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Quantitative DNA measurement by flow cytometry and image analysis of human nonseminomatous germ cell testicular tumors

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Abstract Current clinical staging, which includes the use of serum tumor markers and imaging techniques, fails to identify the 30–40% of clinical stage I (CS I) nonseminomatous germ cell testicular tumor (NSGCT) patients who have occult metastatic disease. Therefore, there is a real clinical need to evaluate new biological parameters of the primary tumor that might be useful as predictors of occult metastatic disease. This study was undertaken to compare quantitative DNA measurements by flow cytometry and image analysis in CS I NSGCT, and to analyze the relevance of these parameters for predicting occult lymph node involvement. Different blocks of formalin-fixed, paraffin-embedded NSGCTs of 62 CS I patients who underwent retroperitoneal lymph node dissection between 1985 and 1989 were prepared according to the Hedley technique, and analyzed by quantitative cytometry. Thirty-six (58.1%) patients had histologically proven lymph node involvement (pathological stage II), whereas 26 (41.9%) patients (pathological stage I) had neither lymph node metastases according to retroperitoneal lymph node dissection (RPLND) specimens nor tumor recurrence during follow-up. Concordant results were found in 76.5% of the samples by both cytometric techniques. For flow cytometry, the percentages of aneuploid cells in the S- and the G2M + S-phase were the most robust predictive parameters for lymph node involvement, whereas for image analysis the 5c exceeding rate (5cER) had the most predictive significance. Based on the experience obtained in this study, both cytometric techniques provide additional information on tumor aggressiveness that might be useful in therapeutic selection of early stage NSGCT patients for either RPLND or surveillance only.

Key words Testicular cancer · DNA content · Flow cytometry · Image analysis

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DNA ploidy analysis has been shown to have prognostic and, in some cases, diagnostic value in the clinical evaluation of patients with different human solid tumors [12, 14, 23]. Since its inception in the mid-1970s flow cytometry (FC) has been commonly used, and it is considered by many to be the standard method for DNA quantification. FC is an accurate and rapid method of DNA measurement, which allows analysis of large numbers of cells. Recent advances in image analysis (IA), especially the introduction of automated video cameras integrated to computer software systems for high-resolution detection of cellular components, have improved this method so that it is now rapid, simple, and economical. Several studies have compared these techniques (FC and IA) recently. These studies have shown a good correlation between these two methods for DNA quantification [5, 15]. In 1983 Hedley et al. [10] described a technique for obtaining nuclear suspensions suitable for analytical cytometry from paraffin blocks. The use of paraffin blocks for FC analysis has played a critical role in establishing the value of DNA ploidy analysis in several human neoplasms, by allowing retrospective studies of patients for whom long-term follow-up of clinical outcome is available [9].

One of the main problems in the care of patients with clinical stage I (CS I) nonseminomatous germ cell testicular cancer (NSGCT) is that of occult retroperitoneal or distant metastases. Current clinical staging, which in-

cludes serum tumor markers and imaging techniques such as computed tomography and sonography, fails to identify the 30–40% of CS I patients who have occult metastases [4, 16]. There is, therefore, a real clinical need to evaluate new biological parameters and combinations of multiparameter measurements that might be useful as predictors of occult metastatic disease.

This study was designed to compare the DNA content obtained by FC and IA in early-stage NSGCT. This type of comparative analysis is necessary to evaluate and to determine the predictive significance of these two analytical techniques for DNA quantification in early-stage NSGCT patients.

Materials and methods

All 62 NSGCT patients investigated in this study were initially CS I (no evidence of metastatic disease according to imaging techniques and serum tumor markers) prior to retroperitoneal lymph node dissection (RPLND). All had been treated by orchiectomy and RPLND and had been followed up for at least 2 years. Thirty-six patients (58.1%) had histologically proven lymph node involvement (pathological stage II), whereas 26 patients (41.9%) (pathological stage I) had neither lymph node metastases according to the RPLND specimens nor tumor recurrence during follow-up.

Single cell preparation

Formalin-fixed and paraffin-embedded tissue from 62 different NSGCT, CS I, (archival material: 132 blocks) were prepared by the Hedley technique to measure DNA content by FC and IA [10]. Briefly, paraffin blocks were recut in areas corresponding to the most representative portions of the tumor previously identified NSGCT. 50-μm sections were cut sandwiched between corresponding 5-μm sections for routine HE staining to reconfirm the presence of tumor tissue. The tissue sections were then deparaffinized in two changes of xylene (5 ml for 10 min) at room temperature. Rehydration was accomplished by exposing the sections to a sequence of 5 ml ethanol solutions (100%, 95%, 70%, 50%) for 10 min each at room temperature. The tissue was then washed twice in distilled water. After decanting the supernatant, the tissue was minced with a pair of iris sissors. Then 2 ml of 0.5% pepsin solution (Pepsin Sigma, activity 2,500–3,200 units/mg protein in 0.9% NaCl, adjusted to pH 1.5 with 2 N HCl) was added to each sample. The quality of tissue digestion was monitored by using an inverted microscope to observe when optimal single cell solutions had been achieved and to avoid overdigestion. Incubation times averaged 40 min and ranged from 30 to 60 min. As recently outlined, different components of scarred tissue, fibrosis, and necrosis have a relevant impact on enzymatic tissue disaggregation [18]. Therefore, the individual sample digestion times varied depending upon the tissue components (cancer, teratoma, scar, and connective tissue). All digestions were carried out in a water bath at 37°C. Tissue disaggregation was enhanced by vortexing the sample at 5-min intervals. Digestion was stopped by adding 2 ml phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The cell suspension was filtered through a 45-μm nylon mesh (TETKO, Briarcliff Manor, N.Y.) and layered over 2 ml 1 M sucrose. After two washes with 2 ml of 1% BSA/PBS solution the nuclei were resuspended in 0.5 ml BSA/PBS. The cell concentration was determined by a hemocytometer and adjusted to 1×10^6 per ml. Quality and cell quantity for each sample was then checked by Diff-Quik staining (American Scientific Products, McGaw Park, Ill.) on one drop from each sample.

In each preparation series, formalin-fixed, paraffin-embedded normal cerebellar tissue from a single patient sample was used as an external diploid control to monitor any potential variation in sample preparation, instrument calibration and alignment, and Feulgen staining that might affect the results.

Flow cytometric DNA measurement

For FC, the samples were stained according to the method of Vindelov et al. [22]. Approximately $1\text{–}2 \times 10^6$ nuclei were stained with 3 ml Vindelov's propidium iodide solution [0.01 M Trizma Base, 10 mM NaCl, 50 mg/ml of propidium iodide (Sigma), 1 mg/ml of RNAse (Sigma)]. After a 30-min incubation in the dark, the samples were centrifuged and then resuspended in 1 ml of Vindelov's propidium solution without RNAse. The samples were held at 4°C and run within 1–3 h of preparation.

FC was performed by using a Coulter profile II flow cytometer (Coulter Electronics, Hialeah, Fla.). The flow cytometer contained an air-cooled argon laser (488 nm excitation wavelength for propidium iodide) and was calibrated with DNA Check beads (Coulter). The emission fluorescence of propidium iodide (> 550 nm) was collected after passing through a series of filters (488 nm long-pass dichroic filter, 488 nm blocking filter, and 550 nm long pass dichroic filter). Approximately 25,000–50,000 events were collected per sample by the use of two-parameter gating. Doublets were eliminated by gating on peak versus linear propidium iodide fluorescence. The DNA histograms were evaluated by Modfit software (Verity Software House, Topshaw, Me.).

Because of the lack of easily identifiable external normal diploid (2c) controls suitable for comparison with all the formalin-fixed, paraffin-embedded samples, the first peak in all the DNA histograms was assumed by convention to be representative of the 2c G0/G1 peak of the non-neoplastic cells present [7, 9, 10]. The DNA index (DI), defined as the ratio of the modal channel number of G0/G1 peak of the malignant cells to that of G0/G1 peak of the normal diploid cells, was used to quantify the amount of DNA and determine the DNA ploidy of the samples. A DNA histogram was classified as diploid ($DI = 1$) if there was a single G0/G1 peak that contained > 80% of the total events. A DNA histogram with one or more G0/G1 peaks (excluding the 2c G0/G1 peak) and $DI \neq 1$ was classified as aneuploid. Aneuploid samples were subclassified according to the value of DI (hyperdiploid: $1 < DI < 1.8$; tetraploid: $1.8 \leq DI \leq 2.2$; hypertetraploid: $DI > 2.2$). In order to differentiate tetraploid samples from proliferating diploid samples, the G0/G1 peak of the tetraploid sample had to contain > 20% of the total events.

Normal testicular tissue contains haploid/diploid compartments. Where the presence of haploid was confirmed by IA, the second peak in the FC histogram was commonly defined as the diploid peak. DNA histograms that contained peaks with a full-peak coefficient of variation (CV) > 10, aneuploid peaks with < 10% of the total events, and closely overlapping peaks with high CV values (> 10) were classified as insufficient data.

Slide preparation and Feulgen DNA staining for image analysis

A 30-G needle was used to deposit three drops of the unstained cell suspension directly onto a glass slide (sedimentation technique). According to a prior study this technique was superior to the cytospin technique [18]. If necessary BSA/PBS solution was used to adjust the cell concentration to give an optimal distribution of single, non-overlapping nuclei [18]. The slides were air-dried and stored at room temperature until Feulgen DNA staining. After postfixation in 10% neutral buffered formalin for 20 min, the slides were stained by the Feulgen reaction with the Roche Image Analysis Systems Feulgen staining kit (Roche Image Analysis Systems, Elon College, N.C.). After DNA staining, the slides were stored in a dark container and measured within 2 weeks. Rat liver touch preparations provided in the staining kit were also stained, to serve as an external control for the DNA stain.

Table 1 Comparison of flow cytometry and image analysis concerning classification of DNA ploidy in 62 human nonseminomatous germ cell testicular tumors (NSGCT) (132 tumor samples)

Flow cytometry	Image analysis				Insufficient data[a]
	Diploid	Aneuploid			
		Hyperdiploid	Tetraploid	Hypertetraploid	
Diploid	10	3			
Aneuploid					
Hyperdiploid	8	66			
Tetraploid	2	5	2		
Hypertetraploid	3	3			
Insufficient data[b]		19			11

[a] CV of the 2c peak > 10
[b] CV of the 2c peak > 10 or not enough events obtained

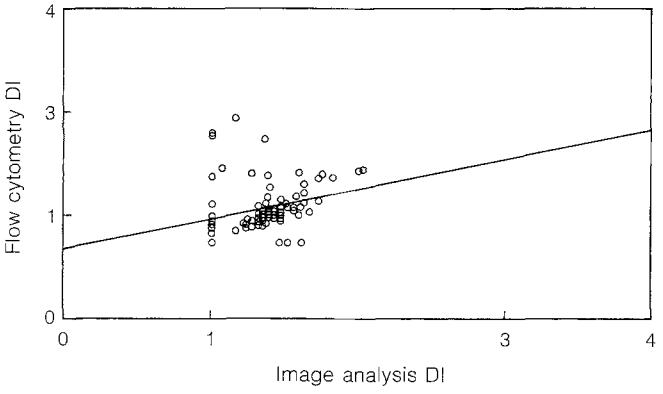

Fig. 1 Correlation between DNA indices (DI) of 56 non-seminomatous germ cell testicular tumors (NSGCT; 102 samples) by flow cytometry and image analysis ($r = 0.88$, $P < 0.0001$)

Cytometric image analysis

IA was performed with the ROCHE Pathology Workstation (Roche Image Analysis Systems, Elon College, N.C.). Generally, 300 viable nuclei were classified interactively for each sample. The slides were examined in a meander fashion to avoid double analysis. Spindle-shaped nuclei of normal testis cells and fibrocyte nuclei were used for internal 2c controls. If only one peak corresponding to the diploid population was identified, the sample was defined as diploid. The tumor was classified as aneuploid if a prominent peak was identified between 2c and 4c that occurred outside the diploid range and contained more than 10% of the total number of nuclei. The aneuploid histograms were subclassified according to the value of DI (hyperdiploid: $1.1 < DI < 1.8$; tetraploid: $1.8 \leq DI \leq 2.2$; hypertetraploid: $DI > 2.2$). A sample was classified as tetraploid when the 2c G2/M peak contained over 20% of the total number of nuclei and when nuclei were present in the 8c position (2c G2/M peak for the tetraploid stemline). The percentage of nuclei with a DNA content over 5c was defined as the 5c-exceeding rate (5cER) [8]. The DNA content determinations by FC and IA were performed independently and blindly by different operators, which means that neither the pathological stage nor the result of each type of analysis was revealed until both cytometric analyses had been completed.

Statistical analysis

For statistical comparison of data collected from the two procedures the univariate chi-square test and the linear regression test were performed. *P*-values less than 0.05 were considered statistically significant. All *P*-values were two-tailed.

Results

Flow cytometry

Of the 132 NSCGT samples processed for FC, 102 (77.3%) had interpretable DNA histograms. Of these 102 neoplastic samples, 13 (12.7%) were diploid and 89 (87.3%) were aneuploid. Of these 89 aneuploid tumors, 74 (83.1%) were hyperdiploid, 9 (10.1%) were tetraploid, and 6 (6.7%) hypertetraploid. The mean CV of the 2c peak for all samples was 6.32 (range 2.99–9.68). There were 18 samples that did not provide enough events, and 12 samples had a CV of the 2c peak > 10 (Table 1).

Image analysis

Of the 132 NSGCT samples, 121 (91.6%) yielded interpretable data by this technique: 23 (19.0%) were diploid, and 98 (81.0%) were aneuploid. The aneuploid tumors consisted of 96 (97.9%) hyperdiploid and 2 (2.1%) tetraploid. Hypertetraploid samples were not observed using this technique, probably because of the small number of nuclei analyzed compared to FC. The mean CV of the 2c peak was 6.96 (range 3.21–9.89). The mean 5cER was 5.33 (range 0.00–25.58). For pathological stage I the mean 5cER was 2.42 (range 0.00–17.75) and for pathological stage II, 7.88 (range 0.00–25.58). Eleven samples provided too few events (not enough nuclei on the slide, or CV of the 2c peak > 10) (Table 1).

Comparison of the two methods

In 102 testicular tumor samples that had interpretable FC and IA data, concordance of DNA ploidy classification was observed in 78 (76.5%), as defined by DI. Linear regression of these data results in a significant correlation ($r = 0.88$, $P < 0.0001$) (Fig. 1). Figures 2 and 3 are examples of concordant diploid DNA histograms and aneuploid DNA histograms, respectively. Figures 4–6 are examples of discordant DNA ploidy results. In Fig. 4 an aneuploid stemline (near diploid, $DI = 1.26$) is detected by FC, whereas by IA a diploid DNA distribution only is obtained. Figures 5 and 6 are examples of a tetraploid and a hypertetraploid tumor in FC, whereas IA exhibits diploid tumors in both cases. The IA results may be due to the small number of analyzed nuclei compared to FC. Table 1 summarizes the DNA ploidy classification by FC and IA of all 132 NSGCT samples. Nineteen of the 30 tumor samples that did not provide sufficient data by FC

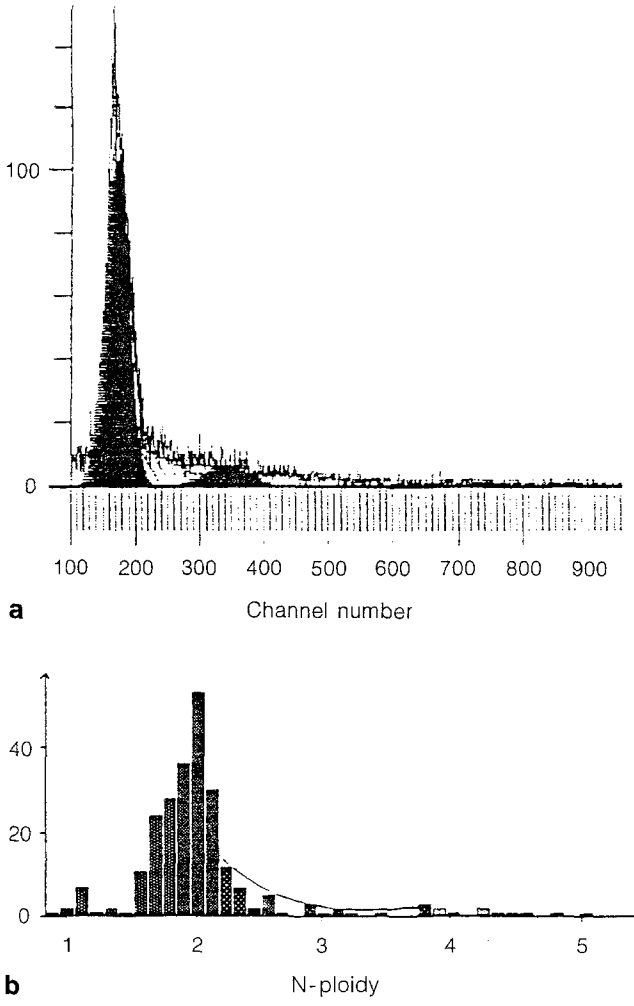

Fig. 2a, b Comparison of concordant diploid DNA histograms generated by **a** flow cytometry (FC) and **b** image analysis (IA) of the same NSGCT sample (*x-axis* DNA content, *y-axis* cell count). DI = 1.0

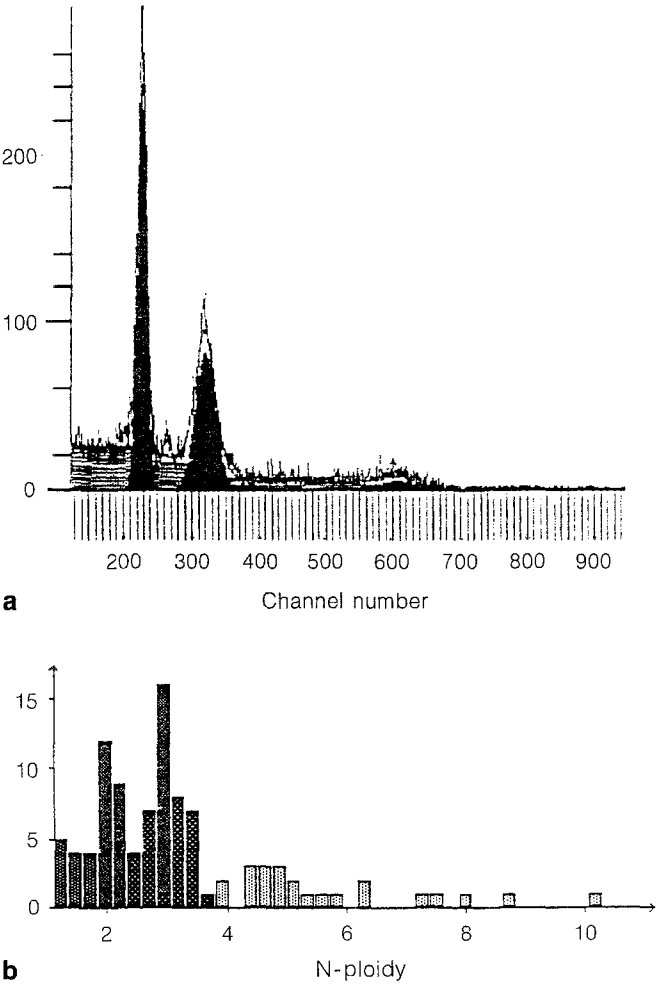

Fig. 3a, b Comparison of concordant aneuploid DNA histograms generated by **a** FC and **b** IA of the same NSGCT sample (*x-axis* DNA content, *y-axis* cell count). The first peak in all histograms was representative of the normal 2c nuclei. **a** DI = 1.42; **b** DI = 1.46

Table 2 Correlation of flow cytometry (FC) and image analysis (IA) parameters with pathological lymph node stages (*I*, no lymph node metastases; *II*, histologically confirmed lymph node metastases) *P* value (Chi-square test)

	P-value (Chi-square test)
DNA-Index[a, b]	> 0.05
Diploid nuceli (%)[a]	> 0.05
Aneuploid nucli (%)[a]	> 0.05
Total S-phase[a]	> 0.05
Total G2M + S-phase[a]	> 0.05
S-phase diploid nuclei[a]	> 0.05
G2M + S-phase diploid nuclei[a]	> 0.05
S-phase aneuploid nuclei[a]	0.0001
G2M + S-phase aneuploid nuclei[a]	0.0001
5cER (exceeding rate)[b]	0.0006

[a] Obtained by FC
[b] Obtained by IA

Table 3 S- and G2M + S-phase of aneuploid cells and 5cER as prognostic markers for occult metastatic disease in early stage NSGCT. Of 62 NSGCT cases, 50 (80.6%) were correctly classified as pathological stage I and II

	Pathological stages		
	I	II	I + II
Low risk	21	7	28
High risk	5	29	34
	26	36	62

(CV > 10, or not enough events) were interpretable by IA. Figure 7 illustrates one example.

Table 2 shows the statistical correlation of the FC and IA data with the 2 pathological stages (I, II). DIs, percentages of diploid and aneuploid cells, total S-phase and S-phase of the diploid cells did not correlate with the

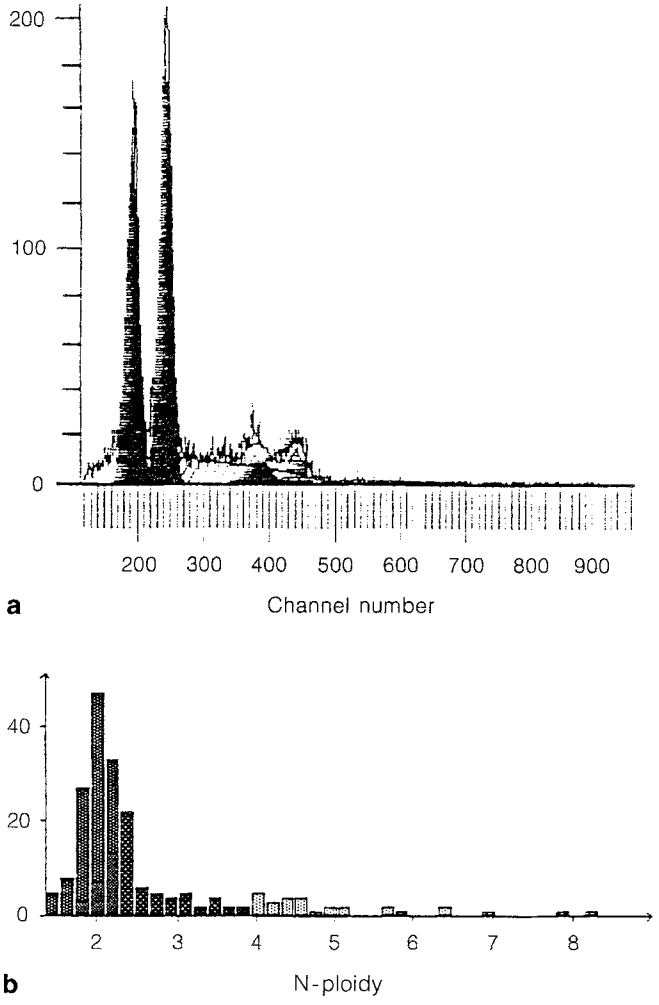

Fig. 4a, b Comparison of DNA histograms generated by **a** FC and **b** IA of the same NSGCT sample (*x-axis* DNA content, *y-axis* cell count). **a** DI = 1.26 (aneuploid/near-diploid); **b** DI = 1.00, 5cER (exceeding rate) = 4.98

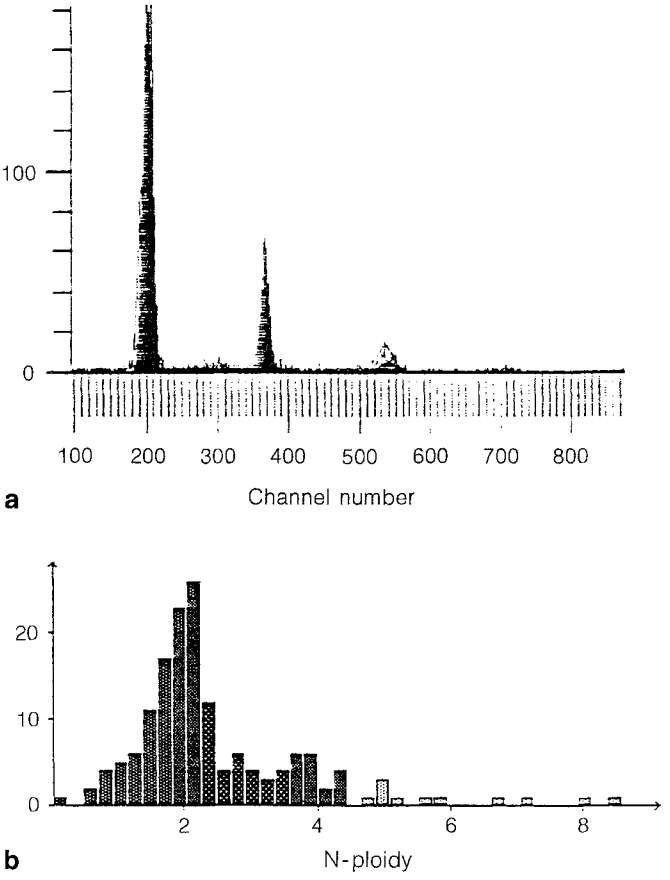

Fig. 5a, b Comparison of DNA histograms generated by **a** FC and **b** IA of the same NSGCT sample (*x-axis* DNA content, *y-axis* cell count). **a** DI = 1.911 (G0/G1 tetraploid peak = 32.7% of all events); **b** Diploid, 11.5% of all events between 3.6c and 4.4c

lymph node status. However, percentages of the aneuploid cells in the S- and G2M + S-phases (parameter only available by FC) and the 5cER (obtained by IA) were strongly correlated with pathologically proven lymph node metastases.

The optimal cut-off was 25% for the S-fraction, 27% for the G2M + S-fraction, and 4.2% for the 5cER. Tumors above the cut-off value of at least one marker were considered as being involving a high risk and those below the cut-offs of all three markers, a low risk of metastatic disease. Using all three parameters (S-and G2M + S-phase of aneuploid cells, 5cER) as prognostic markers, 21 (80.8%) of 26 cases in pathological stage I and 29 (80.6%) out of 36 in pathological stage II were correctly classified (Table 3). Test efficiency (percentage of cases correctly classified for both pathological stages) was 80.6%.

Discussion

The introduction of flow cytometric methods made it possible to perform rapid measurements of large populations of tumor cells [13, 19]. It became possible not only to reveal the presence of aneuploid cells in the tumor, but also to give estimates of cells in the various cell cycle phases. Retrospective studies of nuclei extracted from paraffin-embedded tissue and analyzed by FC have provided the basis of much of our knowledge of the prognostic value of measuring DNA content in tumors [2, 9, 21]. However, the prognostic significance of DNA ploidy and proliferation rates vary between different types of malignancies [9].

Recent advances in IA, especially the introduction of automated video cameras integrated into computer software systems for high-resolution detection of cellular components, have improved this method to the point where it is now rapid, simple, economical and, thus, comparable with FC. With the increased interest in the evaluation of DNA content in human solid tumors, a thorough understanding of the methodological and technical aspects of FC and IA as the two main methods in

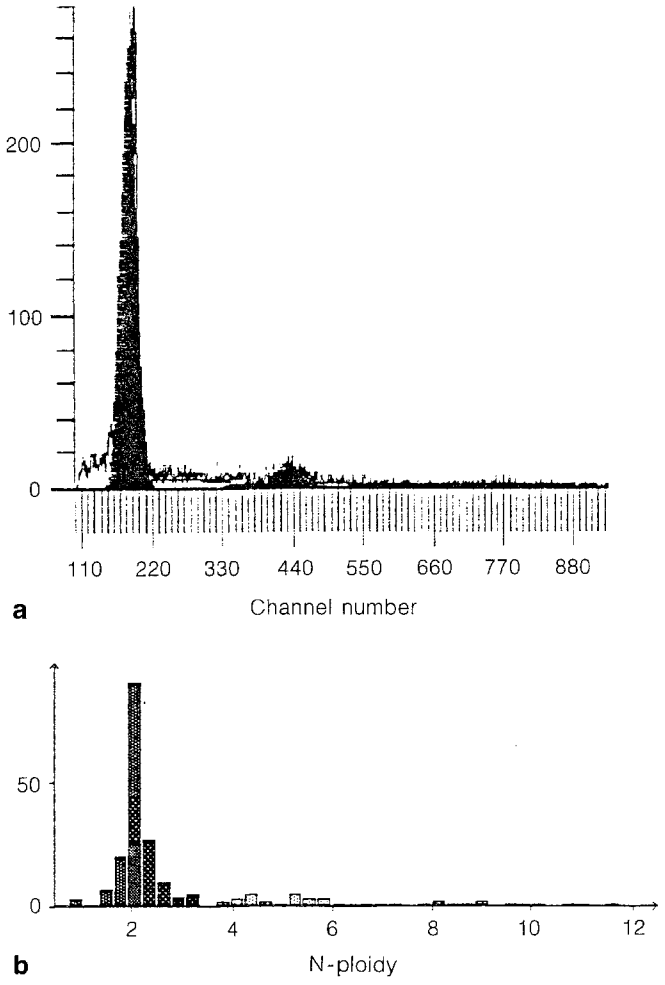

Fig. 6a, b Comparison of DNA histograms generated by **a** FC and **b** IA of the same NSGCT sample (*x-axis* DNA content, *y-axis* cell count). **a** DI = 2.37 (hypertetraploid); **b** diploid population

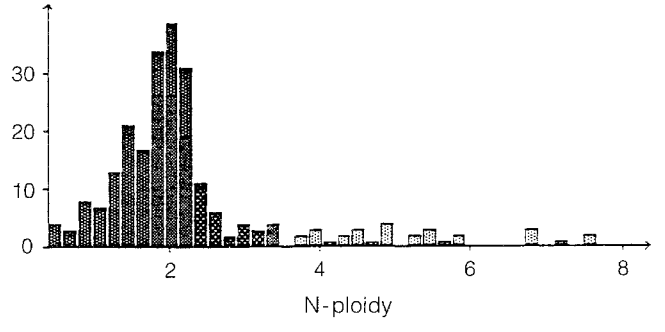

Fig. 7 DNA histogram of a tumor sample (NSGCT); on IA enough data were present (337 nuclei measured. DI = 1.70, whereas there were too few events in FC (*x-axis* DNA content, *y-axis* cell count)

DNA measurements is needed. Most of the comparative studies concerning FC and IA have been performed in breast carcinomas. To our knowledge, no similar comparative data are available in studies of testicular cancer. This study was performed to compare DNA quantifications using both techniques in early-stage NSGCT.

The main problem in the care of patients with early stage (CS I) NSGCT is the detection of occult retroperitoneal metastatic disease. The use of serum tumor markers and imaging techniques, including CT scan and sonography, have not reliably predicted occult disease. If CS I patients are followed up (surveillance strategy), about 35% will develop metastatic disease within 2 years, whereas 65% of such cases will never have any tumor recurrence [4, 16]. If NSGCT patients of this clinical stage undergo RPLND, 30–35% have lymph node metastases. The existing diagnostic criteria needs to be improved in order to detect patients at low and high risk respectively, and thus to minimize over or undertreatment [4, 16]. Conventional histological parameters are presently not efficient in dealing with this issue. Therefore, objective quantitative measurements of new tumor biologic markers, or new approaches to multiparameter analysis of existing diagnostic measurements, are necessary to achieve a better assessment of the malignant potential of individual tumors and thus arrive at better prognostic indicators.

In the present work, flow cytometric and static cytometric DNA measurements of the same nuclear suspensions of testicular tumor samples were compared. The proportion of aneuploid tumors detected by FC and IA in 62 NSGCT cases was similar to the incidence of aneuploidy reported by others [20]. Concerning DNA ploidy as defined by DI, concordant results using the two techniques were obtained in 76.5% of the tissue blocks. These data correspond to similar comparative reports recently published on other human solid malignancies [1, 6, 11]. In most cases of discrepancy between FC and IA results, the difference may be explained by any of several factors. One possible reason is the small number of nuclei analyzed in IA compared with FC. This might also be a relevant factor in causing (on average) higher CVs of the 2c peaks in IA than in FC, as shown by our data. Secondly, the majority of the samples (>90%) were obtained from different hospitals where orchiectomy for the primary tumor was performed. In our experience, the use of different fixatives and variation in fixation procedures have a profound impact on cell preservation and cell debris, which is a major problem for FC [21]. However, this factor can mostly be excluded by interactive IA. This may be the main reason for the higher rate of uninterpretable samples in FC (22.7%) than in IA (8.3%). A similar ratio of uninterpretable histograms was observed in another study recently published [6], in which 19 blocks out of 132 provided insufficient results in FC, whereas by IA sufficient data (>300 nuclei measured, CV of the 2c peak <10) were obtained. In other investigations comparing FC and IA, different tissue and cell preparations were used before cytometric analyses were performed [1]. This may be one source of discrepancy. However, in this study the nuclear suspension measured by FC and IA was prepared according to the same procedure. This study demonstrated that IA is not as effective in identifying aneuploid stemlines within the near-diploid range (see Fig. 4 as an example). Other studies have also found that IA is limited

in comparison to FC in its resolution of peaks in close proximity to each other [2, 3]. Higher CVs of the peaks in IA make it more difficult to differentiate between two stemlines with closely overlapping peaks.

In 63.3% of the samples that provided insufficient data by FC, IA provided sufficient data with no signs of cell destruction (Fig. 7, Tab. 1). Cell debris, too few nuclei within the sample, formation of cell-cell or cell-debris aggregates, and probably cell destruction during the flow cytometric procedure are the main reasons for this phenomenon, which has also been described in studies of other human malignancies [21].

One of the main problems with performing DNA flow cytometric evaluation of paraffin-embedded tissues is the lack of reliable external diploid controls. Since no external standard has been shown to consistently define the diploid reference value of cells from different blocks, the normal cells within a given paraffin section are conventionally used as the diploid control [9, 10]. This study confirmed that the first peak in all flow cytometric DNA histograms was representative of the diploid control nuclei because no hypodiploid tumor stemlines were identified by IA. Furthermore, in FC, the mean channel number (i.e. mean fluorescence) of the diploid (normal) cells did not only vary between different testis tumors, but also between different paraffin blocks of the same tumor. Therefore, in IA as in FC, normal diploid cells also have to be measured as an internal control for each sample individually. Thus, a reference 2c value should not be used as an external diploid control for different blocks of the same tumor in order to save time during the process of measuring the samples.

FC and IA confirmed the heterogeneity in ploidy within the same NSGCT tumor; similar results are found in other human solid malignancies [9, 20]. Therefore, several samples per tumor should be analyzed to minimize the impact of "sampling error" [17]. This aspect has to be taken into consideration when results obtained by different techniques in different blocks of the same tumor for each procedure are compared or when using data obtained from only one block are used to develop a prognosis for an individual patient.

DI, percentages of diploid and aneuploid cells, S- and G2M + S-phase of the diploid cells and total (cumulative) S- and G2M + S-phase showed no correlation with pathological stage (I, II). Only the percentages of aneuploid cells in the S- and the G2M + S-phase exhibited a strong correlation to the lymph node status. Presently, FC is most capable of providing this information. This relates to the number of cells analyzed in IA and the resulting statistical problems. As already mentioned, IA has an important statistical disadvantage, owing to the small number of nuclei analyzed, compared with FC. On the other hand, IA can more readily detect small numbers of nuclei containing a large amount of DNA (5cER) by measuring only preselected, morphologically well-defined atypical cells. This significant prognostic information cannot be obtained by FC. Several factors may be responsible for this. Only cells within the gating range of approximately 1000 channels can be measured by FC at the same time, at any given combination of photomultiplier tube voltage and gain settings. By this gating procedure a small population of cells with a large amount of DNA might be excluded from flow cytometric analysis. They would be accumulated off scale and thus excluded from collecting "bit maps". The sample would need to be rerun at different voltage and gain settings and with different gating criteria to detect these cells. This type of extensive FC analysis is not commonly carried out in most clinical laboratories, since it is time consuming and technically demanding. Furthermore, during the flow cytometric analysis procedure the nuclei are exposed to substantial hydrodynamic forces, which might have a greater disrupting impact on very large tumor cells with a high DNA content than on less fragile, smaller, more compact cells with less DNA content. In support of this explanation is the observation that large cells with a high DNA content are more sensitive to centrifugation forces than are smaller cells with less DNA content, as recently reported in detail [18].

Using the new tumor biological parameters obtained by FC and IA as prognostic markers, about 80% of early stage NSGCT patients (Table 3) were correctly classified for both pathological stage, as opposed to about 50% of cases correctly classified by histological parameters of the primary tumor alone [16]. Based on this experience both techniques, FC and IA, seem to be promising tools in the prediction of occult metastatic disease in low-volume NSGCT cancer.

In conclusion, this study demonstrated a good correlation between DNA-content measurements made by FC and IA in formalin-fixed, paraffin-embedded testicular tumor tissues. It is apparent from these data that the interactive and selective nature of IA can overcome some of the problems encountered in FC analysis of paraffin tissues by eliminating cellular debris, which is one of the major reasons for uninterpretable histograms in FC. Based on this experience in quantitative DNA cytometry, a clinico-pathological study in early stage NSGCT has been initiated at our institution in order to clarify whether the combined use of FC data (S-phase estimate), and IA data (5cER) provides any significant advantage in prognostic assessment independent of conventional histopathological parameters, such as lymphatic and venous invasion, yolk sac and embryonal cell component.

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References

1. Baldetorp B, Ferno M, Fallenius A, Fallenius-Vecchi G, Idvall I, Olsson H, Sigurdsson H, Akerman M, Killander D (1992) Image cytometric DNA analysis in human breast cancer analysis may add prognostic information in diploid cases with low S-phase fraction by flow cytometry. Cytometry 13:577

2. Bosari S, Wiley BD, Hamilton WM, Dugan JM, Cook LB, Lee AKC (1991) DNA measurement by image analysis of paraffin-embedded breast carcinoma tissue: a comparative investigation. Am J Clin Pathol 96:698
3. Dawson AE, Norton JA, Weinberg DS (1990) Comparative assessment of proliferation and DNA content in breast carcinoma by image analysis and flow cytometry. Am J Pathol 136:1115
4. Einhorn LH (1990) Treatment of testicular cancer. J Clin Oncol 8:1777
5. Emdin SO, Stenling R, Rous G (1987) Prognostic value of DNA content in colorectal carcinoma. Cancer 60:1282
6. Falkmer UG, Hagmar T, Auer, G (1990) Efficacy of combined image and flow cytometric DNA assessments in human breast cancer: a methodological study based on a routine histopathological material of 2024 excised tumor specimens. Anal Cell Pathol 2:297
7. Fossa SD, Thorud E (1986) DNA flow cytometry of cells obtained from old paraffin-embedded specimens: a comparison with results of scanning absorption cytometry. Pathol Res Pract 181:200
8. Hanselaar AGJM, Vooijs GP, Van't Hof-Grootenboer AE, Gemmink, JH, De Leeuw H, Pahlplatz MMM (1991) Cytophotometric analysis of corresponding cytological and histological cervical intraepithelial neoplasia grade III specimens. Cytometry 12:1
9. Hedley DW (1989) Flow cytometry using paraffin-embedded tissue: five years on (review article). Cytometry 10:229
10. Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrove EA (1983) Method for analysis of celular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 31: 1333
11. Kaern J, Wetteland J, Trope CG, Farrants GW, Juhng SW, Petterson EO, Reith A, Danielson HE (1992) Comparison between flow cytometry and image cytometry in ploidy distribution assessments in gynecologic cancer. Cytometry 13:314
12. Koss LG, Wersto RP, Simmons DA, Deitch D, Herz F, Freed SZ (1989) Predictive value of DNA measurements in bladder washings: comparison of flow cytometry, image cytophotometry, and cytology in patients with past history of urothelial tumors. Cancer 64:916
13. Lindmo T, Steen HB (1979) Characteristics of a high resolution flow cytometer based on a flow configuration. Biophys J 28:33
14. Merkel D, Dresseler L, McGuire WL (1987) Flow cytometry, cellular DNA content, and prognosis in human malignancy. J Clin Oncol 5:1690
15. Oud PS, Soeters RP, Pahlplatz MMM, Hermkens HG, Beck HLM, Reubsaet-Veldhuizen J, Vooijs GP (1988) DNA cytometry in dysgerminomas of the ovary. Int J Gynecol Pathol 7:258
16. Pizocarro G, Nicolai N, Salvioni R, Piva L (1992) Comparison between clinical and pathological staging in low stage nonseminomatous germ cell testicular tumors. J Urol 148:76
17. Riese W de, Allhoff E, Werner M, Atzpodien J, Kirchner H, Stief CG, Djamilian M, Schlick R, Jonas U (1991) Proliferative behaviour and cytogenetic changes in human renal cell carcinoma. World J Urol 9:79
18. Riese W de, Walker EB, Riese C de, Jones JA, Foster RS, Donohue JP, Messemer J (1993) Predictive value of proliferative parameters in early stage nonseminomatous germ cell tumor (NSGCT). J Urol 149:453A
19. Schuette WH, Shackney SE, MacCollum MA, Smith CA (1983) High resolution method for the analysis of DNA histograms that is suitable for the detection of multiple aneuploid G1 peaks in clinical samples. Cytometry 3:376
20. Sledge GW, Eble JN, Roth BJ, Wuhrman BP, Fineberg N, Einhorn LH (1988) Relation of proliferative activity to survival in patients with advanced germ cell cancer. Cancer Res 48:3864
21. VanDam PA, Watson JV, Lowe DG, Chard T, Shepherd JH (1992) Comparative evaluation of fresh, fixed, and cryopreserved solid tumor cells for reliable flow cytometry of DNA and tumor associated antigen. Cytometry 13:722
22. Vindelov LL, Christensen IJ, Nissen NI (1983) A detergent-trypsin method for the preparation of nuclei for flow cytometry DNA analysis. Cytometry 3:323
23. Williams NN, Daley JM (1990) Flow cytometry and prognostic implications in patients with solid tumors. Gynecol Obstet 171:257