Diagnostic DNA-flow- vs. -image-cytometry in effusion cytology

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Abstract. *Aims:* To determine the sensitivity and specificity of flow- and image-cytometry for the detection of DNA-aneuploidy as a marker for malignant cells in effusions.

Methods: 200 effusions (80 tumor cell-positive, 74 negative and 46 cytologically equivocal) were stained with DAPI-SR for DNA-flow- and with Feulgen-Pararosaniline for -image-cytometry. They were measured using a PAS-flow-cytometer and an AutoCyte-QUIC-DNA-workstation according to the ESACP consensus reports for DNA-flow- and -image-cytometry, respectively [7,23,29,49].

Results: Sensitivity of DNA-aneuploidy for the identification of malignant cells was 32.1% for DNA-flow- and 75.0% for -image-cytometry, specificity of -euploidy in benign cells was 100.0% for both methods. Positive predictive value of DNA-aneuploidy for the identification of malignant cells was 100.0% for both techniques, negative predictive value of DNA-euploidy was 48.6% for DNA-flow- and 72.0% for -image-cytometry.

Conclusions: Searching for DNA-aneuploidy as a diagnostic marker for neoplastic cells in serous effusions image-cytometry revealed superior sensitivity as compared with monoparametric flow cytometry.

Keywords: DNA-flow-cytometry, DNA-image-cytometry, serous effusion, cytopathology

1. Introduction

The sensitivity of conventional cytology for the detection of malignant cells in effusions is unsatisfactory, about 58%; specificity is about 97%. Improvement of diagnostic accuracy is therefore necessary in effusion cytology. Measurements identified DNA-aneuploidy in 95.4–100% of cells in metastatic carcinomas and in 57.1–82.9% of cells in malignant mesotheliomas in malignant effusions. Our analyses achieved a specificity of 100% for the marker DNA-euploidy to confirm absence of malignant cells in effusions. Sensitivity for the detection of tumor cells in cytologically equivocal effusions was 55.9–82.9%, respectively at a specificity of 94.1–94.7%.

The diagnostic value of DNA-cytometry for the identification of malignant cells in effusions has previously also been demonstrated by other authors. DNA-aneuploidy was detected in 49% of tumor cell-positive effusions by image cytometry [21] and in 21–81% by flow-cytometry [16,18,19,30,32,34,35,37,51,56]. Three authors compared the prevalences of DNA-aneuploidy in malignant effusions achieved by DNA-flow- and image-cytometry respectively: 27% and 73% [54], 62% and 77% [1] and 65% and 100% [39]. Yet unfortunately all these authors reported unsatisfactory specificities (Kapusta et al. [39] had 15% false positives). The diagnostic value of DNA-cytometry, especially for the identification of cells from malignant mesotheliomas has also been demonstrated previously, but mostly on single-cell preparations from histological material by DNA-cytometry with prevalences of 48–53% [22,36,52]. Only two authors dedicated their work to the cytometric identification of cells from malignant mesothelioma in effusions [17,

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25]. Both applied DNA-image-cytometry and achieved prevalences of DNA-aneuploidy of 59 and 89% in tumor cell-positive effusions.

DNA-cytometry has also been applied for the identification of malignant cells in cytologically equivocal effusions. Prevalences of DNA-aneuploidy in these were 12.5–60%. Hedley et al. [30], Croonen et al. [14] and Joseph et al. [38] applied DNA-flow-cytometry, while Freni et al. [21], Fischler et al. [20] and Matter-Walstra et al. [42] -image-cytometry. From these studies it may be concluded, that DNA-aneuploidy is a sufficiently sensitive and highly specific marker for the identification of malignant cells in cytologically equivocal effusions.

In this study we examined the effect of the following aspects on diagnostic accuracy of DNA-cytometry concerning the identification of aneuploid tumor cells: (1) improved precision of measurements [26,40], (2) new algorithms (or their combinations) for diagnostic data interpretation, (3) increased number of cells measured. Furthermore, the ability of both methods to distinguish between primary and secondary tumors of the serous membranes was investigated. Finally a comparison of the practicability and costs of both methods was made.

2. Materials and methods

2.1. Specimens and patient population

The subject of our study was 200 effusions of the serous cavities with cytologically tumorcell-positive (80), -negative (74) and equivocal (46) diagnoses. These consisted of pleural (142), peritoneal (48), pericardial (6) effusions and coul de sac specimens (4) routinely investigated between April and October 1998 in the Institute of Cytopathology.

2.2. Staining of specimens

For measurement of DNA-content by flow-cytometry effusions were centrifuged at 340g/5 min. After decanting the supernatant, the cell pellet was rinsed twice in phosphate buffered saline (PBS), re-suspended, once again centrifuged and decanted. The pellets of hemorrhagic effusions were rinsed once in ammoniumchloride (0.3%) and once in PBS. Following the procedures of Heiden et al. [31], the pellets were then fixed in 10% buffered formalin for about 16 h. Centrifugation and decanting of the supernatant

was repeated. The pellet was then fixed in 96% ethanol for 1 h, followed by another centrifugation and decanting, rinsed in tap water for 20 min and again centrifuged. In order to separate cells and to dissolve the, cytoplasm 200 μ l 0.1% protease (Sigma, Deisenhofen, Germany, Nr.: P-8038, protease type XXXiV: bacterial) was added to the pellet for at least 1/2 h at 37°C. The digestion was stopped by adding 1.5 ml DAPI-SR (4'-6-Diamidino-2-Phenylindole (Sigma, Deisenhofen, Germany, Nr.: D9542) and Sulforhodamine 101 (Sigma, Deisenhofen, Germany, Nr.: S7635). Before measurement the specimens were filtered through a nylon net (cell trics, 50 μ m, Partec, Münster, Germany, Nr.: 06-4-2317).

Details of the procedure of cell preparation for DNA-image-cytometry were described in our previous papers [43,46]. In brief, for purposes of routine cytological diagnosis three slides were air-dried and stained according to May-Grünwald-Giemsa (MGG) (five further slides were immediately fixed in a modification of Delauney's solution and stained according to Papanicolaou for optional immunocytochemical staining [46, 47]).

2.3. Cytological diagnosis

The specimens were evaluated according to generally accepted diagnostic criteria [2,3,41] described in our previous paper [45]. 80 (40%) of the cases were diagnosed as tumorcell-positive, 74 (37%) as -negative and 46 (23%) as cytologically equivocal (doubtful or suspicious for malignancy) [9,10].

2.4. DNA-flow-cytometry

The frequency peaks of the fluorescence of normal diploid reference cells (lymphocytes, mesothelial cells, macrophages and granulocytes), which were always contained in the effusions investigated, were set at channel 50. These were used as internal reference cells. The measurement was only accepted, if the coefficient of variation (CV) of reference cells was <6% (σ CV = CV based on the standard deviation) ([49] (half peak CV $\leq$ 3%)).

Trout erythrocytes were used as an external standard (Partec, Münster, Germany, Nr. 06-5-7302. A correct adjustment resulted in narrow peaks (CV $\sim$ 1%) with a doubling of modal values. The first peak was set at channel 25.

In the 80 malignant, 74 benign and 46 cytologically equivocal effusions a minimum of 20,000 cells

were measured per specimen if present. This was the case in 81.5% (163/200) of effusions (in 105/200 cases the measurement was stopped at exactly 20,000 cells and in 58/200 cases more than 20,000 cells were measured, as only minor peaks in abnormal positions were seen in the histogram so that the measurement was continued). In four cases only <5,000 cells, in eight 5,001–10,000, in 17 cases 10,001–15,000 and in eight cases 15,001–19,999 cells were measured, as routine specimens did not yield a higher number of cells. For measurements the PAS II (Particle Analysing System)-cytometer (Partec, Münster, Germany) was used. The performance of the system meets the requirements of the ESACP consensus report on standardization of DNA-flow-cytometry in clinical pathology [49]. The samples were analyzed as described by Heiden et al. [31]. DAPI was excited in the ultraviolet (350–400 nm) and the fluorescence was measured in the blue region (~435 nm). Usually a sample volume of 1.5 ml or at least 20,000 nuclei were analyzed from each sample at a measuring rate of ≤ 100 nuclei/sec. FSC list-mode data storage occurred according to the recommendations published by the Data File Standards Committee of the Society of Analytical Cytology [15].

DNA-aneuploidy was assumed: if (1) the DNA-index of a frequency peak was $\langle 0.9 \rangle$ or $\langle 1.1 \rangle$ and $\langle 1.80 \rangle$ or $\langle 2.20 \rangle$ and $\langle 2.70 \rangle$ or $\langle 3.30 \rangle$ and $\langle 3.60 \rangle$ or $\langle 4.40 \rangle$ (“abnormal stemline”), which was determined by interactive marking its minimum and maximum in the histogram on the screen; or (2) if the stemline at a DNA-index of 2.0 showed a higher peak than that at an index of 1.0 (“predominating tetraploid stemline”); or (3) if a double peak was seen. This was the case, if there was one “empty” channel between two peaks with a frequency value of <75% of the lowest neighboring peak or two respectively three channels with a peak of <80% or <85%, respectively.

2.5. DNA-image-cytometry

For DNA-measurement one previously MGG-stained slide was later uncovered in xylene and subsequently Feulgen-stained in a temperature-controlled staining machine with Schiff’s reagent according to the protocol applied in our previous papers [5–7,13,43,46].

Measurements of nuclear DNA were performed as described in our previous papers [5–7,43,46]. In brief, 30 lymphocytes were measured as reference cells, meeting a coefficient of variation $\leq 5\%$. The coefficient of correlation between nuclear area and integrated optical density (IOD) of reference cells was $r < 0.4$ [29].

Subsequently, if present, 300 atypical or abnormal cells were measured per specimen interactively at random. This was the case in 59.5% (119/200) of the cases. The measurement was stopped at 300 cells in 34 cases and in further 85 cases it was continued measuring >300 cells. Otherwise, only the available cells were measured: four cases revealed <50 cells; 10 cases 51–100 cells; 24 cases 101–150; 13 cases 151–200; 21 cases 201–250 and nine cases 251–299 cells, as routine material did not yield a higher number of cells. The AutoCyte QUIC-DNA-Workstation (AutoCyte Inc., Burlington, NC, USA) was used for the measurements. The performance of the system meets the requirements of the updated consensus report of the ESACP task force on standardization of diagnostic DNA-image-cytometry [7,29]. The data were diagnostically interpreted as described in our previous paper [43]. DNA-aneuploidy was assumed if (1) an abnormal DNA-stemline (STL) was detected (DNA-index $\langle 0.90 \rangle$ or $\langle 1.10 \rangle$ and $\langle 1.80 \rangle$ or $\langle 2.20 \rangle$ and $\langle 3.60 \rangle$ and $\langle 4.40 \rangle$ [6,7,28,29,48], and/or (2) the coefficient of variation (CV) of the first DNA-stemline was >10%, and/or (3) cells >9c occurred (9c exceeding events (9c EE)) [12].

2.6. Feasibility

One of the aims of this study was the comparison of the two methods with reference to the feasibility of measuring cytologically tumorcell-negative, -positive and equivocal effusions taking into account the total number of measurable cells as well as the distribution of different cell types (ratio of tumorcells to non-tumorcells (lymphocytes, mesothelial cells, macrophages and granulocytes)). The different cell types were analyzed by immunocytochemical staining (for details of the procedure [46,47]) of the identical slides and microscopical counting using an ocular grid with 10×10 squares.

2.7. Validation of cytological diagnoses

According to patient follow-up the investigated effusions of the serous membranes were classified as either containing malignant cells or not. We accepted patient histories as presenting sufficient evidence for the presence or absence of tumor cells in effusions. These revealed either histologic follow-up of the serous membranes themselves (34/200 = 17.0%) or of the respective disease (primary tumor or benign disease, for example biopsy of the liver in cirrhosis of the liver)

(81/200 = 40.5%). Clinical evidence for a malignant nature of the effusion was considered valid, applying such diagnostic techniques as radiology and computer tomography (85/200 = 42.5%). Patients presenting abnormal cells in effusions revealed the following primary tumors: carcinomas of the breast (14), the ovary (12), the parotid gland (1), the lung (25), the esophagus (1), the stomach (12), the colon (9), the gallbladder (1), the pancreas (2), the liver (1), the kidney (2), the urinary bladder (2) as well as carcinomas of unknown primary (8). Furthermore, malignant mesotheliomas (9) and leukemias/lymphomas (9) occurred. Non-malignant cases showed the following basic diseases: pneumonia/pleuritis (20), congestive heart failure (25), pneumothorax (2), rheumatic polyarthritisserositis (2), radiation with generalized edema (2), renal insufficiency (2), cirrhosis of the liver (14), hepatitis (1), pancreatitis (1), peritonitis (1), endometriosis (1), rupture of an ovarian cyst (1), postoperative trauma (7) and lymphohistiocytosis (1). Amongst the tumor cell-negative effusions there were 12 cytologically false negative due to carcinoma of the lung (3), the parotid gland (1), the ovary (2), the cervix (1), leukemias/lymphomas (3) and sarcomas of the stomach (2).

3. Results

3.1. Tumor cell-negative effusions of the serous membranes

None of the DNA-histograms of mesothelial and inflammatory cells in non-malignant, inflammatory or reactive effusions revealed any of the above mentioned criteria of DNA-aneuploidy, neither by DNA-flow- nor -image-cytometry and were therefore consequently all interpreted as DNA-euploid. This corresponds to a specificity of both DNA-cytometric methods of 100.0% to detect benignity in normal or reactive mesothelial cells. In DNA-flow-cytometry the DNA-indices were close to channel 50 (mean 49.7; range 45.0–52.0), on which the fluorescence intensity of normal diploid reference cells had been fixed previously. In DNA-image-cytometry the modal values were close to 2c (mean 2.02c; range 1.84–2.18c). The mean CVs of the first DNA-stemlines were 3.70% (sigma-CV) (range 1.88–6.68%) in DNA-flow- and 2.04% (range 0.67–4.92%) in -image-cytometry.

Table 1

Prevalence of DNA-aneuploidy in effusions of primary and secondary tumors serous of the membranes (DNA-flow- and -image-cytometry)

DNA-ploidy-status	Tumor cell-positive effusions <i>n</i> = 80 (100.0%)	
	DNA-flow-cytometry	DNA-image-cytometry
DNA-non-aneuploid	33 (41.3%)	10 (12.5%)
DNA-aneuploid	47 (58.8%)	70 (87.5%)

3.2. Tumor cell-positive effusions of the serous membranes

The DNA-histograms of metastatic carcinomas and malignant mesotheliomas of the serous membranes showed, according to the above mentioned algorithms of either DNA-cytometric method, none, one, two or all aspects of DNA-aneuploidy. On this basis, 47/80 effusions measured by DNA-flow- and 70/80 by -image-cytometry were DNA-aneuploid (Table 1). This corresponds to a prevalence of DNA-aneuploidy in malignant cells in effusions of 58.8% and 87.5%, respectively (metastatic carcinomas: 60.0% and 92.9%, respectively; mesotheliomas: 50.0% and 66.7%, respectively; lymphomas: 50.0% and 25.0%, respectively). Table 2 shows the frequency of occurrence of different aspects of DNA-aneuploidy in effusions due to metastatic carcinomas and mesotheliomas of the serous membranes. It clearly demonstrates that an abnormal stemline in DNA-flow- and 9cEE in -image-cytometry were the most frequent aspects of DNA-aneuploidy and that combined application of different algorithms increased the rate of its detection.

It is obvious that in tumor cell-positive effusions all of the above mentioned aspects of DNA-aneuploidy, with differing sensitivities, are often well enough represented to serve as sensitive criteria for the identification of aneuploidy. One abnormal stemline was observed in DNA-flow-cytometry in 18.8% and in DNA-image-cytometry in 26.3% of the malignant tumors of the serous membranes. Two abnormal stemlines were found in 36.6% and in 42.5%, respectively and multiple DNA-stemlines in 3.8% and 6.3% of cases. Apart from abnormal stemlines (53.8%) in DNA-flow-cytometry a predominant tetraploid stemline (6.3%) was the second most frequent aspect of DNA-aneuploidy. A double peak served as a criterion of DNA-aneuploidy in 3.8% of the tumors of the serous membranes (Table 2). In DNA-image-cytometry, next to 9cEE (77.5%), abnormal stemlines were the second most frequent aspect of DNA-aneuploidy (75.0%). An

Table 2

Prevalence of different aspects of DNA-aneuploidy in effusions of primary and secondary tumors of the serous membranes (DNA-flow- and -image-cytometry) (STL = stemline; CV = coefficient of variation; 9cEE = 9c exceeding events)

Aspects of DNA-aneuploidy	<i>n</i> = 80 (100.0%)
DNA-flow-cytometry	
Abnormal STL	43 (53.8%)
One	15
Two	29
Multiple	3
Stemline 4c > 2c	5 (6.3%)
Double peak	3 (3.8%)
DNA-image-cytometry	
Abnormal STL	60 (75.0%)
One	21
Two	34
Multiple	5
CV of first STL ≥ 10%	19 (23.8%)
9cEE	62 (77.5%)

Table 3

Prevalence of DNA-aneuploidy in cells of cytologically equivocal effusions

DNA-ploidy-status	Cytologically equivocal effusions <i>n</i> = 46 (100.0%)	
	DNA-flow-cytometry	DNA-image-cytometry
DNA-non-aneuploid	37 (80.4%)	25 (54.3%)
DNA-aneuploid	9 (19.6%)	21 (45.7%)

abnormally high CV of the first stemline served as a criterion of DNA-aneuploidy in 23.8% (Table 2).

Whereas in 53.8% of malignant effusions in DNA-flow- and in 75.0% in -image-cytometry DNA-aneuploidy was identified by an abnormal stemline alone, the identification rate increased to 55.0% if a predominant tetraploid stemline and to 58.8% if additionally a double peak was used as a criterion of DNA-aneuploidy in DNA-flow-cytometry. In DNA-image-cytometry the identification rate increased to 87.5%, if additionally 9cEE was used as a criterion of DNA-aneuploidy. An abnormally high CV of the first stemline as a criterion did not further increase the identification rate (Table 3).

Amongst the 80 tumor cell-positive effusions in this study there were only six mesotheliomas, of which three were DNA-aneuploid by DNA-flow- and four by -image-cytometry. In all of these the DNA-histogram of flow-cytometry showed a peridiploid stemline, in two of the aneuploid effusions additionally a hypodiploid and in one a hypotetraploid stemline was ob-

Table 4

Prevalence of different aspects of DNA-aneuploidy in cytologically equivocal effusions (DNA-flow- and -image-cytometry) (STL = stemline; CV = coefficient of variation; 9cEE = 9c exceeding events)

Aspects of DNA-aneuploidy	<i>n</i> = 46 (100.0%)
DNA-flow-cytometry	
Abnormal STL	9 (19.6%)
One	5
Two	4
Multiple	–
Stemline 4c > 2c	–
Double peak	–
DNA-image-cytometry	
Abnormal STL	15 (32.6%)
One	10
Two	5
Multiple	–
CV of first STL ≥ 10%	3 (6.5%)
9cEE	14 (30.4%)

served. In image-cytometry there was no specific pattern to be seen in the histograms.

3.3. Diagnostic accuracy in cytologically equivocal effusions of the serous cavities

9/46 and 21/46 of cytologically equivocal effusions were DNA-aneuploid in DNA-flow- and -image-cytometry, respectively (Table 3). This corresponds to a prevalence of DNA-aneuploidy in cytologically equivocal effusions of 19.6% and 45.7%, respectively. In cytologically equivocal effusions an abnormal stemline was the most frequent aspect of DNA-aneuploidy using DNA-flow-cytometry (19.6% (9/46)). The combined application of three different algorithms did not increase the rate of detection (Table 4). In DNA-image-cytometry an abnormal stemline was the most frequent aspect of DNA-aneuploidy (32.6% (15/46)), followed by 9cEE (30.4% (14/46)) and an abnormally high CV of the first stemline (6.5% (3/46)). The combined application of different algorithms increases the detection rate of DNA-aneuploidy (Table 4).

It is obvious, that all aspects of DNA-aneuploidy, with differing sensitivities, are often enough represented to serve as a sensitive criterion for identification in cytologically inconclusive effusions as well as in effusions suspicious for malignancy. One abnormal stemline was observed in 10.9% (5/46) in DNA-flow- and in 21.7% (10/46) of the cases in -image-cytometry, respectively. Two abnormal stemlines were found in

Table 5
DNA-flow- and -image-cytometry in equivocal effusions

	DNA-flow-cytometry (DNA-aneuploidy)	DNA-image-cytometry (DNA-aneuploidy)
Prevalence	19.6% (9/46)	45.7% (21/46)
Sensitivity	32.1% (9/28)	75.0% (21/28)
Specificity	100.0% (18/18)	100.0% (18/18)
Positive predictive value	100.0% (9/9)	100.0% (21/21)
Negative predictive value	48.6% (18/37)	72.0% (18/25)

8.7% (4/46) and in 10.9% (5/46), respectively; multiple stemlines were not found. A double peak or a predominant tetraploid stemline as criterion for DNA-aneuploidy were not found in cytologically equivocal effusions. Using flow-cytometry 9cEE served as criterion for DNA-aneuploidy in 30.4% (14/46) and an abnormally high CV of the first stemline in 6.5% (3/46) in DNA-image-cytometry (Table 4).

In this study three cytologically equivocal effusions were due to malignant mesotheliomas. DNA-aneuploidy was detected in two of these by DNA-flow- and by -image-cytometry each by detection of an abnormal steamline by either method.

The positive predictive value of the marker DNA-aneuploidy by DNA-flow- as well as -image-cytometry was 100.0% in cytologically equivocal effusions (Table 5). The negative predictive value of DNA-non-aneuploidy was 48.6% and 72.0%, respectively, as only 18 of 37 and 18 of 25 non-aneuploid cases, respectively showed benign histories. Sensitivity for identification of malignancy in equivocal effusions was 32.1% by DNA-flow- and 75.0% by -image-cytometry, specificity of DNA-non-aneuploidy for benignity was 100.0% by both methods. Considering the achieved prevalences of DNA-aneuploidy in cytologically positive ($n = 80$) and negative ($n = 74$) as well as equivocal ($n = 46$) effusions, the total positive predictive value of DNA-aneuploidy was 100.0% by both DNA-flow- and -image-cytometry, the total negative ones were 63.9% and 84.4%, respectively. Total sensitivity was 51.9% and 84.3%, respectively, total specificity 100.0% for both methods. Total diagnostic accuracy was 74.0% and 91.5%, respectively.

3.4. Feasibility

The feasibility of both DNA-cytometrical methods was compared. In 160/200 effusions both methods achieved identical results in the detection of non-aneuploidy or aneuploidy in tumorcell-negative, -positive and cytologically equivocal effusions respectively. In 40/200 cases of tumorcell-containing effusions (26 cytologically tumor cell-positive and 14 cytologically equivocal effusions) image-cytometry detected aneuploidy while flow-cytometry did not. 27.5% (11/40) of these effusions showed an absolute percentage of tumor cells less than 5% (7.5% (3/40) <1%, 12.5% (5/40) 1–2.5% and 7.5% (3/40) 2.5–5%). Another 27.5% (11/40) revealed a high percentage of tumorcells (11.8–99.0% (mean 43.7%)), but not consequently with the same DNA-content and therefore not all represented in one peak, but instead distributed all over the DNA-histogram. Furthermore, in 27.5% (11/40) single cells with a high DNA-content were not detected, in 20.0% (8/40) of these this was the only criterion for aneuploidy in image-cytometry (in 17.5% (7/40) one cell and 2.5% (1/40) three cells with a DNA-content >9c were detected). In 10% (4/40) small double peaks were not detected. In 12.5% (5/40) the absolute number of measurable cells within the effusions was too small (<20,000) and the CV was too high.

4. Discussion

Improvement of the sensitivity for the detection of tumor cells in effusions without loss of specificity is desirable. DNA-cytometry is a promising adjuvant method for this purpose. This study compares the diagnostic accuracy of DNA-flow- vs. -image-cytometry in 200 effusions applying different algorithms for the identification of DNA-aneuploidy using instruments and protocols which promise high precision of measurement.

Image-cytometric analysis of primary and secondary tumors of the serous membranes showed DNA-aneuploidy in 87.5%, -flow-cytometrical investigation in only 58.8% (Table 1). Specificity of both methods was 100.0%.

The detection rate in tumorcell-positive effusions increased by additional application of up to three algorithms. If a high detection rate of DNA-aneuploidy is to be achieved, we recommend using more than one abnormal aspect of the DNA-distribution.

The sensitivity of DNA-cytometry for the identification of malignancy in cytologically equivocal effusions was 32.1% for DNA-flow- and 75.0% for -image-cytometry. Specificity of DNA-non-aneuploidy for the identification of non-neoplastic cells was 100.0% for both methods (Table 5). The positive predictive value of DNA-aneuploidy in cytologically equivocal effusions was 100.0% for both methods. The negative predictive value of DNA-non-aneuploidy was 48.6% for DNA-flow- and 72.0% for -image-cytometry.

A proliferating cell population with an abnormal DNA-content is supposed to represent cells with chromosomal aneuploidies and this is often denominated as an aneuploid DNA-stemline. Whereas cells belonging to a DNA-stemline are supposed to be cytogenetically identical proliferating cells, rare events with DNA-contents $>9c$ most likely represent non-proliferating cells with different chromosomal aneuploidies and abnormally high numbers of chromosomes. The latter cells can only be identified with DNA-image-cytometry including a visual control for diagnostic purposes.

The different prevalences of DNA-aneuploidy found in other studies on malignant effusions may amongst other factors be due to the number and type of algorithms applied for the interpretation of histograms.

Most authors have used flow-cytometry to detect tumor cells in effusions. Yet the sole algorithm applied in most studies was the detection of an abnormal or aneuploid DNA-stemline. Only Stonesifer et al. [56] demonstrated the possibility of increasing the detection rate of DNA-aneuploidy by the analysis of different abnormal aspects of the histograms. They considered the occurrence of $>2.5\%$ of the population in the G2/M-phase of the cell cycle, a wide G0/1-phase-peak as well as an asymmetry or a shoulder of an G1-peak as a further aspect of DNA-aneuploidy, by which they achieved a sensitivity of 88% of all effusions with three false positive cases.

Several authors have applied DNA-image-cytometry. Although Kapusta et al. [39] achieved a prevalence of 100%, their specificity was low (15% DNA-cytometrically false positives). The sole algorithm applied in most of these studies was the detection of an abnormal or aneuploid stemline, and by Freni et al. [21] the observance of DNA-values $>8c$. In our opinion, this approach is not sufficient to achieve an adequately high detection rate of DNA-aneuploidy. As demonstrated in previous studies [43,44] we, have suggested the application of more than one algorithm for the detection of DNA-aneuploidy. Fischler et al. [20]

showed the possibility of increasing the detection rate of DNA-aneuploidy by analysis of different aspects of the histograms. They considered the occurrence of multiple DNA-stemlines and nuclear values $>5c$.

We recommend the application of the following algorithms. For DNA-flow-cytometry: the occurrence of an abnormal stemline, a predominant tetraploid stemline or a double peak. For DNA-image-cytometry we propose the occurrence of an abnormal stemline and DNA-values $>9c$.

4.1. Algorithms for the detection of DNA-aneuploidy by flow-cytometry

DNA-aneuploidy is usually assumed, if the modal value of the G0/1-fraction of the cell population differs more than 10% (or recently only 5%) of that of the diploid reference cell population or one of its integer valued multiples [49]. We believe, that even smaller deviations may be detected (occurrence of a “double peak”). As soon as chromosomal aberrations lead to a change of DNA-content, which differs statistically highly significantly from that of normal, non-neoplastic cells, DNA-aneuploidy may be assumed [8]. In our opinion, the prevalence of DNA-aneuploidy in tumor cell-positive effusions may be increased by a higher precision of DNA-measurements, for example, by use of the PAS-Flowcytometer (Partec, Münster, Germany) applied in this study, the use of DAPI as a DNA-specific fluorochrome instead of propidiumiodide and the simultaneous application of three different algorithms for the detection of DNA-aneuploidy. An abnormal DNA-stemline was the most frequent aspect of aneuploidy in effusions containing tumor cells. As the relation of the modal values of the DAPI-fluorescence of analysis- and reference cells may differ slightly as a result of differences in fixation and staining, an empirically found range must be considered. As, in our study, this ranged from channel 45.0–52.0 for lymphocytes, granulocytes, mesothelial cells or macrophages, we consequently decided on threshold values of DNA-indices $\langle 0.90 \rangle 1.10$, in order to assume DNA-stemline-aneuploidy. This corresponds well with the range of $\pm 5\%$ of the modal values of normal diploid cells mentioned by Ormerod et al. [49]. A further algorithm for the detection of DNA-aneuploidy, applied by us, is the occurrence of a predominant tetraploid stemline, which contains more cells than the respective diploid one. Furthermore, double peaks served as a criterion for the assumption of DNA-aneuploidy in effusions of the serous mem-

branes. In 40 cases of tumor cell-containing effusions (26 cytologically tumor cell-positive and 14 cytologically equivocal effusions) image-cytometry detected aneuploidy while flow-cytometry did not. The main reason was the overlap of a small population of tumor cells by a great number of normal cells (lymphocytes, mesothelial cells, macrophages, granulocytes). This was due to the following facts (more than one cause possible): 27.5% of these effusions showed an absolute percentage of tumor cells less than 5%. Another 27.5% revealed a high percentage of tumor cells, but these did not all have the same DNA-content and consequently were not all represented in one peak, but instead distributed all over the DNA-histogram. Furthermore, in 27.5% single cells with a high DNA-content were not detected because of overlapping of normal cells by doublets, triplets and quadruplets. These problems can be solved by including specific immunologic staining with epithelial antibodies and two wavelength double parameter measurements. In 10% small double peaks were not detected because of insufficient precision of measurement. In 12.5% the absolute number of measurable cells within the effusions was too small ($<20,000$) and the CV was too high. This reflects the situation of routine effusion material.

4.2. Algorithms for the detection of DNA-aneuploidy by image-cytometry

We assume that the high prevalence of DNA-aneuploidy in tumor cell-positive effusions seen by us may be due to the simultaneous application of two different algorithms for the detection of DNA-aneuploidy. We used threshold values of $<1.80c$ and $>2.20c$ to assume DNA-stemline-aneuploidy. A further frequently applied marker for DNA-aneuploidy is the occurrence of single cells with a DNA-content over certain threshold values in cells. In tissues with euploid polyploidisation up to tetraploid cell populations, as the urothelium or mesothelium, only the occurrence of cells with a DNA-content $>9c$ (9c exceeding events) may be considered as a marker for single-cell-DNA-aneuploidy [4,12], as no cells of reactive, proliferating or benign mesothelium will exceed these values. In this aspect, we do not agree with other authors [20,50], who already consider the occurrence of cells with values $>5c$ as a marker for DNA-aneuploidy in cells with euploid polyploidisation. The coefficient of variation of the DNA-stemline depends on the precision of DNA-measurements, the rate of proliferation and the occurrence of aneuploid nuclear DNA-values. In our previous study [43] in the

absence of DNA-aneuploidy, a maximal CV of G0/1-phase cells with a mean of 3.89% was empirically found. Although, in this study here CVs $\geq 10\%$ were found in tumor cell-positive effusions and never in tumor cell-negative ones, we nevertheless no longer recommend [43,44] using a CV $\geq 10\%$ as an indication of DNA-aneuploidy as it does not further increase the detection rate.

While most other authors applied DNA-flow-cytometry, we currently prefer DNA-image-cytometry especially in cytologically equivocal effusions until multiparameter measurements allow the inclusion of immunological markers combined with high precision DAPI-DNA-measurements. Morphologic detection and separate measurement of only few abnormal cells ("rare events") are only possible in image-cytometry. These diagnostically important cells with increased DNA-content are mostly missed by single parameter DNA-flow-cytometry. Studies with a high rate of DNA-aneuploidy in cytologically equivocal effusions [20,21,42] also applied DNA-image-cytometry.

With improved precision of DNA-flow- and -image-cytometry and more sophisticated algorithms for histogram analysis, DNA-aneuploidy can be detected in a higher percentage of cytologically equivocal effusions than previously assumed. This fact should result in an increased sensitivity for the detection of malignant cells and thus decrease the number of cytologically equivocal effusions.

Our results demonstrate that, at this point, DNA-image-cytometry has a higher sensitivity for the detection of malignancy in effusions than DNA-flow-cytometry. Both methods have a specificity of 100.0%. As DNA-flow-cytometry applied to effusions is faster and less expensive to perform than -image-cytometry, the aim should be to improve this method, so that its sensitivity may be increased. In order to do so the rate of detection of DNA-aneuploidy in flow-cytometry, the often very small populations of tumor cells in effusions must be separated from overlaps by non-malignant cells.

Not only in DNA-image but also in -flow-cytometry, measurements for diagnostic purposes are strictly recommended only in combination with a cytological inspection of routine slides including quantitative evaluation of the ratio of the different cell types in each individual effusion. Furthermore, external diploid reference cells should be added in flow-cytometry in order to obtain an absolute calibration. This would also allow the detection of smaller deviations of DNA-stemlines (between 3–5%) from the normal diploid value.

The current advantages of DNA-image-cytometry are the following: the measurement may be performed on existing cytological routine slides; morphologically identifiable cells are individually measurable; remeasurements are possible at any time; a quality control of measured cells is possible on an "image gallery"; there is a relatively broad spectrum of indications (identification of malignant cells especially in borderline lesions and dysplasias, grading of tumor malignancy and monitoring of therapy); multiparametric measurements (combination with antigens); "rare event detections" (i.e., 9cEE) are possible and internal calibrations are possible.

The disadvantages of interactive DNA-image-cytometry are the following: cytodiagnostically trained personnel is necessary; only a limited number of cells are measurable (usually about 300); measurements are relatively time consuming (about 30 min); preparation/staining is time consuming; the proportionality error of Feulgen-staining limits the precision of detection of aneuploid DNA-stemlines near 2c.

The general advantages of DNA-flow-cytometry are: it is easy to measure a representative number of cells (i.e. 20,000); the results are quickly available (10 min); a multiparametric analysis is possible (i.e., additional detection of cytoplasmatic antigens); there is no proportionality error of staining DNA by fluorescence dyes.

The disadvantages of DNA-flow-cytometry currently are: technically trained personnel is necessary; morphologically identifiable cell populations are not individually measurable; there is only a relatively small spectrum of indications (usually only grading of tumor malignancy); "rare event detections" are not possible (i.e., 9cEE); no repetition of measurements are possible; highly precise measurements of DNA using DAPI stain are only possible after enzymatic cell separation (loss of cytoplasm); separate probes are necessary (routine slides cannot be used); internal calibrations often reveal problems.

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