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Routine DNA cytometry of benign and malignant pleural effusions by means of the remote quantitation server Euroquant: a prospective study

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Abstract

Aim—To analyse the practicability and potential assistance of static DNA cytometry performed by means of the remote quantitation server Euroquant and the internet in routine diagnostic analysis of pleural effusions, and to outline the role of DNA cytometry on pleural effusions in distinguishing between benign and malignant (and herein primary versus metastatic) effusions.

Materials and methods—Cytological smears of 294 pleural effusions were stained with the Feulgen method. The DNA content of a minimum of 300 randomly chosen analysis nuclei and 30 reference nuclei (lymphocytes) was measured by internet connection to the remote quantitation server Euroquant. Cytometric features were derived from the histograms, and the time needed for case evaluation, the reliability of staining and measurement procedures, and the contribution to the final diagnosis were evaluated.

Results—Only 120 of 294 pleural effusions could be measured. The total measurement time for each specimen was 60 minutes. The guidelines of the consensus report on DNA measurements were fulfilled. Seventy eight malignant (18 mesotheliomas, 60 metastatic tumours) and 42 benign effusions were measured. Seven of 78 malignant effusions were euploid and none of 42 benign effusions were aneuploid. The sensitivity and specificity were 91% and 100%, respectively, for distinguishing benign from malignant effusions, and 95% and 100%, respectively, for discriminating between benign and malignant effusions caused by metastatic malignant tumours.

Conclusions—Static DNA cytometry using the remote quantitation server Euroquant can be performed reliably in the routine diagnosis of pleural effusions; however, only 40% of effusions meet the technical requirements for static DNA cytometry. Within the measurable cases, static DNA cytometry made an important contribution to the confirmation/exclusion of malignancy.

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Progress in the diagnosis and treatment of various malignant and benign diseases do not always occur contemporaneously, with diagnostic procedures and the functional and biomolecular classification of a disease often preceding its treatment. However, the treatment of malignant pleural effusions requires an accurate differentiation between reactive and proliferative benign pleuritis and mesothelioma, or metastatic adenocarcinoma and mesothelioma, which can be difficult. Therefore, a broad variety of experimental techniques has been reported to support the diagnosis of pleural effusions. These include flow cytometry, immunohistochemistry, ligand histochemistry, syntactic structure analysis, ultrastructural studies, cytogenetic and molecular biology examinations.^{1–6} Only a few of these studies has been undertaken “in the real world” of cytological diagnosis, and it has to be admitted that the application of certain sophisticated techniques fails in routine diagnostic procedures because they often require specifically trained staff members and a certain amount of investment. Therefore, in the routine diagnosis of pleural effusions, only a few techniques are broadly used and contribute greatly to the diagnostic accuracy.^{3–7} These include immunological methods, ligand techniques, and flow and static DNA cytometry.^{8–10} Without doubt, specific staining techniques such as immunohistochemistry and flow cytometry must be performed in the primary diagnostic laboratory, whereas images can easily be sent to any specialised department by electronic mail, and the image information can be extracted by specialists from these transmitted images.

The remote quantitation server Euroquant offers a third way: it permits the interactive quantitation of Feulgen stained cytological specimens and offers, in addition, a continuous control of staining quality, measurement performance, and assistance in histogram classification and interpretation.^{11–12} This option is therefore an attractive adjunct in routine diagnostic cytology.

Because several reports have demonstrated the practical use of DNA cytometry in the difficult tasks of separating benign from malignant, and primary malignant from metastatic malignant diseases of the pleura,^{7–10} our study analyses the usefulness of static DNA cytometry in routine diagnostic cytology methods using pleural effusions and remote measurements performed by means of the Euroquant server. The use of DNA cytometry of pleural effusions to distinguish between benign and

Department of Pathology, Thoraxklinik Amalienstr. 5, D-69126 Heidelberg, Germany
K Kayser
S Blum
M Beyer

Institute of Pathology, Technische Universität Dresden D-01307, Germany
G Haroske
K D Kunze
W Meyer

Correspondence to: Professor K Kayser
kkayser@lung.de

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malignant causes, and between primary and secondary cancer was investigated. In addition to evaluating the specificity and sensitivity of the diagnoses, we have focused on additional aspects, such as quality control of stains and image acquisition, and the practicability of the measurements.

Materials and methods

Pleural effusions sent to the department of pathology, Thoraxklinik, Heidelberg during the period September 1998 to July 1999 were centrifuged ($750 \times g$ 10 minutes), the corresponding smears were formalin fixed for 60 minutes at room temperature, and Feulgen stained according to the technique described by Mikel and Becker.¹⁷ The staining procedure given by these authors¹⁷ was followed strictly, and the hybridisation time at room temperature was set at 30 minutes. According to our experience, this technique is superior (and less expensive) to cytospin preparations. The mean integrated optical density (IOD) of the reference cells (IOD of lymphocytes) was used to control the staining procedure. If the standard deviation of the IOD of lymphocytes was not within the 3% range the slides were excluded from further measurements, in agreement with the consensus report for DNA cytometry.¹¹ Table 1 gives the details of measurement performance using local programs and the software used. A Leitz microscope (SM Lux, 40×0.75) connected to a Cyrix 133 Windows 95 PC (64 Mb RAM) via a CCD camera (JVC TK 1070E) and Leutron Vision RGB frame grabber (resolution 768×512 pixels and 8 bits, only using the red (R) image) was used to acquire and store an average of eight images/effusion. A 56 k V.90 analogue modem served for connection to the remote quantitation server Euroquant via local university facilities. Shading analysis and glare correction were performed by the server as described in detail elsewhere.¹¹ The measurement procedure can be summarised as follows: the images were stored locally, an internet connection with the server was set up, the images were transferred to the server, and the DNA measurements were performed interactively with the use of the server software. A minimum of 300 randomly chosen nuclei of interest and 30 nuclei of lymphocytes were interactively measured. These data again meet the requirements of the consensus report on DNA cytometry.¹¹ The peak of the nuclei of the measured lymphocytes was set to 2.0C. A histogram was graded euploid if the main peak of the integrated optical density (IOD) measured $1.8C \leq \text{IOD} < 2.2C$ ($n = 1, 2, 4$) and no measured nucleus presented an $\text{IOD} > 9C$. The measurement protocol and histograms given by the server were visually interpreted, and a (diagnostic) statement combined with the DNA histogram was printed out for submission to the clinician. Effusions not suitable for measurement were documented separately. The time needed for image acquisition, data transfer, measurement, and documentation were recorded by the computer system. The final diagnosis was evaluated by the histo-

Table 1 Performance of measurements

Procedure	Local programs	Programs provided by the Euroquant server
Image acquisition	X	
Image storage	X	X
IOD measurement		X
Data evaluation		X
Quality assurance		X
Histogram interpretation	X	
Final report	X	

IOD, integrated optical density.

logical analysis of concomitantly performed pleural biopsies or surgical specimens, and thorough evaluation of the clinical history and follow up. The minimum follow up period was 12 months. The statistical analysis was performed using a commercially available program (NCSS, Kaysville, Utah, USA).

Results

Of 294 pleural effusions sent to our department during the study period only 120 cases could be measured successfully (table 2). Most of the non-measurable smears were excluded because of an insufficient number of cells of interest (congestive effusion), followed by those effusions presenting with densely packed inflammatory cells (empyema). Nineteen cases were excluded because of non-reliable staining, which was monitored by the Euroquant server. Technical communication problems occurred on nine days, lasting from 30 minutes to 240 minutes at maximum, caused by server breakdown or disturbances related to the internet provider. Table 3 gives a synopsis of the 120 measured cases. The 120 specimens were from 48 women and 72 men and 78 were malignant and 42 benign effusions. Cell type characterisation of the malignant effusions revealed 18 mesotheliomas, 33 metastatic pulmonary carcinomas (25 adenocarcinomas), 11 metastasising breast carcinomas, and 16 metastases of various origins including kidney, gastric, ovarian, prostate, and thymus carcinomas, as well as non-Hodgkin's lymphomas and sarcomas. The series of measurements was of reliable and time independent performance, as shown in fig 1. The maximum coefficient of variation (CV) of the IOD was $< 5\%$ for all measurements, and no shift to higher or lower mean IOD values

Table 2 Synopsis of material primarily included in our study

No of cases	
Pleural effusions	294
Measured	120 (41%)
Non-measurable	174 (59%)
Too few cells	113 (38%)
Too many cells	15 (5%)
Heavy infiltration/empyema	27 (9%)
Non-reliable staining	19 (7%)

Table 3 Synopsis of measured pleural effusions (no of cases, age in years)

	Women	Men	Median age (years)	Total
Mesotheliomas	1	17	65	18
Different malignomas	35	25	68	60
Benign effusions	12	30	62	42
Total	48	72	65	120

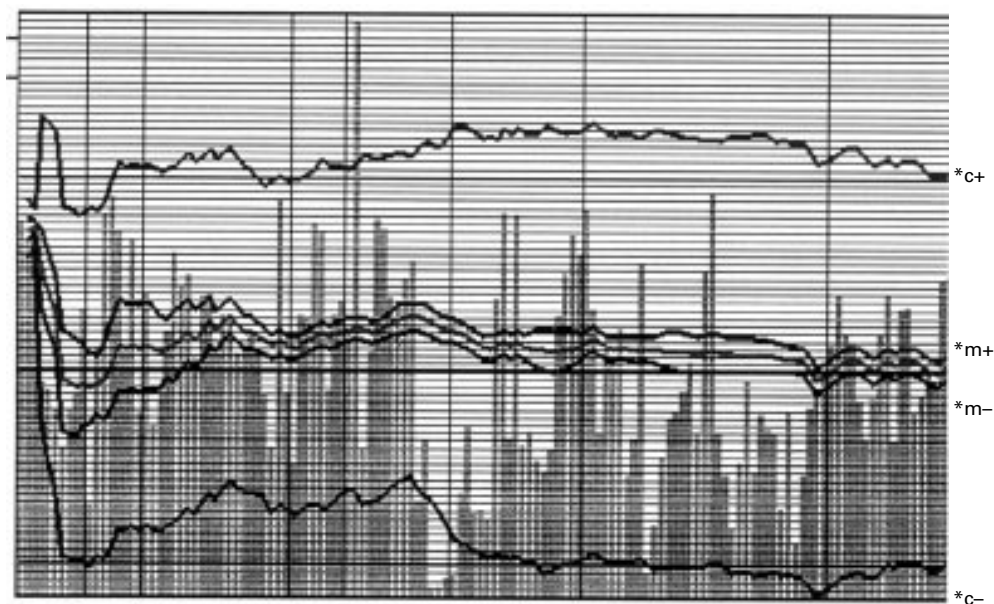


Figure 1 Histogram showing the accuracy of performed measurements. With the exception of six outliers, all mean integrated optical density (IOD) values are within a confidence interval of 95%, and no significant time shift of measurements can be detected. All measurements are grouped according to date. *c+, upper threshold of 95% confidence interval; *c-, lower threshold of 95% confidence interval; *m+, mean +1 standard error of mean; *m-, mean -1 standard error of mean.

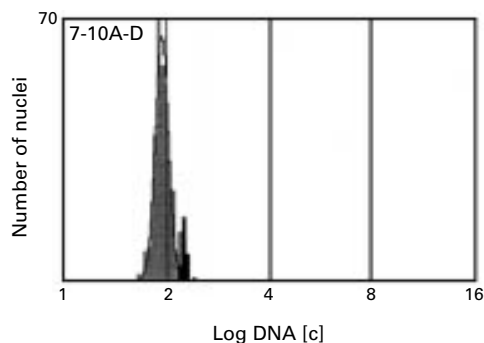


Figure 2 All measured integrated optical density (IOD) values are grouped within the 95% confidence interval of the 2C value indicating a euploid effusion.

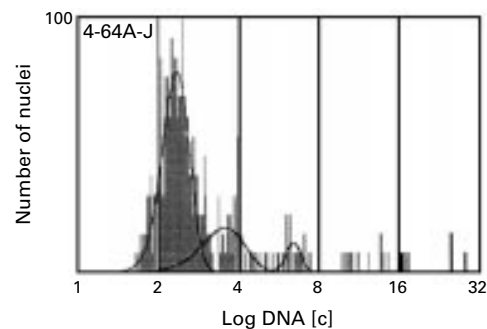


Figure 3 Characteristic DNA histogram of a malignant pleural effusion caused by a metastatic adenocarcinoma. The mean integrated optical density (IOD) value was 2.4C, and two additional peaks (3.8C, 6.4C) indicate an aneuploid effusion.

was seen. The variation of the upper threshold confidence interval was within the normal range, and only six outliers were detected during our study. Characteristic DNA histograms of nuclei measured in pleural effusions of a mesothelioma, a metastasising adenocarcinoma of the lung, and a chronic inflammatory pleuritis are depicted in figs 2–4. Table 4 summarises the obtained ploidy status with respect to the lesion under investigation. Only four of 18 mesotheliomas were euploid, and 11 of the 11 metastatic breast carcinomas were found to be aneuploid (table 4). Table 5 summarises the statistical data on the specificity and sensitivity of static DNA cytometry to distinguish between the different diagnoses and cell types of pleural effusions. Importantly, for patients with a known history of a non-pleural malignancy and pleural effusion of uncertain origin, the calculated sensitivity and specificity were 95% and 100%, respectively.

Discussion

The causes of pleural effusions include acute and chronic pulmonary and pleural inflamma-

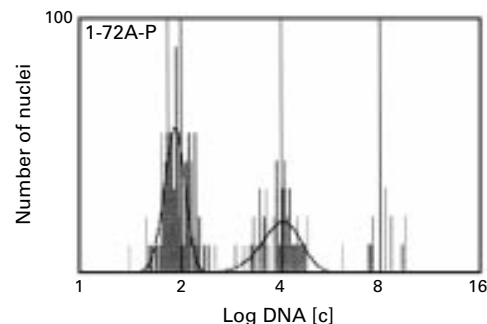


Figure 4 Characteristic DNA histogram of a malignant pleural effusion caused by a mesothelioma. Although the two integrated optical density (IOD) peaks are located at 2C and 4C, singular nuclei with an IOD > 9C clearly indicate an aneuploid effusion.

tory diseases, obstructions of the pleural lymph drainage, infectious diseases (especially pleural tuberculosis), and primary and secondary pleural malignancies. In terms of diagnostic and therapeutic importance, the confirmation of a benign or malignant effusion is the predominant task of cytological body fluid

Table 4 Ploidy status of measured effusions in respect to cell type and nature of the underlying disease

	Euploid	Aneuploid	Total
Malignant	7	71	78
Mesotheliomas	4	14	18
Epithelial	3	5	8
Biphasic	1	9	10
Lung tumours	2	31	33
Adenocarcinomas	2	23	25
Parvicellular carcinoma	0	4	4
Squamous cell carcinoma	0	4	4
Breast cancers	0	11	11
Other malignomas (kidney (2), gastric (1), ovarian (1), thymus (1), and prostate (1) carcinomas, NHL (5) and sarcomas (2), unknown primary (3))	1	15	16
Benign	42	0	42
Acute inflammatory	5	0	5
Tuberculous	5	0	5
Chronic inflammatory	32	0	32
Total	49	71	120

NHL, non-Hodgkin's lymphoma.

Table 5 Sensitivity and specificity of ploidy status

	Sensitivity	Specificity
Malignant/benign	91%	100%
Mesotheliomas/benign	77.8%	100%
Other malignomas/benign	95%	100%

examination. However, this can be handicapped by the great variation in morphology and textural agglutination of reactive mesothelial cells, which can mimic malignancy or benign disease.^{1 13 14 18} In contrast, mesotheliomas, especially at an early stage, often present with quite "harmless" morphology, which contributes to the considerable difficulties of giving a conclusive diagnosis.^{13 19 20} To overcome these well known difficulties in cytology (and histology), numerous techniques have been investigated. These include immuno(cyto)chemistry, ligand cytohistochemistry/histochemistry, electron microscopy, flow and static DNA cytometry, syntactic structure analysis, cytogenetics, and gene rearrangement studies.^{3 7 21} Of the markers used, carcinoembryonic antigen (CEA) and calretinin have been reported to distinguish reliably between metastatic and primary pleural malignancies, and calcylin has been suggested to discriminate between benign reactive pleuritis and mesothelioma.^{5 6 20}

DNA cytometry is a well established, usually fast and simple procedure, which results in an easy to interpret histogram, and numerous studies have used static and flow cytometry as an adjunct in the diagnosis of pleural effusions.^{7-10 15} The technical equipment used in these studies differed in terms of spatial and time resolution, performance, and quality control. Performed by highly specialised teams, several studies have been undertaken retrospectively using paraffin wax embedded specimens and, therefore, are handicapped with the unavoidable constraints of these procedures, such as artifacts in specimen preparation, incomplete clinical data, limited material, etc.²² These problems were behind the idea of creating an internet server named Euroquant, a machine that is offered to any diagnostic cytology department for DNA measurements and quality surveillance.^{11 12} The functionality of the server, including its ability to conform to

the requirements of the consensus report on DNA cytometry has been described in detail elsewhere.¹¹ For those readers who are interested in specific details a web site describing the functions has been installed (euroquant.med.tu-dresden.de). Several groups have tested the access and reliability of the server and its performance, and no major concerns have been noted to our knowledge.^{11 12 16} The construction of the server had to fulfill the regulations given by the consensus report on DNA cytometry.²³ The strict limits of image and staining quality, as well as the minimum number of measurable nuclei set by the server, resulted in the exclusion of numerous smears from quantitative DNA measurement. In addition, these regulations increased the average time of a complete measurement, which lasted about one hour, which is, however, still comparable with measurement times performed with stand alone machines. On the other hand, the built in quality control of staining and image acquisition saved measurement time and manpower in 19 smears that were not accurately stained.

The prospective study described included all pleural effusions sent to the department of pathology of the Thoraxklinik Heidelberg within the period September 1998 to July 1999. In contrast to other reports on static cytometry of pleural effusions, most cases were benign effusions (about 60%). More than 50% of the effusions were congestive effusions, commonly caused by centrally localised lung carcinomas and reactive pleuritis without any evidence of malignant involvement of the pleura. In addition, empyemas were a relatively frequent event, and these are difficult to measure by both flow and static DNA cytometry. Thus, a major part of our material could not be measured for biological reasons and the regulations of the consensus report, and only 19 cases were excluded because of inadequate technical performance.

With respect to the certainty of the diagnosis, the final classification of the disease of all our cases is based upon cytological and histological criteria, the clinical history, and follow up, allowing a thorough confirmation of the nature of the pleural alterations. For example, the five samples from patients with tuberculous pleuritis were confirmed by microbiology and polymerase chain reaction (PCR) analysis, and no patients presenting with a benign pleural effusion had clinical or radiological indications of malignant disease (including during the follow up period). Thus, there is strong evidence that the "gold standard" of benign and malignant pleural effusion is based upon a reliable diagnosis. All benign pleural effusions had euploid DNA values, and most of the malignant pleural effusions were aneuploid. This is in agreement with several reports, which found that benign pleural effusions were 100% euploid.^{7 13} For example, Sikora and colleagues⁷ analysed 14 diploid benign effusions (not described in detail). Similar findings have been reported by Banks *et al*, Rijken *et al*, and Biesterfeld *et al*.^{8 9 24} El-Habashi *et al* reported 14 cytologically

negative cases of peritoneal and pleural effusions, and found seven to be aneuploid.¹⁵ However, only seven of nine cases had a history of concomitant malignant tumour, and one case had no follow up. Using the given classification rules for euploidy/aneuploidy, a malignant pleural effusion can be diagnosed if the DNA distribution is aneuploid, a statement supported by several reports.^{7-9 13 15} Interestingly, Biesterfeld *et al* reported that polyploidy in non-malignant mesothelial cells might reach 6.5%; however, no aneuploidy in terms of abnormal peak position was observed.²⁴ The authors suggest that polyploidy might be introduced by abnormal tissue differentiation, but a final proof is still missing.

Whereas aneuploidy is an extremely rare or even non-existent event in benign pleural effusions, euploidy has been reported in numerous malignant pleural diseases.^{7 16 20} The percentage of euploid malignant effusions varies in the literature between 30% and 5%,^{7-10 13-15} which is comparable with our result of 9%. Although there is agreement that any malignancy can, in principle, display a euploid histogram, the incidence of euploid malignant effusions is difficult to estimate. Several factors such as number of analysed nuclei, the cellular density of the effusion, the number of reactive inflammatory cells, or technical details such as the "age" of the sample or temperature of sample storage can influence the behaviour of the cells, which remain alive in the sample fluid for several hours.²²

In our study, 22.2% of the mesotheliomas were found to be euploid, whereas only three of 60 (5%) pleural metastatic diseases were euploid. Interestingly, all 11 effusions of metastatic breast carcinomas were classified as aneuploid. Motherby *et al* found an abnormal stem cell line in 92% of specimens from pleural metastatic disease, and in 58.5% of mesotheliomas. When taking into account abnormal DNA content (9C exceeding rate or a CV > 10% for one stem cell line) the authors computed that 100% of metastatic adenocarcinomas and 82.9% of mesotheliomas could be grouped into this cohort. Our data are in general agreement with this study: a higher proportion of mesotheliomas are euploid compared with malignant metastatic disease. In addition, pleural effusions associated with a history of breast carcinoma should be subject to static DNA analysis because all malignant effusions were found to be aneuploid.

The computed specificity and sensitivity are within the range reported previously.^{7-10 13-15} A specificity and sensitivity of nearly 100% and greater than 90% can be expected to discriminate between benign and malignant pleural effusions, especially in cases with a known history of non-pleural malignancy. Whereas certain immuno(cyto/histo)chemical and ligand cytohistochemical/histochemical techniques have been proved to be useful in discriminating between primary and secondary pleural malignancies, they are of limited use in distinguishing benign from malignant effusions, despite the analysis of proliferation or abnormal gene expression.¹⁻⁶ Therefore, static DNA cytometry

of pleural effusions can be of value in the clinically important question: are we dealing with a benign effusion in a patient with a history of excised carcinoma?

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