Acta Oncologica Vol. 30 No. 2 1991

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PLOIDY LEVEL AND TUMOR PROGRESSION IN PROSTATIC CARCINOMA

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Abstract

Methods developed for cytophotometric analysis of archival tumor specimens used in retrospective studies were evaluated quantitatively. Up to 20-year-old May-Grünwald-Giemsa stained cytological slides could be Feulgen stained with excellent results. By expressing DNA data from the tumor cells as relative values (c-values) related to the internal staining control (mean value 2c) detailed ploidy level determinations could be made as accurately from measurements of old, destained slides as from measurements of cells from fresh tumor material, which were directly Feulgen-stained. Ploidy level determinations of prostatic carcinoma in 213 patients selected on the basis of survival time were analyzed. By studying the tumors in two extreme groups (extreme group I: 131 patients who died from prostatic cancer within 3 years of diagnosis, and extreme group II: 82 patients who survived more than 15 years after diagnosis) it was possible to evaluate in detail the optimal limits for defining the diploid 2c-region and tetraploid 4c-region. Using these limits to determine the percentage of aberrant tumor cells, i.e. non 2c and non 4c, and combining this with the modal value (in c units) of the tumor cell population the tumors could unambigously be divided into near-diploid-D-, near-tetraploid-, T- and highly aneuploid A-types. The prognostic significance of ploidy level was studied in prostatic carcinoma in a non-selected group of patients subjected to endocrine therapy and long-term clinical follow-up (up to 23 years). This patient group consisted of all of the patients who were diagnosed as having prostatic carcinoma by means of fine-needle aspiration biopsy at the Karolinska Hospital during 1966. The A-type tumors progressed rapidly and killed 96% of the patients within 5 years (and all patients within 7 years). D- and T-type tumors progressed much more slowly. None of the patients with these tumors died from the tumor disease within the first 5 years after diagnosis, and 12% of the patients (crude survival) were still alive 15 years after diagnosis. Ploidy level was superior to morphological grade and clinical stage (tumor size) as a prognostic indicator.

Key words: Prostate cancer, ploidy, tumor progression.

In order to study the relationship between the clinical course of human tumors and the gross ploidy level, determined from the nuclear DNA content of individual tumor cells, procedures have previously been introduced for retrospective studies (1–3). These procedures allow quantitative cytophotometric Feulgen-DNA analysis of old May-Grünwald-Giemsa stained cytological smear-preparations used in clinical cytopathological diagnostics and stored in our files for 10 to 20 years. In retrospective studies the cytophotometric DNA-data could be directly related to the known clinical course of various human tumors. Different cytophotometric DNA criteria were used for separating low-grade from high-grade malignant variants. Using the frequency of cells with elevated DNA values as a gross measure of degree of aneuploidy, it was found that prostatic tumors with diploid or near-diploid DNA contents were less malignant in terms of survival rate than tumors with highly aneuploid DNA values (3–6).

In most studies performed with slide cytophotometric methods, frequency of cells with elevated DNA values above the normal diploid level (usually more than 2.5 c-units) was successfully used as a parameter of aneuploidy. However, in prostatic carcinoma (3–6) as well as in breast cancer (4, 6, 7) some of the tumors with abnormally high DNA contents, possibly representing tetraploid (or near-tetraploid) tumors, show a more favorable clinical course than most of the tumors with abnormally increased, clearly aneuploid DNA values.

The degree of prognostic information provided by ploidy level, as determined from nuclear DNA content in relation to other prognostic parameters, is still somewhat unclear. One reason for this is the fact that different methods for DNA determinations and different definitions of ploidy derived from DNA data have been used (3–13).

Using the methods referred to in the present paper (1–3, 14) and the criteria discriminating near-diploid (or near-tetraploid) tumors from highly aneuploid tumors

Presented at the Conference on Early Prostatic Cancer. The WHO Collaborating Centre for Urological Tumours, Karolinska Hospital, Stockholm, March 21–23, 1990.

Accepted for publication 29 November 1990.

(15), ploidy level determination is an extremely powerful prognostic parameter.

Material and Methods

Destaining. Fine-needle aspiration biopsy material from 12 patients with prostatic carcinomas was selected. The biopsy material was ejected onto a glass slide and then divided in two halves by means of a cover slip. One half was rapidly smeared on a slide, briefly air dried, fixed in 10% neutral buffered formalin and stained according to the modified Feulgen procedure, involving acid hydrolysis in 5 N HCl for 1 h at room temperature, 22°C (1, 16). The remaining half was processed for conventional morphologic analysis, i.e. air dried and May-Grünwald-Giemsa (MGG) stained. After a storage time of two years the MGG-smears were destained in absolute methanol (99.8%), refixed in 10% neutral buffered formalin and subsequently Feulgen stained (2, 3). Treatment in absolute methanol for 1–3 weeks efficiently removed most of the previous stain. The small amount of stain remaining after this procedure was completely removed during the acid Feulgen hydrolysis. After the methanol treatment the cells were refixed in 10% neutral buffered formalin for a period of 12–24 h. This refixation stabilizes the nuclear chromatin and thereby minimizes the risk of losing DNA during the acid Feulgen hydrolysis (2–3). Further details of the staining procedure have been published elsewhere (14).

Clinical material. I. Extreme groups. During the period 1960–1969, 3 874 patients with a primary diagnosis of prostatic carcinoma were registered in the Swedish Cancer Register from the Stockholm county. From the material 213 patients, diagnosed by fine-needle aspiration biopsy, were selected on the basis of survival time. The only treatment these patients received was estrogen. Two extreme groups were chosen. Extreme group I consisted of 131 patients with progressive tumor disease and death from cancer in less than 3 years after diagnosis as documented in clinical records and autopsy protocols. Extreme group II consisted of 82 patients who survived more than 15 years after diagnosis. In group II, 54 patients had died from cancer after more than 15 years and 28 were still alive at the time of this study (1988).

Clinical material. II. Consecutive patient material. The material consisted of 145 patients with prostatic carcinoma. All patients were diagnosed by fine-needle aspiration biopsy (FNA) at the Karolinska Hospital and registered in the Swedish Cancer Registry in 1966. All the 145 patients had initially received estrogen therapy. Furthermore, orchidectomy was performed in 7 cases, and in 11 cases radiation therapy was given after tumor progression. None of the patients underwent surgery in the form of total or partial prostatectomy. Seventy-four patients died from prostatic cancer, 5 were still alive after more than 22 years, and the remaining 66 patients died from intercurrent diseases. Cytological grading was performed in 142 cases. The smears were graded according to a previously described system (17–19). Clinical stage of the tumors were classified according to the UICC 1987 (20) as T (size of primary tumor) and M (distant metastasis). Twelve cases were primarily not classified with respect to tumor size. Only 6 patients had distant metastases at diagnosis. No information about the nodal status (Nx) of the tumor was available.

Cytochemical procedures. The original May-Grünwald-Giemsa (MGG) stained smears, on which the primary diagnosis of prostatic carcinoma was based, were used for the cytophotometric DNA-analysis. The slides were destained in absolute methanol for 1–3 weeks, refixed in 10% neutral buffered formalin for 12–24 h (2, 3, 14) and restained according to a modified Feulgen staining procedure (acid hydrolysis in 5N HCl for 60 min at 22°C) (1, 16). The cytophotometric measurements of Feulgen stained cell nuclei were performed using a modified technique (see below) based on a previously described photographic method (21). A Leitz photomicroscope with a plan apochromatic 40 × 1.0 oil objective (refractory index 1.518) in monochromatic light, wavelength 546 nm, was used. The sensitivity used for the film (Kodak Technical Pan 2415) was 29 DIN/640 ASA, the development time was 4 min at 22°C (Kodak D19), and the films were fixed for 8 min at 22°C (Kodak F24). Representative tumor areas were selected for photography for each slide. In general, 75 tumor cells were measured. Granulocytes were used as internal staining controls. The median value (P50) of the control cells was calculated in order to determine the normal diploid, 2c, content of DNA. The 2c value was determined from the measurements of 10–20 control cells. All measured values of the tumor cells were expressed in c-units as defined by the DNA-content of the corresponding (granulocyte) control cells. To distinguish near-diploid and near-tetraploid tumor cell populations from highly aneuploid tumors, the near-diploid 2c-region (<2.5c) and the near-tetraploid 4c-region (3.5c–4.5c) were determined for each slide. The modal value was defined as the most frequent c-value using a class width of 0.5c. D- or T-type tumors were defined as tumors in which more than 50% of the tumor cells had DNA values within the diploid and tetraploid region (15). A-tumors were defined as tumors in which more than 50% of the tumor cells had DNA values deviating from these regions (15).

Statistics. Crude survival was defined as survival without regard to cause of death and relative survival as survival with regard to death from cancer. Expected survival was survival derived from life tables of the Swedish male population in 1966 (22), adjusted for age and geographic area.

Results

The accuracy of the MGG-destaining Feulgen-restaining procedure is illustrated in Figs 1 and 2. Both in the near-diploid (DNA-values predominantly around 2c, Fig.

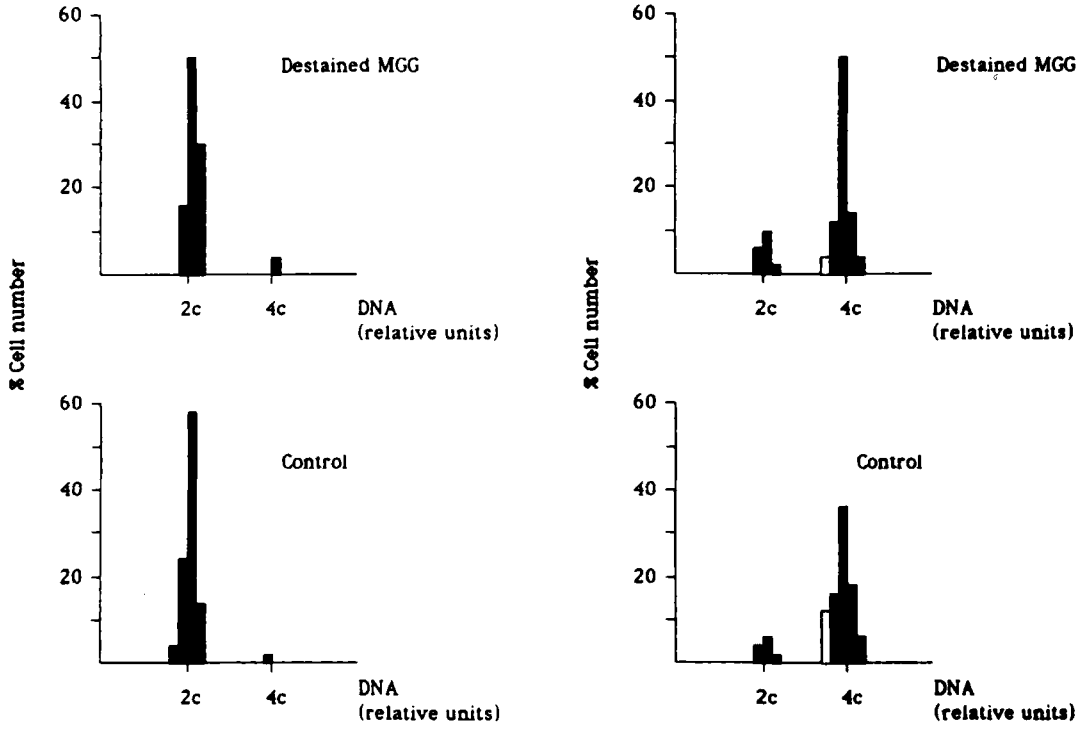

Fig. 1. Feulgen-DNA histograms (expressed in c-units) of a diploid (or near-diploid) prostatic carcinoma (left) and a tetraploid (or near-tetraploid) prostatic carcinoma (right). The top histograms (destained MGG) represents Feulgen-stained cells after MGG-destaining. The bottom (control) represents freshly Feulgen-stained smears from the same tumor.

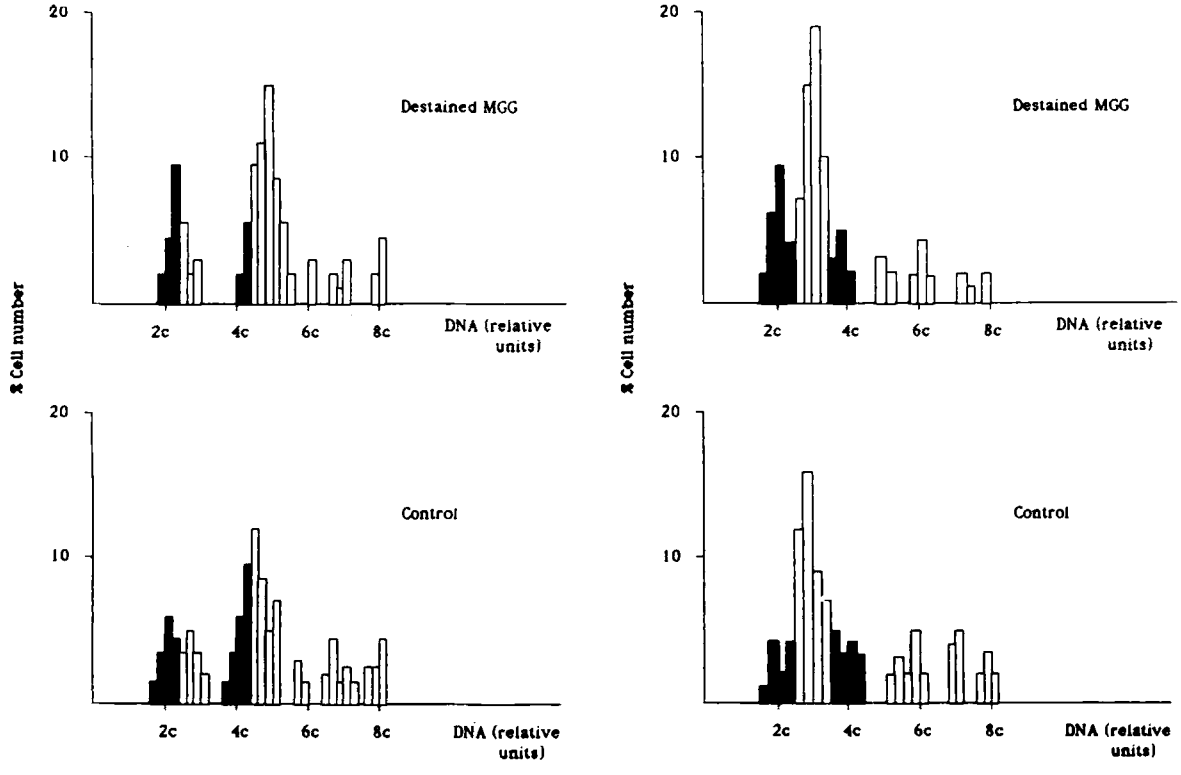

Fig. 2. Feulgen-DNA histograms expressed in c-units of two highly aneuploid prostatic carcinomas. To the right a tumor cell population with DNA-values predominantly between the 2c- and 4c-region, and a tumor cell population with DNA-values predominantly above the 4c-region (left). The top histograms are measurements of tumor cells of a Feulgen-stained destained MGG-smear. The bottom histograms (controls) are tumor cells of a freshly Feulgen-stained smear preparation from the same tumor.

1 left) and the near-tetraploid (DNA-values predominantly around 4c, Fig. 1 right) tumors and in the highly aneuploid tumors (DNA-values predominantly between the 2c-region and the 4c-region, Fig. 2 right, or DNA-values predominantly above the 4c-region, Fig. 2 left) the Feulgen DNA-distribution curves obtained from the destained MGG-smears are essentially identical to corresponding control smears that were Feulgen-stained directly after fixation without any previous MGG-staining. Thus, separation into near-diploid, 2c-tumors (D), near-tetraploid, 4c-tumors (T) and highly aneuploid tumors (A) can be as accurately performed in old, destained MGG-smears from prostatic carcinoma as in fresh tumor specimens that are Feulgen-stained directly after fixation.

The accuracy with which D-, T- and A-tumors can be discriminated from each other is dependent on which limits are used for defining the 2c- and 4c-region. This was studied in detail by comparing highly malignant tumor variants (extreme group I, death from cancer within 3 years) with considerably less malignant variants (extreme group II, alive more than 15 years after diagnosis). Different upper limits for the 2c window (2.25c, 2.5c and 2.75c) were tested as well as the range of the 4c window (3.6c–4.4c, 3.5c–4.5c and 3.2c–4.8c). In order to determine the limits that give the best discrimination between extreme groups I and II the percentage of tumor cells outside these windows (non 2c plus non 4c) was used as a denominator of ploidy abnormality. Fig. 3 shows that clear separation of the two extreme groups is obtained by using 2.5c as an upper limit for the 2c-region and a width of the 4c-region of 3.5c–4.5c.

The tumors were further characterized by combining the percentage of cells outside the 2c- and 4c-regions with the modal DNA-value of the tumor population (Fig. 4). Three different categories were found. Extreme group I (highly

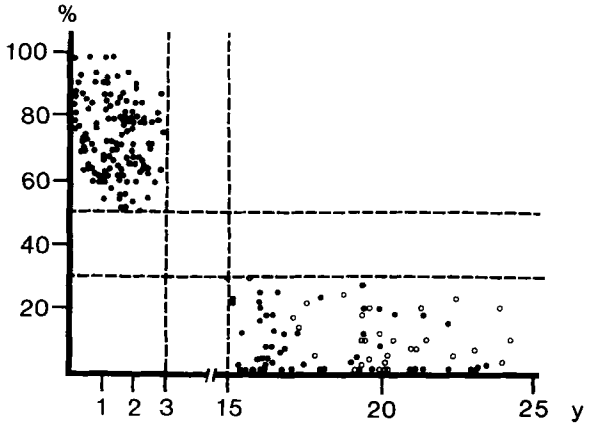

Fig. 3. The relation between survival time (in years) and percentage of tumor cells, which are non 2c and non 4c in 213 cases of prostatic carcinoma. Extreme group I (dead <3 years) 131 cases; extreme group II (alive >15 years) 82 cases. 2c-region: 2.5c, 4c-region: 3.5c–4.5c. ● = dead patient; ○ = patient still alive.

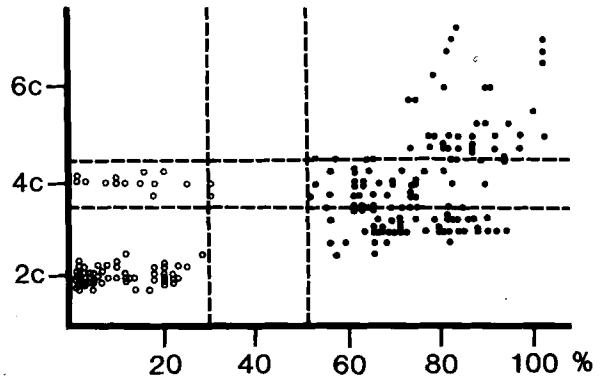

Fig. 4. The relation between percentage of tumor cells which are non 2c and non 4c and the modal value in c units of the tumor cell population. ● = Extreme group I (dead < 3 years) 131 patients. ○ = Extreme group II (alive > 15 years) 82 patients. 2c-region: <2.5c, 4c-region: 3.5c–4.5c.

malignant, filled circles) showed modal values between 2.5c and 7.25c. In extreme group II, (low-malignancy, open circles) tumors with modal values around 2c (1.75c–2.5c) or around 4c (3.75c–4.25c) were found. By combining these two parameters the tumors can be accurately separated into D-, T- and A-tumors.

Using the procedures described above for cytophotometric DNA determinations in cytological slides the prognostic significance of ploidy level was studied in a clinical material of 145 patients with prostatic carcinoma (see further Material and Methods, clinical material II). The relation between ploidy level and tumor stage is illustrated in Table 1. D-, T- and A-tumors were all represented among T2 and T3 categories. However, D- and T-tumors were slightly more common among T2, while A-tumors were more common among T3.

Table 2 shows the distribution of ploidy among the three

Table 1

Classification of patients by ploidy and by T- and M-categories

Tumor type	No. of patients (%)				No. of patients M1
	Total	T2	T3	Not T-classified	
D and T	76 (52)	53 (37)	22 (15)	1	–
A	69 (48)	31 (21)	30 (21)	8 (6)	6
Total	145 (100)	84 (58)	52 (36)	9 (6)	6

Table 2

Classification of patients by ploidy and by grade

Tumor type	G I (%)	G II (%)	G III (%)	Not graded (%)	Total (%)
D and T	28 (19)	44 (30)	4 (3)	0	76 (52)
A	10 (7)	32 (22)	24 (17)	3 (2)	69 (48)
Total	38 (26)	76 (52)	28 (20)	3 (2)	145 (100)

different grades. Again, D-, T- and A-tumors were found among all grades. However, D- and T-tumors were more common among grade I-tumors while A-tumors dominated among the grade III-tumors. D- and T-tumors on one hand and A-tumors on the other hand were approximately equally represented among the grade II-tumors. Fig. 5 shows crude survival curves and expected survival curves for matched control populations (see definition in Material and Methods) of patients with A-tumors (triangles) and D- or T-tumors (circles). Only 4% of the patients with A-tumors were alive 5 years after diagnosis, which differs significantly from the expected survival of a control population (68%). In contrast, no increased mortality was found among the patients with D- or T-tumors in relation to a control population within the first 5 years after diagnosis. However, an increased mortality was found among the D- and T-tumors after the first 5-year-period, although as many as 12% of these patients were still alive 15 years after diagnosis, which constitutes 43% of the expected survival of the control population (28%).

In order to further analyze the correlation between ploidy and cause of death the relative survival curve (death from cancer calculated (23) from the crude survival curve in Fig. 5 and death from intercurrent disease) was compared with a survival curve based on a selected patient group (36 A-tumors and 34 D- and T-tumors) in which death from cancer was recorded from clinical records and autopsy protocols. These results are presented in Fig. 6. A close agreement is found between the survival curve (solid line) based on calculated relative survival and the survival curve (dotted line) based on the selected patient group observed to die from progressive cancer disease. Crude survival with respect to grade and stage was studied for A-, D- and T-tumors separately (Figs 7–8). It is evident that tumors of D- or T-type show a more

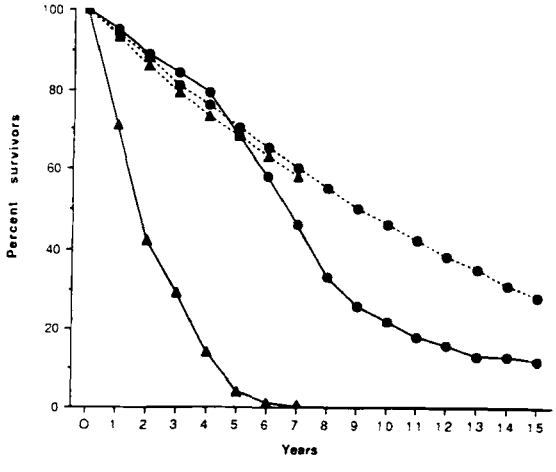

Fig. 5. Life-table showing crude survival of patients with A-type (▲) and D- or T-type (●) tumors. The dotted lines represent expected survival for each ploidy type. A-type = 69 cases and D- or T-type = 76 cases.

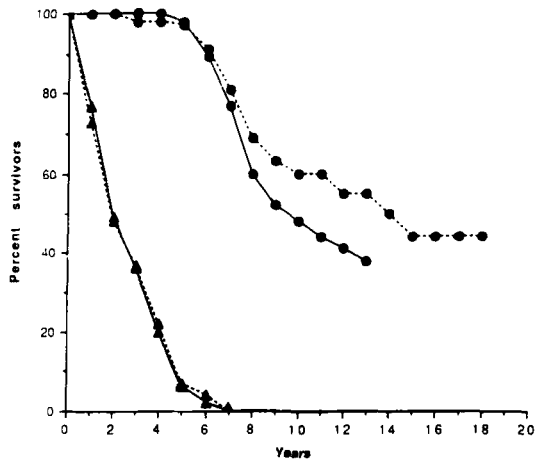

Fig. 6. Life-table showing relative survival (RS) of patients with A-type (▲) and D- or T-type (●) tumors. The dotted line shows the RS of a selected patient material and the continuous lines show the RS calculated from crude survival data. A-type n = 53, D- or T-type n = 27 cases.

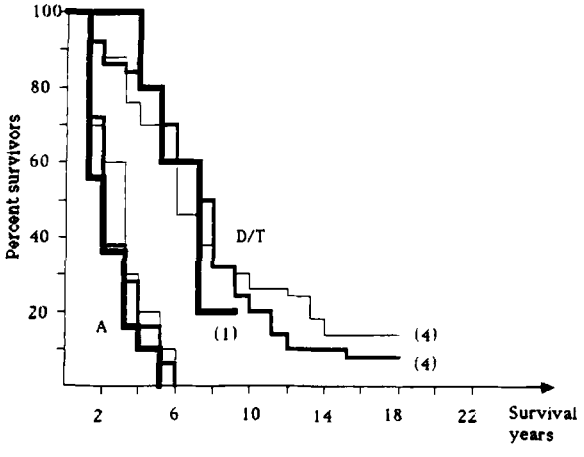

Fig. 7. Life-table showing crude survival for patient with D- or T-type and A-type tumors with respect to grade. Number of patients still at risk within parentheses. — G1; — G2; ▬ G3.

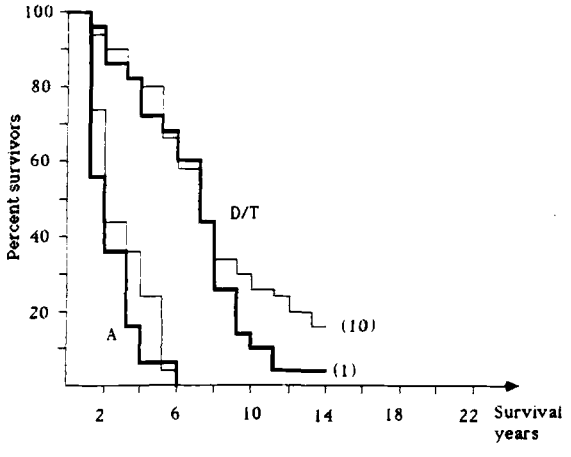

Fig. 8. Life-table showing crude survival for patients with D-/T-type and A-type tumors with respect to tumor size. Number of patients still at risk within parentheses. — T2; — T3.

favorable prognosis than tumors of A-type, irrespective of grade (Fig. 7). An analogous result was found comparing tumor stage (T-categories) and ploidy (Fig. 8). Thus, survival was primarily related to ploidy level rather than to grade or stage.

Discussion

The possibility of using old, archival tumor specimens in studies of the prognostic value of cytophotometric DNA analysis has obvious advantages. In retrospective studies the cytophotometric DNA data can be directly correlated to the known clinical course of the tumor disease, thereby circumventing the need for long-term prospective investigations. By using procedures for retrospective studies on cytological slides (2, 3, 14) meaningful prognostic information could be obtained from cytophotometric DNA analysis of prostatic carcinoma.

In a recent study (15) cytophotometric DNA-criteria have been determined to discriminate between high-grade (death from prostatic cancer within 3 years) and low-grade (survival with the tumor disease at least 15 years after diagnosis) malignant tumors. Using these criteria, which are based on ploidy level and degree of deviation from normal prostatic epithelium and/or benign prostatic lesions, high-grade malignant carcinomas of the prostate could unequivocally be classified as highly aneuploid A-type tumors, and low-grade malignant tumors could be classified as either near-diploid, D-type or as near-tetraploid, T-type (15).

In the present study the relationship between ploidy level and tumor progression was investigated in prostatic carcinoma. The relation between morphological grade, clinical stage, ploidy level and clinical course of the tumor disease was studied in a patient material with at least 20 years' follow-up. The material consisted of all of the patients with the diagnosis prostatic carcinoma made by fine-needle aspiration biopsy at the Karolinska Hospital during 1966. There is no reason to believe that this patient material is selected or non-random as the material had the same distribution with respect to age and cytological grade as a previous investigation, which consisted of a consecutive patient material irrespective of source of diagnosis (17). The two patient materials corresponded thus in this respect. The relation between ploidy level and clinical course was studied by two independent methods: a) on selected patients who died from progressive cancer disease as judged from clinical and autopsy protocols, and b) on an unselected patient group in which the cancer mortality was calculated from crude survival data. A good agreement was found between the two methods, suggesting that the ploidy data could indeed be correlated with death.

Highly aneuploid, A-type, tumors progressed and killed nearly all patients within 5 years (Fig. 6) whereas few patients with D- or T-type tumors died from their tumor disease within the first 5 years. However, after the first 5-year period tumors of the D- and T-type did in fact also progress, metastasize and eventually kill the patients. The rate at which the D- or T-tumors progressed was, however, considerably lower than that of the A-tumors. Whether this is an effect of the endocrine therapy or a result of the inherently low degree of biological malignancy among the D- and T-tumors remains to be investigated.

The prognostic significance of morphological grade was compared with that of ploidy level. Previous observations (5) showing a correlation between grade and ploidy were confirmed in this study. Grade I tumors were commonly of D- or T-type, and grade III tumors were of A-type. Among grade II tumors the D- or T-type and the A-type were approximately equally common. Although grade has a prognostic importance as shown previously (17) and furthermore confirmed in this study, a multivariate analysis showed that morphological grade provided no additional prognostic information to that obtained from ploidy data. This was also illustrated by the fact that grade I tumors of A-type had a poor prognosis, whereas grade III tumors of the D- or T-type showed as favorable a prognosis as the rest of the D- or T-tumors. In other words, the clinical course of the patients was mainly related to ploidy level, while grade was of secondary importance. Whether this finding is true also when comparing ploidy level with other grading systems (24) remains to be proved but it seems most probable.

The mechanism of aneuploidy formation and the biological nature of the difference between the near-diploid (or near-tetraploid) D-tumors (or T-tumors) and the highly aneuploid A-tumors is unclear. As has been suggested in other studies (25–28), the frequency of cells with S-phase DNA-values tends to be comparatively higher among highly aneuploid tumor variants, and S-phase data have also been suggested to have prognostic significance (25, 28). This could reflect a higher proliferative rate with more cells actually replicating DNA in the highly aneuploid tumors, as suggested from bromodeoxyuredin (BrdU) and thymidine labelling data, but it could also reflect an increased heterogeneity in ploidy or reflect cells arrested in the S-phase due to defects in the control of DNA-replication. Whether related to abnormalities in DNA-replication or not it is, however, likely that the difference between the D- or T-tumor variants and the A-tumor variants also depends on an underlying difference in the stability of the genome. It is not unlikely that A-tumors more frequently undergo rearrangement mutations, such as deletions or gene amplifications, which in turn could facilitate tumor progression. Irrespective of the genetic background behind abnormality in ploidy level and its relation to malignancy and tumor progression, the cytophotometric identification of D-, T- and A-tumors provides a powerful tool in grading the malignancy of prostatic carcinoma, which has its obvious place in the clinical routine of tumor diagnostics.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Medical Research Council, the Swedish Cancer Society, the Cancer Society in Stockholm and the research funds of the Karolinska Institute, Stockholm, Sweden.

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