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Representativity and Reproducibility of DNA Malignancy Grading in Different Carcinomas

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The reproducibility of the determination of the "DNA malignancy grade" (DNA-MG) was tested in 56 carcinomas of the colon, breast and lung while its representativity was tested on 195 slides from 65 tumors of the colon, breast and lung. DNA measurements were performed on Feulgen-stained smears with the TAS Plus TV-based image analysis system combined with an automated microscope. The variance of the DNA values of tumor cells around the 2c peak, the "2c deviation index" (2cDI), was taken as a basis for the computation of the DNA-MG, which ranges on a continuous scale from 0.01 to 3.00. The representativity, analyzed by comparison of the DNA-MGs measured in three different areas of the same tumor ≥1.5 cm apart from each other, yielded an 81% agreement. No significant differences between DNA-MGs of these areas were found. The intraobserver and interobserver reproducibilities of the DNA grading system, investigated by repeated DNA measurements, were 83.9% and 82.2%, respectively. In comparison, histopathologic grading of the 27 breast cancers studied yielded 65% intraobserver and 57% interobserver reproducibilities and 66% representativity.

Grading of tumor malignancy, based on morphologic criteria of differentiation, has been used since the time of Virchow as a prognostic index for tumor patients. In 1925, the first histopathologic grading system with statistically proven prognostic validity was published by Broders.[22] Since then, many attempts have been made to establish clinically relevant morphologic grading systems for different tumors.[9,11,12,24,30] The fact that various grading systems have been proposed for the same tumor type reflects the difficulties of creating reproducible and prognostically valid grading systems based on morphologic criteria alone.

Besides its prognostic validity, a grading system must produce reproducible and representative results on a single biopsy of the tumor to be clinically useful. The reproducibility of histopathologic grading systems is relatively low due to the subjective evaluation of the morphologic criteria.[26,28,38] The representativity of morphologic grading systems has been questioned due to the great morphologic heterogeneity of many tumors.[9,11,32,34] Another disadvantage of morphologic grading systems is their rough grouping of malignant tumors in only two to four groups; a continuous grading system would better represent the biologic reality of cancer malignancy.

One nonmorphologic parameter with well-known prognostic validity is the nuclear DNA distribution pattern.[2,5,10,21,41] The nuclear DNA content of tumor cells may be easily and reproducibly quantified after Feulgen staining using various methods.[6,23,36,37,40]

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We have developed a continuous grading system, based on the variation of the nuclear DNA content of tumor cells around the normal diploid value, the "DNA malignancy grade" (DNA-MG),[13,14] which has been proven to be prognostically valid in different malignant tumors.[7,15-20] The aim of this study was to investigate the reproducibility and representativity of the DNA grading system for carcinomas of the colon, breast and lungs.

Materials and Methods

Material

The reproducibility studies utilized material from 9 patients with colon carcinoma, 24 with breast carcinoma and 23 with lung carcinoma; the representativity studies were performed on 14 colon carcinomas, 27 breast carcinomas and 24 lung carcinomas (which included all cases used in the reproducibility studies). The specimens had been collected in the Department of Pathology, Aachen University of Technology, from 1977 to 1983. All colon carcinomas were adenocarcinomas of different differentiation. The breast carcinomas were all of the ductal type. The lung carcinomas, mostly squamous cell carcinomas and adenocarcinomas, included one small cell carcinoma and one bronchioloalveolar carcinoma. In 19 cases, tumors with a minimum size of 4.5 cm were selected, and imprint smears were prepared from three different areas 1.5 cm apart from each other. In 46 cases, monolayer smears were prepared as described below from three different blocks of paraffin-embedded tissues of the same tumor that originated 1.5 cm apart from each other. For comparison of the reproducibility of the DNA grading results with histopathologic grading results, the breast cancer grading system of Bloom and Richardson[11] was used to grade hematoxylin-and-eosin-stained sections from all 27 breast tumor specimens.

Specimen Preparation and Staining

The preparation of monolayer smears from paraffin-embedded tissue was performed as previously described.[27,43] Imprint smears and monolayer smears were fixed with an 85:10:5 volumetric mixture of methanol, formalin and acetic acid. Feulgen staining was performed automatically in a modified Shandon Varistain 24 staining machine,[25] using hydrolysis with 4 N HCl for 55 minutes at 27.5°C and staining with basic pararosaniline.

DNA Measurements

Measurements of the nuclear DNA content were performed with a TV-based image analysis system (Leitz TAS Plus) combined with an automated microscope (Leitz Orthoplan).[6] Tumor cells were chosen at random for measurement. Measurements of at least 20 lymphocytes or granulocytes served as a reference for the diploid (2c) DNA content. The final number of measured reference cells depended on the relative coefficient of variation (CV) of their DNA values: only a value <3% was accepted as sufficient for calibration. Based on previous studies, a correction factor of 1.19 was used between the DNA values of lymphocytes or granulocytes and those of epithelial cells.[13]

DNA Grading of Malignancy

The "2c deviation index" (2cDI) is defined as the variance of the DNA contents of single cells around the normal diploid (2c) DNA peak:

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

The 2cDI scale ranges from 0.1 to 51, which was the highest 2cDI measured up to now (found in an osteosarcoma[13]). For better comparability with other grading systems, and in accordance with the grading proposals of the UICC,[31] a logarithmic transformation of the 2cDI was performed to produce a continuous scale, ranging from 0 to 3.0, for the DNA-MG[13,14]

$$\begin{aligned} \text{DNA-MG} &= 3 \times \lg(2cDI + 1)/\lg 51 \\ &= 1.757 \times \lg(2cDI + 1) \end{aligned}$$

Statistics

The representativeness of the DNA-MG, tested in three different areas of each of the 65 tumors, was estimated with an analysis of variance (ANOVA) with simple hierarchic classification, presupposing a normal distribution of the single values. Since the DNA-MG represents a logarithmic transformation of the 2cDI, which as a variance follows a chi-square distribution, it is statistically correct to run an ANOVA. The intraobserver reproducibility was tested by remeasurement of 56 cases by the same operator; the interobserver reproducibility was tested by remeasurement of these cases by a second operator. Coefficients of correlation (CCs) between the resulting DNA-MGs were calculated and analyzed by an ANOVA run.

In addition, the continuous DNA-MG scale was divided into three percentile groups (respectively containing 25%, 50% and 25% of the patients) to compare the results in breast cancers with those obtained with the morphologic grading system. For evaluation of the reproducibility of this subjective grading system, the same 27 breast cancer sections were graded (1) three times by the same pathologist and (2) by three different pathologists.

Results

The lowest observed DNA-MG was 0.18 in a case of well-differentiated ductal breast carcinoma. The highest DNA-MG was 2.80, measured in a squamous cell carcinoma of the lung. The results of the DNA measurements for the assessment of the representativity (homogeneity) are shown in Figure 1. The mean difference between the DNA-MGs of three smears from the same tumor was 0.21±0.11 grades (0.20±0.09 in colon carcinoma, 0.24±0.13 in lung carcinoma and 0.19±0.11 in breast carcinoma). The F-test did not reveal differences in the homogeneity of the DNA-MG in colon, lung or breast carcinomas. The highest observed DNA-MG difference was 0.58 in a case of poorly differentiated ductal breast carcinoma; the lowest difference was 0.04 in an adenocarcinoma of the lung. The mean DNA-MG difference was the same in low-grade and high-grade tumors. The results of the ANOVA for the assessment of the representativity of the DNA-MG in three different areas of the same tumor (n=65) are shown in Table I. CVs of 9.9%, 14% and 7.7% were obtained for measurements in the three different areas of the specimens in colon, breast and lung cancers, respectively.

Table I *Representativity of the DNA Malignancy Grade in Colon, Breast and Lung Cancers*

	DNA-MG		
	Colon (n=14)	Breast (n=27)	Lung (n=24)
Mean	1.41	1.01	1.48
Variance	0.019	0.02	0.013
SD	0.14	0.14	0.11
CV	9.9%	14.0%	7.7%

The CC between DNA-MG measurements performed by the same operator (intraobserver reproducibility) was 0.973 for all cases together (0.951 for colon, 0.979 for breast and 0.953 for lung) (Figure 2). Between two different operators (interobserver reproducibility) the CC between the DNA-MG grading results was 0.923 (Figure 3) (0.810 for colon, 0.910 for breast and 0.917 for lung). The results of the ANOVA of the DNA-MG grading results are shown in Tables II and III. CVs of 5.9%, 7.2% and 6.3% were produced by remeasurements by the same operator, while CVs of 19.6%, 14.2% and 8.8% were produced by measurements of the same section by two operators in colon, breast and lung tumors, respectively.

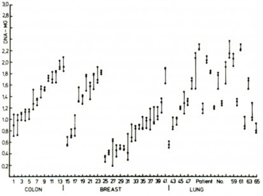

Figure 1
Representativity of the DNA-MG in 65 cancer cases (14 colon, 27 breast and 24 lung carcinomas). DNA measurements were performed in three different areas of the same tumor in each patient, resulting in three DNA-MGs per patient, as shown by the three connected dots per patient.

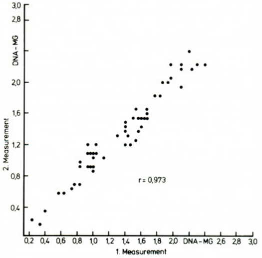

Figure 2
Intraobserver reproducibility of the DNA-MG in 56 cancer cases (8 colon, 25 breast and 23 lung carcinomas) (r=coefficient of correlation).

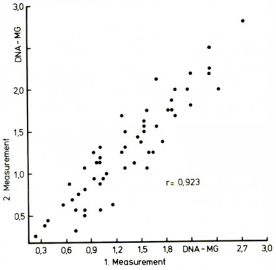

Figure 3
Interobserver reproducibility of the DNA-MG in 56 cancer cases (9 colon, 24 breast and 23 lung carcinomas) (r=coefficient of variation).

With the breast carcinoma cases divided into three percentile groups, the intraobserver reproducibility of the DNA-MG grading was 83.9% while the interobserver reproducibility was 82.2%; the representativity was 81%. In comparison, the reproducibilities of the Bloom and Richardson[11] grading for breast cancer were 65% (intraobserver) and 57% (interobserver). The representativity of the morphologic grading system was 60% in the three different areas of the breast cancer tumors.

Discussion

A grading system for malignant tumors can only be prognostically valid if the malignancy grade of a small part of the tumor, as obtained by biopsy, is representative of the whole tumor. A significant variation of the malignancy grade within a tumor would require the assessment of many tissue samples, which is clinically not practicable. Morphologic criteria have been the basis for malignancy grading systems so far. However, since most malignant tumors contain a great morphologic heterogeneity and variability of their growth patterns in different areas, grading based on morphologic criteria alone is often not representative, and has even been thought to be impossible.[32,34]

The nuclear DNA content is a nonmorphologic parameter of prognostic significance. Despite morphologically different growth patterns in different areas of the tumor, the representativity of the DNA distribution pattern within tumors has been demonstrated for carcinomas of the cervix uteri,[3] for renal carcinomas[8] and for ovarian carcinomas.[29]

Few attempts have been made to objectively quantify the nuclear DNA distribution to arrive at a prognostic index or malignancy grade; most studies have relied on a subjective interpretation of DNA histograms.[1,4] Our DNA malignancy grade (DNA-MG) is based on an objective quantification of the variation of the tumor cell DNA contents around the diploid peak, the 2cDI value. The DNA grading, which ranges on a continuous scale from 0 to 3.0, implies that patients with the same tumor type whose tumors have low DNA-MG values have a better prognosis than do patients whose tumors have high DNA-MG values. This correlation between the DNA-MG and patient survival has been demonstrated for various malignant tumors in previous investigations.[7,15-20] The present study sought to investigate the reproducibility and representativity of the determination of this prognostic index, not just the homogeneity of the DNA distribution and its reproducibility in tumor material.

The wide range of DNA-MGs in our patients indicates that the DNA grading system provides the possibility of detecting the extremes of very high and very low malignancy grades. However, the differences of the DNA-MGs between three different areas of a tumor were very low, with a mean of 0.21 DNA-MGs and a maximum difference of less than 0.6

Table II *Intraobserver Reproducibility of the DNA Malignancy Grade in Colon, Breast and Lung Cancers*

	DNA-MG		
	Colon (n=8)	Breast (n=25)	Lung (n=23)
Mean	1.26	1.04	1.66
Variance	0.0057	0.0057	0.011
SD	0.075	0.075	0.104
CV	5.9%	7.2%	6.3%

Table III *Interobserver Reproducibility of the DNA Malignancy Grade in Colon, Breast and Lung Cancers*

	DNA-MG		
	(n=9)	(n=24)	(n=23)
Mean	1.22	1.05	1.68
Variance	0.056	0.023	0.022
SD	0.24	0.15	0.15
CV	19.6%	14.2%	8.8%

DNA-MGs. These small differences within a tumor did not increase in higher-graded tumors. The differences of the DNA-MGs were even very small in the breast carcinomas, despite the morphologic heterogeneity of these tumors. Earlier investigations demonstrated no significant differences in the DNA distribution patterns between breast carcinomas and their metastases, indicating a monoclonal origin of these tumors.[4,18,33] In the carcinomas of the lung and colon, which are assumed to be of heterogeneous clonal origin,[35,39,42] the differences of the DNA-MGs within the same tumor were (1) quite low and (2) not significantly different from those in breast carcinomas. It can be concluded that the measurement of the nuclear DNA contents in one area of a tumor provides sufficient representativity for the whole tumor.

Morphologic grading of the breast tumors gave a representativity of 60% in three different tumor areas, a 65% intraobserver reproducibility and a 57% interobserver reproducibility; these results are in good agreement with previous investigations.[26,28,38] When the continuous DNA-MG scale was divided into three percentile ranges for comparison with the subjective grading, the DNA grading gave an 83.9% intraobserver reproducibility and an 82.2% interobserver reproducibility. ANOVA revealed low intraobserver and interobserver differences in the DNA-MG grading results.

It is concluded that the DNA malignancy grading yields sufficiently reproducible and representative grading results in carcinomas of the colon, lung and breast. Since the prognostic validity of the DNA-MG has been proven for various tumors, the continuous-scale DNA grading system may provide a valuable prognostic index for clinical purposes.

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