ANTICANCER RESEARCH 8: 129-136 (1988)

# DNA-Grading of Prostatic Carcinoma: Prognostic Validity and Reproducibility

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**Abstract.** *The prognostic significance of the DNA-Malignancy-Grade (DNA-MG) was tested in 52 prostatic carcinoma patients in comparison with a cytomorphological grading system (10) and the clinical staging. Papanicolaou- or MGG stained smears from transrectal aspiration biopsies were automatically restained according to Feulgen in a modified Shandon staining machine. The DNA-MG, based on the variation of the nuclear DNA content of tumor cells around the normal DNA (2c) peak, ranges on a continuous scale from 0.01 to 3.0. It was determined with a TV-image analysis system (Leitz, TAS plus), combined with an automatic microscope. The DNA-MG revealed a high prognostic validity, the clinical stage showed only a minor influence on the survival time and the cytopathologic grading system was of insufficient prognostic information. Significant differences in survival time could be demonstrated between patients with DNA-MGs of 0.01 - 1.5 and 1.5 -3.0, as well as with DNA-MGs 1.0 - 2.0 and 2.0 -3.0. The intra- and interobserver variations of the DNA grading system were found to be $\sigma = 0.014$ and $\sigma = 0.036$ respectively. The reproducibilities were 86.7% for intra-and interobserver measurements, when the continuous DNA-MG scale was divided in three groups.*

Histogenetic classification, grading and staging of malignant tumors yield relevant information for the clinical management and treatment planning of cancer patients. This also holds for prostatic carcinoma patients. During the past decades many attempts have been made to establish histopathological, and later also cytopathological, grading systems for the morphologic grading of prostatic carcinomas (1-9). However, contradictory results of intra- and interobserver reproducibilities have been published for some of these grading systems. The intraobserver reproducibilities of morphologic grading systems for prostatic carcinomas range from 71% to 90%. Interobserver reproducibilities were 38 to 86% for different prostatic carcinoma grading systems (1, 2, 10-14). Besides problems with their reproducibility, grading systems based on the subjective evaluation of morphologic criteria alone allow only a rough grouping of malignant tumors in three or four malignancy grades, instead of providing a continuous scale, which would better represent the malignant potential of a tumor. Therefore, an objective and continuous parameter which is easy to quantitate is needed as a basis for a malignancy grading system.

The nuclear DNA distribution has been demonstrated in several investigations to be of prognostic validity for various tumors (15-19). The prognostic validity of DNA distribution patterns has also been shown for prostatic carcinomas (20-29).

The aim of this study was to test the prognostic validity and reproducibility of the previously developed DNA Malignancy Grade (DNA-MG) (16, 30, 22, 18, 19) for prostatic cancer patients, in comparison with a conventional cytopathologic grading system(2).

## Materials and Methods

*Clinical material.* The clinical material used in this investigation consisted of cytologic smears from 52 patients, obtained by transrectal aspiration biopsies of the prostate, which were collected in the Department of Urology, University of Munich, from 1963 to 1983. These smears, routinely stained according to May-Grünwald-Giemsa or Papanicolaou, were reviewed in order to perform a cytologic grading (Table I) according to Böcking (2). In all cases, the morphological and the DNA-grading was performed on specimens which were collected prior to the beginning of treatment. For the assessment of the intra- and interobserver reproducibilities of the DNA-grading system, 15 cases of prostatic carcinoma were chosen from the Department of Pathology, Aachen, University of Technology, FRG (cytopathologic grade 1 (5 cases), 2 (5 cases) and 3 (5 cases)). Taking these new cases was necessary, since at the time we performed the reproducibility measurements, the smears used for the assessment of the prognostic validity of the DNA-grading system were no longer suitable for remeasurements because of fading of the Feulgen

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*Key Words:* Grading of malignancy, prostatic carcinomas, DNA-image-cytometry, DNA-grading of malignancy.

Table I. *Datafile of 52 prostatic carcinoma patients. Status: A = alive, D = dead. DNA-MG = DNA Malignancy Grade. Morphologic grading system (2). Clinical Staging (31).*

| Pat No. | Age (Years) | Status | Survival time months | DNA MG | Morpho-logical grade | Clinical stage |
|---|---|---|---|---|---|---|
| 1 | 74 | A | 96 | 0.20 | 1 | B |
| 2 | 67 | A | 156 | 0.90 | 2 | C |
| 3 | 75 | D | 49 | 0.13 | 1 | C |
| 4 | 82 | D | 28 | 0.30 | 2 | B |
| 5 | 79 | D | 37 | 0.22 | 2 | D |
| 6 | 64 | D | 110 | 0.28 | 3 | B |
| 7 | 61 | D | 47 | 1.13 | 2 | C |
| 8 | 56 | D | 127 | 1.41 | 1 | C |
| 9 | 63 | D | 130 | 0.21 | 2 | B |
| 10 | 69 | D | 82 | 0.68 | 2 | C |
| 11 | 66 | D | 49 | 0.31 | 2 | B |
| 12 | 63 | D | 76 | 0.69 | 1 | C |
| 13 | 60 | D | 66 | 1.90 | 2 | C |
| 14 | 56 | D | 109 | 1.35 | 3 | C |
| 15 | 65 | D | 26 | 2.55 | 3 | D |
| 16 | 70 | D | 111 | 1.26 | 2 | B |
| 17 | 73 | D | 130 | 1.09 | 3 | D |
| 18 | 66 | D | 46 | 0.61 | 1 | C |
| 19 | 71 | A | 112 | 1.23 | 2 | C |
| 20 | 69 | D | 32 | 0.21 | 3 | B |
| 21 | 74 | D | 62 | 1.67 | 2 | C |
| 22 | 70 | D | 108 | 1.51 | 3 | C |
| 23 | 54 | A | 125 | 0.68 | 1 | B |
| 24 | 71 | D | 61 | 1.56 | 2 | A |
| 25 | 74 | D | 73 | 0.39 | 2 | C |
| 26 | 63 | D | 35 | 0.70 | 1 | D |
| 27 | 62 | D | 55 | 1.29 | 3 | C |
| 28 | 62 | A | 118 | 0.93 | 1 | C |
| 29 | 59 | D | 32 | 1.50 | 3 | C |
| 30 | 51 | D | 50 | 1.75 | 3 | D |
| 31 | 75 | D | 83 | 0.63 | 2 | C |
| 32 | 60 | D | 105 | 0.45 | 1 | C |
| 33 | 56 | D | 58 | 0.17 | 1 | B |
| 34 | 69 | D | 52 | 1.33 | 2 | C |
| 35 | 74 | D | 6 | 2.81 | 3 | D |
| 36 | 73 | D | 73 | 0.71 | 1 | D |
| 37 | 55 | D | 28 | 2.19 | 3 | D |
| 38 | 60 | D | 97 | 1.59 | 3 | C |
| 39 | 62 | D | 64 | 2.27 | 3 | A |
| 40 | 67 | D | 110 | 0.92 | 1 | C |
| 41 | 62 | D | 152 | 0.71 | 1 | C |
| 42 | 64 | D | 72 | 1.85 | 3 | B |
| 43 | 62 | D | 30 | 2.08 | 2 | D |
| 44 | 77 | D | 7 | 2.50 | 3 | D |
| 45 | 67 | D | 9 | 2.42 | 2 | D |
| 46 | 63 | D | 111 | 0.43 | 1 | B |
| 47 | 68 | D | 83 | 1.05 | 2 | D |
| 48 | 62 | D | 134 | 0.71 | 2 | A |
| 49 | 60 | D | 104 | 0.83 | 1 | D |
| 50 | 70 | D | 43 | 0.95 | 1 | Missing |
| 51 | 80 | D | 9 | 1.23 | 3 | C |
| 52 | 76 | D | 17 | 1.16 | 1 | B |

stain. For determination of the intraobserver reproducibility, three different operators each measured 5 out of the 15 cases twice; for determination of the interobserver reproducibility, these operators measured all 15 cases independently. The clinical stage of the patients was assessed according to Jewett (31) (Table I).

All patients were treated by orchiectomy, radiation of mammillae and with 1.5 g/d. fosfestrol-tetrasodium (Salt) for 10 days, followed by 100 mg estradiol undecylate at 2 to 4 week intervals. Regional radiation was performed in 4 patients. 5 patients were treated additionally with 300 mg estramustinphosphate for 3 weeks, and in 2 cases the patients were treated with a combination of radiation and cytostatic drugs.

*Feulgen staining.* The Papanicolaou or May-Grünwald-Giemsa stained cytologic smears were destained with xylene, after the coordinates of tumor cell complexes had been recorded at the cross stage of a conventional microscope. After rehydration, the cells were hydrolysed (4 N HCl, 28° C, 45 minutes) and stained with Schiff's reagent. The whole staining procedure was performed automatically in a modified Shandon M 24 staining machine as previously described (32).

*DNA Measurements.* DNA measurements of the Feulgen stained nuclei were performed with a TV image analysis system (TAS plus, Leitz, FRG) combined with an automatic microscope. The configuration of the image analysis system and measurement performance with this equipment, controlled by a software program developed in our institute, have been described in detail elsewhere (33). Measurements of at least 20 lymphocytes served as an individual and tissue specific reference system for the determination of the diploid (2c) peak. The number of lymphocytes measured depended on the error of the mean of the lymphocyte DNA values, which should be less than 5% to be acceptable as sufficient for the gaging of the 2c peak. A correction factor of 1.19 was used according to previous studies (30) between the DNA values of lymphocytes and epithelial cells. After random measurements of 100 morphologically detected tumor cells (Figure 1), the computer loads an algorithm program for the computation of the DNA Malignancy Grade (30). The grading result, including a DNA-frequency histogram, is then printed out. Measurement of one smear, including computation and printout of DNA-diagnoses, takes about 30 to 40 minutes.

*DNA Grading of malignancy.* The DNA Malignancy Grade (DNA-MG) is based on the 2c Deviation Index (2cDI), obtained from the single tumor cell DNA values. The 2cDI is defined as the mean square deviation of the measured cells (c) around the diploid (2c) DNA peak (11):

$$2cDI = \frac{1}{N} \sum_{i=1}^{N} (c_i - 2c)^2$$

This 2cDI, depending on the proliferation activity of the tissue (S-and G-2 phase cells) and the amount and the variation of nuclear DNA, ranges on a scale from 0.01 to 51.51 is the highest 2cDI known, so far measured in an osteosarcoma (34). For a better comparability with other grading systems and in accordance with the grading proposals of the UICC, we performed a logarithmic transformation of the 2cDI, in order to obtain a continuous scale for the DNA Malignancy Grade, ranging form 0.01 to 3.00:

$$\text{DNA-MG} = 3 \times \lg (2cDI + 1)/\lg 51$$
$$= 1{,}757 \times \lg (2cDI + 1)$$

*Statistics.* Several statistical tests for exploratory data analysis were employed. The Spearman test (35) was applied to test the association between two variables. The Cox Proportional Regression Model (36) was used to analyse the prognostic relevance of (a) the DNA Malignancy Grade; (b) the cytopathological grading system(2); (c) the clinical stage, and (d) the age of the patient. Repeated stepwise regression procedures were performed in the Cox regression model, once allowing all covariates to be entered in the model and to be removed again and once starting with a model which already included all covariates. To compare survival curves between specified groups of patients whose survival times were estimated by the product limit method of Kaplan-Meier (37), generalised Wilcoxon-Breslow tests were performed (34). For the estimation of the reproduci-

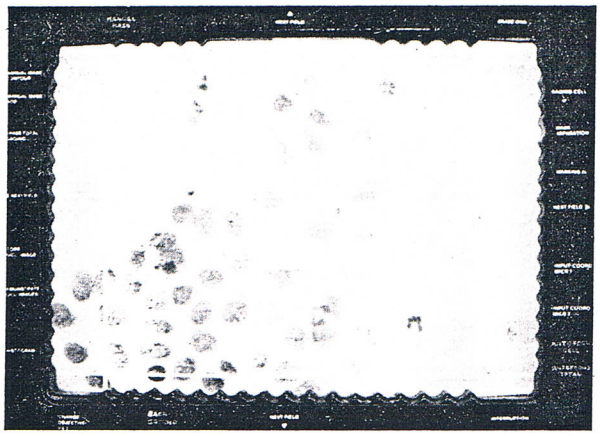

Figure 1. *Feulgen stained prostatic carcinoma nuclei on the monitor of the TV-image analysis system (TAS plus, Leitz, FRG). DNA measurements of cell nuclei are performed interactively on the monitor. Nucleus to be measured is covered by a shape adapted white mask.*

bility of the DNA grading system, variance component models with one factor balance nested classification (38) (interobserver) and two fold nested classification (intraobserver) (25) were used. 95% confidence limits were calculated for the variation obtained. Dividing the continuous DNA-MG scale into three groups (DNA-MG ranging from 0.0 -1.0-, 1.0 - 2.0, 2.0 - 3.0), the reproducibility could also be indicated as a percentage. An analysis of variance was performed to test differences between the reproducibilities of the three operators.

## Results

At the end of the study, 47 patients had died and 5 patients were still alive (Table I). The median survival time of all patients (mean age 66.4 years, ranging form 51 to 82 years) was 64 months (75th quantile: 35 months; 25th quantile: 109 months). The mean cytological grade was 1.98, the mean clinical stage 2.90 (Table I). The mean DNA Malignancy Grade (DNA-MG) was 1.09 (SD: 0.73, CV: 0.67, ranging from 0.03 to 2.81).

The DNA-MGs measured in the given cytopathological grades are listed in Talbe II and shown in Figure 2. It can be seen that the DNA-MGs within given morphological grades were considerable.

Table III and Figure 3 show the DNA-MGs obtained in given clinical stages. No constant increase of DNA-MG's with increasing stages can be observed, even if the highest mean DNA-MG was measured in clinical stage D. The variations of DNA-MG's within given clinical stages were also considerable. In the Spearman test no association was found between the age of the patients divided into the following groups: <60, 69-70 and<70 years and (a) the DNA-MG (p=0.666), (b) the cytopathological grade (p=0.297), and (c) the clinical stage (p=0.948). Thus, the age of the patient did not significantly influence either the results of the two grading systems or the clinical stage. Between the cytopathological malignancy grades 1 to 3 (survival curves in

Table II. *Variation of the DNA Malignancy Grade (DNA-MG) in given morphologic grades according to Böcking (2). SD = standard deviation, CV = coefficient of variation.*

| Morphological Grade | n | DNA Malignancy Grade | | | |
|---|---|---|---|---|---|
| | | Mean | SD | CV | Range |
| 1 | 17 | 0.69 | 0.34 | 0.50 | 0.13 - 1.41 |
| 2 | 19 | 0.99 | 0.71 | 0.72 | 0.03 - 2.42 |
| 3 | 16 | 1.62 | 0.74 | 0.46 | 0.21 - 2.81 |

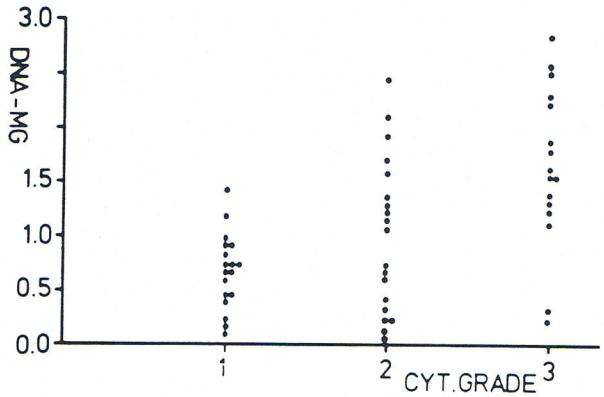


Figure 2. *DNA-Malignancy-Grades (DNA-MG) in given cytologic malignancy grades according to Böcking (10).*

Table III. *Variation of the DNA Malignancy Grade (DNA-MG) in given clinical stages according to Jewett (31). SD = standard deviation, CV = coefficient of variation.*

| Stage | n | DNA Malignancy Grade | | | |
|---|---|---|---|---|---|
| | | Mean | SD | CV | Range |
| A | 3 | 1.51 | 0.78 | 0.52 | 0.71 - 2.27 |
| B | 12 | 0.57 | 0.56 | 1.00 | 0.03 - 1.85 |
| C | 23 | 1.02 | 0.50 | 0.49 | 0.09 - 1.90 |
| D | 13 | 1.61 | 0.87 | 0.54 | 0.22 - 2.81 |

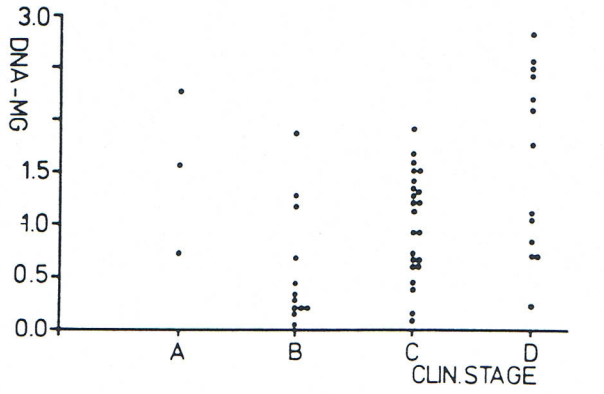


Figure 3. *DNA-Malignancy-Grades (DNA-MG) in given clinical stages according to Jewett (27).*

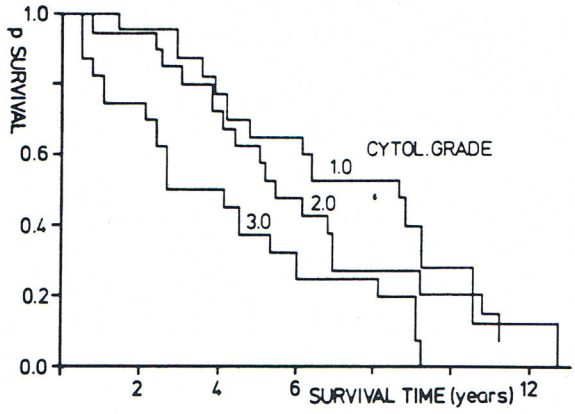


Figure 4. *Survival curves (Kaplan-Meier) of prostatic carcinoma patients, grouped according to their cytopathologic malignancy grades.*

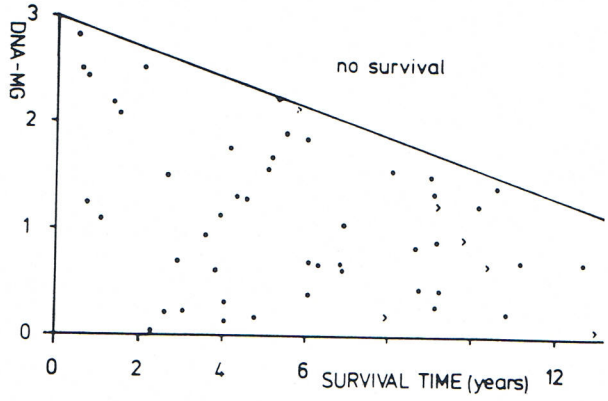


Figure 5. *Correlation between the DNA Malignancy Grade (DNA-MG) and the survival time of 52 prostatic carcinoma patients (• = non-survivors, > = survivors).*

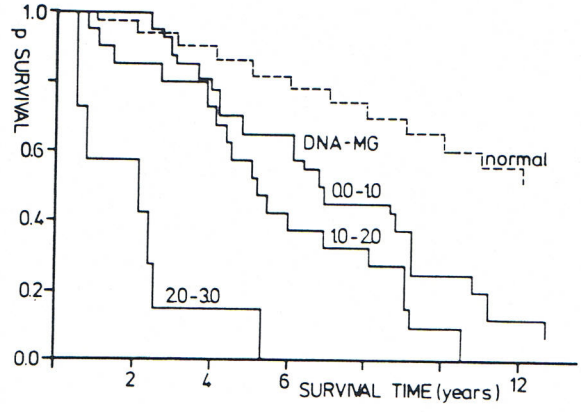


Figure 6. *Survival curves of prostatic carcinoma patients grouped according to the DNA Malignancy Grade (DNA-MG), ranging from 0-1, 1-2 and 2-3.*

Table IV. *Median survival times of prostatic carcinoma patients. Significance of differences between the malignancy grades and clinical stages (Breslow test).*

| Covariates | | Survival time (months) | | | Breslow test |
|---|---|---|---|---|---|
| | n | Median | 75th | 25th | p-value |
| Morphological grade | 1 | 17 | 104 | 49 | 127 | 0.435 |
| | 2 | 19 | 66 | 47 | 111 | |
| | 3 | 16 | 32 | 13 | 72 | |
| Clinical stage | A | 3 | 64 | 61 | 134 | 0.674 |
| | B | 12 | 72 | 322 | 111 | 0.944 |
| | C | 23 | 82 | 52 | 110 | 0.0002 |
| | D | 13 | 30 | 13 | 50 | |
| DNA-MG | 0.1-1.5 | 38 | 76 | 46 | 111 | 0.0092 |
| | 1.5-3.0 | 14 | 50 | 26 | 66 | |
| DNA-MG | 0-1 | 26 | 82 | 49 | 130 | 0.186 |
| | 1-2 | 19 | 62 | 47 | 108 | 0.001 |
| | 2-3 | 7 | 26 | 7 | 30 | |

Figure 4) no significant differences could be demonstrated in the Breslow test (Table IV).

Significant differences in survival time between clinical stages could only be demonstrated between stage C and D (Table IV). Figure 5 shows the relationship between the DNA-MG for each individual patient and his survival time. It can be seen that a lower DNA-MG was correlaterd with a longer survival time. Beyond the hypothetical curve approaching the x-axis, no survival was observed. Significant differences between the survival times of patients classified into two DNA-MG groups (DNA-MG 0 - 1.5 and 1.5 - 3.0) could be demonstrated (p=0.0092) (Table IV). Dividing the DNA-MG scale into three groups (DNA-MG: 0-1, 1-2, 2-3), significant differences in survival time could still be demonstrated between the two higher graded groups (Table IV, Figure 6). In the Cox model several stepwise forward regression procedures led to the results listed in Table V. It could be demonstrated that the DNA-MG (p=0.001) and the age of the patient (p=0.004) had a strong influence on the survival probability, while the clinical stage (p= 0.039) had a weaker influence and the cytopathological grade no influence on the survival time (Table V, line I). When the age of the patient and the clinical stage were *a priori* entered into the Cox model, the DNA-MG further influenced probability of survival (Table V, line II), and when finally the age, the clinical stage and the morphological grade were entered into the model, the DNA-MG was able to provide further prognostic information.

The reproducibility of the DNA grading system was tested in 15 different prostatic carcinoma cases with DNA-MG values ranging from 0.01 to 2.66, the global mean DNA-MG was 1.06. The interobserver reproducibility resulted in a variation of $\sigma$= 0.036 between the three operators (95%

Table V. *Results of the Cox regression model (global and improvement chi square p-values), testing the influence of the parameters on the survival time of prostatic carcinoma patients.*
*Line I: no covariate initially entered in the model.*
*Line II: age and clinical stage initially entered in the model.*
*Line III: age, clinical stage and morphological grade initially entered in the model (n.e. = not entered).*

| | Entered covariates | Global $x^2$ p-value | Additional entered covariates | | | (Improvement $x^2$ p-value) |
|---|---|---|---|---|---|---|
| | | | DNA-MG | Age | Cl. stage | Morphological grade |
| I | | | 0.001 | 0.004 | 0.039 | n.e |
| II | Age<br>Clinical stage | 0.0094 | 0.0001 | – | – | n.e. |
| III | Age<br>Clinical stage<br>Morph. grade | 0.0007 | 0.004 | – | – | – |

confidence limit 0.023 = σ) and a variation of σ = 0.436 between the 15 cases. Taking the total variation of the DNA-MG in the 15 cases (1.480 = 100 %), the variation between the cases amounted to 90.8% and between the operators to only 9.2%. Dividing the continuous DNA-MG scale into three groups (DNA-MG: 0 - 1, 1 - 2, 2 - 3), the reproducibility was 86,7%. In the intraobserver reproducibility the variation amounted to (total variation: 0.503 = 100%) σ = 0.014 (= 2.8%) between the remeasurements (95% confidence limits 0.008 ≤σ≤ 0.034), to σ = 0.151 (= 30%) between the cases and to σ = 0.338 (= 67.2%) between the operators. No differences between the reproducibilities of the three operators could be demonstrated (p = 0.1937). When the continuous DNA-MG scale was divided into three groups (see above), an intraobserver reproduciblity of 86.7% resulted.

## Discussion

The purpose of clinical tumor staging, histogenetic tumor classification and grading of tumor malignancy is to provide basic information for the clinical management of the patient. Grading of malignancy should provide an index for the malignant potential of tumors, independently of the histogenetic classification and the clinical staging. A malignancy grading system should be:
(a) of high prognostic validity,
(b) of high representativity (grades obtained from biopsies should be representative for the tumors as a whole),
(c) of high reproducibility (*e.g.*low intra-and interobserver variation).

To date, all histopathologic and cytopathologic grading systems were based on the subjective evaluation of morphologic parameters and are therefore not sufficiently reproducible. The interobserver reproducibilities of prostatic carcinoma grading systems (30, 12) range, from 69 to 86% (2,14). The representativity of grading results obtained from prostate biopsies has been doubted by various authors (40, 41). The prognostic validity of conventional histopathologic and cytopathologic grading systems seems limited, as some only reveal significant differences in survival time between their malignancy grades when many patients are observed. Conventional histopathologic and cytopathologic grading systems only allow a rough grouping into 2 to 4 grades (2,7). This does not take into consideration the fact that the malignant potential of tumors represents a continuous and not a discontinuous parameter. Thus, other systems should be developed which allow a more reproducible, representative, continuous and prognostically valid grading of tumor malignancy.

A non-morphologic parameter which can reproducibly be determined and which is known to correlate well with the malignant potential of a tumor is the nuclear DNA content (15, 42). The prognostic significance of nuclear DNA distributions in prostatic carcinomas has already been described by some authors (20-29). But to date, only a few attempts have been made to derive a numerical prognostic index from the DNA single cell values (27). Most authors merely described the DNA distribution in tumors by means of histograms and grouped the casses subjectively according to different patterns.

In order to create a reproducible prognostic index based on single cell DNA values, we defined a DNA Malignancy Grade (DNA-MG). The DNA-MG, ranging on a continuous scale form 0.01 to 3.00, is based on the variation of the tumor cell DNA values around the diploid 2c peak (2c Deviation Index = 2cDI). This DNA-MG has already been demonstrated to be of high prognostic validity in urinary bladder carcinomas (16), malignant non-Hodgkin lymphomas (18, 19) and also in prostatic carcinomas, as demonstrated previously for a smaller group of patients (22). The representativity and reproducibility of the DNA-MG has been tested for various other carcinomas and found to be sufficient (11). Routine application of single cell DNA-cytometry up to now lasted many hours with conventional scanning cytophotometers,

and manual Feulgen-staining was circumstantial. For this reason we developed a temperature controlled staining machine for automated Feulgen staining, based on the Shandon Varistain 24 (32). Furthermore, we use a TV image analysis system (Leitz, TAS plus) combined with an automated microscope for rapid interactive DNA cytometry (33). With this system, measurements of 100 tumor cells and at least 20 reference cells (lymphocytes or granulocytes from the same smear), including the computation of data and printout of the DNA Malignancy Grade, takes up to 40 minutes for one smear.

The results of this investigation revealed that DNA-MG has a strong influence on the survival time of treated prostatic cancer patients in the Cox model. This also holds for the age of the patients. The clinical stage showed only a weak influence on survival time; this is probably due to the small numbers of patients in each stage, especially stage A (three patients), and it might be assumed that some of the early patients in the series were mis-staged 15 years ago. The cytopathological grading system provided no significant prognostic inforamation, also probably due to the small number of patients. The DNA-MG provided prognostic information even when the age of the patient, the clinical stage and the morphologic grade were introduced *a priori* into the Cox regression model. The variation of the DNA-MGs within each of the four clinical stages and each of the three different cytopathological grades was considerable. This indicates that clinical stages as well as morphological grades represent a rather heterogenous grouping of prostatic cancer patients. Significant differences in survival time could be demonstrated between patients with DNA-MGs of 0.01-1.5 and 1.5-3.0 and between groups with DNA-MGs of 1.0-2.0 and 2.0-3.0. Yet no significant differences in the survival probabilities could be demonstrated between patients with different cytopathological grades. With regard to clinical stage, significant differences could only be demonstrated between stages C and D. Again, the low number of patients in each stage has to be taken into consideration in the interpretation of these results. Thus the DNA-grading of prostatic carcinoma patients seems to be of superior prognostic validity when compared with the subjective cytopathologic grading. It seems possible that the DNA-MG for an individual patient with defined age and clinical stage can predict a maximal survival time as indicated in Figure 6. The inter- and intraobserver reproducibilities of the DNA grading system were 86.7% when the continuous DNA-MG scale was divided into three groups. Compared with the reproducibilities of morphological grading systems, which yield 38 to 86% for the interobserver variation and 71 to 90% for the intraobserver variation (2, 13, 31), the DNA grading for prostatic carcinoma patients seems to be reasonably reproducible.

In conclusion, it could be demonstrated that the DNA Malignancy Grade is a reasonably reproducible and prognostically valid index, which provides clinically relevant information for the individual prostatic carcinoma patient.

## Acknowledgements

This investigation was supported by grants from the Federal Ministry for Labor and Social Affairs, Bonn, FRG.

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*Received June 26, 1987*
*Accepted November 16, 1987*