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Flow cytometric evaluation of colorectal carcinoma as completion of conventional tumor examination

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The DNA-ploidy and the carcinoembryonic antigen (CEA) were measured simultaneously by flow cytometry on single cell suspensions to determine their significance for colorectal tumor identification and evaluation of prognosis. Additionally, cellular CEA was compared with immunohistochemically determined CEA and serum CEA.
59% of the carcinomas were DNA-aneuploid and all mucosa samples were DNA-euploid. 97% of the tumors could be identified by simultaneous evaluation of DNA-ploidy and CEA-surface density. The CEA-density varied considerably for different tumors; in total, it was about twice as high for tumors as for mucosa. Flow cytometric and immunohistochemical determination of cellular CEA were significantly correlated. Cellular CEA and serum CEA were not correlated, but cellular CEA-negative tumors never developed elevated serum levels. At the time of surgery DNA-aneuploid tumors were in more advanced Dukes stages. The postoperative course showed a significantly poorer prognosis for DNA-aneuploid and for low-CEA-density tumors.
Flow cytometric analysis in colorectal cancer can serve as a model for automated tumor identification, enables further classification of tumor malignancy and improves efficiency of serum-CEA controls.
The purpose of this study was to measure at the cellular level simultaneously the DNA-content and the CEA-antigen expression in colorectal cancer patients. Subsequently the biological relevance of these data with respect to automated tumor identification and classification was analysed. Furthermore correlation of flow cytometric with immunohistochemical and serum CEA-maesurements were determined.

Key words: Flow cytometry, Colorectal carcinoma, Tumor examination.

Classification of colorectal carcinoma and estimation of its prognosis is generally based on histopathologic findings such as grade of differentiation, tumor type or stage of extension (23, 29). Serum carcinoembryonic antigen (CEA) levels are useful for the assessment of the tumor and its follow up (14, 15, 18, 29). For optimal cancer management, however, further refinement of methods for detection and definition of the biologic tumor behaviour and the individual risk, respectively, would be desirable. Many of the tumor's primary determinants, such as DNA-content or DNA-ploidy, cell kinetics, antigen expression or hormone receptors are manifestations at the cellular level (3, 4, 6). Flow cytometric analysis permits to detect and differentiate quantitatively these parameters (4, 25).

Materials and Methods

Fresh, unfixed tissue samples (0,1 — 0,5 g) were removed from colorectal carcinoma as well as from normal mucosa in 170 patients undergoing surgery

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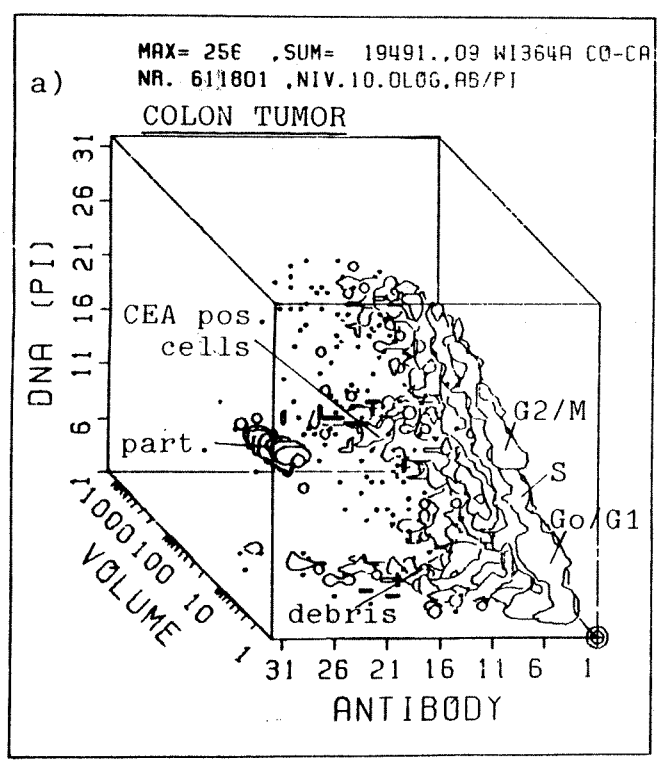
and processed to single cell suspensions as required for flow cytometry. The preparation and staining of the cells was described in detail previously (25). In short, the specimens were minced mechanically (electric tissue chopper), sieved through a steel mesh (40 um mesh width) and suspended (at 0°C) in 5 mM Hepes buffered saline pH 7,4. The cells were fixed in 1,7% formaline, washed twice and labeled with an NCA (non specific cross reacting antigen)-free, absorbed and specific CEA-antiserum (DAKO, Copenhagen, Denmark) in an indirect immunofluorescence assay (with fluorescein isothiocyanate (FITC) as label of the second antibody). After repeated washing and resuspension (cell concentration 1 — 5 x 10^7/ml), the DNA of the cells was additionally stained with propidium iodide (PI). Finally, the cell volume, the FITC — and the DNA-fluorescence of these double stained cells were measured simultaneously with a FLUVO-METRI-CELL-flow cytometer (13). All measurements were standardized with monodisperse, fluorescent latex particles to maintain long term stability of evaluation (Fig. 1).

The automated computer analysis from list mode data (25) consisted in the calculation of a three dimensional cube for visual inspection (Fig. 1a), a two-parameter histogram (FITC-antibody versus PI-DNA) and three one-parameter distribution curves of cell volume (Fig. 1b), PI-DNA (Fig. 2), and FITC-antibody (Fig. 3). Only morphological intact cells characterized by their simultaneous presence of cell volume and DNA, and only the epithelial cells of each specimen were used for further evaluation. Epithelial cells are larger and well separated from the small inflammatory cells (lymphocytes and granulocytes) by automatic gating of volume distribution curves (Fig. 1b).

DNA-ploidy was detected by evaluating the PI-DNA-histograms. The position of G 0/G 1 peak in the DNA-distribution curves of the small inflammatory cells served as the internal standard for DNA-ploidy. (This procedure was found to be more reliable than to relate the DNA-distributions to the G O/G 1 peak of a standard suspension of fixed human or rat lymphocytes, since variations within 10 to 15% in the absolute positions of the G 0/G 1



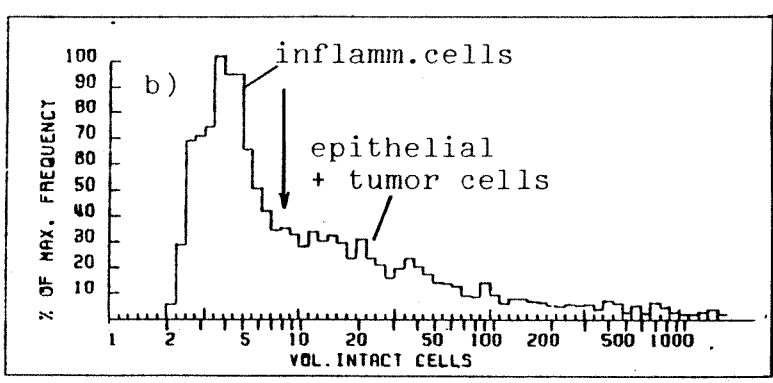


Fig. 1. a) Three dimensional representation of a simultaneous cells volume, and CEA-antibody measurement of colon cancer cells. The contour lines surround the areas where particles and cells are located. The channel contents are standardized to the maximum logarithmic channel content and plotted for the 10% level. One class on the logarithmic volume scale corresponds to 30 fl. 19419 cells were measured.
b) Volume distribution curve of the morphologically intact cells, obtained from the measurement in panel a). The smaller inflammatory cells can be well distinguished from the larger epithelial cells. For DNA-distribution curves (fig. 2) and cellular CEA-surface density calculation (fig. 3) only the epithelial cells were evaluated. (↓ distinction between inflammatory and epithelial cells)

peaks of normal cells occurred in various patients; the latter was due to staining variations which could not be influenced without proteolytic digestion of the cells). A sample was considered DNA-aneuploid, when a separated second DNA peak or a clearly distinguishable shoulder in the DNA distribution of the epithelial cells, which had an amplitude of more than 20% of the G 0/G 1 peak of the DNA-euploid normal cells, was observed.

Histograms were divided according to their DNA-index (2, 7) in DNA-aneuploid (index ⅔1,2) and in DNA-euploid (or nearly euploid) samples (index $>1,2$), as has been found useful for clinical correlation studies (7, 21).

The percentage CEA-positive cells of each specimen was determined from the number of antigen positive celles versus the total number of cells. The antigen surface density was calculated by dividing the fluorescence intensity of each cell through the cell surface. The cell surface (S) was obtained from the cell volume (V) as: $S=4,84 \times V^{0,67}$, assuming a spheroid cell shape. For each specimen the mean cellular antigen surface density was automatically determined. The tumors were divided in two groups, according to their low or high mean cellular CEA-surface density (threshold: median of all samples).

The CEA on the cell surface was estimated for 29 colorectal carcinomas with both techniques for correlation of flow cytometric and immunohistochemical CEA-determination. The same tumor and identical CEA-antiserum (DAKO) were used for flow cytometry as well as histochemistry. Localization of CEA on paraffin sections was performed using the indirect immunoperoxidase technique (17, 20, 27). The stained sections were evaluated semiquantitatively for CEA-positive cells and for the CEA-staining intensity (3 degrees: widespread strongly positive +++, uniformly moderate or focal strongly positive ++, only focal moderately or weakly positive +).

Evaluation of flow cytometric and histochemical determinations was accomplished without knowledge of the patients' clinical data.

Serum-CEA controls were done in all patients pre — and postoperatively, using a standardized radioimmunological assay (16). Preoperative serum-CEA was correlated with flow cytometrically determined cellular CEA.

Postoperatively after complete pathohistological evaluation all tumors were staged according to the Dukes classification (9). The follow up (6 to 50 months, median 32 months) was done for all patients in intervals of three months during the first two years and in intervals of 6 months for the following years, according to a standardized scheme (including clinical, endoscopical, and x-ray examination, serum-CEA, liver sonography, CT-scan of the pelvis as well as histological examination of suspicious lesions).

Tumor DNA-ploidy, and tumor CEA-surface density were correlated with tumor stage, tumor relapse (local recurrence and metastases) and tumor dependent deaths.

Results

The precision of the flow cytometric volume determination (peak position at the distribution curves) was 2,7 % on 5 repeated measurements of the same fluorescent, monodisperse latex beads. Coefficients of variation (CV's) of 1,8 % for the fluorescence distribution were reached with this beads. The CV's for the DNA-distributions of fixed, morphologically intact intestinal cells were always in the order of 8 to 15%, depending on the cell sample. These high CV's could not be diminished by using other DNA stains (e.g. mithramycin). They were, however, reduced by digestion of the cell body with pepsin, but then antigen information on the cell surface was lost.

The normal bowel mucosa always revealed DNA-distributions (Fig. 2) with only one higher G 0/G 1-DNA-peak (amplitude 100% of maximal frequency) and one lower G 2/M-peak (amplitude $< 10\%$). Tumors with abnormal, additional, higher DNA-peaks (DNA-index $> 1,2$; amplitude $> 25\%$) or with DNA-aneuploid cells, respectively, were found in 59,4% of the 170 colorectal cancer patients (Fig. 1). In all other patients (40,6%) the carcinoma specimens showed euploid DNA-distributions. Only 59,4% of the tumors could be identified by automated DNA-measurement alone.

The cellular CEA-surface-density distribution curves (Fig. 3) showed a symmetrical shape for each individual sample. In total, the median CEA-antigen density was about twice as high for the tumors as for the normal mucosa (Fig. 4). The evaluation of CEA-positive cells was less discriminative. Tumor samples showed between 0 % to 90 % positive cells while 0 % to 50 % CEA-positive cells were observed in normal mucosa. For the CEA-density distribution the threshold between normal and abnormal samples was set using a ROC-curve (19). 91% of the tumor samples were correctly recognized with 11% of false positive results for normal mucosa samples.

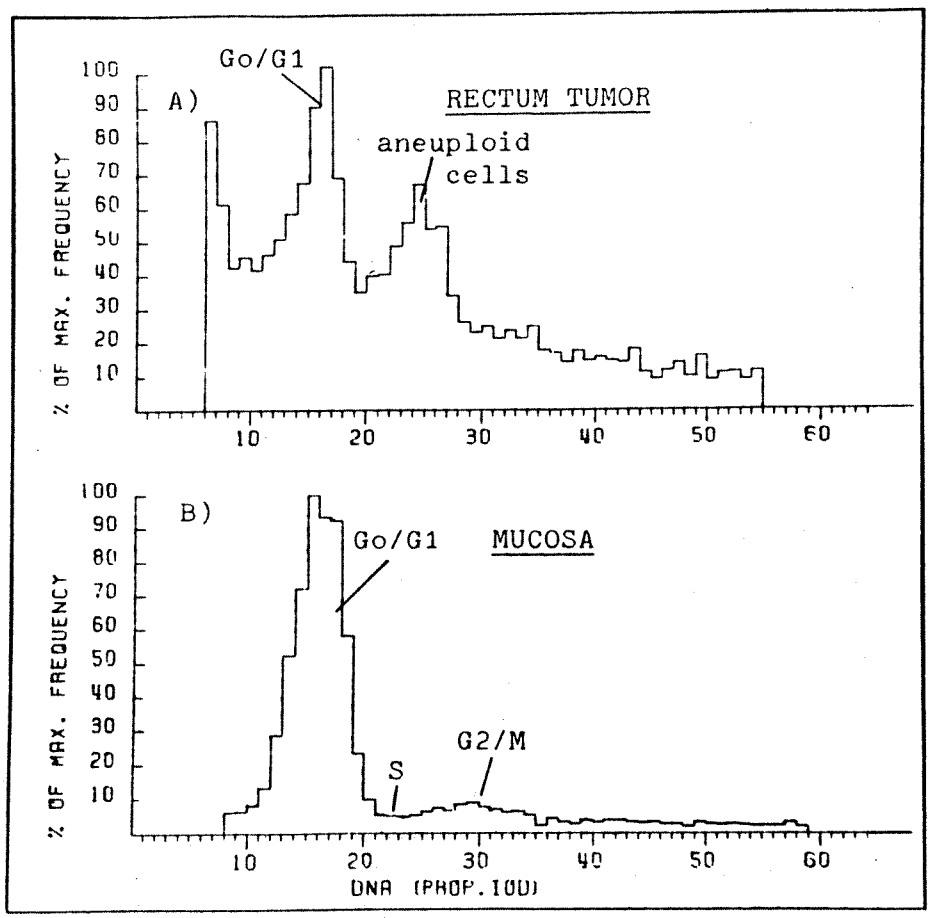


Fig. 2. DNA-histograms (epithelial cells) of rectum tumor (A) and normal mucosa (B). Abscissa: Cellular DNA-content. Ordinate: Cells, % of maximum frequency. The DNA-aneuploid tumor cells (in A) are visible as a separate high DNA-peak (DNA-Index 1,52).

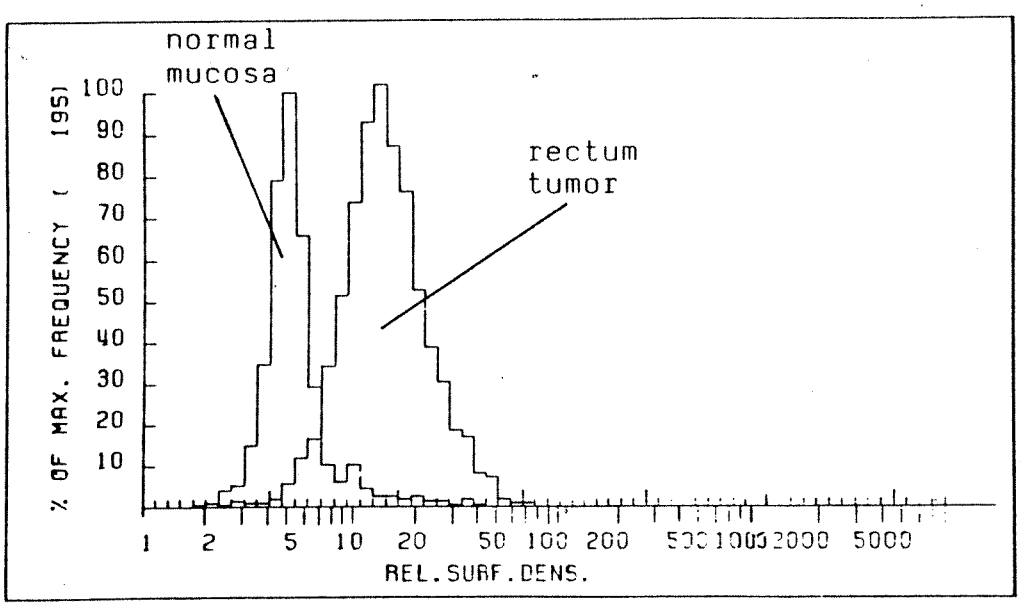
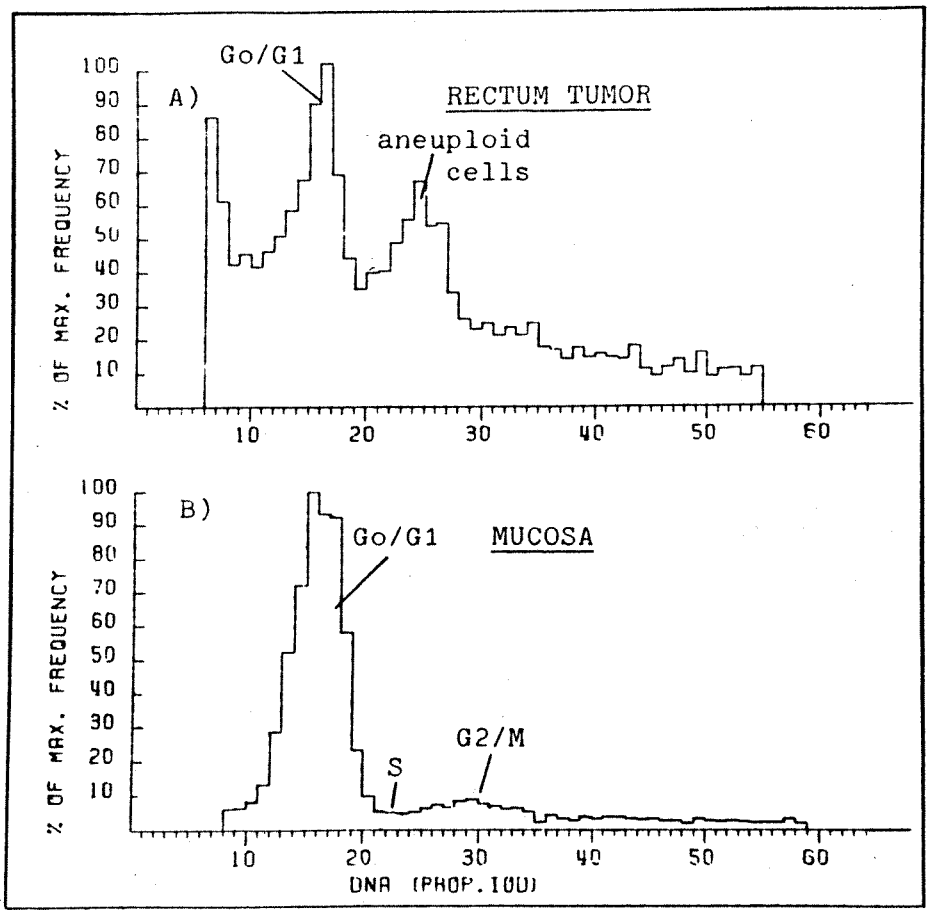


Fig. 3. CEA-surface density histograms of rectum tumor and normal mucosa. Abscissa: Relative CEA-surface density. Ordinate: Cells, % of maximum frequency. The mean CEA-density for tumor cells is about three times higher than for normal mucosa cells.



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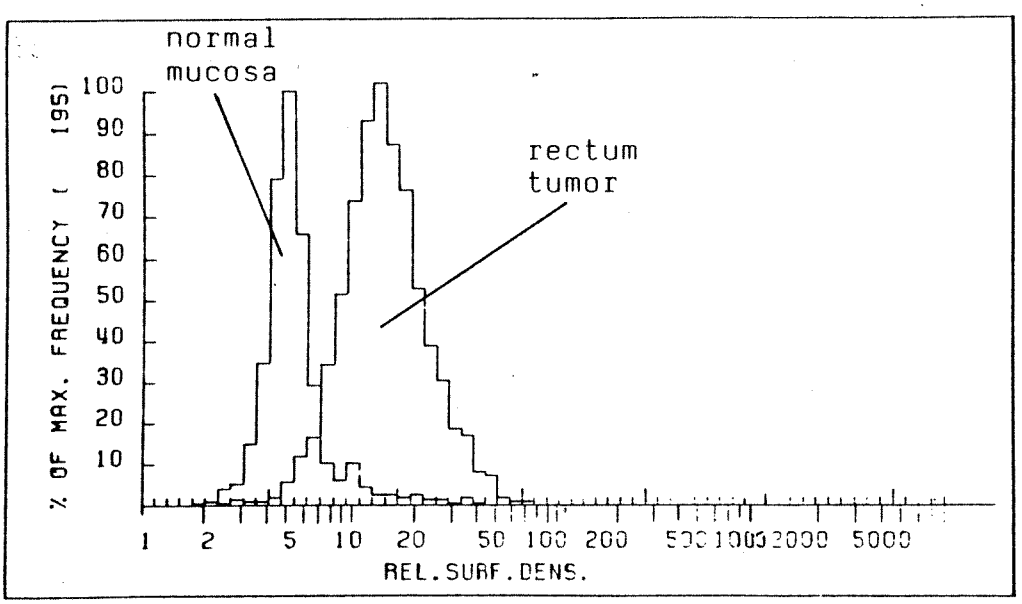

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Simultaneous automatic assessment of DNA-ploidy and CEA-surface density increased the tumor identification rate to 97%.

The metastases behaved identically as the primary tumors, concerning their DNA-ploidy and their CEA-expression, both in the individual case and for the entire population (Fig. 4).

There was no statistical correlation between DNA-ploidy and CEA-density.

The tumors with parallel flow cytometric and immunohistochemical CEA-evaluation showed from 26% to 90% CEA-positive cells for the cytometric and from 10% to 80% CEA-positive cells for the histochemical determination (Fig. 5). The results of both methods were significantly correlated (Spearman-test). There was also a significant correlation for cytometrically and histochemically determined cellular CEA-surface density (Kendall-test) (Fig. 3).

No significant correlation was found for serum CEA and cellular CEA (Fig. 6), not even in consideration of the individual tumor stages (Dukes A to D). Some tumors with little or no cellular CEA (lower left in Fig. 5) were of particular interest. Here, to date, no elevation of serum-CEA was confirmed, neither initially in the presence of advanced tumor stage nor later in case of tumor recurrence.

Tumor stage and DNA-ploidy revealed a significant correlation (Fig. 7). In DNA-aneuploid carcinomas the more advanced Dukes' stages were found with a higher incidence.

The postoperative course of curatively resected patients showed a significantly increased tumor recurrence or poorer prognosis, respectively, for DNA-aneuploid tumors as compared with DNA-euploid tumors (Fig. 8a). For example, 80% of the patients with DNA-euploid tumors were free of recurrence as compared with only 55% of the patients

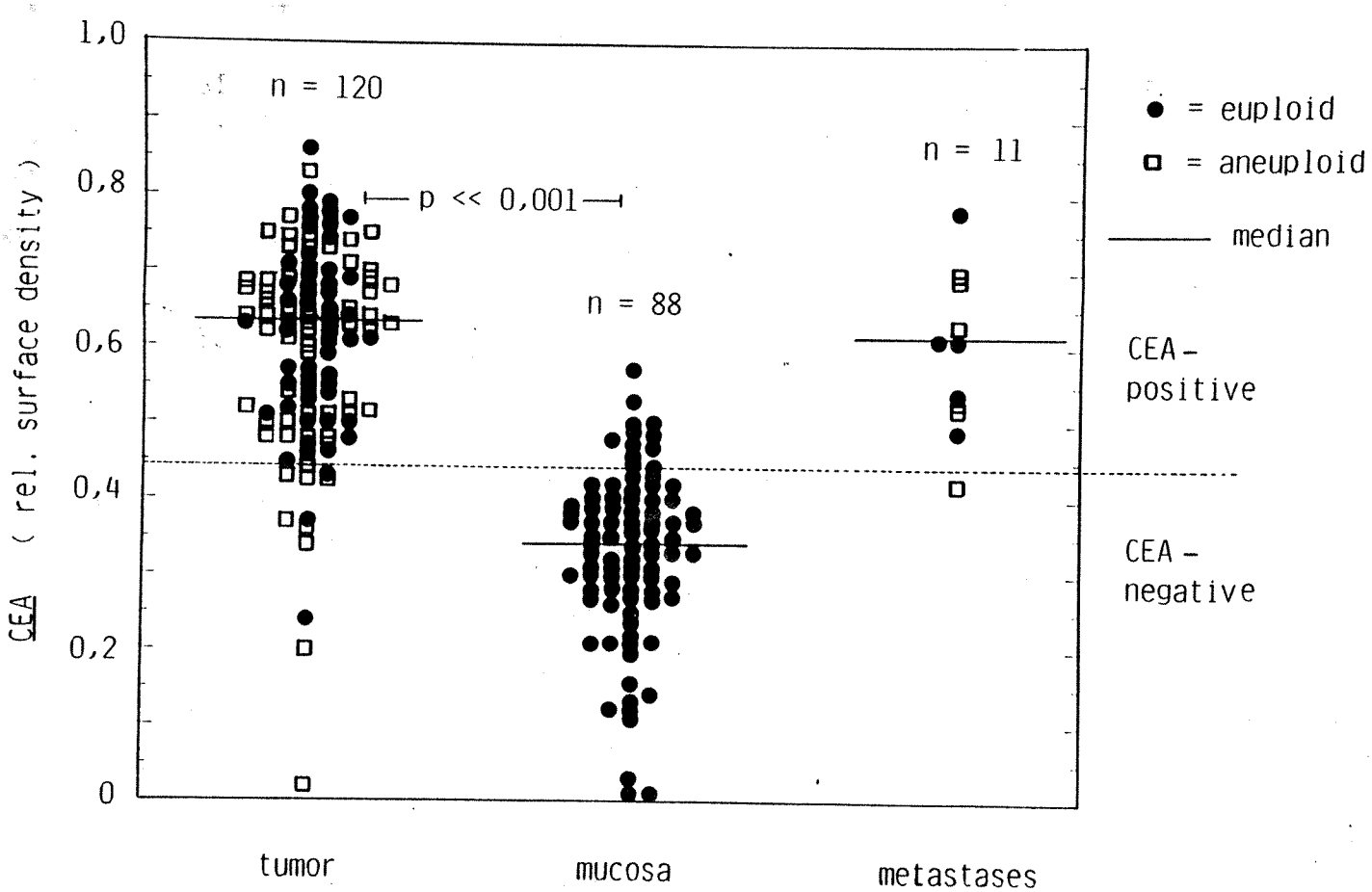

Fig. 4. Simultaneous flow cytometric determination of cellular CEA-surface density and DNA-ploidy in colorectal carcinomas (n=120), associated metastases (n=11) and in normal mucosa (n=88). The best threshold for differentiation between CEA-positive and CEA-negative samples was derived from a ROC-curve.

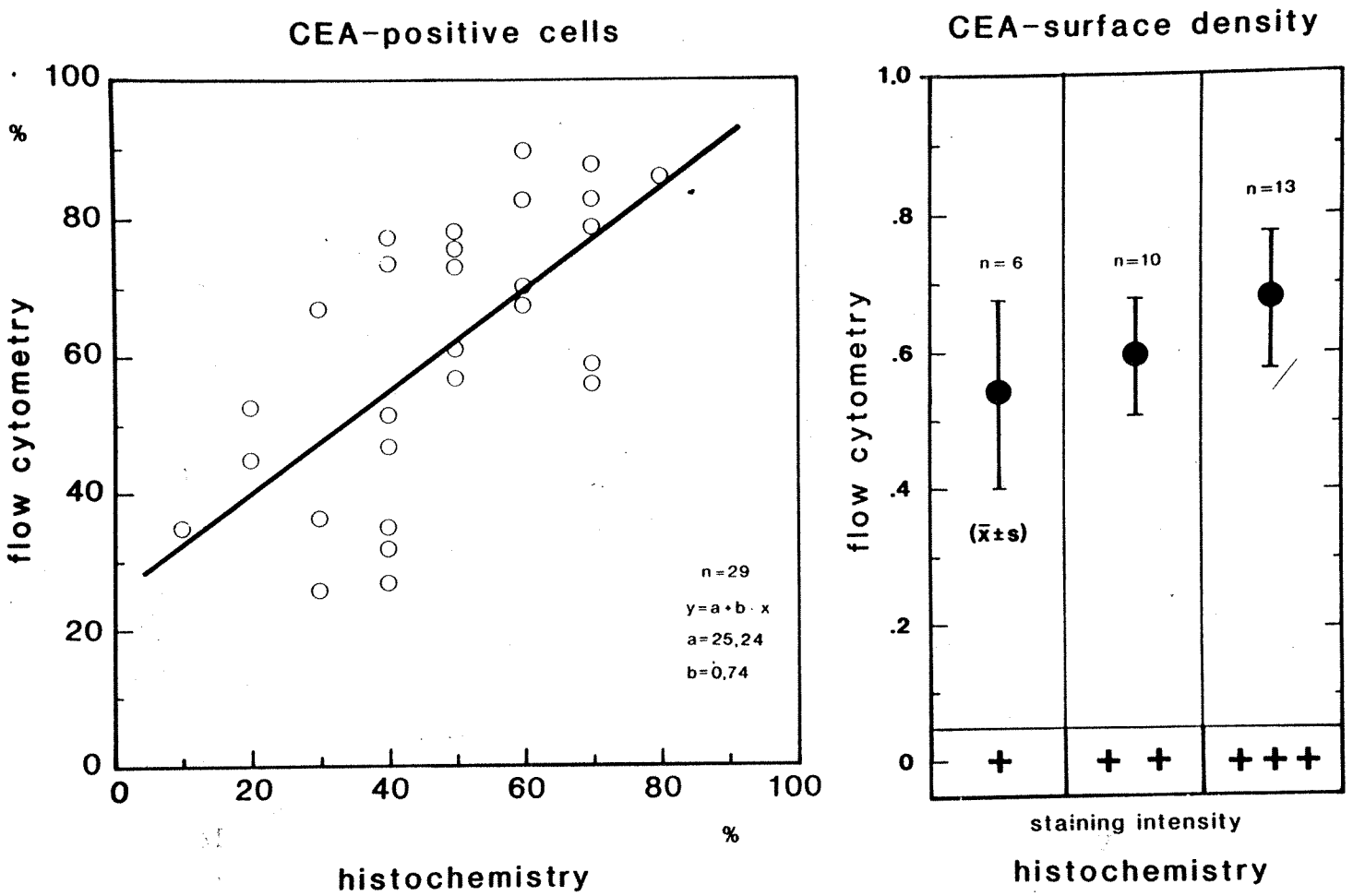

Fig. 5. Correlation between flow cytometrical and immunohistochemical determination of cellular CEA in colorectal carcinomas (n = 29). Left: CEA-positive cells (correlation: r = 0.668, p < 0.001). Right: Histochemical CEA-staining intensity (+ to +++) and flow cytometrical CEA-surface density (mean + s) (correlation: r = 0.320, p < 0.05).

with DNA-aneuploid tumors at 36 months after surgery.

Likewise, significantly more tumor dependent deaths occurred in patients with DNA-aneuploid tumors (Fig. 8b).

Additionally, the CEA-surface density was of some importance for the late prognosis. Curatively resected patients with low-CEA density on the tumor cells showed more often tumor relapses during the follow up period than those with high-CEA density (Fig. 9).

Discussion

The pathohistological diagnosis of malignancy is, in general, easily made by observation of qualitative changes in tissue structure and the relations between the different cells (22). The cytological differentiation of «benign» and «malignant», based on determination of the intracellular architecture, is more difficult (2, 23). Specimens can also be determined as malignant by measurement of abnormal DNA-content or DNA-aneuploidy, respectively, (2, 3, 4). In recent years changes in the cellular DNA-content were found for many human tumors (6), which are now easily analysed by flow cytometry (2, 3, 4). The 59,4% DNA-aneuploidy rate for the colo rectal carcinomas in this study, which is in the range of other series (2, 10, 21), was not sufficient for a reliable automated cancer screening. Therefore, for further improvement, our concept was to evaluate the DNA-ploidy and, additionally, other tumor associated para-

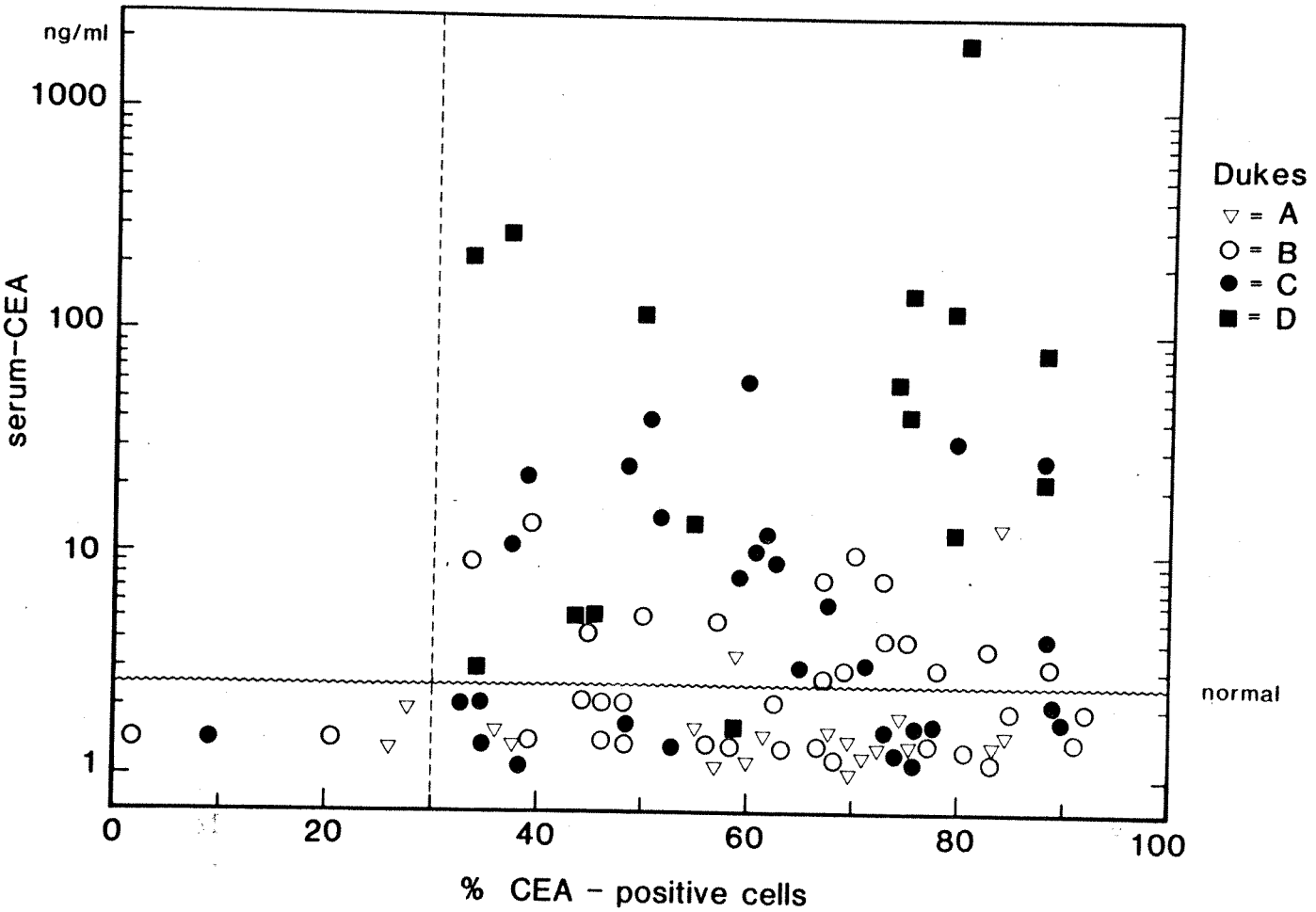

Fig. 6. Correlation between flow cytometrically determined cellular CEA-expression (percent CEA-positive cells) and preoperative serum-CEA in colorectal cancer patients (n = 97). Tumors are differently marked, according to their Dukes-stage. (Tumors left of the vertical dotted line are classified cellular CEA-negative).

meters like CEA with a three parameter flow cytometry technique (25). This enabled simultaneous determination of CEA, DNA-ploidy (Fig. 2 and 3) and cell volume and, thus, automatic restriction (Fig. 1b) on the relevant epithelial cells of colorectal tissue.

Flow cytometric determination of CEA at the cellular level (Fig. 3 and 4) again confirmed its lack of tumor specificity and the high individual variability of its expression (14, 15).

The higher CEA-surface density of carcinomas and the lower density of normal mucosa revealed remarkable overlapping (Fig. 4). Yet, the differentiation between malignant and benign specimens was more distinctive than for immunohistochemical investigations (21).

The 97% tumor identification rate for simultaneous evaluation of DNA-ploidy and CEA-density is considerably higher than for DNA-ploidy alone, indicating, that the loss of resolution in the DNA-measurement without proteolytic digestion is overcompensated by the gain of information with the CEA-measurement; additionally, these 97% are a comparable high rate for automated cancer screening (2, 4). Moreover, there are possibilities for improvement, especially by use of new and more specific tumor associated antingens. Although, to date, this flow cyto metric measurement has no clinical significance for the early detection of colorectal cancer, it may serve as a model for the automated identification of other tumors, e.g. lung, bladder, and cervix uteri cancers.

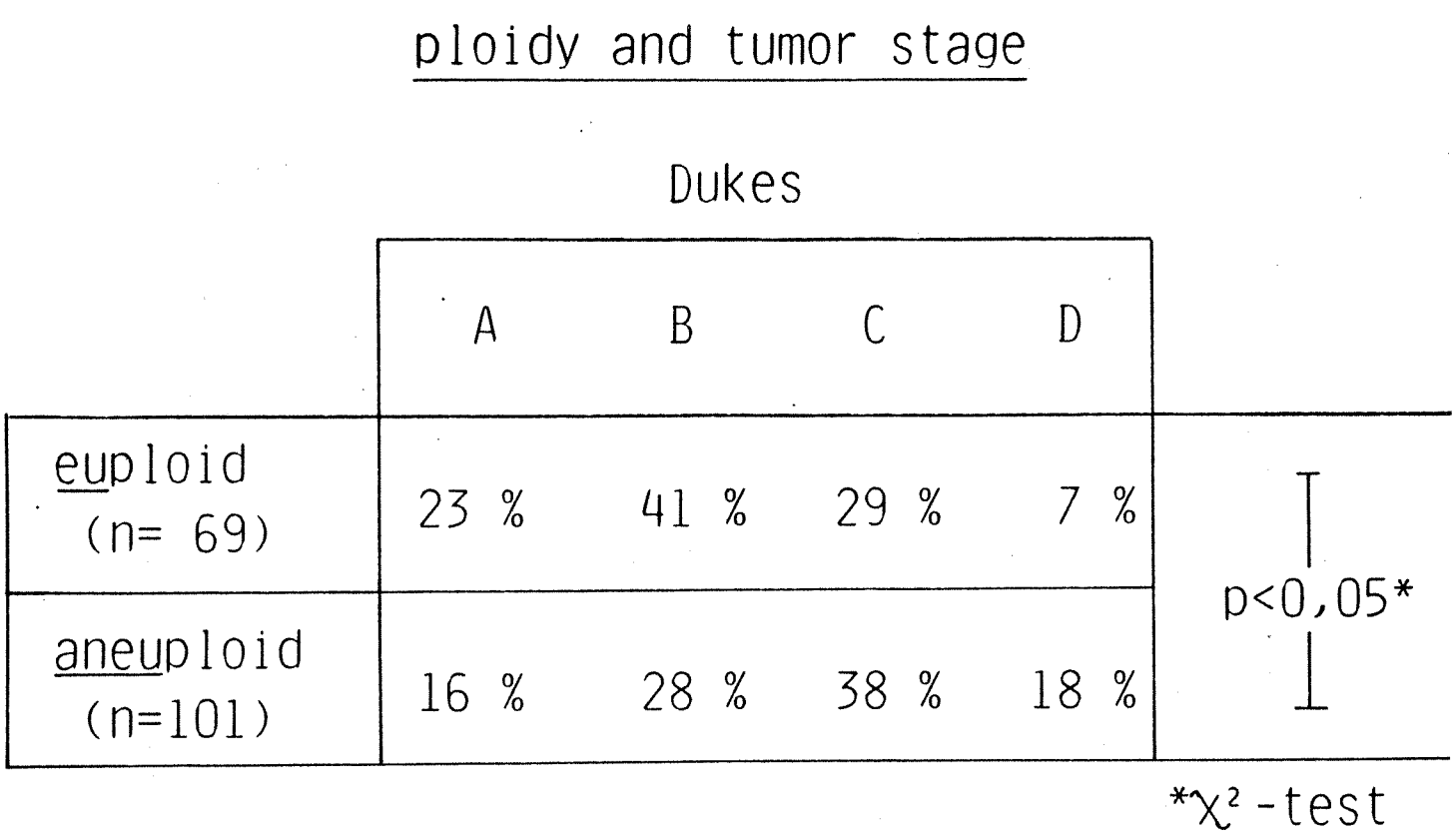

Fig. 7. Correlation between tumor DNA-ploidy and tumor stage (Dukes A to D) for colorectal carcinomas (n = 170) at the time of surgery.

Flow cytometrically determined cellular CEA-density (Fig. 4) showed no significant changes between colorectal primary and metastatic tumors. This is in accordance with histochemical investigations (11, 24) and appears particularly meaningful for new diagnostic measures such as radio-immunoimaging. Furthermore, the evaluation of whether or not a tumor contains CEA-producing cells may be helpful for the interpretation of routine serum-CEA determinations (12).

Thus, in earlier histochemical investigations (27), colorectal tumors without CEA-stain never revealed elevated serum-CEA levels.

Analysis of cellular CEA is usually made by immunohistochemical investigations (11, 12, 14, 17, 20, 21). Flow cytometry offers an easy, exact and even molecularily quantitative evaluation of cell surface antigens in colorectal carcinomas (25); nevertheless, perhaps due to problems with single cell preparation, cellular CEA determinations have only been done by few investigators (5, 8).

Our series with parallel histochemical and cytometrical CEA-analysis of the same samples now shows significant correlations for both determination methods (Fig. 5). The lower statistical significance for comparison of cellular CEA-surface densities may be due to histochemistry and its difficulties to approximate antigen density.

Cellular CEA and serum-CEA (Fig. 6) were not significantly correlated. Also histochemical evaluations did not reveal any correlation (26). The serum-CEA level seems to be not only influenced by cellular CEA and tumor stage, but, additionally, by factors like antigen secretion, vascular supply, degree of tissue necrosis or CEA-metabolism (15, 26, 27, 29). Yet, it should be taken into account for routine CEA-determinations that tumors without or with only few CEA-positive cells never developed elevated serum levels.

The biological importance of DNA-ploidy for tumor classification and prognosis has been established by cytophotometric (1, 6) and, currently, flow cytometric analyses (3, 4,

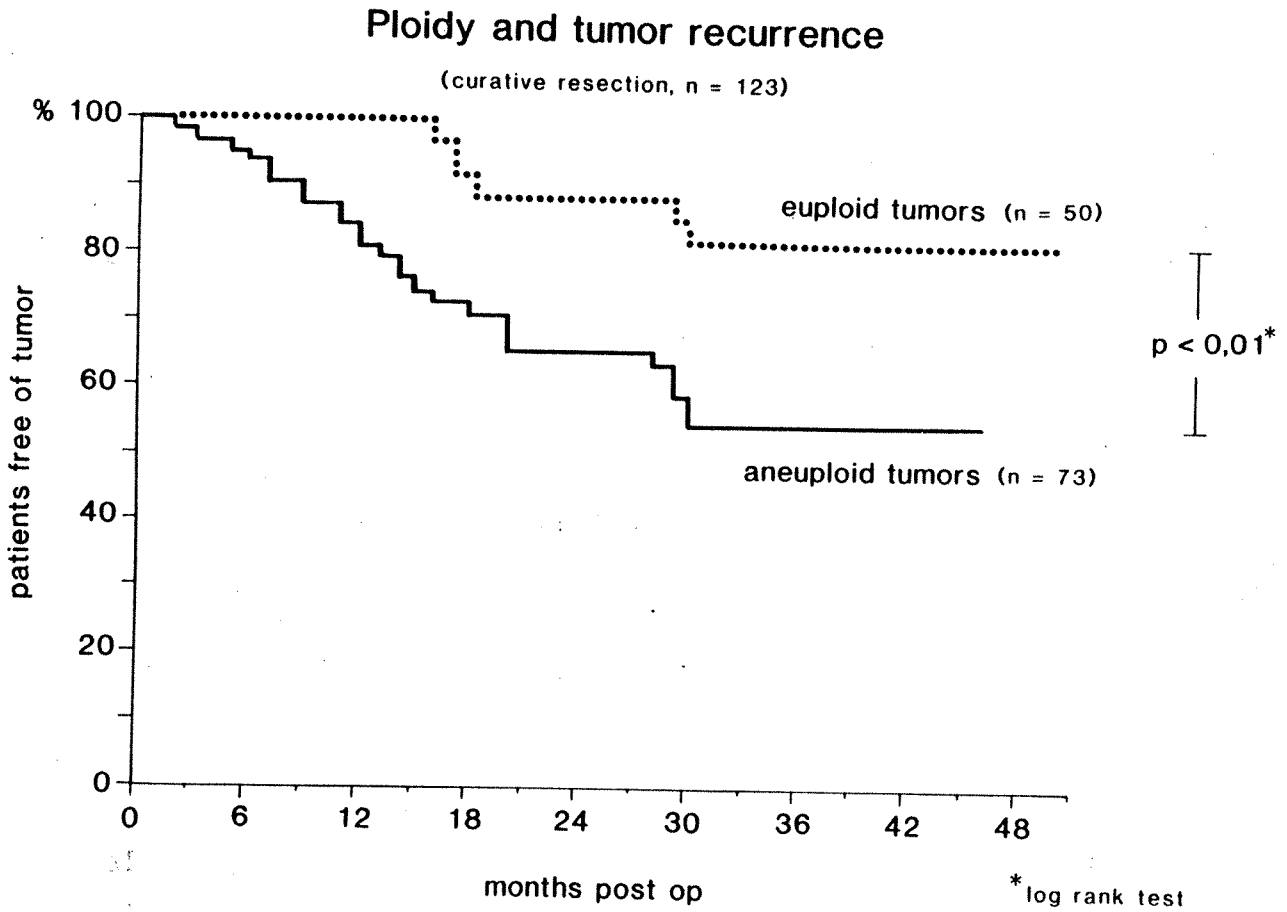

Fig. 8. a) Tumor relapse of curative resected colorectal cancer patients with DNA-euploid (n = 50) and DNA-aneuploid (n = 73) tumors.

7, 10). In most of these series DNA-aneuploidy was combined with a poorer prognosis. This study of 170 large bowel cancers clearly pointed out the significant correlation of DNA-aneuploidy and increased malignancy of more advanced tumor stage, respectively (Fig. 7). For smaller numbers of tumors this correlation has not yet been found (10, 21). The poorer prognosis of the DNA-aneuploid colorectal tumors was particularly apparent in consideration of the higher incidence of tumor recurrence (Fig. 8a) and tumor dependent death (Fig. 8b). This unfavorable prognosis was not only due to an even more advanced tumor stage at the time of surgery (28).

The main relevance of serum-CEA measurements is the patients' follow up and the early detection of tumor relapse (14, 18, 23). The pretherapeutic serum-CEA level as a prognostic parameter is restricted to the 45% to 60% of colorectal cancer patients with elevated CEA at that time (14, 15, 23, 29). On the other hand, cellular CEA can be determined by flow cytometry immediately after surgery and may be used for further evaluation of prognosis in all patients.

In consideration of tumor relapses (Fig. 9) also cellular CEA surface density proved to be a factor of prognostic relevance. First indications for this correlation between cellular CEA and clinical course were given by histochemical investigations (17). One reason for the poorer prognosis of the low-CEA density colorectal cancers might be their lower degree of tumor cell differentiation, which has been demonstrated histochemically (12, 27).

In conclusion, this investigation of colorectal carcinomas clearly demonstrated, that tumor DNA-ploidy and cellular CEA are biologically relevant parameters, which can be measured quantitatively by flow cytometry.

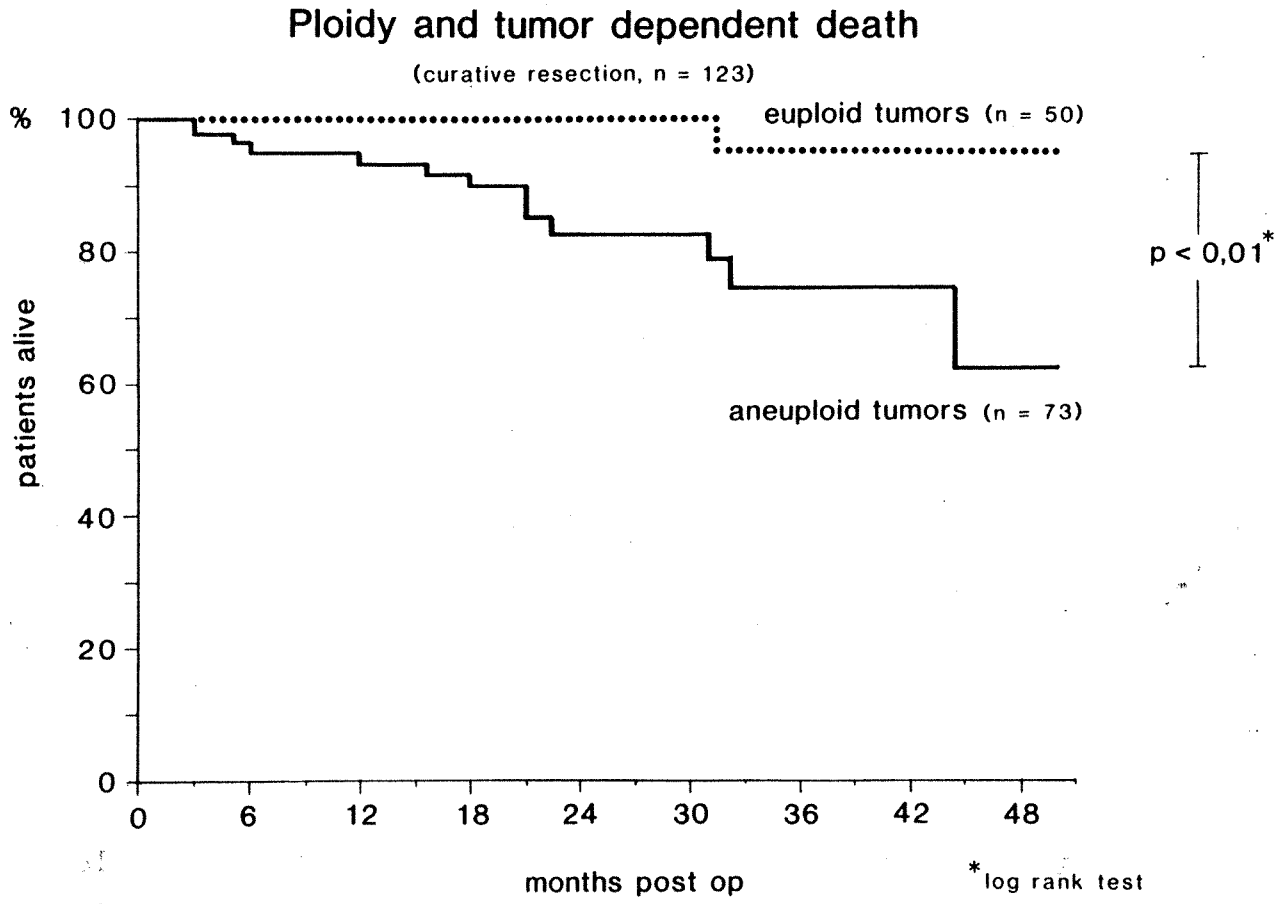

Fig. 8. b) Tumor dependent death of curative resected colorectal cancer patients with DNA-euploid (n = 50) and DNA-aneuploid (n = 73) tumors.

Thus, determination of DNA-ploidy and cellular CEA-expression may provide a completion of conventional tumor examination, better evaluation of the patients' individual risk as well as additional guidelines for theatment modalities and for follow up.

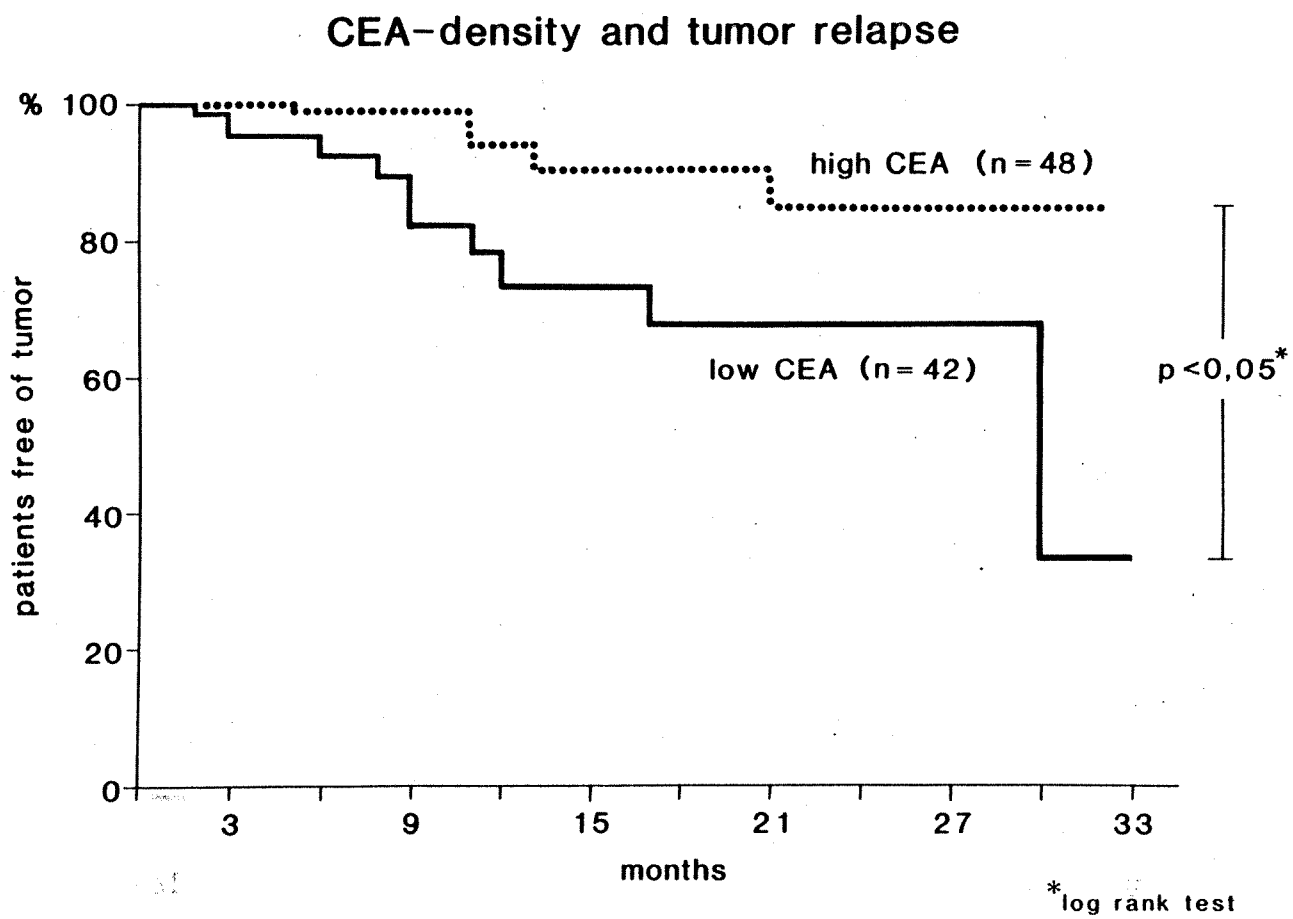

Fig. 9. Tumor relapse for curative resected colorectal cancer patients and tumors with high (n = 48) and low (n = 42) cellular CEA-surface density.

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