DNA content and histologic subtypes of osteosarcoma.

**Clinically** to (1) investigate the relationship between DNA content and clinical course; (2) assess the diagnostic value of DNA analysis in osteosarcoma.

# Material and methods

A total of 175 primary bone tumors were analyzed by microspectrophotometry (MSP) of tissue sections and imprint preparations, and/or flow cytometry (FCM) of cell suspensions. In addition, data from MSP analysis of 184 normal cell populations in tissue sections, and 30 in imprint preparations, were collected. The distribution of the material according to specific investigation and mode of DNA analysis is shown in Figure 1. Since the same DNA data were utilized to address various methodological and clinical issues, there was a considerable overlapping of the material amongst the different investigations. Details are given in each chapter.

| | |
|---|---|
| Upper limit of diploidy | MSP / FCM |
| 184 Normal cell population | |
| 5 Chondrosarcoma | |
| 37 Osteosarcomas | |
| Demineralization in EDTA | MSP |
| 1 Muscle tissue specimen | |
| 3 Osteosarcomas | |
| Stainability of archival tissue | MSP |
| 21 Osteosarcomas | |
| Histologic areas | MSP |
| 12 Osteosarcomas | |
| Biopsy and surgical specimens | FCM |
| 20 Osteosarcomas | |
| Primary and recurrent lesions | MSP / FCM |
| 19 Osteosarcomas | |
| 28 Recurrences | |
| DNA content and histology | FCM |
| 47 Osteosarcomas | |
| Prognostic factors | MSP / FCM |
| 60 Osteosarcomas | |
| 23 Osteosarcomas - No DNA data | |
| Diagnostic aspects | MSP / FCM |
| 166 Benign and malignant tumors | |

Figure 1. Distribution of the material according to cytochemical analysis.

## Histopathologic assessment

Histological reports, original slides and corresponding paraffin blocks of all cases were collected. The histologic slides were reviewed, without access to clinical data, by Claes Silfverswärd, M.D., at the Department of Tumor Pathology, Karolinska Hospital.

The tumors were evaluated according to the principles proposed by WHO (1972). Osteosarcomas were classified according to histologic grade (I-IV) (Broders et al. 1939), and subtype (osteo-, chondro-, fibroblastic, and telangiectatic) (Dahlin 1975). Parosteal osteosarcomas were distinguished as a separate entity.

## Slide DNA analysis

### Tissue preparation

Tissue sections were prepared according to the technique described by Kreicbergs and Zetterberg (1980). Fresh tumor tissue was fixed in 10% neutral buffered formalin (24h) and paraffin embedded. Sections (4 µm) from archival and fresh specimens were deparaffinized in xylol (20 min), and refixed in 10% formalin before Feulgen staining. Additional sections were stained in hematoxylin-eosin for histologic identification of areas for DNA measurement.

Imprint preparations were obtained from fresh tissue, subsequently air dried and fixed in 10% neutral buffered formalin (24h) before staining (Kreicbergs et al. 1981a).

Staining of cell nuclei in both tissue sections and imprint preparations was performed according to a modified Feulgen procedure using acid hydrolysis in 5 M HCl, 1h, 22°C before staining (De-Cosse and Aiello 1966; Eneroth and Zetterberg 1974).

## Microspectrophotometry

The measurements were made in a rapid scanning microspectrophotometer (SMP 05/Zeiss, Oberkochen, West Germany). Immersion oil (refractive index 1.518) was used for the objective (plan 100/1.25). The condenser setting was plan 40/0.6. The diameter of the scanning beam was 1 μm and the step length 1μm. The total extinction at 546 nm was determined in each cell nucleus as a measure of the relative DNA content. Background transmission was set at 100%. The calculated total extinction (TE) of each cell nucleus was based on transmission values in the interval 5 to 85% in analysis of tissue sections, and 5 to 95% in analysis of imprint preparations. Hence, in tissue sections, the upper light transmission limit was lowered to 85% to exclude non-specific light losses caused by background inhomogeneity (Kreicbergs 1981; Weid et al. 1970).

Fifty normal connective tissue cells and 100 tumor cells were analyzed in each tissue section. In imprint preparations, the analysis included 10-20 granulocytes and 50 tumor cells.

## DNA content

The median (50th percentile) DNA content of each control cell population was given the arbitrary value DNA Index (DI) 1.0, denoting the diploid DNA content. The percentage of cells with DNA values exceeding DI 1.25 (% cells >DI 1.25) was determined for each control and each tumor cell population (Erhardt et al. 1984).

Several additional DNA variables were derived from the tumor DNA histograms, apart from determining the % cells >DI 1.25. As illustrated by the DNA histogram in Figure 2, the following were considered: percentage tumor cells >DI 2.5 (% >DI 2.5) (Erhardt et al. 1984), median DNA value (50th percentile), and extreme DNA value (90th percentile) (Kreicbergs et al. 1982).

## Nuclear size

Nuclear size (projected area) in $\mu m^2$ was obtained from computerized transcripts of the microspectrophotometric DNA measurement of each Feulgen stained nucleus (Kreicbergs et al. 1981b). For each tumor cell population the median (50th percentile) and extreme (90th percentile) nuclear size was calculated (Figure 3).

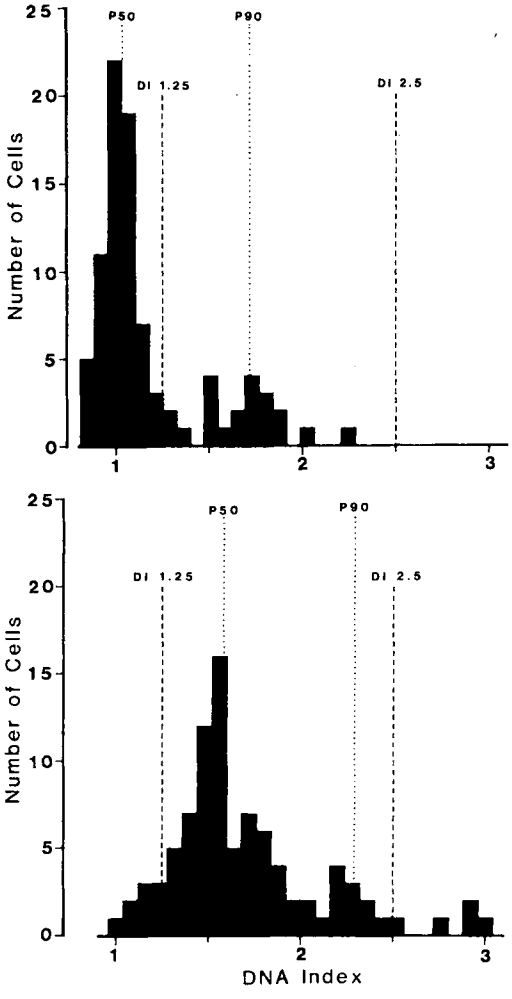



Figure 2. Examples of DNA histograms of a diploid (top) and hyperploid (bottom) cell population.

## Flow DNA analysis

### Tissue preparation

Specimens for flow DNA analysis were obtained by squeezing fresh tumor tissue through fine mesh nylon gauze with Tris-EDTA buffer pH 7.5. After centrifugation, the cell material was fixed in 96% ice cold ethanol. The fixed cells were washed in Tris-EDTA buffer together with 1 mg/ml RNAase to remove all RNA. Suspensions of single cell nuclei were obtained by pepsin treatment. After washing in the buffer, the nuclei were stained in $2.5 \times 10^{-5}$ Methidium bromide in Tris-EDTA buffer with a molarity of 395 mOsm.

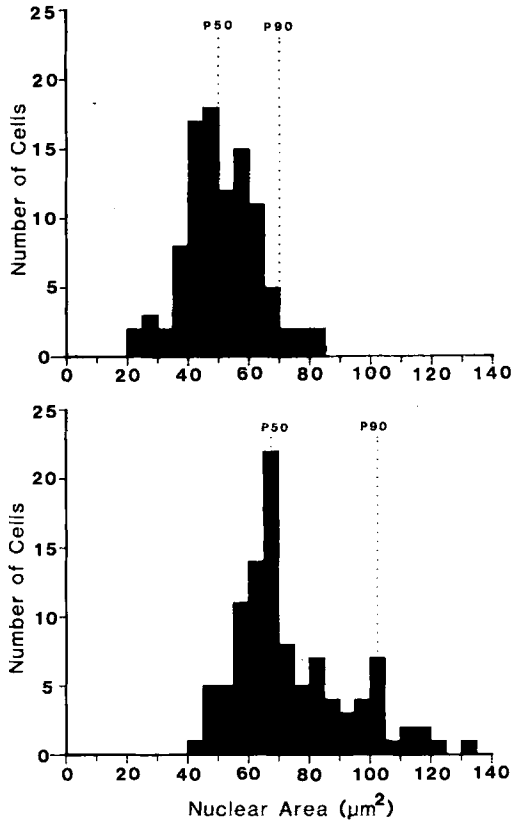



Figure 3. Nuclear size histograms from the same two cell populations shown in Figure 2.

### Flow cytometry

The DNA content of the cell nuclei was analyzed using a rapid flow cytofluorometer ICP 11 (Phywe, West Germany, now Ortho Instruments, Westwood, MA, U.S.A.) equipped with a XB-0 75 W lamp. The excitation and emission wave lengths were 455 to 490 nm and 590 to 630 nm, respectively. The output was sorted with a 256 multichannel analyzer. For each tumor sample, a minimum of 30,000 cells was analyzed. For details see Tribukait (1987).

### DNA content

Human lymphocytes were used as staining controls of the normal diploid (DI 1.0) DNA content. The coefficient of variation was <3%. The DNA values of the analyzed tumor cells were expressed in relation to the DNA value of the control lymphocytes. Tumors with a unimodal peak within 10% of the diploid standard were classified as diploid. Tumors were classified as aneuploid in the presence of non-diploid peaks. However, tumors with a peak at DI 1.9-2.1 containing >15% of the analyzed cells, and a corresponding ($G_2$/M) peak in the octaploid region were classified as tetraploid.

Tumors with 2 aneuploid peaks were considered to have 2 stem lines when the second aneuploid peak value was not a multiple of the first. Moreover, if a second aneuploid peak, representing a multiple of the first, contained >15% of cells, and had a corresponding $G_2$/M peak, the tumor was also classified as having 2 stem lines. No attempts were made to distinguish between diploid-tetraploid and exclusively tetraploid tumors, since it is not possible to determine by FCM, whether the diploid peak represents a mixture of normal and neoplastic cells, or only normal cells. Examples of DNA histograms from FCM of cell suspensions are shown in Figure 4.

The proportion of S and $G_2$/M phase cells of each tumor cell population was determined after correction for background, as described by Baisch et al. (1975).

## Clinical assessment

In the clinical part of this study, the following patient and tumor characteristics were assessed: sex, age, tumor location and size, surgical procedure, and time of follow-up. In addition, the system of muskuloskeletal tumor staging according to Enneking (1986) was applied.

The location of the tumors was categorized into 3 groups: shoulder/trunk/hip, distal humerus/femur, and lower arm/leg. Tumor size was defined by the largest diameter (cm), as determined by macroscopic examination of the surgical specimen or original radiographs. The operative procedure was classified as either local or ablative surgery.

Tumor stage and surgical margin were assessed by reviewing radiographic, surgical, and histopathologic reports (Enneking 1986). The tumors were staged as Low (I) or High (II) Grade, Intracompartmental (A) or Extracompartmental (B), forming the following groups: IA, IB, IIA and IIB. Tumors associated with metastasis at time of diagnosis constituted Stage III. The margins obtained at surgery were classified as intralesional, marginal, wide, or radical.

Clinical follow-up focused on recurrence and survival. The follow-up covered the period from the day of diagnosis to January 1, 1988. Patients who died of non-tumor related causes were included in the prognostic analysis until time of death.

## Statistics

The estimated 7-year survival and local recurrence rates were determined by Kaplan-Meier (life-table) analysis of censored data (Peto et al. 1977). Prognosis was related to the following clinicopathologic features: sex, age, tumor location and size, histologic subtype and grade. However, the retrospective assessment of surgical stage and margin was not considered sufficiently reliable for inclusion in the prognostic analysis. Age and tumor size were dichotomized into ≤18 and >18 years, ≤10 and >10 cm, respectively. The prognostic significance of DNA content and nuclear size, treated as continuous variables, was analyzed separately without regard to the clinicopathologic features.

Multivariate Cox (1972) proportional regression analysis was used to test the relative significance of the different variables. Estimated survival for subgroups of patients with none, one or several identified clinicopathologic risk factors was determined by life-table analysis. Survival rates of different subgroups of patients were compared by either log rank (Mantel-Haenzsel) test or test for trend in prognosis (Peto et al. 1977).

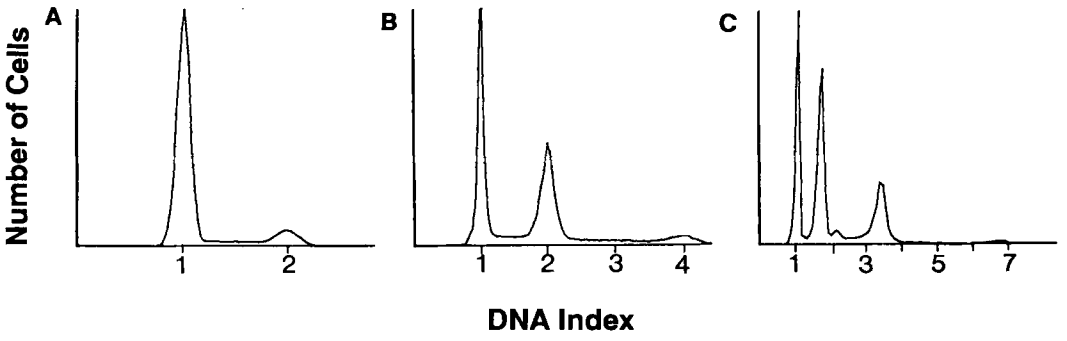



Figure 4. Examples of DNA histograms from flow cytometry of cell suspensions.
A. Diploid; B. Tetraploid; C. Aneuploid cell populations.

# Microspectrophotometry of tissue sections

The aim of this investigation was to establish an upper limit of diploidy for MSP analysis of mesenchymal cell populations in tissue sections (Bauer et al. 1986b). Furthermore, Feulgen DNA stainability of fresh tissue after demineralization in EDTA and the reliability of analyzing weakly stained archival tissue were studied (Bauer and Kreicbergs 1987).

## Upper limit of diploidy

MSP of tissue sections is associated with the methodological error of determining the DNA content of sectioned cell nuclei. This methodological error causes problems in discriminating between diploid and hyperploid tumors. Several means of interpreting histograms from DNA measurements in tissue sections have been proposed (Bennington and Mayall 1983; Eneroth and Zetterberg 1974; Kreicbergs and Zetterberg 1980; McCready and Papadimitriou 1983), but none has gained wide acceptance. In fact, the possibility of distinguishing between diploid and hyperploid tumors by DNA analysis in tissue sections has been questioned (Berryman et al. 1984).

DNA analysis in tissue sections (4 μm) disclosed that 145 of 184 normal mesenchymal cell populations (fibroblasts, myocytes, lipocytes, chondrocytes) exhibited cells with DNA values exceeding DI 1.25 (Figure 5). The percentage of cells >DI 1.25 ranged from 0 to 31% (Figure 6).

Corresponding analysis, in imprint preparations, showed that 5 of 30 granulocyte populations had cells with DNA values exceeding DI 1.25; range 0-20% (Figure 6).

The maximum percentage of normal cells >DI 1.25 demonstrated in each type of preparation was subsequently used as an upper limit of diploidy. Thus, tumors with a higher percentage of cells >DI 1.25, i.e., 31% in tissue sections and 20% in imprints, were classified as hyperploid.

In the analysis of 37 osteosarcomas and 5 chondrosarcomas in tissue sections, 6 were diploid and 36 hyperploid (Figure 7). Complete agreement in ploidy classification was found for the 30 sarcomas also analyzed in imprints. Comparison of the percentages of cells >DI 1.25 in each type of preparation showed a good correlation (r=0.89).

To further assess the reliability of discriminating between diploid and hyperploid tumors in tissue sections, specimens of the same 42 tumors were analyzed by FCM. In complete agreement with the MSP analysis, 6 sarcomas were diploid and 36 hyperploid.

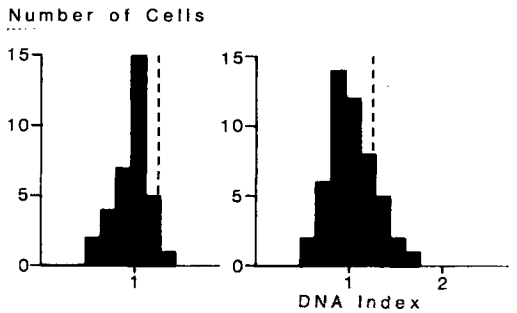



Figure 5. Representative DNA histograms of two fibroblast cell populations analyzed in tissue sections, with 5% (left) and 25% (right) cells >DI 1.25, respectively.

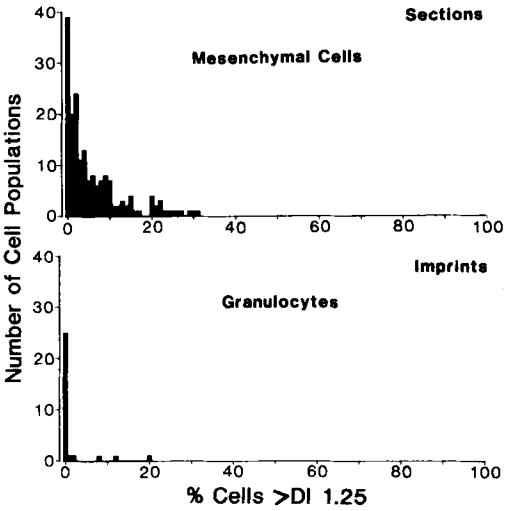



Figure 6. Percentages of cells >DI 1.25 in 184 normal cell populations analyzed in tissue sections (top), and 30 granulocytes populations in imprints (bottom).

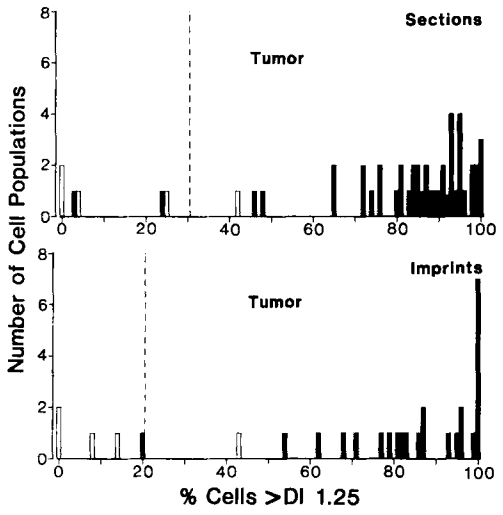



Figure 7. Percentages of cells >DI 1.25 in bone sarcomas analyzed in tissue sections, n=42, (top), and imprints, n=30, (bottom). The dotted line denotes the upper limit of diploidy in each type of preparation. Empty bars, chondrosarcomas; filled bars, osteosarcomas.

According to FCM, 4 of the 36 hyperploid tumors were tetraploid. Notably, 2 of these 4 lesions exhibited a bimodal diploid-tetraploid distribution according to MSP in tissue sections and imprints (Figure 8B), whereas the other 2 were exclusively tetraploid (Figure 8C).

In conclusion, the 3 methods of DNA analysis compared, provided identical ploidy discrimination (diploid versus hyperploid) of the tumors. Hence, the upper limit of diploidy suggested for MSP analysis in tissue sections, appears reliable for distinguishing between diploid and hyperploid sarcomas.

## Demineralization in EDTA

MSP analysis of archival bone tumors is often impeded by previous acid demineralization, which destroys Feulgen DNA stainability. To find an alternative to acid for prospective DNA studies of bone tumors in tissue sections, Feulgen stainability of fresh osteosarcoma specimens after demineralization in neutral EDTA was investigated.

Four fresh specimens, 3 osteosarcomas and 1 piece of normal connective tissue, were demineralized for 14 days in EDTA 240 g/l (Anderson 1984), adjusted to pH

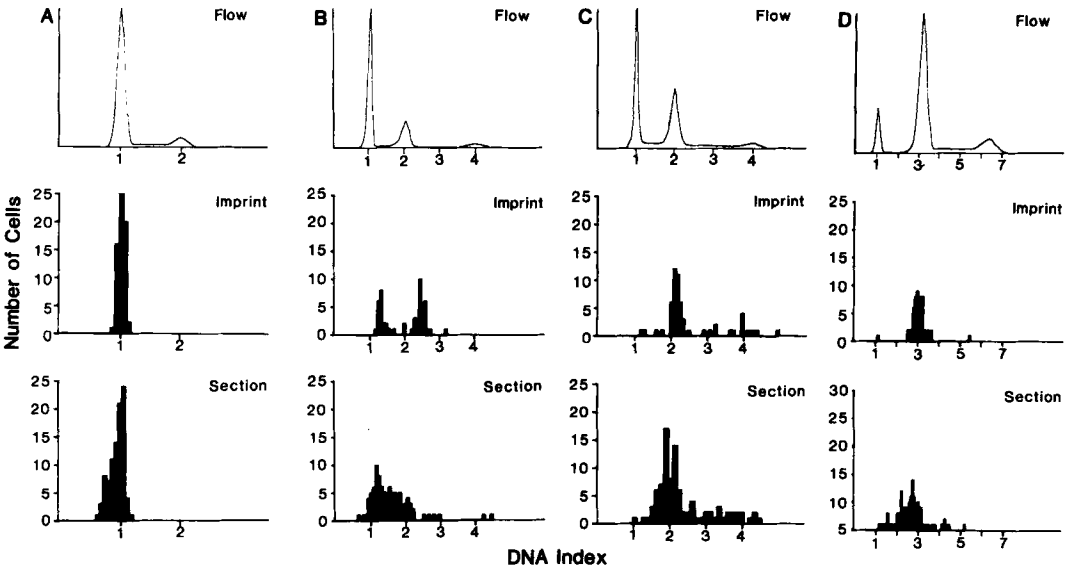



Figure 8. Representative DNA histograms of bone tumors analyzed by MSP in tissue sections and imprints, and by FCM in cell suspensions.

7.4 by 5 mmol/l NaOH. After demineralization, the specimens were embedded, cut, and stained as previously described. For comparison, additional tissue from each of the 4 fresh specimens was prepared similarly, although without previous demineralization in EDTA.

Demineralization in EDTA slightly reduced nuclear Feulgen stainability. However, the staining relationship between control and tumor cell populations was not affected. 1 muscle tissue specimen and 1 osteosarcoma were diploid in both EDTA demineralized and non-demineralized sections (Figure 9A). Another 2 osteosarcomas were clearly hyperploid in both types of preparations (Figure 9B).

To investigate hydrolytic properties after EDTA treatment, demineralized and non-demineralized sections of 2 osteosarcomas were hydrolyzed in 5 N HCl for 10, 20, 40, 60, and 120 minutes, before Feulgen staining. Twenty control and 25 tumor cells were analyzed in each section. The course of the hydrolysis curves of EDTA- and non-demineralized specimens was almost identical. This applied to both the control cell and tumor cell populations of the 2 tested specimens, i.e., 1 diploid and 1 hyperploid osteosarcoma (Figure 10).

The results show that, for bone tumors requiring demineralization, EDTA offers a means of retaining Feulgen DNA stainability.

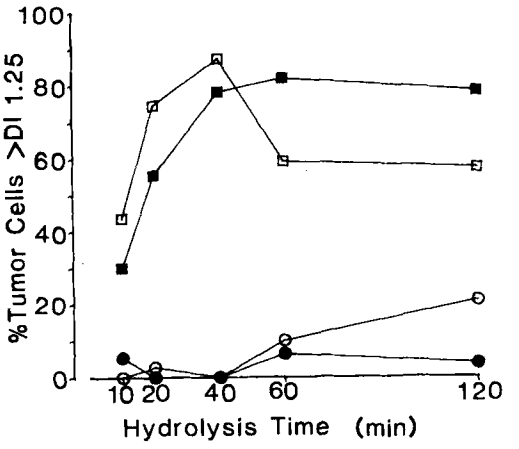



Figure 10. Comparative hydrolysis test of non-demineralized (open symbols) and EDTA demineralized (closed symbols) osteosarcomas. Percentage tumor cells >DI 1.25 in relation to hydrolysis time of the diploid (circles) and hyperploid (squares) osteosarcomas.

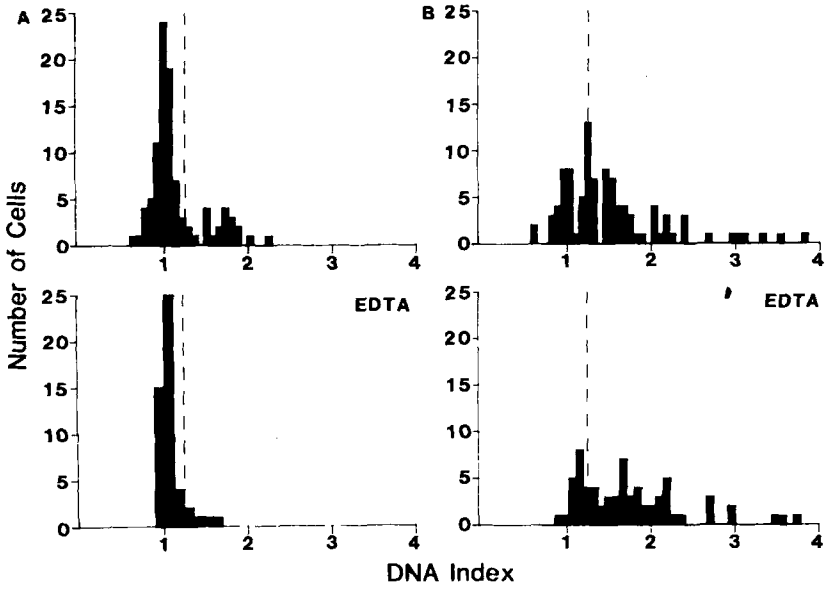



Figure 9. Comparative DNA analysis of non-demineralized and EDTA demineralized specimens. A. Diploid osteosarcoma; B. Aneuploid osteosarcoma. The dotted line denotes DI 1.25.

## Stainability of archival tissue

In DNA studies based on MSP of archival tissue, the specimens exhibit varying Feulgen stain intensity. For weakly stained tissue sections, background inhomogeneity, causing light scattering, can be assumed to affect the recorded extinction to such an extent that the measurements become unreliable. To test the significance of background disturbances in relation to nuclear stain intensity, different upper limits of light transmission were applied in the analysis of the same slides (Bauer and Kreicbergs 1987).

Twenty-one archival osteosarcoma specimens of varying Feulgen stainability were analyzed. Setting 3 different upper limits (75, 85, and 95%) of light transmission was generally not found to affect the staining relationship between control cells and tumor cells of the same specimen (Figure 11). In 4 cases, however, tumor DI was clearly affected, when the upper transmission limit was altered; in 1 there was even a shift from diploidy to hyperploidy. All 4 specimens were poorly stained; the median total extinction value for the control cell populations was <6, corresponding to less than one third of the maximum control median total extinction encountered, i.e., 18.

The results show that, among weakly Feulgen stained sections, those inappropriate for ploidy determination can be identified.

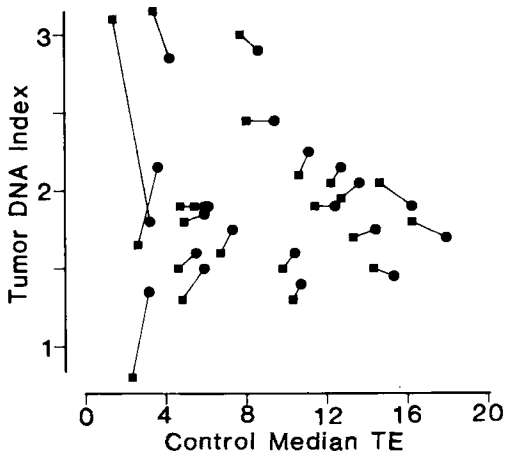



Figure 11. Tumor DI of 21 specimens of varying Feulgen stainability determined with the upper transmission limit set at 75% (squares) and 95% (circles), respectively. For each specimen, the change in tumor DI is denoted by a line.

## Comments

DNA determinations of tumors are commonly related to a diploid staining reference based on analysis of normal cells. Accurate assessment of diploidy can be obtained by analysis of whole cell nuclei, by MSP of imprints or FCM. However, in tissue sections only part of the nuclear DNA content is measured. Since the nuclei are sectioned to a varying degree, the DNA values of diploid cells will display a wide range. Within a given section thickness, this range may also vary between different types of normal cell populations because of differences in nuclear size, form, and orientation. The influence of all these factors can not be calculated (Berryman et al. 1984), and, therefore, an accurate assessment of diploidy can probably not be made by measurement in tissue sections.

The observed variability of DNA values of normal cell populations in tissue sections implies that individual internal control cell populations, although mandatory as staining controls, comprise an unreliable basis for classifying tumors as diploid or hyperploid. However, collective data from analysis of a large number of normal cell populations can be used to assess the overall methodological error of measuring diploid cells in tissue sections. The maximum percentage of normal cells >DI 1.25 encountered proved to be a reliable limit for discriminating between diploid and hyperploid sarcomas. Employing this upper limit of diploidy, the risk of classifying diploid tumors as hyperploid is negligible, although there may be a risk of classifying hyperploid tumors erroneously as diploid, particularly near-diploid variants. Nevertheless, in the present comparative study, there was no disagreement in the ploidy classification of the tumors. It must be emphasized, that the upper limit of diploidy for MSP in tissue sections should be determined for each tissue type separately, since the section artefact may vary between different cell types (Berryman et al. 1984).

FCM has become increasingly applied in DNA analysis of fresh tumor tissue (Barlogie et al. 1983; Tribukait 1987), because of high speed and resolution. FCM offers accurate peak determination in aneuploid tumors, and permits estimation of the proportion of cells in different phases of the cell cycle. However, FCM of solid tumors, particularly bony and highly collagenous lesions, may present difficulties (Kreicbergs et al. 1981a; Kreicbergs et al. 1987). Insufficient cellular yield and background disturbances caused by tissue debris may lead to inconclusive DNA histograms. Apart from these aspects, a limitation of FCM is the problem of differentiating between tumor cells and admixed normal cells, particularly in the interpretation of histograms with diploid and tetraploid peaks. Since

MSP permits analysis of normal cells and tumor cells separately, it provides a means of distinguishing between diploid-tetraploid and exclusively tetraploid tumors, as observed in 4 cases of the present study. Another advantage of MSP is the possibility of analyzing different histologically defined tumor areas within the same tissue section.

A substantial number of bone tumors can not be analyzed cytophotometrically nor histologically, without previous demineralization. However, the use of acid for demineralization causes DNA degradation. As an alternative to acid, EDTA offers a means of demineralizing bone tumors without significantly affecting Feulgen DNA stainability. As opposed to the hydrolytic effect of acid, EDTA acts as a chelating agent (Anderson 1984). Recently, EDTA treatment has also been employed in the preparation of tissues for FCM analysis (Hiddeman et al. 1987).

In previous retrospective DNA studies, based on MSP of tissue sections, the upper limit of transmission was set at 85%, instead of 95% as commonly used for imprint preparations, to exclude non-specific light losses (Kreicbergs 1981). This was considered to be of particular importance in the analysis of sarcomas, often containing abundant collagen, which may cause light scattering. In the present study, changing the upper transmission limit from 75% to 95% was not found to affect significantly the total extinction relationship between control and tumor cells, except in some of the weakly stained specimens. The unreliability of analyzing poorly stained sections was evidenced by altering the upper transmission limit. This caused a significant change in tumor DI of 4 specimens, all with a median total extinction value of the control cells <6.

The findings may be attributed to several factors inherent in MSP of tissue sections. The methodologic problems involve background inhomogeneity, and to some extent also differences in chromatin distribution and density; the latter partly a function of nuclear size. The proportional effect of all these factors may be assumed to increase with decreasing Feulgen stain intensity. In weakly stained specimens, non-specific light losses may compose a significant part of the nontransmitted light, contributing decisively to the recorded total extinction. Lowering the upper limit of transmission excludes more background light losses, but in poorly stained sections also a significant part of the Feulgen specific absorption. Raising the upper limit would certainly include more specific absorption, but then non-specific light losses may comprise a large part of the recorded total extinction. Control and tumor cell populations may be affected unequally in these respects because of differences in background and nuclear features. However, the significance of these differences is mostly negligible, unless the contrast between background and stain is very low.

The reason for low Feulgen stainability of occasional archival paraffin embedded specimens is probably related to tissue autolysis, primary tissue fixation, and storage. Among weakly stained specimens, those which are unsuitable for DNA analysis can be identified by the described test. Presumably, the test is applicable not only to sarcoma specimens, but also to cancer specimens of weak Feulgen stainability.

In conclusion, the present methodological study shows that MSP analysis in tissue sections permits reliable discrimination between diploid and hyperploid bone tumors; bone specimens demineralized in EDTA retain Feulgen stainability; weakly stained specimens, inappropriate for ploidy determination, can be identified. In the following studies of the relationship between DNA content and morphology, the established upper limit of diploidy was employed for discriminating between diploid and hyperploid sarcomas in tissue sections.

# DNA content of individual tumors

Individual osteosarcomas often display histologic heterogeneity, both with respect to the degree of nuclear anaplasia and type of tissue differentiation. It is unsettled whether this morphologic variability within the same lesion can be attributed to cytochemical heterogeneity.

## Histologic areas

Twelve high grade osteosarcomas, with histologically well defined areas of both chondroblastic and osteo/fibroblastic differentiation, were analyzed by MSP (Bauer et al. 1988b). In all, both the chondroblastic and osteo/fibroblastic areas were hyperploid (Table 1). The median DNA values of the respective tissue areas were clearly correlated (r=.76). As to median nuclear size, however, no significant relationship could be demonstrated between the two types of tissue (r=.37). When comparing the median nuclear size of the chondroblastic area of different tumors, there proved to be a considerable variation (range 35-100 $\mu m^2$). The same applied to the osteo/fibroblastic area of different tumors (range 35-90 $\mu m^2$).

These results indicate that individual osteosarcomas, in spite of histologic heterogeneity with respect to differentiation and nuclear size, have a uniform DNA content.

## Biopsy and surgical specimens

In 20 tumors, comparative FCM analysis of specimens from biopsy and surgery showed complete agreement in the classification into diploid, tetraploid, and aneuploid lesions (Bauer et al. 1988b). Closer analysis revealed the same peak DNA values in 19 tumors (Figure 12). However, in 1 case, analysis of the biopsy specimen showed 2 aneuploid peaks, whereas in the corresponding surgical there was only 1, which, moreover, differed from the 2 peak values of the biopsy specimen.

This investigation shows that FCM analysis of a single tumor sample can be relied upon as representative of the whole tumor, substantiating the concept of uniformity in ploidy level of individual osteosarcomas.

Table 1. Microspectrophotometry of different histologic areas within the same tumor

| | Median values (DI) | | Cells > DI 1.25 (Per cent) | | Nuclear area ($\mu m^2$) | |
|---|---|---|---|---|---|---|
| Case No. | Chondro | Osteo/Fibro | Chondro | Osteo/Fibro | Chondro | Osteo/Fibro |
| 14 | 1.6 | 1.9 | 77 | 86 | 67 | 59 |
| 21 | 1.9 | 1.7 | 79 | 78 | 53 | 44 |
| 28 | 1.4 | 1.7 | 70 | 83 | 44 | 57 |
| 37 | 1.9 | 2.2 | 96 | 98 | 45 | 55 |
| 38 | 2.1 | 2.6 | 89 | 95 | 45 | 92 |
| 45 | 1.2 | 1.2 | 44 | 34 | 35 | 35 |
| 51 | 1.5 | 1.5 | 86 | 77 | 46 | 57 |
| 52 | 1.1 | 1.5 | 38 | 78 | 55 | 66 |
| 100 | 1.6 | 1.8 | 88 | 85 | 70 | 90 |
| 101 | 1.4 | 2.0 | 67 | 96 | 45 | 65 |
| 102 | 1.3 | 1.5 | 59 | 79 | 100 | 70 |
| 103 | 1.6 | 1.5 | 82 | 68 | 55 | 65 |
| Regression | | | | | | |
| r | .76 | | .56 | | .37 | |
| p | 0.003 | | >0.05 | | >0.05 | |

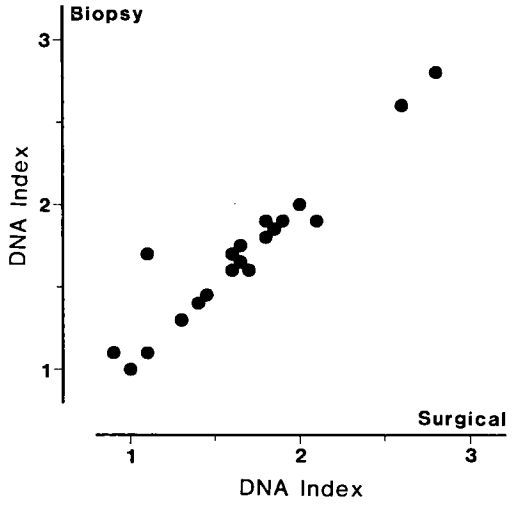



Figure 12. Comparative FCM analysis of peak DNA values of specimens from biopsy and definitive surgery in 20 cases. Regression analysis: Y = 0.3 + 0.87X; r = .95; p<0.001.

## Primary and recurrent lesions

The DNA content of local recurrences and metastases was compared to that of the corresponding primary lesions to investigate whether progression of tumor disease is associated with changes in ploidy (Bauer et al. 1988b).

In 5 recurrences, 4 local and 1 lung metastasis, the aneuploid peak value, as assessed by FCM, was exactly the same as that of the primary tumor (Table 2). However, in case 28, the lung metastasis was diploid, although the primary lesion exhibited 2 aneuploid peaks, i.e., DI 1.4 and 1.6. Histologic examination of the specimen obtained at thoracotomy, revealed regressive changes with few tumor cells. Prior to thoracotomy, the patient had received Interferon and lung radiation therapy.

In 9 local recurrences and 15 metastases, corresponding to 15 hyperploid primary lesions, MSP analysis in tissue sections disclosed that all 24 recurrences were hyperploid. There was, however, a considerable variability in percentage cells >DI 1.25 and in median DNA values between the recurrent and primary lesions.

The results indicate that local recurrences and metastases in general retain the hyperploid characteristic of the primary tumor.

## Comments

According to MSP analysis of chondroblastic and osteo/fibroblastic tumor areas, there were no decisive differences in DNA content within the same lesion. The minor variations in median DNA values of the compared areas should mainly be attributed to the methodological error of determining DNA content in tissue sections. The comparison was confined to chondroblastic and osteo/fibroblastic areas, since the former are well demarcated, whereas the latter areas commonly intermingle. In contrast to DNA content, the size of the cell nuclei of the compared histologic areas showed no significant correlation. Thus, individual tumors exhibiting histologic heterogeneity, both with respect to tissue differentiation and nuclear size, proved to be cytochemically uniform.

Determination of the DNA content in osteosarcoma, based on a single tumor sample, seems to be reliable. Comparative FCM analysis of biopsy and surgical specimens from the same tumors disclosed not only complete agreement with respect to the classification into diploid, tetraploid, and aneuploid lesions, but also a strong correlation between the peak values of the compared specimens. These findings indicate that, contradictory to a previous report (Hiddeman et al. 1987), the individual osteosarcoma has uniform DNA content.

Comparative DNA analysis of primary and recurrent lesions showed that osteosarcomas probably retain their individual hyperploid feature during progression of disease, as reported for other tumor entities (Auer et al. 1979; Friedlander et al. 1984). According to MSP, all recurrences were hyperploid as were the corresponding primary lesions. The poor correlation between the median DNA values of the primary and recurrent tumors should mainly be attributed to the increased methodological error of comparing results from MSP analysis of different tissue sections. In fact,

Table 2. Flow cytometry analysis of primary and recurrent lesions. L, Local recurrence; M, Metastasis

| Case No. | Peak value (DI) | | |
|---|---|---|---|
| | Primary | Recurrence | |
| 28 | 1.4 / 1.6 | M | 1.0 |
| 37 | 1.8 | L | 1.9 |
| 47 | 1.5 / 1.8 | L | 1.8 |
| 50 | 1.9 | L | 1.1 / 1.9 |
| 63 | 1.8 | L | 1.7 |
| 104 | 1.4 | M | 1.4 |

FCM analysis of recurrences disclosed exactly the same peak DNA values as their primary tumors in 5 out of 6 cases. The single case of a diploid lung metastasis from an aneuploid primary lesion merely questions the validity of the analyzed metastatic specimen, particularly, since histologic examination revealed regressive changes. This investigation of primary and recurrent lesions would seem to substantiate that individual osteosarcomas are cytochemically uniform.

The combined findings of these comparative studies indicate that a single tumor sample for DNA analysis can be relied upon as representative for the tumor as a whole. This observation formed the basis for the following study, relating DNA content to histologic subtype and grade in a consecutive series of high grade osteosarcomas.

# DNA content and histologic subclassification

In this flow cytometric study, the relationship between peak DNA values and histologic subtypes of osteosarcoma was investigated (Bauer et al. 1988b). In addition, the fraction of S-phase cells was determined, as an estimate of the proliferative activity.

## Ploidy and differentiation

Among high grade osteosarcomas there is a great morphologic variability, which is reflected by histologic subtyping and grading (Dahlin 1975). So far, it has not been demonstrated whether morphologic variants of osteosarcoma differ in DNA content. Inversely, it remains unsettled whether osteosarcomas, classified identically with respect to histologic subtype and grade, exhibit the same ploidy level.

The study was based on 49 consecutive cases of high grade osteosarcoma treated at the Karolinska Hospital from 1980 to 1987. In 2 cases, tissue was not procured for DNA measurement leaving 47 for the present investigation.

Histologically, the majority was of the osteoblastic type, i.e., 32 out of 47. All tumors were of high grade, evenly distributed among Grade III and IV.

FCM analysis disclosed that 2 were diploid and 45 non-diploid, out of which 8 were tetraploid (DI 1.9-2.1). Tumors with a peak in the triploid region (DI 1.2-1.8) predominated, i.e., 28 of 47. Two lesions were hypodiploid (DI 0.9). Of the 45 non-diploid lesions, the osteoblastic variants (Figure 13A), and Grade IV lesions (Figure13B), appeared to have a higher peak DNA value compared to other subtypes, although this was not statistically significant. Thus, no clear relationship could be demonstrated between peak DNA values on one hand, and histologic subtype or grade on the other. Noteworthily, the only 2 diploid lesions were fibroblastic, Grade III tumors.

Two stem lines were detected in 13 of the 40 aneuploid tumors (Table 3). The second peak value was a multiple of the first in 9 tumors, eg. DI 1.6 and 3.2, whereas in 4 not, eg. DI 1.6 and 2.3. Notably, the 2 hypodiploid (DI 0.9) tumors exhibited a second peak at DI 1.8.

Determination of the fraction of S-phase cells was feasible in 38 out of 47 tumors. Mean percentage of S-phase cells was 19.5 (SD 8.4). The fractions of S-phase cells displayed the same distribution among tumors of different subtype and grade. However, the proportion of S-phase cells appeared related to tumor DNA Index in the sense that it was higher for lesions in the triploid and pentaploid range, compared to those in the tetraploid (Figure 14). Regression analysis of the 30 tumors in the triploid and tetraploid range (DI 1.2-2.1) disclosed a negative correlation (r=-.52) between peak DNA value and fraction of S-phase cells.

This study shows that the vast majority of high grade osteosarcomas is hyperploid. A high percentage of S-phase cells also appears to be a characteristic fea-

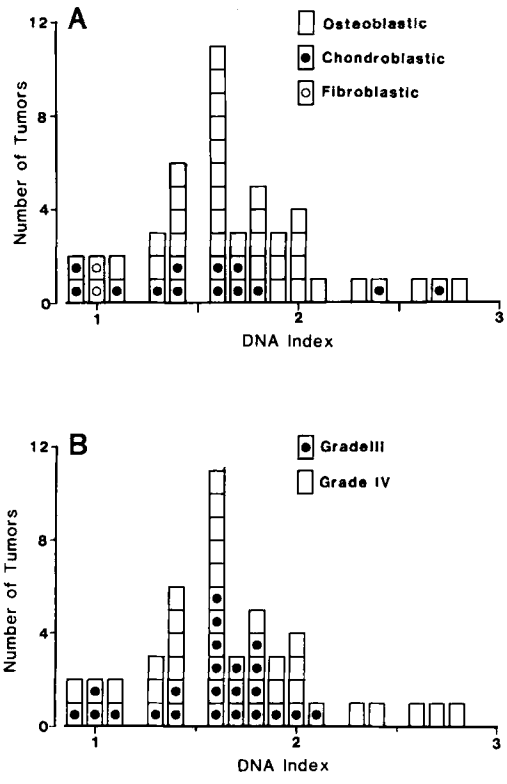



Figure 13. Distribution of peak DNA values according to A. histologic subtype and B. histologic grade of 47 high grade osteosarcomas analyzed by FCM. Wilcoxon rank sum test of peak DNA values in relation to subtype and grade: p = 0.14 and 0.52, respectively.

Table 3. Osteosarcomas with two aneuploid peaks

| Case No. | Type [a] | Grade | Peak value (DI) #1 | #2 |
|---|---|---|---|---|
| 19 | O | IV | 1.6 | 3.2 |
| 20 | O | III | 1.6 | 2.3 |
| 24 | O | IV | 1.9 | 3.8 |
| 25 | O | IV | 1.6 | 3.0 |
| 27 | O | IV | 1.5 | 1.8 |
| 28 | C | IV | 1.4 | 2.6 |
| 30 | O | IV | 1.1 | 2.1 |
| 35 | C | IV | 0.9 | 1.8 |
| 42 | O | IV | 1.6 | 3.2 |
| 47 | O | III | 1.4 | 1.6 |
| 53 | O | III | 1.6 | 3.2 |
| 100 | C | III | 0.9 | 1.7 |
| 105 | C | III | 1.1 | 2.2 |

[a] Type: O, Osteoblastic; C, Chondroblastic.

ture. However, neither the peak DNA value of the tumors nor the fraction of S-phase cells seem to be related to histologic subtype or grade.

## Comments

The results show that high grade osteosarcomas, from a cytochemical point of view, represent a heterogeneous tumor entity. This heterogeneity was not found to articulate with the histologic subclassification. Cytochemical differences are either not expressed phenotypically or not discernible microscopically. In fact, osteosarcomas classified identically, with respect to subtype and grade, displayed a wide range in peak DNA values. Inversely, osteosarcomas of different histologic subtype and grade were found to have the same DNA content.

The majority of the analyzed lesions exhibited peak DNA values in the triploid-tetraploid range, as found in several other malignant entities (Barlogie 1983; Tribukait 1987), including soft tissue sarcomas (Kreicbergs et al. 1987). One may speculate, if the predominance of lesions in the triploid-tetraploid range is the result of polyploidization of diploid variants into tetraploid, subsequently followed by DNA losses (Nielsén 1976; Tribukait 1984). The hypothesis of polyploidization as a step in the development of aneuploidy, would seem to be substantiated by the finding that, in most of the osteosarcomas with 2 stem lines, the second peak value was a multiple of the first. Subsequent loss of the first of 2 stem lines has been observed experimentally in serial passage of transplanted tumors (Baisch et al. 1986; Mittelman 1972; Vindelöv et al. 1982), including osteosarcomas (Bauer et al. 1986a). Presumably, such a change reflects a selection in favour of the faster growing cell population. In a few osteosarcomas of this study, the 2 stem lines were not multiples of each other. This may be a sign of further chromosomal derangement. In other tumor entities, it has been shown that the presence of multiple aneuploid peaks is an ominous prognostic feature (Tribukait et al. 1987).

The proliferative activity of tumors has been reported to be of prognostic significance (Tribukait 1987). However, the methodological error in assessing the proliferative activity of osteosarcomas, representing solid, often mineralized tumors, is probably considerably greater than in e.g. hematopoietic malignancies. Hence, the percentages of S-phase cells in the present study should be regarded as relative values representing rough estimates of cycling cells. The percentage of S-phase cells was somewhat higher for aneuploid lesions than tetraploid, indicating a relationship between peak DNA value and proliferative activity. A similar relationship has been demonstrated for bladder tumors, and it has been suggested that the higher proliferative activity of aneuploid lesions, compared to tetraploid, may account for poorer prognosis (Tribukait 1984).

This investigation, based on a consecutive series, shows that hyperploidy is a characteristic feature of high grade osteosarcoma. However, there is a considerable variability in the hyperploid DNA content. Whether such differences in ploidy level can be related to prognosis was investigated in the following study.

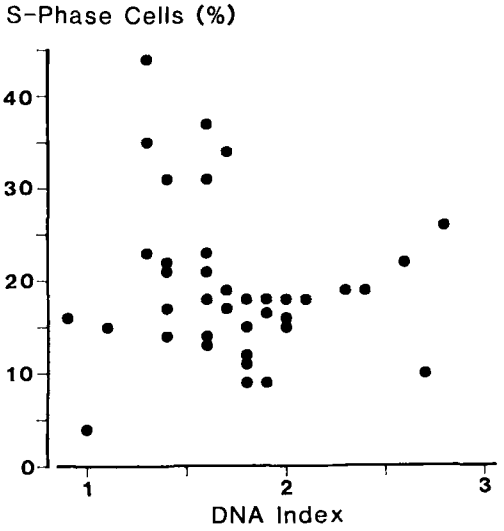



Figure 14. Distribution of percentage of S-phase cells in relation to peak DNA values of 38 osteosarcomas analyzed by FCM. Regression for the 30 tumors in the triploid to tetraploid range (DI 1.2 - 2.1): Y = 52 - 19X; r = -.52; p = 0.004.

# Prognostic factors

A problem in the evaluation of osteosarcoma treatment is the heterogeneity of the series, with respect to different patient and tumor features. Improved tumor characterization, preferably by objective means, remains a major concern for meaningful comparison of treatment results. In the present prognostic study, based on a consecutive series of osteosarcoma patients, all treated by surgery and adjuvant Interferon, the clinical course was related to various clinicopathologic features and tumor DNA content (Bauer et al. 1989).

## Patients

The study included 94 consecutive patients with primary osteosarcoma referred to the Department of Orthopedics at the Karolinska Hospital 1971-1986. Eleven patients had lung metastasis on admission according to plain radiography. These were excluded, leaving 83 cases for analysis. There were 51 males and 32 females. Median age was 17 (5-74) years (Figure 15). Prior to admission, open biopsy had been performed in 20 cases.

The most common tumor locations were distal femur and proximal tibia (Figure 15). Median tumor size was 9 (2-20) cm. Histologic subtype and grade is shown in Table 4. According to the system of muskuloskeletal tumor staging (Enneking 1986), there were 4 IB, 3 IIA, and 76 IIB lesions. The relationship between surgical procedure and surgical margin achieved is shown in Table 5.

All patients were treated by surgery and adjuvant Interferon. Ablative surgery was performed in 47 cases and local surgery in 36. Natural Interferon-alpha was given by im injections starting at the time of diagnosis and continued for 18 months (Strander et al. 1984). The dose was $3x10^6$ daily the first month, and then $3x10^6$ 3 times weekly for the ensuing 17 months. From January 1985, the daily dosage of $3x10^6$ is continued throughout the whole 18 months period (Strander et al. 1988).

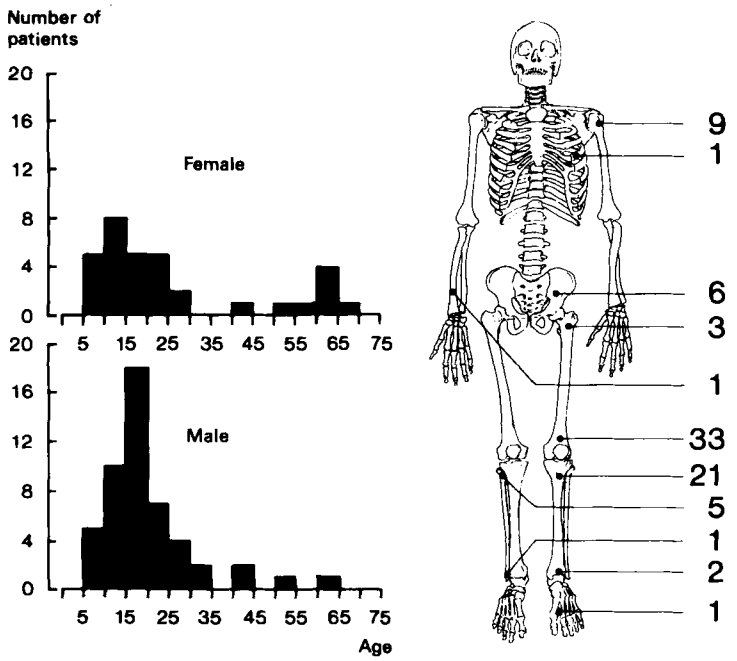



Figure 15. Tumor location and patient distribution according to sex and age.

Table 4. Histologic grade and subtype

| | Grade | | | |
|---|---|---|---|---|
| | II | III | IV | Total |
| Osteoblastic | 1 | 22 | 27 | 50 |
| Chondroblastic | 0 | 13 | 5 | 18 |
| Fibroblastic | 3 | 5 | 6 | 14 |
| Telangiectatic | 0 | 0 | 1[a] | 1 |
| Total | 4 | 40 | 39 | 83 |

[a] The single telangiectatic lesion was included in the osteoblastic group in the prognostic analysis.

Table 5. Surgical margins according to procedure

| | Surgery | |
|---|---|---|
| | Local | Ablative |
| Intralesional | 12 | 0 |
| Marginal | 11 | 2 |
| Wide | 13 | 24 |
| Radical | 0 | 21 |
| Total | 36 | 47 |

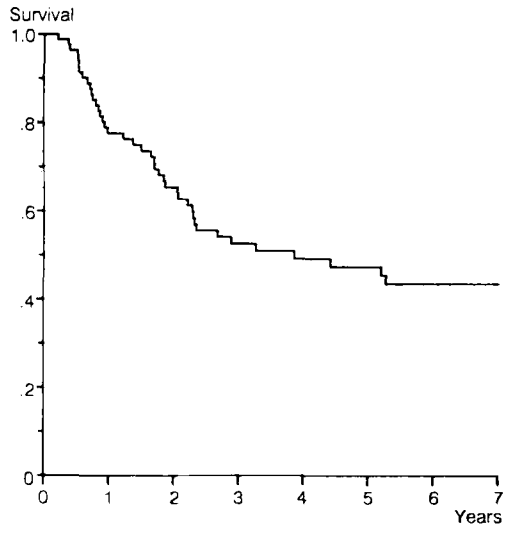



Figure 16. Life table survival curve for the whole series of 83 patients. Twenty-one patients were still at risk at 7-years.

Local recurrences were treated by surgery and adjuvant interferon. Patients developing metastases were not treated according to a standardized protocol. The therapy included Interferon, Adriamycin, high-dose Methotrexate, cis-Platinum, and radiation in different combinations, and also surgery.

Mean follow-up from time of diagnosis was 8 (0.3-16) years. No patient was lost to follow-up. Two patients committed suicide at 3 and 6 months after diagnosis. Autopsy showed no evidence of disease. All other deaths, except 2, were due to metastatic disease. The only exceptions were 2 patients, who died in local pelvic recurrence after intralesional excision and radiation.

Determination of nuclear DNA content and size, by MSP analysis in tissue sections, was feasible in 60 cases. Exclusion of 23 cases was due to deficient nuclear DNA stainability in 21 and loss of paraffin blocks in 2.

DNA data obtained by FCM of cell suspensions were available for 35 of 37 cases treated since 1980. As follow-up of these patients was short (mean 3.5 years), the prognostic analysis was confined to 3-year metastasis free survival.

## Survival

The 7-year survival rate for the whole series of 83 patients, as estimated by life-table analysis, was 0.44 (Figure 16). Comparison of survival for patients treated 1971-1979 and those treated 1980-1986 disclosed no difference.

According to multivariate analysis, the significant factors associated with a poor prognosis were histologic grade IV, proximal tumor location, and male sex (Table 6). Age, tumor size, and histologic subtype had no independent prognostic influence.

The identified risk factors, i.e. histologic Grade IV, proximal tumor location, and male sex, were considered in different combinations to form 8 subgroups of patients in a Venn diagram (Figure 17). Life-table estimates of 7-year survival was determined for each subgroup. The survival rate was highest (0.80) for females with distal Grade III tumors, and lowest (0.13) for males with proximal Grade IV tumors. As can be seen from the Venn diagram, the survival rate was approximately the same for subgroups with equal number of risk factors. Hence, the series could be divided according to the number of risk factors present. The 7-year survival rates for patients with 0, 1, 2 or 3 risk factors was 0.80, 0.59, 0.42, and 0.13, respectively (Figure 18).

Table 6. Multivariate Cox regression analysis of risk factors for tumor related death

| Covariate | Definition 1 | Definition 0 | Multiple regression coefficient | p |
|---|---|---|---|---|
| Sex | Male | Female | .74 | 0.042 |
| Age | ≤18 yrs | >18 yrs | .53 | 0.11 |
| Location | Proximal | Distal | .98 | 0.008 |
| Size | >10cm | ≤10cm | .04 | 0.91 |
| Grade | IV | II / III | .96 | 0.005 |
| Type | Osteo/Chondro | Fibro | 1.05 | 0.057 |

Table 7. Bivariate Cox regression analysis of DNA content and nuclear size in relation to death in tumor disease. Each covariate was analysed separately

| Covariate [a] | Regression coefficient | p |
|---|---|---|
| Median (P50) DNA value | .24 | 0.42 |
| Extreme (P50) DNA value | .24 | 0.18 |
| Percent cells > DI 1.25 | .006 | 0.51 |
| Percent cells > DI 2.5 | .010 | 0.27 |
| Median nuclear area | .011 | 0.48 |
| Extreme nuclear area | .002 | 0.80 |

[a] All covariates were treated as continuous.

DNA analysis by MSP in tissue sections, feasible in 60 cases, disclosed that the vast majority, i.e., 56 tumors, was hyperploid. The 7-year survival rate for the 56 patients with hyperploid lesions was 0.40. Three out of 4 diploid cases remain free of disease at 4, 9 and 12 years; 1 died in local pelvic recurrence 10 months after diagnosis. Multivariate analysis disclosed that none of the tested DNA variables, nor nuclear size, gave prognostic information (Table 7). Only the extreme (P90) DNA value appeared related to survival. In fact, estimated survival according to life-table analysis was 0.12 for the 9 patients with a tumor P90 DNA value exceeding DI 4.0, as compared to 0.52 for the other 51 (log rank p=0.03).

In 35 cases, treated from 1980 to 1986, FCM analysis disclosed no relationship between peak DNA value and 3-year metastasis free survival; nor was the presence of multiple aneuploid peaks related to metastatic rate. However, the proliferative activity, as estimated by the percentage of S-phase cells, feasible in 28 cases, appeared to be of predictive value. Thus, for the 7 patients with less than 15% S-phase cells, the 3-year metastasis free survival was 0.71, compared to 0.27 for the 21 patients with ≥15% S-phase cells (p=0.019) (Table 8).

## Local recurrence

The estimated 7-year local recurrence rate for the whole series was 0.29. Median time to local recurrence was 6 months; the longest lag 37 months.

According to surgical procedure, 3 out of 47 patients had local recurrence after ablative surgery, and 17 out of 36 after local surgery. The estimated risk of local recurrence was 0.07 after ablative and 0.54 after

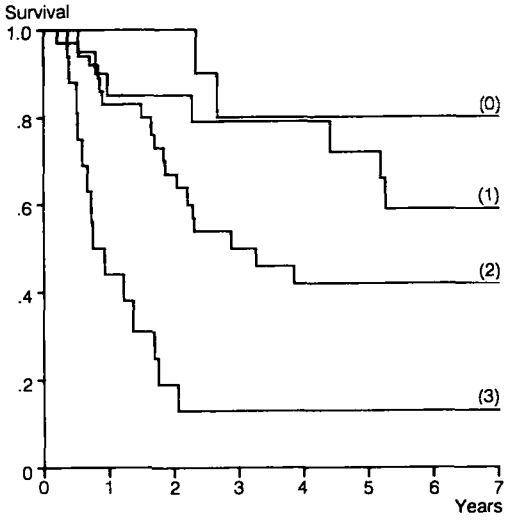



Figure 17. 7-year survival based on the risk factors male, sex, proximal tumor location, and histologic grade IV. Ten patients had none of these characteristics. Number of patients within parenthesis.

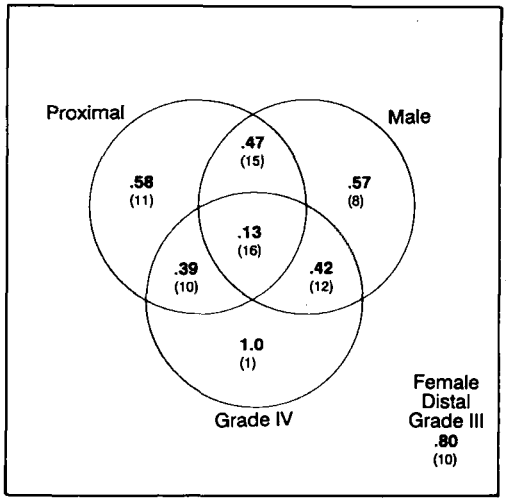



Figure 18. Life table survival curves, based on the number (within parenthesis) of risk factors, for subgroups of patients. There were 10, 20, 37, and 16 patients in the subgroups. Chi-square test for trend in survival yielded 23.05; d.f. = 1; p<0.001.

Table 8. 3-year metastasis free survival rate in relation to percentage of S-phase cells and presence of two aneuploid peaks as determined by flow cytometry

| Covariate | | n | Rate | p[a] |
|---|---|---|---|---|
| S-phase (%) | <15 | 7 | 0.71 | 0.019 |
| | ≥15 | 21 | 0.27 | |
| Two peaks | no | 24 | 0.50 | 0.52 |
| | yes | 11 | 0.28 | |

[a] Log rank test.

local surgery (Figure 19). Taking into account the type of surgical procedure in multivariate analysis, other factors such as tumor size, location and histologic grade were not significantly related to local recurrence.

To assess the significance of local recurrence for survival, patients who developed local recurrence within 1 year of diagnosis, but not metastasis, were compared to patients free of disease at 1 year. Hence, 38 patients were excluded from this analysis either because of death or metastatic disease within 1 year, or shorter follow-up than 1 year. The estimated 7-year survival rate for the 10 patients with local recurrence within 1 year of diagnosis was 0.48 as compared to 0.86 for the 35 patients free of disease at 1 year (Log rank test, p=0.046).

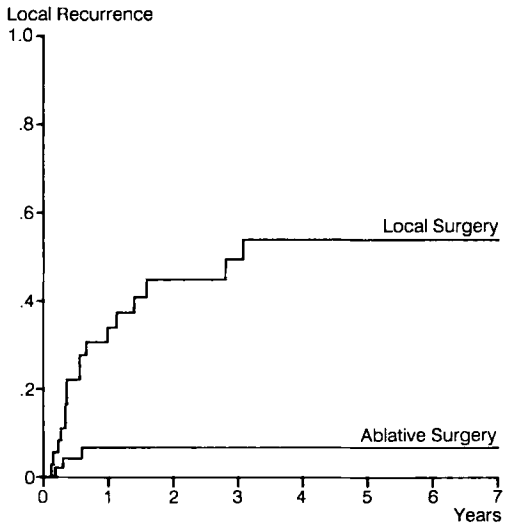



Figure 19. Life table analysis showing risk for local reccurrence after local and ablative surgery, respectively. Log rank test p<0.001.

## Comments

In view of the large number of trials being conducted on adjuvant chemotherapy in osteosarcoma, the present is of particular interest by comprising a contemporary non-chemotherapy study. Moreover, it was based on a consecutive series, treated at one centre by surgery and adjuvant Interferon, over a period of 15 years. The patients of this study, although not based on a defined population, can be considered representative with respect to different clinicopathologic features compared to major series of osteosarcoma (Dahlin and Coventry 1967; Price 1961). There was a male predominance, peak age incidence in the second decade, and a preponderance of lesions about the knee. Histologically, the vast majority was high grade and the most common subtype osteoblastic.

The number of patients alive at 7 years, and the observed plateau of the survival curve, allowed estimation of 7-year survival (Peto et al. 1977), instead of recurrence free rate considered more appropriate for short term studies. The 7-year survival rate (0.44) is comparable to that reported from randomized trials of different adjuvant chemotherapy regimens (Bacci et al. 1986; Edmonson et al. 1984; Eilber et al. 1987; Link et al. 1986). However, this study was not focused on adjuvant Interferon therapy in osteosarcoma, but rather on the prognostic significance of different host and tumor features.

In the vast literature on osteosarcoma, various clinicopathologic features have been reported to be prognostically relevant. However, the findings have been contradictory (Broström et al. 1980; Dahlin and Coventry 1967; Gilchrist et al. 1981; Lockshin and Higgins 1968). To some extent, this may be explained by the lack of appropriate statistical methods. In a recent study from the Mayo Clinic, based on multivariate analysis, Taylor et al. (1985) were able to identify the following unfavourable characteristics: age less than 10 years, male sex, tumor diameter exceeding 15 cm, osteo/chondroblastic subtype, involvement of femur or humerus, and less than 2 months duration of symptoms.

In the present study, male sex, proximal tumor location, and histologic Grade IV were the only independent risk factors. Male sex has been reported to be a risk factor also in soft tissue sarcoma (Rydholm 1983). Noteworthily, males and females with osteosarcoma exhibit not only differences in survival, but also in incidence and age, the latter coinciding with puberty. According to multivariate analysis, proximal location, but not large tumor size, was a risk factor. However, these 2 variables were intercorrelated. Excluding location from the analysis, tumor size emerged as a signifi-

cant prognostic variable, as reported in other studies (Broström et al. 1982; Taylor et al. 1985). Although, it may be that size, rather than location, reflects inherent tumor properties, proximal lesions, presumably, are detected later than distal, and are also more difficult to manage surgically.

The most important prognostic factor proved to be histologic malignancy grade. The observed significance of distinguishing between Grade III and Grade IV osteosarcomas appears somewhat surprising, since this distinction has been claimed to be almost impossible, due to overlapping of different morphologic features (Dahlin and Coventry, 1967).

DNA analysis by MSP in tissue sections was not found to provide prognostic information, except for tumors with extremely high DNA values, which were associated with a very low survival rate. Other variables such as percentage of hyperploid tumor cells, and nuclear size, were not prognostically discriminative. In chondrosarcoma, the distinction between diploid and hyperploid variants has been reported to be of significant prognostic value (Kreicbergs et al. 1982). However, in osteosarcoma this distinction is of limited predictive value, since the vast majority is hyperploid. The almost consistent finding of hyperploidy appears to reflect the highly malignant nature of the tumor entity. Notably, none of the 4 cases with diploid lesions developed metastatic disease. Hence, it would seem appropriate to separate diploid variants from hyperploid in future prognostic studies of osteosarcoma.

Differences in degree of hyperploidy do not seem to be prognostically relevant in osteosarcoma. Not even peak DNA values, as determined by FCM, could be related to the clinical course. Thus, in the presence of hyperploidy, the DNA value per se does not seem to provide information about specific malignant properties. Instead, it appears that, in ostesarcoma, prognostic information can be obtained by estimating the proliferative activity. In fact, tumors with <15% S-phase cells were associated with a more favourable clinical course. This conclusion should be regarded as tentative, since based on a limited number of cases.

The 3 independent risk factors, i.e. male sex, proximal tumor location and histologic Grade IV, were approximately of equal strength according to multivariate analysis. By considering all conceivable combinations of these risk factors, the estimated 7-year survival rate was found to be related to the number, rather than the type, of risk factors present. Hence, the prognostication model was created according to the number of risk factors involved, all reasonably well defined. The subgroups, representing 4 risk levels, were associated with a stepwise decreasing 7-year survival rate ranging from 0.80 to 0.13. Hence, in the present series of high grade osteosarcomas 4 prognostically different groups could be identified. Similar conclusions were reached by Taylor et al. (1985), although not considering histologic grade. The validity of the prognostication model has also been substantiated in soft tissue sarcoma (Rööser 1987). Interestingly, male sex and histologic Grade IV proved to be of similar significance as now demonstrated in osteosarcoma, but, instead of tumor location, size was of prognostic importance.

Local recurrence was strongly related to surgical procedure, in the sense that it almost exclusively occurred after local surgery. The high rate, obviously, was due to inadequate surgical margins. Other factors, such as tumor location and size were not found to be of decisive importance. In several studies, the local recurrence rate has been reported to be 5-20% after local surgery combined with chemotherapy (Bacci et al. 1986; Rosen et al. 1985; Simon et al. 1986). The superior results may be attributed to improved local tumor control by adjuvant chemotherapy. The findings of this study would seem to suggest, that local surgery and adjuvant Interferon therapy in high grade osteosarcoma, should not be applied, except in cases carefully selected with respect to the feasibility of obtaining adequate margins.

The importance of local tumor control for survival was clearly reflected in the present study. In fact, the 7-year survival rate was 0.46 for patients with local recurrence as compared to 0.86 for those without, when considering specifically patients without metastasis within the first year of diagnosis. Thus, local recurrence seems to be a decisive risk factor emphasizing the importance of safe surgical margins (Simon 1984). Relenting the requirements for wide or radical procedures can not be justified, until adjuvant chemotherapy or radiation are convincingly proven effective for local tumor control.

To summarize, in osteosarcoma conventional clinicopathologic features and local tumor control remain the most important prognostic factors. A prognostication model, based on sex, tumor location, and histologic grade, appears to be useful for identifying prognostically different subgroups of high grade osteosarcoma patients. DNA analysis does not seem to be of additional predictive value. However, the almost consistent finding that osteosarcomas are hyperploid, suggested that this feature might be used for differential diagnostic purposes.

# Diagnostic aspects

Osteosarcoma, commonly, exhibits characteristic histologic features indicative of high grade malignancy. Nevertheless, diagnostic errors occur. These are sometimes even made by experienced pathologists, since other primary bone tumors, occasionally, exhibit histologic similarities to osteosarcoma. Tumor entities which may cause differential diagnostic problems include osteoblastoma, fibrous dysplasia, giant cell tumor, and aneurysmal bone cyst (Ackerman 1976; Dahlin 1975; Dorfman et al. 1973; Lichtenstein 1950; Meryweather et al. 1980; Mirra et al. 1976). The aim of the present study was to investigate whether DNA analysis can be used as a diagnostic adjunct to histologic assessment of primary bone tumors (Bauer et al. 1988a).

## Histologic diagnosis

The series included 166 bone tumors, diagnosed between 1971 and 1986. Based on the primary, final, and review histologic assessments the material was divided into a diagnostically concordant and discordant group. The former group included all cases that received the same diagnosis at primary, final, and review assessments. The discordant group comprised cases where there was a discrepancy between the different histopathologic evaluations. In the diagnostically discordant group, the clinical course was reviewed and related to tumor DNA content.

There was complete agreement between the primary, final, and review diagnoses in the vast majority of the present series. Thus, the diagnostically concordant group comprised 149 (90%) lesions, whereas the discordant 17 (10%).

## Ploidy and diagnosis

The DNA content was determined by MSP in 79 tumors, by FCM in 49, and in 38 by both methods. The lesions were classified as diploid or hyperploid according to the principles described previously.

### Concordant Group

The diagnostically concordant group included 43 benign and 106 malignant lesions (Table 9). All 43 benign tumors were diploid. The malignant series included both diploid and hyperploid tumors. All 4 low grade parosteal osteosarcomas were diploid, whereas only 5 out of 102 osteosarcomas had a normal DNA content. Hence, hyperploidy was found in 95% of the osteosarcomas analyzed. As to the 5 exceptions, i.e., the patients with diploid osteosarcomas, 4 have remained free of disease 4, 6, 9, and 12 years, respectively; whereas 1 with a pelvic tumor, excised intralesionally, died in local recurrence and chronic infection 10 months postoperatively.

### Discordant Group

The diagnostically discordant group included 17 cases, with a minimum follow-up of 2 years. There were 9 lesions primarily classified and treated as benign, and 8 as malignant (Table 10 A and B). As can be seen, 7 were diploid and 10 hyperploid. None of the 7 patients with diploid lesions had local recurrence or distant metastasis. Out of 10 patients with hyperploid tumors, 8 had local recurrence. 3 patients with hyperploid lesions have died of tumor disease and 1 is alive with lung metastases. Hence, in the diagnostically contro-

Table 9. Ploidy of diagnostically concordant cases

| | Diploid | Hyperploid |
|---|---|---|
| Benign | | |
| Giant cell tumor | 19 | - |
| Aneurysmal Bone Cyst | 13 | - |
| Osteoblastoma | 5 | - |
| Fibrous dysplasia | 6 | - |
| Malignant | | |
| Parosteal osteosarcoma | 4 | - |
| Osteosarcoma | 5 | 97 |

versial group, local recurrence or death was consistently related to hyperploidy. The clinical history of 2 illustrative cases is presented below.

Case 50 had a lytic lesion of the distal femur diagnosed after open biopsy as an aneurysmal bone cyst and treated by curettage (Figure 20). The specimen obtained from the latter procedure was also suggestive of aneurysmal bone cyst, but some tumor areas aroused the suspicion of a telangiectatic osteosarcoma. Hence, the material was referred to the pathologists of 3 bone tumor centres. 2 classified the lesion as aneurysmal bone cyst and 1 as telangiectatic osteosarcoma. One month after curettage, the patient had local recurrence and lung metastases; he died 6 months later. DNA analysis of the primary lesion showed that the tumor was hyperploid.

Case 37 involved a sclerotic lesion of the distal femur, primarily diagnosed as parosteal osteosarcoma and treated by local surgery (Figure 21). The lesion was reevaluated as high grade osteosarcoma after local recurrence and treated by ablation. DNA analysis of the primary lesion disclosed hyperploidy. The patient is free of disease 4 years after the last procedure.

Table 10 B. Summary of clinical course and ploidy level of diagnostically discordant cases

| Primary diagnosis | Diploid | Hyperploid |
|---|---|---|
| Benign | | |
| Free of reccurence | 4 | - |
| Local recurrence | - | 2 |
| Metastasis / Death[a] | - | 3 |
| Malignant | | |
| Free of reccurence | 3 | 2 |
| Local recurrence | - | 2 |
| Metastasis / Death[a] | - | 1 |

[a] All these patients also had local recurrence.

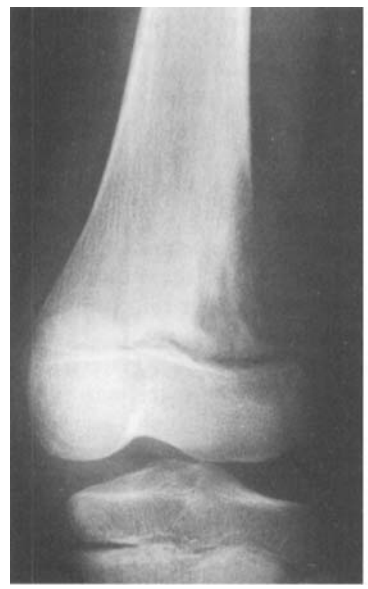
## Comments

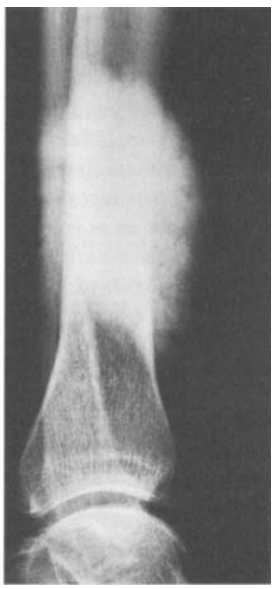
The distinction between high grade osteosarcoma and other primary bone tumors, commonly, does not present difficulties. This was reflected by the results of the comparative histologic classification of the present series. In fact, 90% of the tumors were diagnostically noncontroversial.

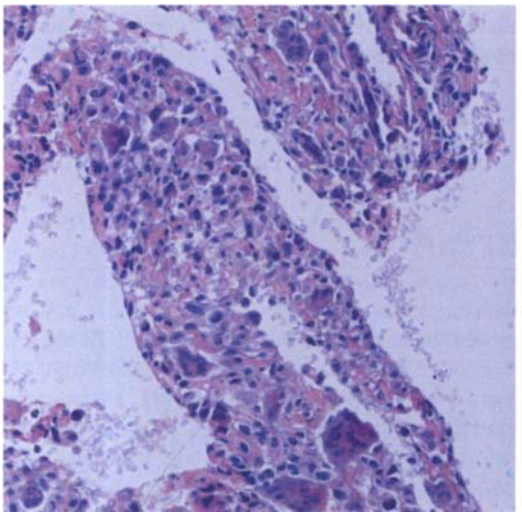
Table 10 A. Clinicopathologic data and ploidy of diagnostically discordant group

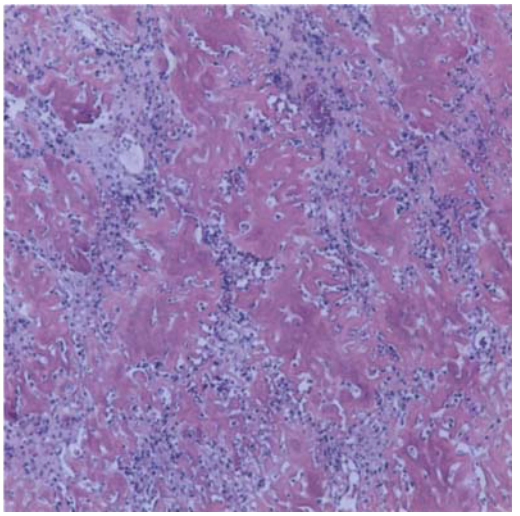
| Case No. | Age | Location | Primary diagnosis[a] | Local recurrence (months) | Final diagnosis | Review diagnosis | Follow-up (years)[b] | Ploidy[c] |
|---|---|---|---|---|---|---|---|---|
| Primarily diagnosed as benign | | | | | | | | |
| 106 | 15 | Clavicle | OBL | - | OBL | OS | NED 12 | D |
| 107 | 19 | Tibia | OBL | - | OBL | OS | NED 9 | D |
| 108 | 31 | Sacrum | OBL | 9 | OS | OBL | D 2 | H |
| 49 | 14 | Tibia | OBL | 6 | OS | OS | NED 10 | H |
| 109 | 13 | Radius | ABC | - | ABC | OS | NED 9 | |
| 110 | 19 | Pelvis | ABC | - | ABC | OS | NED 4 | D |
| 4 | 20 | Tibia | ABC | 3 | OS | OS | NED 11 | H |
| 50 | 8 | Femur | ABC | 1 | OS | OS | D 1 | H |
| 111 | 31 | Femur | GCT | 12 | GCT | OS | M 2 | H |
| Primarily diagnosed as malignant | | | | | | | | |
| 112 | 24 | Femur | POS | - | OS | POS | NED 3 | D |
| 37 | 59 | Fibula | POS | 12 | OS | POS | NED 3 | H |
| 38 | 22 | Pelvis | POS | 7 | OS | OS | D 2 | H |
| 63 | 13 | Tibia | POS | 18 | OS | OS | NED 3 | H |
| 113 | 19 | Femur | OS | - | OS | ABC | NED 12 | D |
| 114 | 9 | Tibia | OS | - | ABC | OS | NED 10 | D |
| 17 | 22 | Femur | OS | - | OS | OBL | NED 7 | H |
| 56 | 11 | Fibula | OS | - | ABC | OS | NED 12 | H |

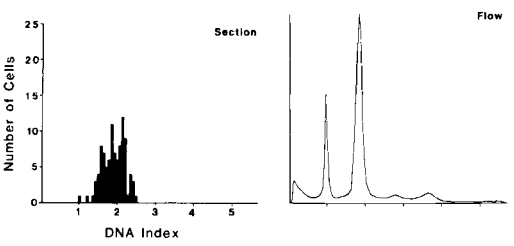
[a] OBL, osteoblastoma; ABC, aneurysmal bone cyst; GCT, giant cell tumor; POS, parosteal osteosarcoma; OS, osteosarcoma.
[b] NED, no evidence of disease; M, metastases; D, death in tumor.
[c] D, diploid; H, hyperploid.











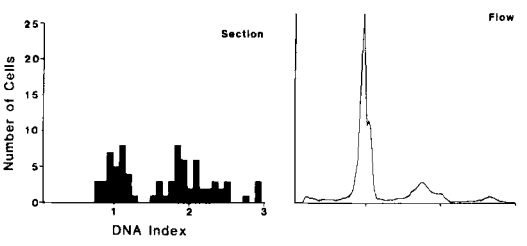




Figure 20. Case 50. Tumor of distal femur, primarily diagnosed as an aneurysmal bone cyst and treated by curettage. Radiography consistent with both osteosarcoma and aneurysmal bone cyst. Histologically controversial. DNA analysis by both MSP (left) and FCM (right) showed hyperploidy.

Figure 21. Case 37. Tumor of distal fibula, primarily diagnosed as a parosteal osteosarcoma. At local recurrence reevaluated as a high grade osteosarcoma. The radiographic and histologic appearance of the primary lesion was typical of parosteal osteosarcoma. DNA analysis by MSP (left) and FCM (right) disclosed hyperploidy.

In the diagnostically concordant group, ploidy was strongly related to histologic classification. Thus, all benign tumors were diploid as well as those of low grade malignancy, i.e., parosteal osteosarcomas, whereas osteosarcomas of high grade almost consistently were hyperploid. Collective data from other DNA studies of primary bone tumors substantiate these findings (Table 11). As can be seen, this applies not only to the consistent finding of diploidy among benign tumors and parosteal osteosarcomas, but also, to the overwhelming predominance of hyperploidy among high grade osteosarcomas.

The combined results indicate that the incidence of diploid osteosarcomas is about 5 %. Although, morphologically high grade, diploid osteosarcomas may prove to be associated with a better prognosis than hyperploid, as has been found in chondrosarcoma (Kreicbergs et al. 1982). This is not contradicted by the single case, among 5 diploid of this series, who died in local pelvic recurrence.

The diagnostically discordant group of this study illustrates that difficulties, occasionally, are encountered in the differential diagnosis of osteosarcoma. Although the number of such cases is small, diagnostic errors may have serious clinical implications. In the present series, 17 tumors, i.e., 10 %, were diagnostically controversial. Notably, all 7 patients with diploid lesions had a benign clinical course. None of the lesions recurred. It may be questioned whether they actually were osteosarcomas, although diagnosed, primarily or at review, as such. Out of 10 patients with hyperploid lesions 3 have died and 1 has lung metastases. As many as 8 had local recurrence. The 4 lesions associated with a malignant clinical course, beyond doubt, should be regarded as high grade osteosarcomas. As to the other 6 hyperploid lesions, it is reasonable to assume that they also represent high grade osteosarcomas, although the clinical course does not provide definite proof.

The results of the present and other studies suggest that quantitative DNA measurements can be used as a valuable adjunct in the routine diagnosis of primary bone tumors. However, it must be emphasized that ploidy determination can not be used to discriminate benign lesions from all malignant, since many histologically low grade tumors are diploid.

In most instances, ploidy determination offers confirmatory information. Applied routinely, DNA analysis may contribute to increased diagnostic certainty by yielding objective support for the histopathologic assessment. Moreover, DNA analysis may be employed to resolve diagnostic problems, when histopathologic alternatives of decisive therapeutical implication are considered. This applies particularly to the distinction between benign lesions and those of high grade malignancy.

Cases displaying histological and cytochemical discrepancy deserve particular attention. Obviously, tumors histologically evaluated as benign, but exhibiting a hyperploid DNA content should be considered malignant. Such lesions prompt histologic reevaluation.

The inverse situation, i.e., morphologically high grade tumors with a diploid DNA content, likewise calls for histologic reevaluation. However, it also necessitates closer analysis of the cytochemical assessment. The representativeness of the analyzed specimen may be questioned and, therefore, additional tis-

Table 11. Collective data on ploidy level of primary bone tumors associated with osteoid formation

| Study | Method | Benign lesions [a] | | Parosteal osteosarcoma | | Osteosarcoma | |
|---|---|---|---|---|---|---|---|
| | | Diploid | Hyperploid | Diploid | Hyperploid | Diploid | Hyperploid |
| Heliö et al 1985 | FCM | 24 | 0 | - | - | 2 | 13 |
| Mankin et al 1985 | FCM | 49 | 0 | 8 | 0 | 2 | 41 |
| Hiddeman et al 1987 | FCM | - | - | 3 | 0 | 3 | 18 |
| Xiang et al 1987 | FCM | - | - | 3 | 0 | 0 | 16 |
| Present study [b] | MSP / FCM | 43 | 0 | 4 | 0 | 5 | 97 |
| Total | | 116 | 0 | 18 | 0 | 12 | 185 |

[a] Osteoblastoma, fibrous dysplasia, giant cell tumor, aneurysmal bone cyst.
[b] Only diagnostically concordant cases included.

sue material should be procured for DNA measurement. Under these circumstances MSP in histologic sections is preferable to FCM. As opposed to FCM, MSP permits cell analysis under visual control and, hence, exclusion of normal tissue and necrotic tumor tissue. Provided the histological and cytochemical inconsistency persists, the histologic diagnosis of high grade malignancy should be adhered to. Hence, the existence of highly malignant tumors with a normal DNA content can not be ruled out.

In conclusion, hyperploidy is a characteristic feature of high grade osteosarcoma. Benign bone tumors, representing entities which histologically may be mixed up with osteosarcoma, are diploid. The results suggest, that hyperploidy should be considered a prerequisite for inclusion in clinical trials on osteosarcoma. Provided DNA analysis is generally adopted in the diagnosis of osteosarcoma, it would allow more meaningful comparison of treatment series from different tumor centres.

# Summary and conclusions

The present work was aimed at analyzing the relationship between cytochemical features and histomorphology in osteosarcoma, and investigating the clinical significance of DNA content. For DNA analysis, both flow cytophotometry (FCM) of cell suspensions and microspectrophotometry (MSP) of tissue sections were utilized.

DNA measurement in tissue sections is associated with the methodological problem of determining the DNA content of sectioned cell nuclei. This causes difficulties in the interpretation of DNA histograms for discriminating between diploid and hyperploid tumors. To establish an upper limit of diploidy, the distribution of DNA values of 184 normal mesenchymal cell populations was analyzed. The maximum percentage of normal cells with DNA values exceeding DNA Index (DI) 1.25 was 31. Hence, tumors exhibiting more than 31 % cells >DI 1.25 were classified as hyperploid. Applying this upper limit of diploidy in the analysis of 42 sarcomas by MSP in tissue sections, 6 lesions were found to be diploid and 36 hyperploid. The reliability of this ploidy determination was tested by analyzing unsectioned nuclei of the same lesions by MSP of imprint preparations and FCM of cell suspensions. The comparison disclosed complete agreement in ploidy classification (diploid *versus* hyperploid) between all 3 methods of DNA analysis.

DNA analysis of archival bone tumor tissue is often impeded by previous demineralization in acid, which destroys Feulgen DNA stainability. To find an alternative to acid for prospective DNA studies of bone tumors, Feulgen stainability of fresh osteosarcoma specimens after demineralization in neutral EDTA was investigated. Demineralization in EDTA slightly reduced Feulgen DNA stainability, but did not affect the determination of tumor ploidy level. Hence, for DNA studies of bone tumors requiring demineralization, EDTA offers a means of retaining nuclear Feulgen stainability.

Archival tumor tissue, occasionally, exhibits poor Feulgen stainability. In tissue sections, decreased contrast between background and nuclear stain, may lead to erroneous extinction determinations, mainly because of light scattering caused by background inhomogeneity. Therefore, the reliability of ploidy determination in relation to stain intensity was investigated for specimens of varying Feulgen stainability. The results showed that the influence of background disturbances, in general, did not affect ploidy determination. However, for specimens, exhibiting less than one third of the maximum stainability encountered, ploidy determination was unreliable.

Individual osteosarcomas often display morphologic heterogeneity. Whether this is related to ploidy variability was investigated. MSP analysis of chondroblastic and osteo/fibroblastic areas within the same tissue sections, disclosed no decisive difference in nuclear DNA content, indicating that individual osteosarcomas have a uniform DNA content. Peak DNA values of biopsy and surgical specimens from the same tumors, as assessed by FCM, showed a strong correlation, substantiating cytochemical uniformity. Furthermore, comparison of recurrent lesions with corresponding primary osteosarcoma, by MSP and FCM, showed that local recurrences and metastases, in general, retain the hyperploid feature of the primary tumor. The combined results indicate that a single tumor sample for DNA analysis can be relied upon as representative for the tumor as a whole.

In a consecutive series of 47 osteosarcomas, analyzed by FCM, the relationship between DNA content and histologic subclassification (type and grade) was investigated. Lesions with peak DNA values in the triploid to tetraploid region predominated. Only 2 were diploid. The DNA content of the tumors was not related to histologic subtype or grade. Assessment of the percentage of S-phase cells, indicated that the proliferative activity was higher in triploid than in tetraploid lesions.

In a prognostic study, based on a consecutive series of 83 osteosarcoma patients treated by surgery and adjuvant Interferon, the significance of various clinicopathologic features and DNA content was investigated. Estimated 7-year survival rate for the whole series was 0.44. Multivariate analysis identified 3 independent risk factors for tumor related death, i.e., male sex, proximal tumor location, and histologic grade IV. DNA analysis by MSP gave no significant prognostic information, although the few tumors with extremely high DNA values were associated with a very low survival rate. A prognostication model was created, based on the number of clinicopathologic risk factors.

Hence, the patient series was divided into 4 subgroups, with 0, 1, 2, or 3 risk factors present. The corresponding 7-year survival rates were 0.80, 0.59, 0.42, and 0.13. These results show that it is feasible to identify subgroups of osteosarcoma patients with different prognosis.

Local recurrence was strongly related to surgical procedure. The risk of local recurrence was 0.07 after ablative surgery and 0.54 after local surgery. Other clinicopathologic factors were not related to local recurrence. Local tumor control was of decisive importance for survival. The findings confirm that local surgery in osteosarcoma should be confined to patients carefully selected with respect to the feasibility of obtaining safe surgical margins.

In a study of 166 primary bone tumors, the applicability of DNA analysis for differential diagnostic purposes was investigated. The series, apart from high grade osteosarcomas, included parosteal osteosarcomas and benign bone tumors. The benign lesions represented entities, which may be mixed up histologically with osteosarcoma, such as giant cell tumor, osteoblastoma, fibrous dysplasia, and aneurysmal bone cyst. Out of 166 tumors analyzed, 149 (90%) were histologically noncontroversial, whereas 17 (10%) posed diagnostic difficulties.

Among 149 diagnostically non-controversial tumors, all benign and parosteal osteosarcomas proved to be diploid, whereas 97 of 102 osteosarcomas were hyperploid. Hence, hyperploidy seems to be a characteristic feature of high grade osteosarcoma. The results show that ploidy determination, in general, offers confirmatory information in the histopathologic assessment of primary bone tumors.

The diagnostically controversial group comprised 17 cases, where there had been diagnostic uncertainty as to benignity or malignancy. Analysis of the clinical course, disclosed that all, who developed local recurrence or died, had hyperploid lesions. In contrast, none of the diploid lesions recurred. These findings suggest that DNA analysis is of great value, when diagnostic alternatives of decisive therapeutical implications are considered.

In conclusion, hyperploidy is a characteristic feature in osteosarcoma, although there is a wide variability of the hyperploid DNA content. Individual osteosarcomas, however, are cytochemically uniform, despite morphologic heterogeneity. The hyperploid feature may be assumed to reflect the highly malignant nature of osteosarcomas. The clinical significance of DNA analysis in osteosarcoma is mainly diagnostic. Applied routinely in the diagnosis of primary bone tumors, ploidy determination can contribute to increased diagnostic accuracy. Characterization of osteosarcomas according to DNA content would permit more meaningful comparison of treatment series from different centres.

# Acknowledgments

This study was supported by grants from the Jonas Björnberg Memorial Foundation, the Cancer Society of Stockholm, the King Gustav V Jubilee Fund, Inga-Britt and Arne Lundberg's Research Foundation, the Swedish Cancer Society, and the Karolinska Institute's Funds.

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# Appendix

## Prognostic status of 83 consecutive osteosarcoma patients

**Patient and tumor characteristics**

- A Age at diagnosis (years)
- B Sex 0=Male, 1=Female
- C Year of diagnosis (71-86)
- D Location
  - 0=Rib, 1=Pelvis,
  - 2=Proximal humerus,
  - 3=Distal radius,
  - 4=Proximal femur,
  - 5=Distal femur,
  - 6=Proximal tibia,
  - 7=Proximal fibula,
  - 8=Distal tibia,
  - 9=Distal fibula,
  - 10=Foot
- E Compartmentalization
  - 0=Intracompartmental,
  - 1=Extracompartmental
- F Malignancy grade (1-4)
- G Histologic subtype
  - 0=Osteoblastic,
  - 1=Chondroblastic,
  - 2=Fibroblastic,
  - 3=Telangiectatic
- H Size, largest diameter (cm)

**DNA data**

- I Ploidy (MSP/FCM)
  - 0=Diploid, 1=Hyperploid
- J Median (P50) DNA value (MSP)(DI)
- K Extreme (P90) DNA value (MSP)(DI)
- L Cells >DI 1.25 (MSP)( %)
- M Cells >DI 2.5 (MSP)( %)
- N Median (P50) nuclear area (MSP)($\mu m^2$)
- O Extreme (P90) nuclear area (MSP)($\mu m^2$)
- P Peak value (FCM)(DI)
- Q S-phase cells (FCM)(%)
- R Multiple aneuploid peaks (FCM)
  - 0=No, 1=Yes

**Treatment and follow-up**

- S Surgical procedure for primary tumor
  - 0=Local, 1=Ablative
- T Surgical margin
  - 0=Intralesional, 1=Marginal,
  - 2=Wide, 3=Radical
- U Time of first local recurrence
  - months
- V Time of pulmonary metastases
  - months
- W Time of death (months)
- X Cause of death
  - 0=Tumor,
  - 1=Other disease
- Y Follow-up time (years)

| Case | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V | W | X | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 15 | 1 | 73 | 5 | 1 | 2 | 4 | 10 | 1 | 1.8 | 2.9 | 78 | 14 | 55 | 80 | | | | 1 | 2 | | | | | 14 |
| 2 | 16 | 0 | 75 | 5 | 1 | 0 | 4 | 20 | 1 | 1.5 | 2.3 | 72 | 8 | 76 | 110 | | | | 1 | 2 | | 8 | 21 | 0 | 12 |
| 3 | 29 | 0 | 75 | 5 | 1 | 1 | 3 | 6 | 1 | 1.8 | 3.2 | 72 | 22 | 72 | 128 | | | | 0 | 1 | | | 6 | 1 | 0.5 |
| 4 | 20 | 1 | 75 | 6 | 1 | 0 | 4 | 9 | 1 | 2.8 | 4.7 | 100 | 65 | 60 | 94 | | | | 1 | 3 | | | | | 12 |
| 5 | 30 | 0 | 76 | 6 | 1 | 0 | 4 | 6 | 1 | 2.0 | 3.5 | 79 | 31 | 60 | 100 | | | | 0 | 0 | | 43 | 109 | 0 | 11 |
| 6 | 28 | 0 | 75 | 1 | 1 | 0 | 4 | 12 | 1 | 1.7 | 3.4 | 76 | 20 | 60 | 100 | | | | 0 | 2 | | | | | 12 |
| 7 | 16 | 0 | 76 | 2 | 1 | 0 | 4 | 15 | 1 | 2.4 | 4.4 | 86 | 47 | 75 | 132 | | | | 1 | 1 | 2 | 9 | 24 | 0 | 10 |
| 8 | 62 | 1 | 76 | 5 | 1 | 2 | 3 | 9 | 1 | 1.2 | 1.9 | 41 | 1 | 80 | 105 | | | | 0 | 2 | | | | | 11 |
| 9 | 24 | 0 | 77 | 5 | 1 | 0 | 3 | 11 | 1 | 2.0 | 3.0 | 95 | 29 | 40 | 60 | | | | 0 | 0 | 4 | 9 | 22 | 0 | 10 |
| 10 | 19 | 0 | 77 | 5 | 1 | 0 | 4 | 10 | 1 | 2.4 | 3.5 | 94 | 42 | 68 | 97 | | | | 1 | 2 | | 6 | 20 | 0 | 10 |
| 11 | 10 | 0 | 77 | 4 | 1 | 1 | 4 | 16 | 1 | 2.9 | 4.6 | 99 | 74 | 111 | 154 | | | | 1 | 2 | | 3 | 4 | 0 | 10 |
| 12 | 14 | 0 | 77 | 6 | 1 | 0 | 4 | 7 | 1 | 4.0 | 5.9 | 99 | 79 | 74 | 107 | | | | 0 | 0 | 7 | 12 | 27 | 0 | 10 |
| 13 | 13 | 0 | 78 | 5 | 1 | 0 | 3 | 10 | 1 | 2.5 | 4.4 | 100 | 51 | 50 | 70 | | | | 1 | 1 | | 8 | 27 | 0 | 9 |
| 14 | 14 | 1 | 78 | 5 | 1 | 1 | 3 | 12 | 1 | 1.6 | 2.9 | 80 | 14 | 62 | 87 | | | | 1 | 2 | | 2 | 6 | 0 | 8 |
| 15 | 24 | 0 | 79 | 6 | 1 | 2 | 4 | 15 | 1 | 1.3 | 1.8 | 59 | 3 | 54 | 87 | | | | 1 | 2 | | 6 | 26 | 0 | 8 |
| 16 | 16 | 0 | 78 | 6 | 1 | 2 | 2 | 9 | 1 | 1.3 | 2.2 | 60 | 1 | 45 | 60 | | | | 1 | 3 | | | | | 9 |
| 17 | 22 | 1 | 79 | 5 | 1 | 0 | 3 | 13 | 1 | 1.4 | 1.6 | 70 | 1 | 34 | 50 | | | | 1 | 2 | | | | | 8 |
| 18 | 50 | 1 | 79 | 6 | 1 | 0 | 3 | 11 | 1 | 1.8 | 2.9 | 89 | 27 | 61 | 87 | | | | 1 | 3 | | | | | 8 |
| 19 | 15 | 0 | 79 | 6 | 1 | 0 | 3 | 8 | 1 | 1.7 | 3.0 | 92 | 19 | 50 | 75 | 1.6 | | 1 | 1 | 3 | | | | | 8 |
| 20 | 16 | 0 | 79 | 7 | 1 | 0 | 4 | 6 | 1 | 1.9 | 2.9 | 73 | 27 | 60 | 95 | 1.6 | | 1 | 1 | 3 | | 3 | 20 | 0 | 8 |
| 21 | 15 | 0 | 80 | 6 | 1 | 1 | 3 | 14 | 1 | 1.6 | 2.8 | 71 | 20 | 50 | 80 | 1.6 | 18 | 0 | 1 | 3 | | 8 | 52 | 0 | 7 |
| 22 | 16 | 1 | 79 | 2 | 1 | 1 | 4 | 10 | 1 | 2.6 | 3.4 | 99 | 58 | 82 | 105 | 2.7 | 10 | 0 | 0 | 2 | | | | | 8 |
| 23 | 19 | 1 | 80 | 5 | 1 | 0 | 4 | 8 | 1 | 2.2 | 4.3 | 75 | 37 | 57 | 93 | 1.3 | 23 | 0 | 1 | 2 | | 1 | 22 | 0 | 7 |
| 24 | 9 | 1 | 80 | 5 | 1 | 0 | 4 | 15 | 1 | 2.6 | 3.2 | 87 | 24 | 72 | 135 | 1.6 | 14 | 1 | 1 | 3 | | | | | 7 |
| 25 | 17 | 0 | 80 | 6 | 1 | 0 | 4 | 11 | 1 | 2.4 | 3.9 | 92 | 45 | 70 | 120 | 1.6 | | 1 | 1 | 3 | | 3 | 10 | 0 | 7 |
| 26 | 20 | 0 | 80 | 7 | 0 | 2 | 4 | 3 | 0 | 1.2 | 1.7 | 23 | 3 | 61 | 95 | | | | 0 | 2 | | | | | 7 |
| 27 | 10 | 1 | 80 | 6 | 1 | 0 | 3 | 9 | 1 | 1.9 | 3.6 | 94 | 26 | 45 | 65 | 1.6 | 23 | 1 | 1 | 3 | | 14 | 32 | 0 | 7 |
| 28 | 8 | 0 | 81 | 5 | 1 | 0 | 3 | 8 | 1 | 1.5 | 2.5 | 88 | 18 | 50 | 75 | 1.4 | 14 | 1 | 1 | 2 | | 30 | | | 6 |
| 29 | 9 | 1 | 82 | 2 | 1 | 0 | 3 | 15 | 1 | 1.9 | 2.2 | 92 | 5 | 55 | 80 | 1.8 | 15 | 0 | 0 | 2 | | 3 | 12 | 0 | 5 |
| 30 | 7 | 0 | 82 | 5 | 1 | 1 | 4 | 8 | 1 | 1.5 | 2.3 | 79 | 6 | 55 | 75 | 0.9 | 16 | 1 | 1 | 2 | | 4 | 15 | 0 | 5 |
| 31 | 65 | 1 | 84 | 10 | 1 | 0 | 3 | 4 | 1 | 1.9 | 2.2 | 98 | 0 | 55 | 80 | 1.9 | 9 | 0 | 1 | 3 | | | | | 3 |
| 32 | 13 | 1 | 83 | 6 | 1 | 0 | 3 | 5 | 1 | 2.0 | 3.1 | 94 | 25 | 60 | 95 | 1.7 | 34 | 0 | 1 | 3 | | | | | 4 |
| 33 | 62 | 0 | 83 | 2 | 1 | 0 | 3 | 8 | 1 | 2.5 | 3.8 | 96 | 48 | 75 | 145 | | | | 0 | 2 | | | | | 4 |
| 34 | 10 | 1 | 84 | 5 | 1 | 0 | 3 | 7 | 1 | | | | | | | 1.6 | 21 | 0 | 1 | 2 | | 6 | | | 3 |
| 35 | 16 | 0 | 84 | 5 | 1 | 1 | 3 | 10 | 1 | 1.9 | 3.8 | 90 | 23 | 55 | 90 | 0.9 | | 1 | 0 | 1 | 4 | 2 | 6 | 0 | 3 |
| 36 | 50 | 0 | 85 | 1 | 1 | 0 | 3 | 10 | 1 | 2.0 | 3.6 | 82 | 34 | 80 | 180 | 2.0 | 18 | 0 | 0 | 2 | | | | | 2 |
| 37 | 59 | 1 | 82 | 9 | 1 | 0 | 3 | 6 | 1 | 2.0 | 3.2 | 97 | 20 | 50 | 70 | 1.8 | 9 | 0 | 0 | 1 | 13 | | | | 5 |
| 38 | 22 | 1 | 77 | 1 | 1 | 1 | 4 | 12 | 1 | 2.2 | 3.6 | 88 | 40 | 60 | 105 | | | | 0 | 0 | 7 | 13 | 20 | 0 | 10 |
| 39 | 20 | 0 | 78 | 2 | 1 | 0 | 4 | 8 | 1 | 1.2 | 2.3 | 48 | 7 | 65 | 102 | | | | 0 | 0 | 4 | 2 | 5 | 0 | 9 |
| 40 | 14 | 0 | 78 | 5 | 1 | 1 | 3 | 12 | 1 | 1.5 | 3.1 | 86 | 18 | 55 | 72 | 1.7 | 19 | 0 | 0 | 1 | 3 | 8 | 10 | 0 | 9 |
| 41 | 17 | 0 | 83 | 2 | 1 | 0 | 4 | 12 | 1 | 3.0 | 4.4 | 98 | 76 | 85 | 125 | 1.4 | 44 | 0 | 0 | 0 | 2 | 2 | 6 | 0 | 4 |
| 42 | 62 | 1 | 82 | 5 | 1 | 0 | 4 | 10 | 1 | 1.4 | 2.2 | 64 | 7 | 55 | 90 | 1.1 | 15 | 1 | 1 | 2 | | | 4 | 1 | 0.3 |
| 43 | 10 | 0 | 83 | 5 | 1 | 0 | 4 | 12 | 1 | 1.3 | 2.4 | 75 | 9 | 50 | 90 | 2.0 | 15 | 0 | 1 | 3 | | 3 | 8 | 0 | 4 |
| 44 | 9 | 1 | 83 | 6 | 1 | 2 | 3 | 8 | 0 | 1.0 | 1.7 | 2.4 | 0 | 50 | 70 | 1.0 | | 0 | 1 | 3 | | | | | 4 |
| 45 | 30 | 0 | 75 | 5 | 1 | 0 | 4 | 12 | 1 | 1.5 | 2.2 | 69 | 7 | 50 | 65 | | | | 1 | 2 | 7 | 8 | 11 | 0 | 12 |
| 46 | 60 | 1 | 84 | 5 | 1 | 0 | 4 | 10 | 1 | 1.4 | 2.2 | 71 | 6 | 70 | 100 | 1.4 | 17 | 0 | 1 | 3 | | 2 | 3 | 0 | 3 |
| 47 | 19 | 0 | 80 | 2 | 1 | 0 | 4 | 7 | 1 | 1.8 | 2.7 | 89 | 13 | 70 | 90 | 1.8 | 18 | 1 | 1 | 2 | 4 | 8 | 9 | 0 | 7 |
| 48 | 40 | 0 | 84 | 0 | 0 | 0 | 3 | 2 | 1 | 1.9 | 2.5 | 85 | 10 | 60 | 80 | | | | 0 | 2 | | | | | 3 |
| 49 | 13 | 1 | 76 | 8 | 0 | 0 | 3 | 5 | 1 | 1.3 | 1.9 | 57 | 2 | 57 | 88 | | | | 0 | 2 | | | | | 11 |
| 50 | 7 | 0 | 85 | 5 | 1 | 3 | 4 | 9 | 1 | 1.9 | 3.0 | 64 | 10 | 55 | 80 | 1.9 | 18 | 0 | 0 | 0 | 3 | 2 | 9 | 0 | 2 |
| 51 | 23 | 1 | 78 | 3 | 1 | 1 | 3 | 5 | 1 | 1.8 | 3.0 | 90 | 20 | 63 | 88 | | | | 0 | 1 | 19 | 60 | 94 | 0 | 9 |
| 52 | 20 | 0 | 78 | 5 | 1 | 1 | 3 | 8 | 1 | 1.4 | 2.4 | 76 | 8 | 65 | 105 | | | | 1 | 2 | | 8 | 18 | 0 | 9 |
| 53 | 22 | 0 | 85 | 7 | 1 | 1 | 3 | 6 | 1 | | | | | | | 1.1 | | 1 | 0 | 2 | | | | | 2 |
| 54 | 26 | 1 | 85 | 1 | 1 | 0 | 3 | 6 | 1 | | | | | | | 1.6 | | 0 | 0 | 0 | | | | | 2 |
| 55 | 44 | 1 | 85 | 1 | 1 | 2 | 3 | 6 | 0 | 1.0 | 1.0 | 3 | 0 | 35 | 50 | 1.0 | 4 | 0 | 0 | 0 | | | 10 | 0 | 2 |
| 56 | 11 | 0 | 74 | 7 | 1 | 2 | 3 | 3 | 1 | 1.4 | 1.8 | 83 | 3 | 70 | 90 | | | | 0 | 2 | | | | | 13 |
| 57 | 5 | 0 | 85 | 5 | 1 | 1 | 4 | 7 | 1 | 1.8 | 3.8 | 83 | 26 | 65 | 140 | 1.6 | 31 | 0 | 1 | 2 | | | | | 2 |
| 58 | 9 | 0 | 85 | 5 | 1 | 1 | 4 | 11 | 1 | 2.0 | 4.5 | 84 | 32 | 65 | 120 | 1.4 | 31 | 0 | 1 | 3 | | 4 | 6 | 0 | 2 |
| 59 | 13 | 0 | 86 | 5 | 1 | 1 | 3 | 10 | 1 | | | | | | | 1.7 | 17 | 0 | 1 | 2 | | | | | 1 |
| 60 | 29 | 1 | 86 | 5 | 1 | 0 | 4 | 11 | 1 | 2.0 | 3.6 | 80 | 33 | 70 | 115 | 2.8 | 26 | 0 | 0 | 2 | | | | | 1 |
| 61 | 43 | 0 | 86 | 5 | 1 | 0 | 3 | 8 | 1 | 2.5 | 4.7 | 97 | 51 | 75 | 100 | 2.1 | 18 | 0 | 1 | 2 | | 5 | 11 | 0 | 1 |
| 62 | 18 | 0 | 84 | 5 | 1 | 0 | 3 | 6 | | | | | | | | | | | 0 | 1 | 4 | | | | 3 |
| 63 | 13 | 1 | 84 | 6 | 1 | 1 | 3 | 8 | 1 | | | | | | | 1.8 | 12 | 0 | 0 | 1 | 8 | | | | 3 |
| 64 | 14 | 1 | 86 | 5 | 1 | 1 | 3 | 9 | 1 | 1.2 | 2.2 | 41 | 2 | 60 | 85 | 1.3 | 35 | 0 | 1 | 2 | | | | | 1 |
| 65 | 13 | 1 | 76 | 6 | 1 | 0 | 3 | 7 | | | | | | | | | | | 1 | 3 | | 10 | 28 | 0 | 11 |
| 66 | 74 | 0 | 80 | 6 | 1 | 0 | 4 | 9 | | | | | | | | | | | 1 | 3 | | 13 | 24 | 0 | 7 |
| 67 | 16 | 0 | 76 | 5 | 1 | 0 | 4 | 3 | | | | | | | | | | | 0 | 0 | 1 | 3 | 7 | 0 | 11 |
| 68 | 14 | 0 | 74 | 5 | 1 | 2 | 3 | 7 | | | | | | | | | | | 1 | 2 | | 13 | 34 | 0 | 13 |
| 69 | 18 | 0 | 72 | 6 | 1 | 2 | 2 | 9 | | | | | | | | | | | 0 | 1 | 33 | | | | 15 |
| 70 | 14 | 0 | 73 | 7 | 1 | 1 | 3 | 10 | | | | | | | | | | | 0 | 2 | | 8 | 27 | 0 | 14 |
| 71 | 15 | 0 | 72 | 5 | 1 | 0 | 4 | 12 | | | | | | | | | | | 1 | 3 | | 10 | 16 | 0 | 15 |
| 72 | 19 | 1 | 71 | 1 | 1 | 0 | 2 | 10 | | | | | | | | | | | 0 | 0 | 37 | | 62 | 0 | 16 |
| 73 | 9 | 1 | 74 | 2 | 1 | 2 | 2 | 10 | 0 | 1.0 | 1.0 | 5 | 0 | 60 | 80 | | | | 0 | 1 | | | | | 13 |
| 74 | 29 | 0 | 74 | 6 | 1 | 0 | 4 | 6 | | | | | | | | | | | 0 | 1 | | 8 | 11 | 0 | 13 |
| 75 | 17 | 0 | 71 | 6 | 1 | 1 | 3 | 8 | | | | | | | | | | | 0 | 0 | 17 | 51 | 63 | 0 | 16 |
| 76 | 17 | 0 | 72 | 6 | 1 | 0 | 4 | 10 | 1 | 1.6 | 2.1 | 81 | 5 | 52 | 72 | | | | 1 | 3 | | 24 | 46 | 0 | 15 |
| 77 | 26 | 0 | 72 | 8 | 1 | 2 | 4 | 5 | | | | | | | | | | | 1 | 2 | | | | | 15 |
| 78 | 11 | 0 | 74 | 2 | 1 | 0 | 3 | 14 | | | | | | | | | | | 1 | 3 | | | | | 13 |
| 79 | 7 | 1 | 75 | 5 | 1 | 0 | 4 | 13 | | | | | | | | | | | 1 | 2 | | 3 | 9 | 0 | 12 |
| 80 | 12 | 0 | 75 | 6 | 1 | 0 | 4 | 14 | | | | | | | | | | | 1 | 3 | | | | | 12 |
| 81 | 21 | 0 | 76 | 6 | 1 | 0 | 4 | 7 | | | | | | | | | | | 0 | 1 | 12 | 28 | | | 11 |
| 82 | 16 | 1 | 77 | 5 | 1 | 2 | 4 | 8 | | | | | | | | | | | 1 | 2 | | 11 | 39 | 0 | 10 |
| 83 | 22 | 1 | 76 | 5 | 1 | 0 | 3 | 12 | | | | | | | | | | | 1 | 2 | | | | | 11 |