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Nuclear DNA Analysis: DNA Heterogeneity in the Monitoring of Patients with Locally Advanced Prostatic Carcinoma

Hussein Al-Abadi
Reinhard Nagel

Department of Urology, Clinical Cytology and DNA Cytometry, Charlottenburg Medical Center, Free University of Berlin, FRG

Key Words
Nuclear DNA analysis
DNA heterogeneity
Prostatic carcinoma, diagnosis
Therapy control

Abstract
Single-cell DNA cytophotometry was employed to analyze the tumors of 271 patients with locally advanced prostatic carcinoma as to DNA ploidy and heterogeneity and the distribution of the phases of the cell cycle before and during therapy, with the intention of establishing prognostic factors apart from those already known (stage, grade). Follow-up periods ranged from 1 to 9 years. 198 (73%) of the 271 patients had carcinoma stage T_3 N0 M0, and 73 (27%) of them had carcinoma stage T_3/T_4 N+ M1. The tumors were evaluated cytologically to establish the grades of malignancy. 11.8% were grade I carcinoma, 64.3% were grade II and 23.9% were grade III carcinoma. Single-cell DNA cytophotometry demonstrated aneuploidy rates of up to 73% and diploidy rates of up to 23.8% for the higher grades of malignancy, whereas the diploidy rate established for grade I carcinoma was 71% and the respective aneuploidy rate was 15.2%. These differences are significant ($p < 0.001$). There was a significant correlation between the results of DNA cytophotometry and the clinical course of the disease. Patients with diploid tumor cell nuclei developed no metastases and no local tumor progression during the follow-up period of 9 years, whereas patients with aneuploid tumor cell nuclei showed metastases and local tumor progression within 8–22 months, despite changes in therapy. These patients died of carcinoma after an average 18 months following primary diagnosis.

Introduction

The pathomorphological classification of malignant tumors of the prostate is often difficult, even today. Carcinoma of the prostate is highly heterogeneous, with overlapping histological characteristics, variable biological behavior and clinical course.

Treatment could be improved by developing criteria of malignancy apart from the histological ones, and in addition to the determination of hormone receptors, histochemical and immunological analyses and analyses of molecular genetics. The DNA ploidy and the proliferative activity of the different tumors could be such criteria of differentiation [1–5, 7, 9, 14, 18, 19, 24].

Hussein Al-Abadi
Urologische Klinik der FU Berlin
Klinikum Charlottenburg
Spandauer Damm 130
1000 Berlin 19 (FRG)

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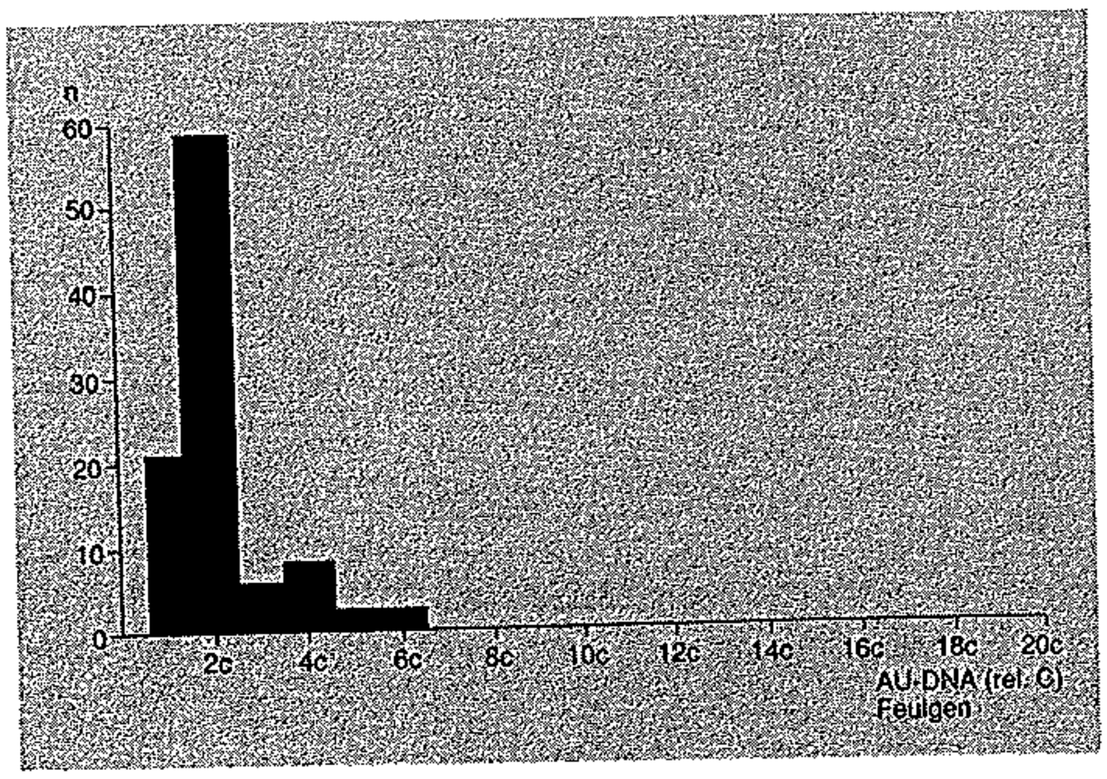


Fig. 2. Grade (of malignancy) I. DNA histogram with frequency peak in the diploid range with values scattering up to 7c.

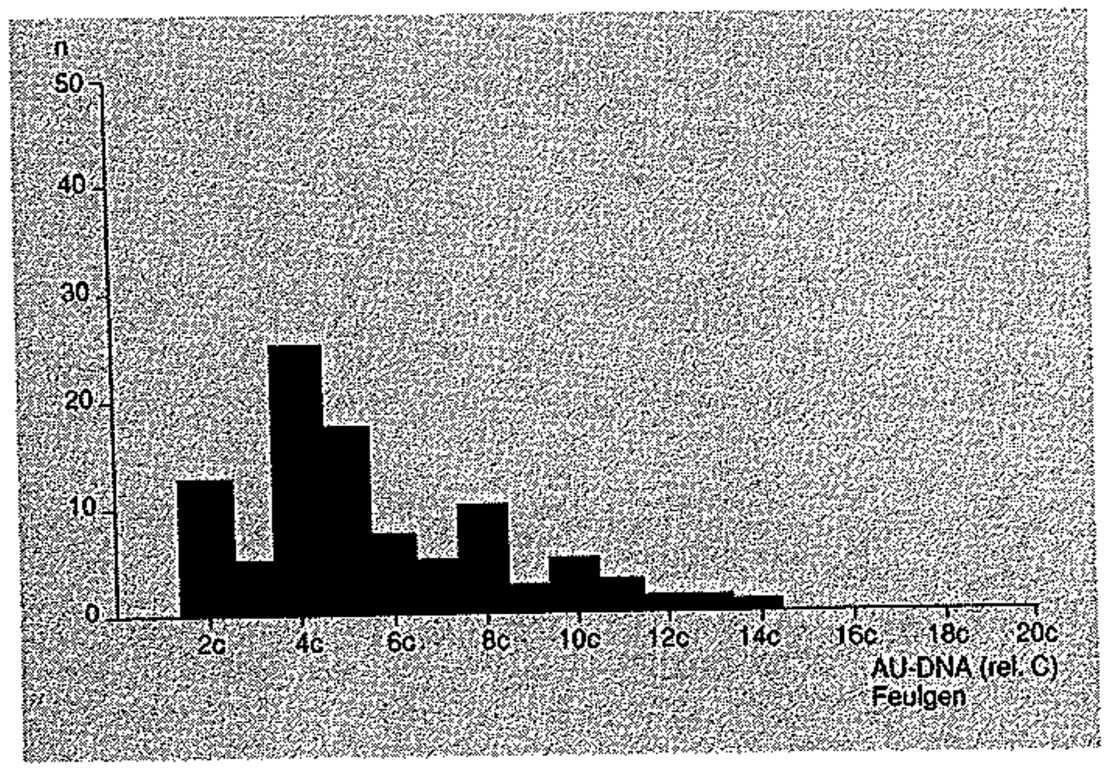


Fig. 3. Grade (of malignancy) II: DNA histogram with highly heterogeneous distribution pattern ranging from 2c to 15c.

The DNA content of a tumor cell population is higher than that of normal tissue, which means a change in the number of chromosomes towards aneuploidy [12, 27]. This fact has been established and confirmed by studies on malignant tumors in various organs. The DNA heterogeneity of tumor cell nuclei in malignant tumors of different organs has become very important clinically during the last few years because of its relation to tumor behavior before and during therapy [1–5, 7, 8, 10, 13, 19, 22, 23, 26, 30, 31, 33, 35].

In an experimental study – from 1980 to 1989 – the authors analyzed tumor cell material obtained by aspiration biopsy, with the intention of establishing prognostic factors in addition to those already known. The DNA ploidy and heterogeneity and the proliferative activity of prostatic carcinoma were analyzed, by means of absorption scanning cytophotometry, before and during therapy.

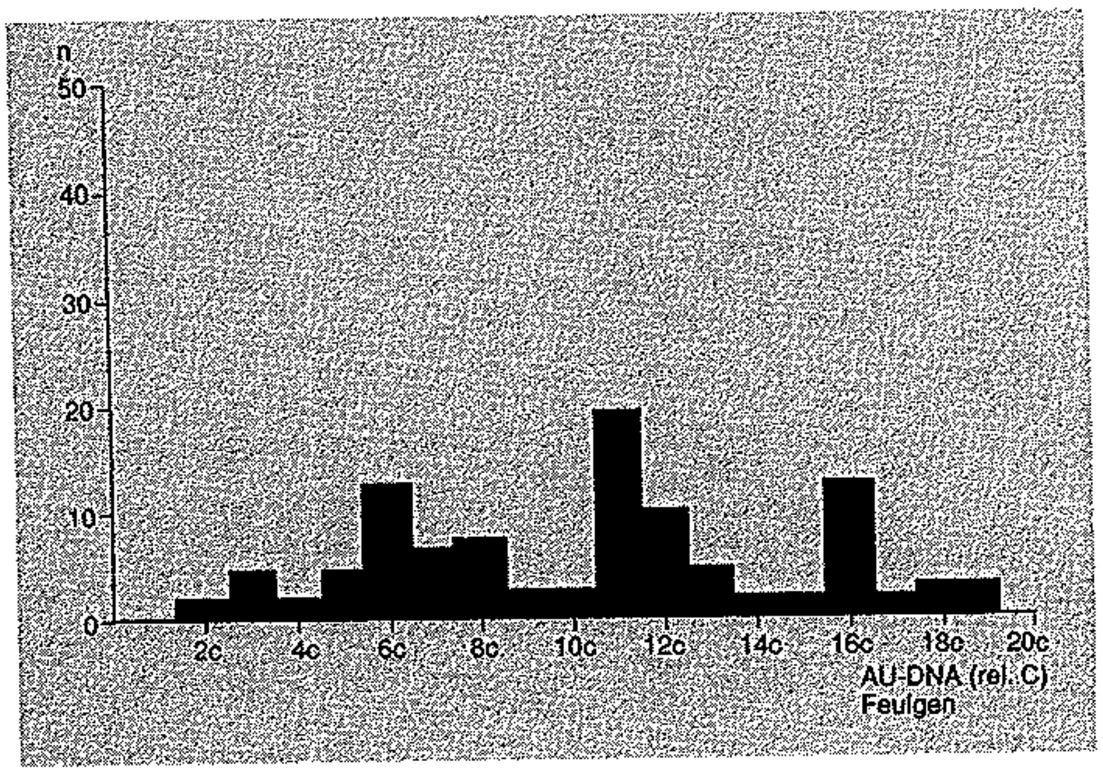


Fig. 4. Grade (of malignancy) III. Heterogeneous DNA histogram with frequency peaks in 6c, 8c, 12c and 16c and values scattering up to 19c.

Patients and Methods

From January 1980 to December 1989, single-cell scanning absorption cytophotometry was employed to analyze the tumors of 271 patients with locally advanced prostatic carcinoma, and the clinical course of the disease in the respective patients was documented. Follow-up periods ranged from 1 to 9 years. The average age of the patients was 68.9 years (42–88 years).

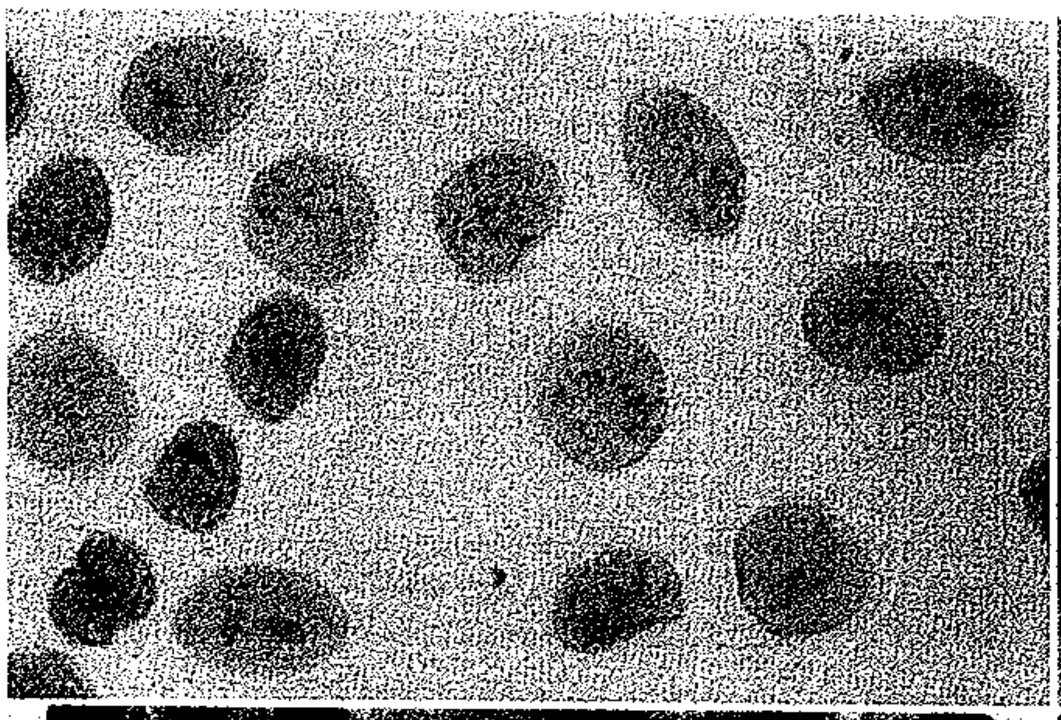
The cellular material for DNA analysis was obtained by means of transrectal fine-needle aspiration biopsy [17]. The preparations were stained with Papanicolaou's stain. It was then assessed cytologically to establish the diagnosis and differentiation of the tumor, according to the recommendations of the Uropathological Study Group on Prostatic Carcinoma [20]. For the determination of DNA, the preparations were stained by Feulgen's reaction (30-min hydrolysis in 5 *N*

Fig. 1. Feulgen's reaction: isolated, clearly distinguishable cell nuclei without overlapping or impurities. × 1,000.

Fig. 5. a K.H.: primary cytological diagnosis prior to buserelin therapy. Cytological sample obtained by aspiration. Grade I carcinoma. **b** Same patient, after 2.5 years of buserelin therapy. Regression grade II. **a, b** Papanicolaou's stain. × 1,000.

Fig. 6. a W.K.: primary cytological diagnosis prior to buserelin therapy. Cytological sample obtained by aspiration. Grade II carcinoma. **b** Same patient, after 2.5 years of buserelin therapy. Regression grade IV. **a, b** Papanicolaou's stain. × 1,000.

Fig. 7. a F.Sch.: primary cytological diagnosis prior to buserelin therapy. Cytological sample obtained by aspiration. Grade III carcinoma. **b** Same patient, after 6 months of buserelin therapy. Regression grade X. **c** Same patient, after 2.5 years of secondary Estracyt therapy. Regression grade VI. **a–c** Papanicolaou's stain. × 1,000.



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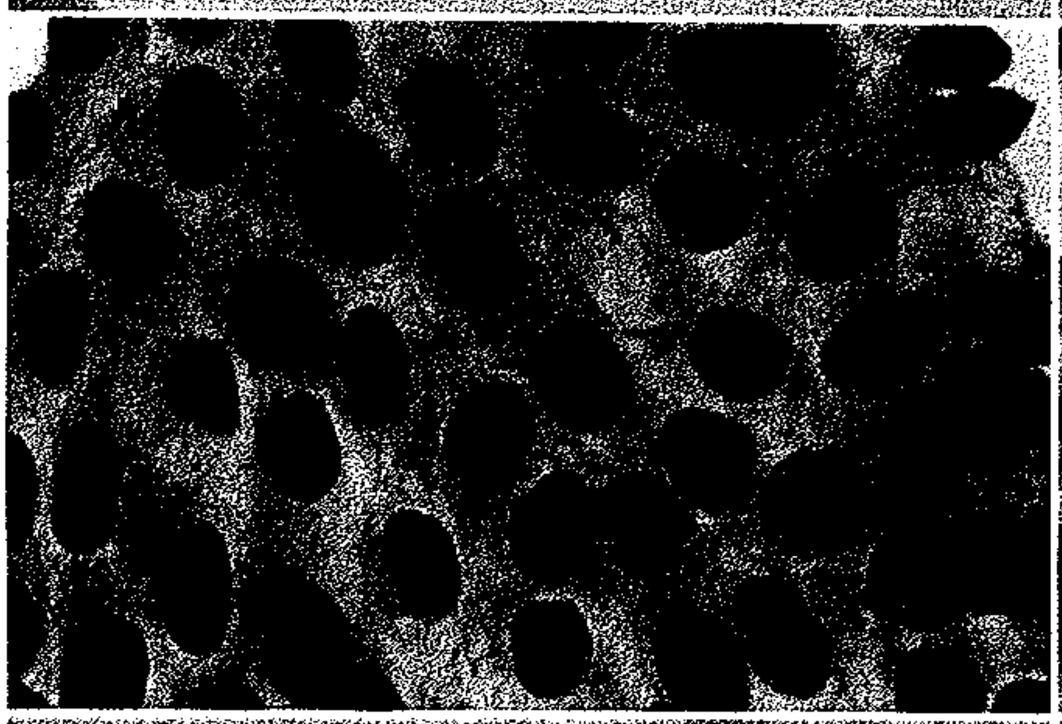

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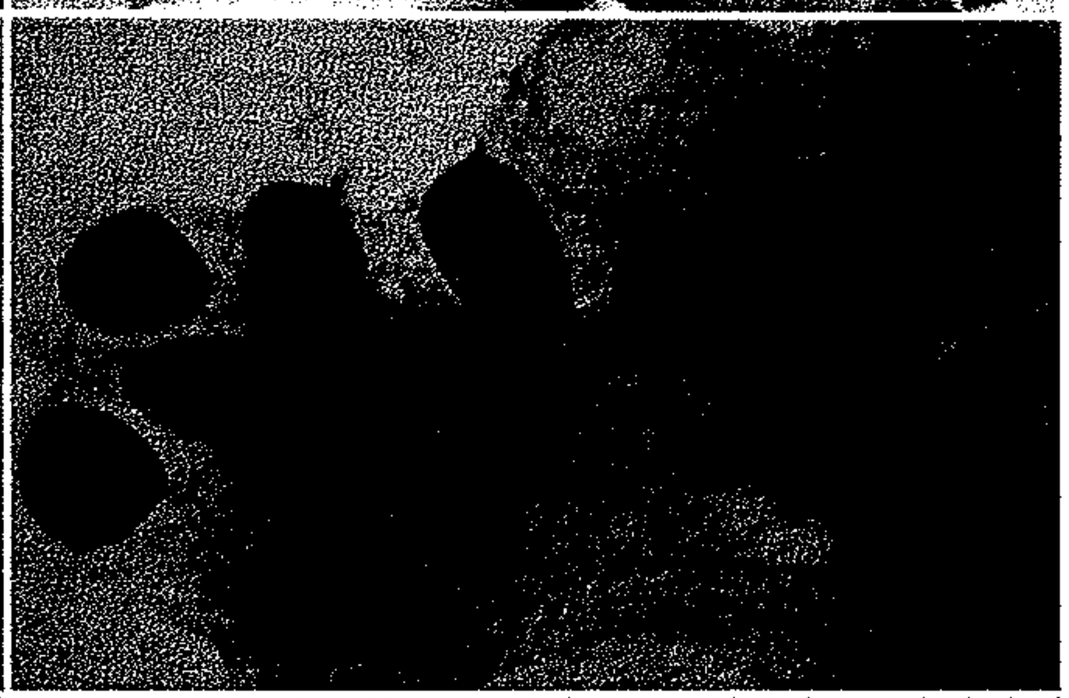

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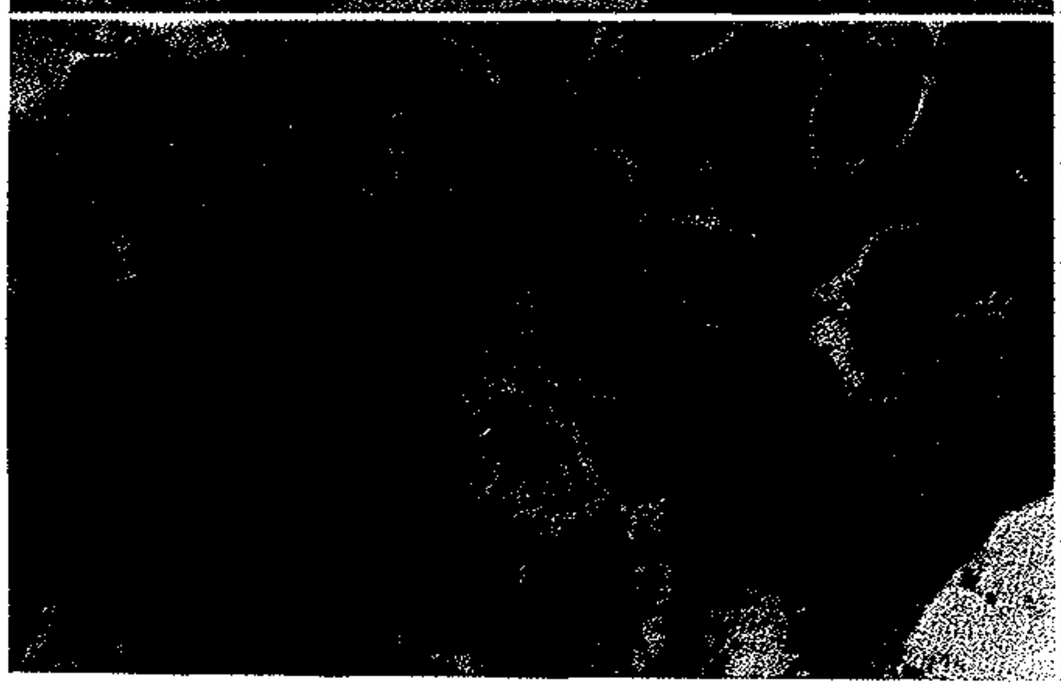


6a

7c

hydrochloric acid at room temperature) [16], which is specific for DNA (fig. 1). Leukocytes from peripheral blood from healthy donors treated in the same manner as the test material were used as reference preparations for the determination of DNA diploidy. As a rule, approximately 100 tumor cell nuclei were measured, occasionally more. The DNA content of these nuclei was determined by means of single-cell absorption photometry.

The fine scanning procedure with a Leitz MPV-2 cytophotometer was used to measure the cell nuclei. The scanning stage, measuring procedure and recording of the absorption were controlled by a Digital pdp 8a process computer. The respective total extinction values measured were printed together with the mean value, standard deviation, variance and coefficient of variation. The results are printed in the form of a histogram in which the number of tumor cell nuclei measured is marked as n on the ordinate, and the relative DNA content of the individual cell populations, given in relative c and expressed in arbitrary units, is plotted against the abscissa. The figures 2–4 show typical DNA histograms. The tumors exhibit either a diploid or several aneuploid or tetraploid DNA cell lines.

Data Analysis

The computer of the University Institute for Medical Statistics and BMDP Software were usd for documentation and data analysis. The statistical analysis of survival curves was made according to the Kaplan-Meier model for censored data [21]. The individual survival curves were compared by means of the multivariate analysis according to the methods of Mantel [25] and Cox [15].

Results

Tumor Stages and Grades of Malignancy

The staging in this study follows the recommendations of the UICC applying the TNM classification [32]. When first diagnosed, 198 (73%) of the patients had carcinoma stage T_3 N0 M0, and 73 (27%) patients had carcinoma stage T_3/T_4 N + M1 (table 1). The total number of cases studied shows the cytological grade of malignancy grade II to prevail with 64.3%, followed by grade III with 23.9% and grade I with 11.8% (table 2).

Cytological Criteria of Tumor Response to Therapy

The case material described by the authors is evidently different from the cases reported by other authors as only 27% of the patients included in this study had metastases whereas 73% had none although they had stage T_3 carcinoma.

This is explained by the fact that the authors have been able to monitor the effect or failure of therapy in the primary tumor itself by means of cytology and DNA cytophotometry, and performed more than 18,000 aspiration biopsies in the last 15 years.

At present, these two methods – and the histomorphological examination of material gained by punch biopsy – are the only objective parameters which permit an evaluation of the response of locally advanced, nonmetastatic carcinoma to treatment.

The most important signs of tumor regression are found in the cell nucleus (pyknosis, reduction in size) and in the nucleoli (reduction in size and number) [14, 23]. The cytological criteria of regresion correspond with those defined for the regression grading by histological methods [14, 23]. The cytological regression grading adopted by the Uropathological Group in Germany distinguishes six grades (table 3). The six grades are defined by characteristic signs of regression. The regression grades

Table 1. Frequency of stages in 271 patients with locally advanced prostatic carcinoma at the time of diagnosis

Stage	n	%
T_3 N0 M0	198	73
T_3/T_4 N+ M1	73	27

Table 2. Frequency of grades of malignancy in 271 patients with prostatic carcinoma

Grade of malignancy	n	%
I	32	11.8
II	174	64.3
III	65	23.9

Table 3. Regression grading according to the recommendations of the Uropathological Group in the FRG

Grade of regression	Effect of therapy	Cytomorphiological criteria
0	very good	no carcinoma, marked regressive changes
II	good	epithelial atypias, carcinoma?, marked regressive changes
IV	satisfactory	few and small carcinoma cell clusters ('residual carcinoma'), marked regressive changes
VI	sufficient	carcinoma with marked regressive changes
VIII	poor	carcinoma with poor regressive changes
X	none	carcinoma without regressive changes

II–VI indicate good to sufficient therapy response of the tumor whereas the regression grades VIII and X signal poor or no response of the tumor to therapy. The difference between good (grades II–VI) and poor regression (grade VIII or X) is statistically significant [14, 23].

The regression signs occur irrespective of the form of therapy. This was shown in an earlier study the authors undertook with 600 patients on six different forms of therapy. What has been established furthermore is the point of time when a poor or lacking therapeutic effect can be unmistakably demonstrated in the tumor itself for the different forms of therapy. When the tumor shows poor or no regression after 6 months of antiandrogen treatment such as orchiectomy, therapy with estrogens, LH-RH analogues or antiandrogens, it is unlikely to respond to continued therapy. The therapeutic effect of estramustine phosphate (Estracyt) or cyclophosphamide can be reliably evaluated after only 3 months of treatment [23].

According to the authors' observations, the regression grades VIII and X signal that local tumor progression or metastases are very likely to occur within a few months. The interindividual reproducibility of the cytological regression grading averaged 85%, which is a remarkably high percentage.

The three cases described below show different responses to buserelin therapy and the respective characteristic cytological changes.

Case 1. K.H., 63 years, prostatic carcinoma stage T_3 NxM0. Prior to therapy: moderate nuclear polymorphism, pronounced nuclear hypochromatism, prominent nucleoli, grade I carcinoma (score 9) (fig. 5a). After 2.5 years of buserelin therapy: reduction in nuclear size, nuclear rarefaction, diminution of nucleoli, pyknotic nuclei, fine vacuolation of cytoplasm and nuclear chromatin. Overall, good therapeutic effect, corresponding to regression grade II (fig. 5b).

Case 2. W.K., 65 years, prostatic carcinoma stage T_3 NxM0. Prior to therapy: marked nuclear polymorphism and hyperchromatism, disturbance of nuclear arrangement, prominent nucleoli displaying loss of circularity; some nuclei contain several nucleoli. Grade II carcinoma (score 13) (fig. 6a). After 2.5 years of buserelin therapy: reduction in nuclear size, localized pyknotic nuclei, scarcely distinguishable nucleoli. Regression grade IV (fig. 6b).

Case 3. F.Sch., 51 years, prostatic carcinoma stage T_3 NxM0. Prior to therapy: markedly polymorphic and hyperchromatic nuclei, disturbance of nuclear arrangement, prominent nucleoli displaying loss of circularity, conspicuously dissociated nuclei. Grade III carcinoma (score 17) (fig. 7a). After 6 months of buserelin therapy: the nuclei are still hyperchromatic and polymorphic, with prominent nucleoli; rare and only localized regressive changes. Regression grade X, therefore, crossover to secondary therapy with Estracyt (fig. 7b). After 1.5 years of Estracyt therapy: nuclear rarefaction and reduction in size, localized cytoplasmic vacuolation, nucleoli reduced in size. Sufficient therapeutic effect. Regression grade VI (fig. 7c).

Single-Cell Scanning Cytophotometry

While cytology yields morphological results and thus permits a qualitative rating of the effect of therapy, the analysis of the DNA content of tumor cell nuclei by single-cell scanning cytophotometry permits a quantitative grading of regression in the primary tumor. Both methods, particularly DNA cytophotometry, can contribute greatly to a better understanding of biological tumor activity.

It has been known for years that tumor cell populations, corresponding to a change in the number of chromosomes towards aneuploidy, show higher DNA contents than normal tissues. This has been confirmed by investigations of different organs, particularly the mammas, lungs, and urinary bladder.

It also applies to prostatic carcinoma whose DNA content differs substantially from that of adenoma. This fact assigns DNA cytophotometry its place in the treatment and therapy control of prostatic cancer [24, 28, 35]. As far as is ascertainable, the first sequential studies assessing the response of nuclear DNA in prostatic carcinoma to various therapeutic measures were done at this clinic [14, 22, 23].

The authors have observed that a statistically significant fall of the grade of ploidy from aneuploid towards diploid occurs in prostatic carcinoma when therapy is successful. If an aneuploidy remains unchanged during therapy, a negative clinical course of the disease may be predicted. Furthermore, the authors have shown that therapy-resistant carcinomas of the prostate differ in their nuclear DNA content significantly from tumors with positive response to therapy.

Results of DNA Cytophotometry

The prognostic value of DNA histograms for buserelin therapy is the same as for any other therapy; the results are statistcally significant and can be reproduced in the sample at any time. Figures 8–10 show the histograms of 3 patients with different responses to therapy.

Case 2. W.K., T_3 NxM0, grade II carcinoma (see fig. 6a, b). Prior to therapy, there is a broad DNA frequency peak between 8c and 10c, with some values spreading to 12c. After 1.5 and 7.5 years of buserelin therapy, there are slim peaks in the diploid (2c) range. The histograms correspond with the morphological findings and the clinically stable condition of the patient after 8 years of therapy (fig. 8).

Case 3. F.Sch., T_3 NxM0, grade III carcinoma (see fig. 7a–c). Prior to therapy, the DNA frequency peak reads between 4c and 8c, with some values spreading to 12c. After 6 months of buserelin therapy, the DNA frequency peak is still between 6c and 8c (fig. 9). As no signs of regression were demonstrated by cytological examination, either (fig. 7b) therapy was changed to estramustine phosphate. 1.5

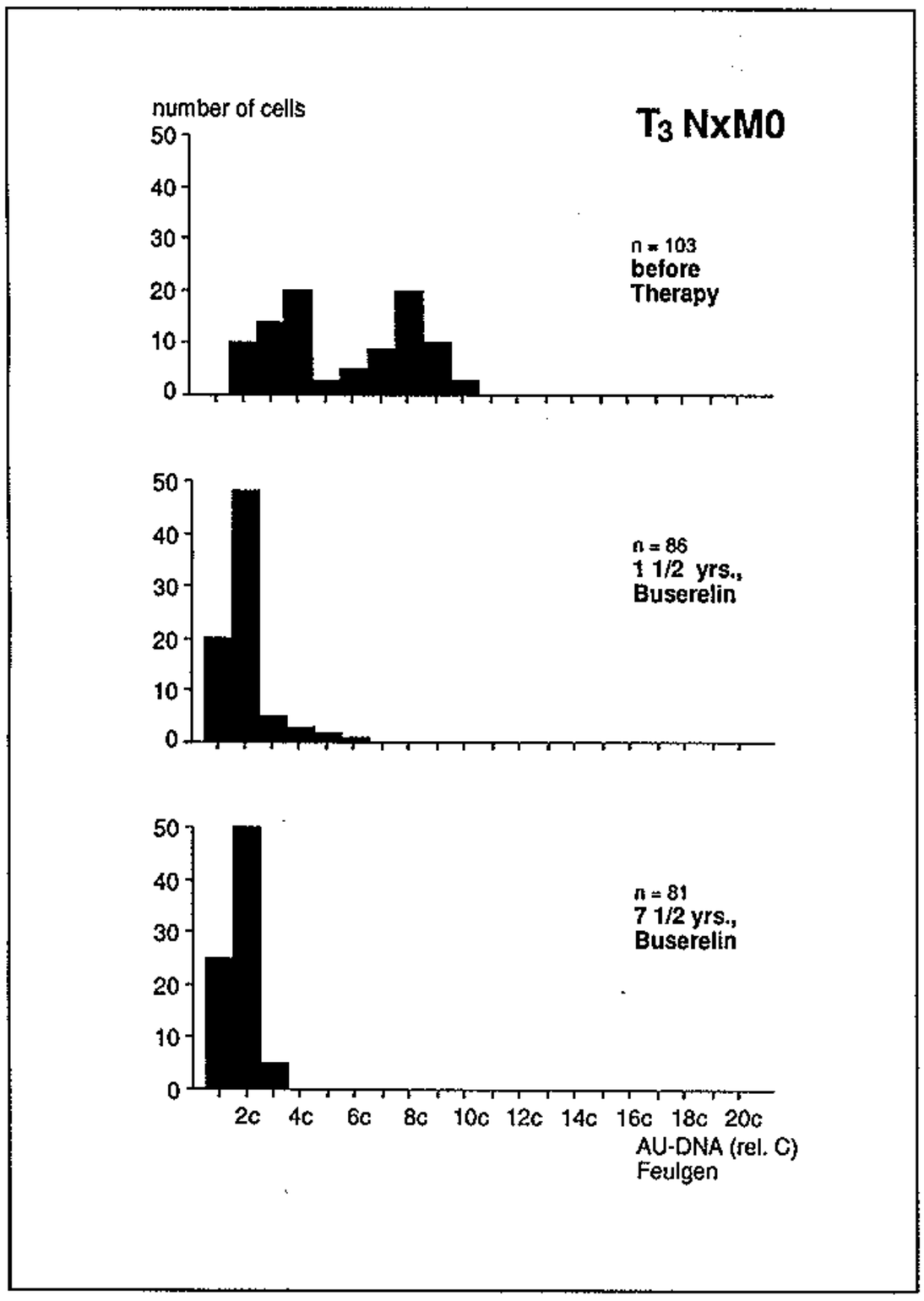


Fig. 8. DNA histograms for patient W.K. (T_3 NxM0) prior to therapy, after 1.5 years of buserelin therapy, and after 7.5 years of buserelin therapy. Good therapeutic effect.

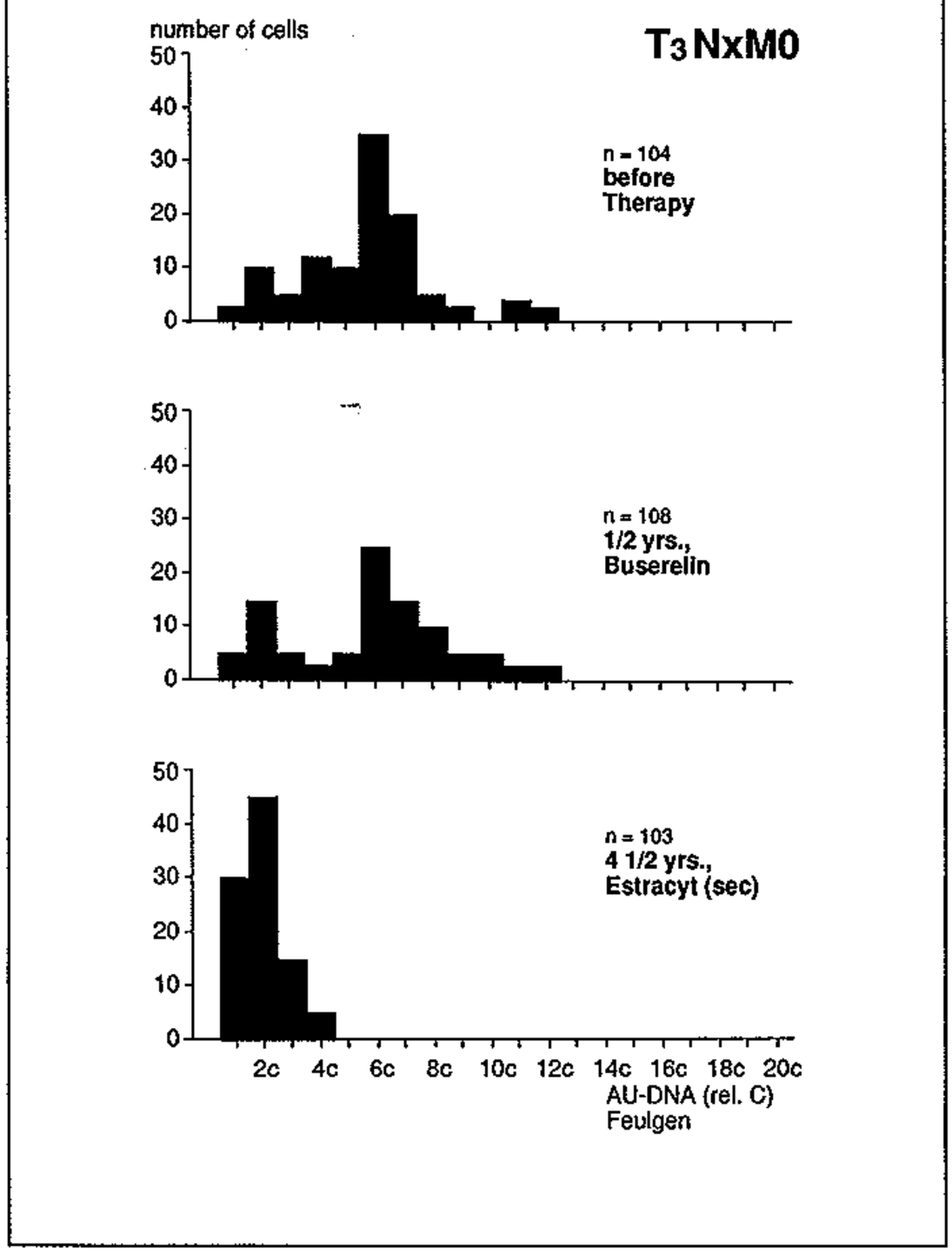


Fig. 9. DNA histograms for patient F.Sch. (T_3 NxM0) prior to therapy and after 6 months of buserelin therapy: unsatisfactory therapeutic effect; after 1.5 years of secondary treatment with Estracyt: satisfactory therapeutic effect.

years after the change in therapy, there is a statistically significant DNA frequency peak in the diploid range signalling good regression. This result corresponds with the morphological findings (fg. 7c) and the clinically stable condition of the patient, i.e. there is no evidence of metastatic development.

Case 4. F.H., T_3 NxM0, grade III carcinoma (score 17). This is an example of primary resistance to buserelin and resistance to secondary Estracyt therapy as well (fig. 10). Prior to buserelin therapy, there are DNA frequency peaks near 5c and 7c, another near 9c, and some values reach as far as 16c. After 6 months of buserelin therapy, the DNA frequency peak is in the octaploid range, with values spreading to 19c. As such a DNA distribution pattern definitely signals a very poor response to treatment, therapy was changed to Estracyt. After 6 months of secondary Estracyt therapy, the tumor proved to be resistant to Estracyt as well. It is demonstrated that the DNA distribution beyond the 19c range. The histogram shows a pronounced aneuploidy.

This finding was confirmed clinically by a breakthrough of the tumor into the urinary bladder which was detected for the first time then, although no bone metastases were found yet at this point. Therefore therapy was changed to cyclophosphamide, the third form of treatment. Despite this change in therapy the patient died from widespread metastases after 8 months of treatment with cyclophosphamide.

DNA Ploidy, Heterogeneity of DNA Distribution and Grade of Malignancy

The DNA distribution in prostatic carcinoma shows DNA values ranging from 2c (diploid) to 19c. The ploidy values of DNA frequency peaks most frequently seen were in the 5c, 6c, 9c, 10c and 12c ranges.

The DNA analyses in the 32 patients with grade I carcinoma showed diploid (2c) DNA distribution in 71%,

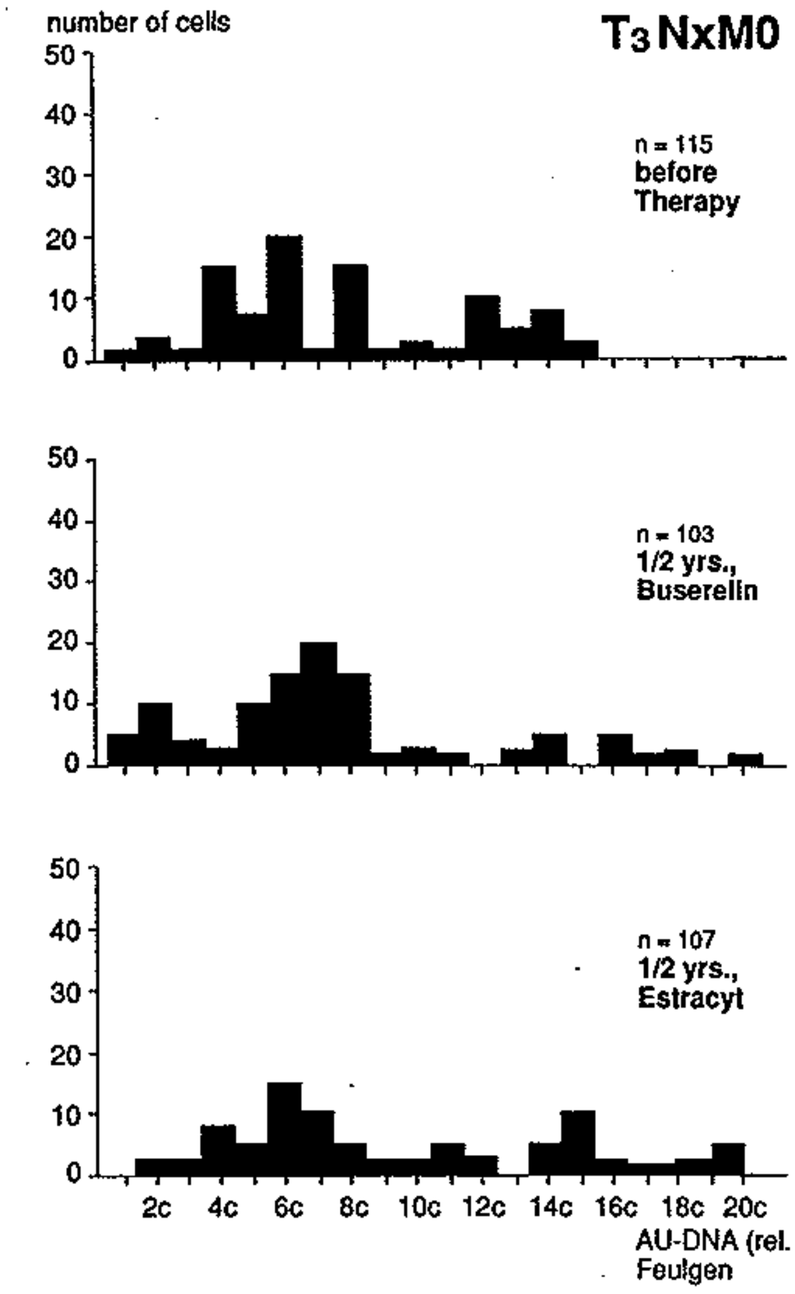
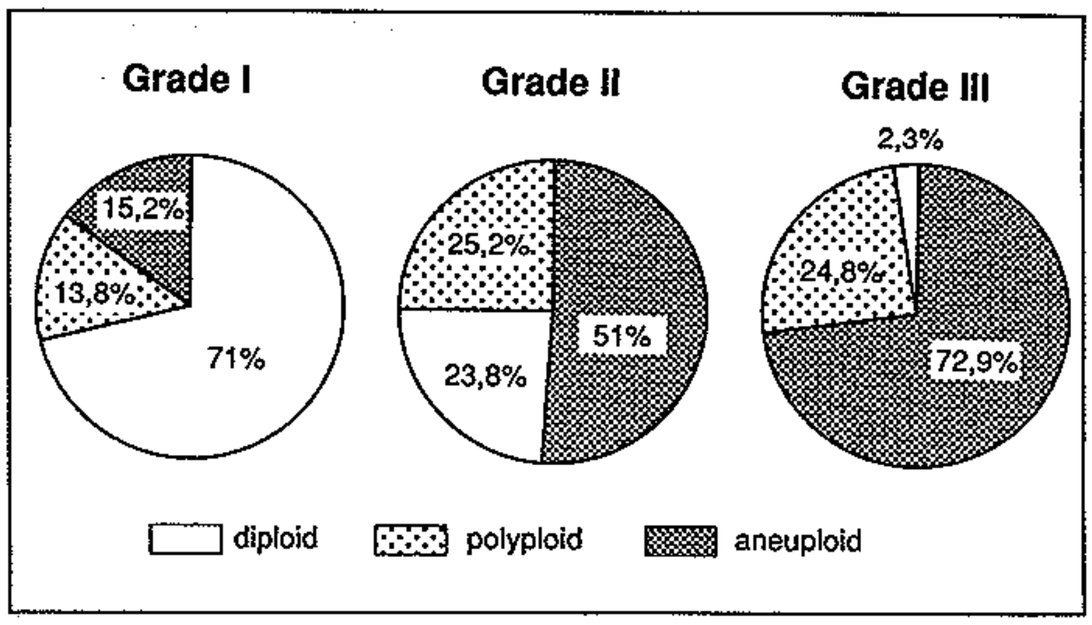


Fig. 10. DNA histograms for patient F.H. (T_3 Nx M0) prior to therapy, after failure of buserelin therapy, and after 6 months of unsuccessful secondary treatment with Estracyt.



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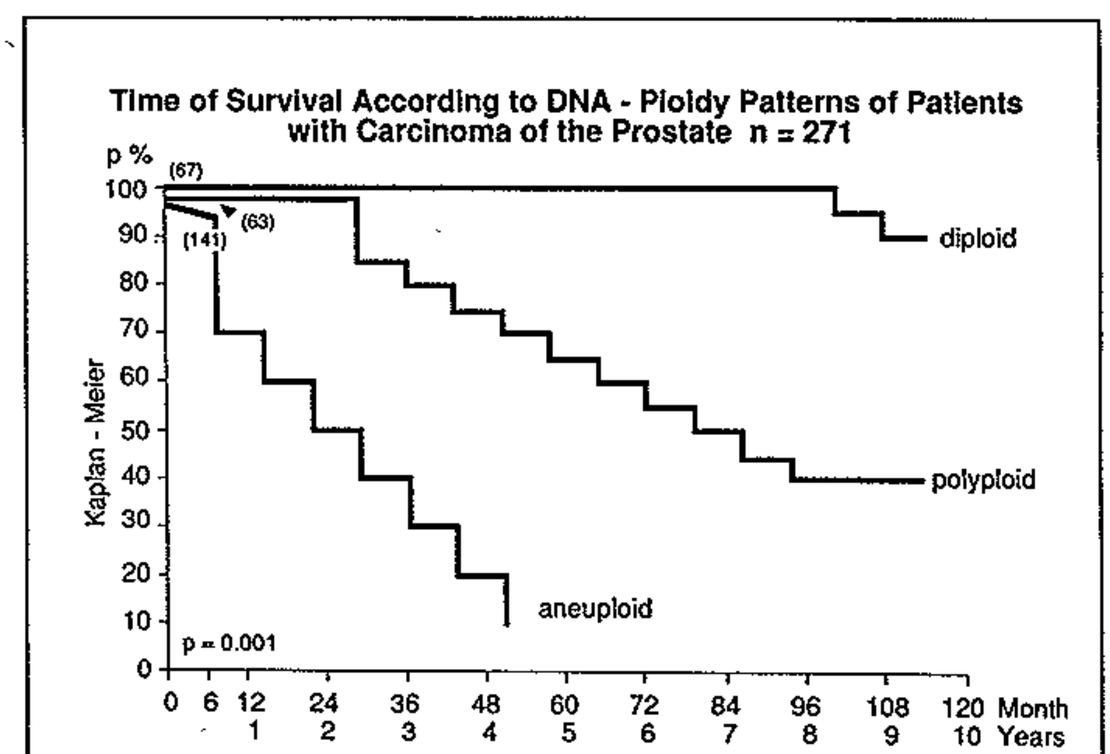

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Fig. 11. Correlation between the grades of malignancy and the DNA contents of several tumor cell nuclei for 271 patients with prostatic carcinoma.

Fig. 12. Survival time of patients with carcinoma of the prostate, grouped according to DNA ploidy (n = 271).

tetraploid (4c) DNA distribution patterns in 13.8% and aneuploid (3c and 5c) DNA distribution in 15.2% of the tumor cell nuclei measured (fig. 2, 11).

The 174 patients with gade II carcinoma showed highly varying DNA frequency patterns although the cytomorphological differentiation of the tumors was uniform.

As can be seen from figure 11, the DNA values measured were in the diploid (2c) range 23.8%, in the polyploid (4c) range 25.2% and in the aneuploid (3c, 5c) range 51% of the tumor cell nuclei measured.

As for the 65 patients with grade III carcinoma, only 2.3% of the tumor cell nuclei measured exhibited diploid DNA distribution, whereas 24.8% showed polyploid DNA values and 71% exhibited aneuploid DNA values with more than two DNA frequency peaks and scattered DNA ploidy values up to 19c (fig. 4, 11).

Grade of Malignancy and 4.5c Exceeding Rate

A reliable diagnosis of the malignancy of a tumor can be made by using the 4.5c exceeding rate, a statistical parameter indicating the percentage of clearly aneuploid tumor cell nuclei. Table 4 shows that grade I carcinomas contain 15.2% of aneuploid tumor cells whereas grade II and grade III carcinomas exhibit an increase in this rate to 51 and 71%, respectively. Patients with a high rate of aneuploidy suffered clinical progression earlier than patients with minor rates of aneuploidy.

Prognostic Relevance of DNA Ploidy

During follow-up periods of up to 9 years, 67 (25%) patients with a DNA frequency peak in the diploid (2c) range (fig. 2) developed no metastases and showed no tumor progression, as can be seen in the tables 5 and 6.

65 of the 141 patients (52%) with two or more DNA frequency peaks or with widely scattered aneuploid DNA distribution (fig. 3, 4) already had distant metastases when they were first diagnosed, and they died of carcinoma within the first 22 months. 73 of the remaining 76 patients in this group developed bone metastases after periods ranging from 9 to 36 months following diagnosis; 67 of them died of carcinoma within the follow-up period.

33 of the 63 patients with polyploid DNA distribution tending towards diploid had distant metastases and died of carcinoma despite a change in therapy; another 6 patients in this group remained clinically stable (fig. 12).

Discussion

The results of the DNA analyses presented in this study are indicative of the variable biological behavior of prostatic carcinoma and thus of its heterogeneous nature. The few studies published to date on investigations of prostatic carcinoma by means of both single-cell cytophotometry and flow-through cytophotometry show differing results and report high DNA values particularly for poorly differentiated tumors [1–3, 6, 11, 22, 23, 28, 29].

This investigation established the rate of DNA aneuploidy for highly differentiated tumors at 15% and for poorly differentiated tumors at 71%. The results of the DNA analyses of cell nuclei reported in this study and the findings of other authors have shown that the measuring of the DNA content of cells forms a reliable basis for a quantitative evaluation of the success of failure of the therapy in question. A shift towards euploidy or an increase in the hypodiploid DNA distribution and a decrease in the aneuploid DNA values seen prior to therapy indicate a therapy-induced remission of the tumor. If the DNA distribution pattern remains aneuploid during therapy, it is a sign of tumor progression [11, 14, 22, 23, 28].

The prognosis tends to be more negative when cytophotometry shows widely scattered DNA values. The relation of diploid and aneuploid cells is of similar significance [1–5, 7, 11, 14, 22, 34]. This relation is especially relevant in respect of grade II (grade of malignancy) carcinomas which are morphologically homogeneous but have highly varying malignant potentials.

DNA histograms show the distribution of the cell cycle phases in a cell population measured. The individual tumor cell nuclei are assigned to the different phases of the cell cylce (G0/G1, S phase and G2/M phases) according to the DNA content. Changes in cellular genetics result in corresponding changes in the DNA histograms [2–4, 8–10].

If the rate of diploid cells (G0/G1 phase) is $> 60\%$, the prognosis for the patient is more favorable than with a rate of $< 60\%$. In such a case, a high rate of cells in the G0/G1 phase can be taken to signal a low growth rate of the tumor. The same differences in respect of survival periods can be seen in the groups with grade II and grade III carcinoma showing a decrease in diploid cells and an increase in aneuploid cells.

Table 4. Distribution of 4.5c exceeding rate according to grade of malignancy

Grade	n	4.5c exceeding rate, %
I	32	15.2
II	174	51.0
III	65	73.0

Table 5. Nuclear DNA ploidy pattern obtained by single-cell cytophotometry compared with clinical findings and bone metastases in 271 patients with prostatic carcinoma

	Total		M1		Died		Clinically stable	
	n	%	n	%	n	%	n	%
Diploid	67	25	–	–	8[a]	12	59	88
Polyploid	63	23	33	52	33	52	24	38
Aneuploid	141	52	138	98	137	97	4	3
Total	271		171	63	170	63	87	32
					14[a]	5		

[a] Cardiocircular failure.

Table 6. Survival chances of 271 patients with prostatic carcinoma, grouped according to DNA ploidy

	Survival probabilities, %		
	3 years	5 years	9 years
Diploid	99	97	88
Polyploid/diploid	71	51	38
Aneuploid	34	6	–

According to Kaplan-Meyer: $p = 0.001$.

The single cell cytophotometric analyses of 271 patients in this study enabled the authors to draw valid clinical and prognostic conclusions on the basis of the rates obtained for the ploidy, the DNA frequency peaks and the phases of the cell cycle.

Acknowledgements

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