Comparison of nucleolar organiser regions and DNA flow cytometry in the evaluation of pleural effusion

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Abstract

Background – In conventional cytological diagnosis of pleural effusions the assessment of morphological features plays an important part. However, false negative and false positive results may occur. In this study conventional cytology was compared with flow cytometric DNA analysis and the argyrophil staining technique for nucleolar organiser regions (AgNOR) to characterise benign and malignant effusions.
Methods – Pleural effusions from 71 patients (38 with benign lung disease, 33 with proven adenocarcinoma of lung) were studied by conventional cytology, flow cytometric DNA analysis, and the AgNOR technique. Tumour cell ploidy was determined by flow cytometry. In an attempt to detect the cell proliferative state, flow cytometric S phase fraction and the AgNOR technique were used. The correlations among conventional cytology, flow cytometric DNA ploidy, S phase fraction analysis, and nucleolar organiser regions were investigated.
Results – All the 38 benign pleural effusions were diploid. There were 17 (52%) aneuploid and 16 (48%) diploid malignant pleural effusions. Based on these results this type of DNA analysis had a sensitivity of 52% and a specificity of 100%. The mean (SD) numbers of flow cytometric S phase fractions of benign and malignant cases were 5·32 (1·67)% and 12·45 (3·93)% respectively. The mean numbers of S phase fractions of diploid malignant cases were higher than diploid benign cases. In each case the number of AgNORs was counted in 100 cells. The mean number of AgNOR dots per nucleus was 12·57 (3·64) for malignant pleural effusion cells and 3·96 (1·39) for benign pleural effusion cells. The mean number of AgNOR dots was 14·45 (3·36) for aneuploid malignant pleural effusion cells and 10·57 (2·82) for diploid malignant pleural effusion cells. The AgNOR numbers were higher in diploid malignant cells than in diploid benign cells. There was a significant correlation between the S phase fraction determined by flow cytometry and the mean number of AgNORs per nucleus in malignant cases.
Conclusions – Both flow cytometry and the AgNOR methods provide comparable measurements in the diagnosis of pleural effusion. The study also indicates that the AgNOR method, which is rapid and easy to perform, may be a useful adjunct to flow cytometry, S phase fraction analysis and conventional cytology in the routine diagnosis of malignant pleural effusion.

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The diagnosis of a pleural effusion is often difficult. In making a cytological diagnosis, the assessment of morphological features plays a very important part. However, false negative and false positive results can occur due to scarcity of recognisable malignant cells and abnormalities of mesothelial cells and macrophages in the sediment.[1] For these reasons various diagnostic methods have been used to identify tumour cells.

Several experimental techniques have been used to improve the diagnosis of effusions such as transmission and scanning electromicroscopy,[2] cytochemistry,[3] and cytogenetics.[4] Chromosomal abnormalities strongly suggest that a problem exists[5–7] and, because these abnormalities may be expressed as an abnormal DNA content, measuring DNA for diagnostic purposes has been repeatedly attempted by flow cytometry.[8–10] DNA analysis using automated flow cytometry is rapid and permits evaluation of a large number of cells from an effusion.

Nucleolar organiser regions (NOR) are loops of DNA in the nucleus which code for ribosomal RNA and are thus significant in the synthesis of protein.[11] These regions are associated with acidic non-histone proteins that can be visualised by argyrophilic staining (AgNOR).[12] The use of the AgNOR technique in histopathology[13–17] in numerous neoplastic tissues has shown that the distribution pattern of AgNORs is helpful in the diagnosis of malignancy.

In this study the pleural effusions were analysed by conventional cytology, DNA flow cytometry, and the AgNOR technique and the results were compared.

Methods

Pleural effusion samples from 71 patients admitted to the Department of Internal Medicine of Kaohsiung Medical College Hospital were analysed by conventional cytology, flow cytometry DNA analysis, and the AgNOR technique. In all patients the diagnosis was confirmed either by histology or clinical data.

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CONVENTIONAL CYTOLOGY

Fluids were centrifuged at 600 g for 10 minutes. The supernatant was discarded and a drop of the sediment was placed on a clear glass slide. Smears were made and fixed in 95% ethanol for 30 minutes. The Papanicolaou stain was used, and the smears were screened and evaluated routinely as either negative for malignant cells, insufficient for diagnosis, suspicious, or positive for malignant cells. The "positive" cytology result was designated when the cytological examination revealed a suspicious or positive result.

FLOW CYTOMETRY

Specimens were prepared for flow cytometric studies by the technique of Schneller *et al*[18] Briefly, specimens of fresh pleural fluid were centrifuged at 600 g for 10 minutes. After centrifugation the supernatant was decanted and cell pellets were resuspended in 10 ml of cold citrate buffer. The suspension was then incubated at room temperature for 20 minutes, with periodic agitation every five minutes. The material was subsequently washed two or three times in cold citrate buffer. Each wash was centrifuged until a clear supernatant was obtained. Cells were filtered through a 30 µm pore nylon mesh filter and centrifuged at 200 g at 4°C for 15 minutes to pellet the nuclei. The nuclear pellet was resuspended in cold citrate buffer and a 0·5 ml aliquot containing 5×10^5 cells was resuspended in 1·7 ml of 0·1% sodium citrate containing 0·05 mg/ml propidium iodide (Sigma, St. Louis, Missouri, USA). The suspensions were allowed to equilibrate with propidium iodide at 4°C for 30 minutes in the dark. The specimens were analysed on a Becton Dickinson FASCan (San Jose, California, USA) equipped with a 15 mW, 488 nm argon ion laser. At least 10 000 cells were measured with the use of Consort 30 software (Becton Dickinson) for data acquisition and the DNA cell cycles analysis software for DNA ploidy and S phase fraction analysis. Normal human lymphocytes served as a normal DNA (diploid) content control. The flow cytometry results were categorised as either normal (diploid) or abnormal (aneuploid).[18] Aneuploidy was defined in this study as having one or more distinct separate peaks compared with the G_0/G_1 and G_2M peaks of the diploid cell population. The DNA index was obtained from the ratio of channel numbers of the abnormal peak to the modal channel numbers of the diploid peak. The coefficient of variation of G_0/G_1 cells of both normal and abnormal populations ranged from 1·1% to 7·6% with a mean of 3·8%. DNA histograms with a coefficient of variation of more than 9% were excluded from this study. The "positive" flow cytometry result was designated when aneuploidy was detected.

AgNOR TECHNIQUE

Fresh fluid was received by the laboratory and each sample was centrifuged at 600 g for 10 minutes. The sediment was placed on a clear glass slide. All smears were fixed in 95% ethanol for 30 minutes. The AgNOR staining was performed by the method of Smith and Crocker.[13] Briefly, all specimens were rehydrated with distilled deionised water. The AgNOR solution was prepared by dissolving gelatin in 1 g/dl aqueous (distilled deionised) formic acid at a concentration of 2 g/dl. This solution was mixed (1:2 volumes) with 50 g/dl aqueous (distilled deionised) silver nitrate solution to make the final working solution. This was immediately poured over the specimens and left for 30 minutes at room temperature in the dark. The silver colloid was washed off with distilled deionised water and the specimens dehydrated through graded ethanol to xylene and mounted in a synthetic medium. All specimens were examined using a × 100 oil immersion objective for a total magnification of × 1000. Atypical cells and/or reactive mesothelial cells were selected at random for the counting procedure. In each specimen the number of AgNOR dots was counted in 100 cells with the aid of a graticule, adjusting the focus carefully to enable clear identification of an AgNOR dot. The mean (SD) number of AgNORs per nucleus was calculated for each specimen.

STATISTICAL ANALYSIS

The data were analysed by the Student's unpaired t test and χ^2 test. Linear regression was used to compare the data obtained with the AgNOR technique, DNA index, and flow cytometric S phase fraction analysis methods. Statistical significance was defined as $p < 0.05$.

Results

Seventy one pleural effusions were studied and the relation between NOR and flow cytometry DNA analysis was also investigated. Thirty eight patients had pleural effusions caused by benign disease and 33 had adenocarcinoma of the lung. The results were confirmed by histopathology or patient follow up.

CYTOLOGICAL ANALYSIS

Four of 33 patients with malignant tumours had negative results at cytological examination (table 1). Also, two of the 38 patients with benign diseases were considered to have cells suspicious of malignancy. After following up these two cases, an inflammatory cause was diagnosed and the effusions subsided with appropriate management. Conventional cytology therefore had a 95% specificity and an 88% sensitivity (table 2).

Table 1 Results of analysis of pleural effusions by conventional cytology and flow cytometry

Effusions	Cytology			Flow cytometry	
	Negative	Suspicious	Positive	Diploid	Aneuploid
Malignant	4	3	26	16	17
Benign	36	2	0	38	0
Total	40	5	26	54	17

Table 2   Comparative results with conventional cytology and flow cytometry

	Cytology	Flow cytometry	Combined
Sensitivity	88%	52%	94%
Specificity	95%	100%	100%
Predictive value of negative result	90%	70%	95%
Predictive value of positive result	94%	100%	100%

Sensitivity = true positive (TP)/TP + false negative (FN))
Specificity = true negative (TN)/TN + false positive (FP))
Predictive value of negative result = TN/(TN + FN)
Predictive value of positive result = TP/(TP + FP)

FLOW CYTOMETRIC DNA ANALYSIS

All 38 samples from patients without clinical evidence of malignancy were diploid. There were 17 aneuploid (52%) and 16 diploid (48%) DNA ploidy patterns in the 33 patients with malignancy (table 1). The DNA indices of the aneuploid tumours ranged from 1·93 to 1·11 with a mean (SD) of 1·42 (0·25). Four of 33 patients with malignancy were cytologically negative for malignant cells, and two of these had aneuploid DNA results. However, another two had diploid DNA content by flow cytometry (table 3). The flow cytometry DNA analysis showed no false positive results and yielded 100% specificity. There were 16 false negative cases and the sensitivity was 52%. The results obtained by both flow cytometry and conventional cytology were compared and yielded 94% sensitivity and 95% predictive value of a negative result. The combined specificity was 100% and the combined predictive value of positive results was 100% (table 2).

FLOW CYTOMETRY S PHASE FRACTION ANALYSIS

In three of 17 aneuploid DNA histograms S phase fraction analysis could not be performed due to complex histograms or excessive debris in the sample. The S phase fraction was significantly increased in malignant cases (n = 30) compared with the benign cases (n = 38) (12·45 (3·93)% v 5·32 (1·67)%; p<0·0001). The aneuploid malignant cases had increased S phase

fraction compared with the diploid malignant cases (15·89 (2·36)% v 9·44 (2·09)%; p<0·0001) (table 4). There was also an increase in the S phase fraction associated with the diploid malignant cases compared with the diploid benign cases (9·44 (2·09)% v 5·32 (1·67)%; p<0·0001) (table 4).

AgNOR ANALYSIS

The mean (SD) number of AgNOR dots per nucleus was 3·96 (1·39) (range 8·31–1·93) for cells from benign pleural effusions and 12·57 (3·64) (18·94–5·67) for the malignant pleural effusion cells (p<0·001; table 5). The AgNOR dots were irregularly distributed and their size was highly variable in patients with malignant disease. The mean (SD) number of AgNOR dots was 14·45 (3·36) (range 18·94–8·93) for aneuploid malignant pleural effusion cells and 10·57 (2·82) (16·24–6·03) for diploid malignant pleural effusion cells (p<0·01; fig 1). The AgNOR numbers were greater in diploid malignant effusion cells (10·57 (2·82)) than in diploid benign pleural effusion cells (3·96 (1·39); p<0·001) (fig 1). The best cutoff point of the mean number of AgNOR dots per nucleus was determined by a receiver operative characteristic curve[19] for predicting a malignant tumour in a patient with a pleural effusion. The selected value was 8·0, and there were two false negative cases and one false positive case. The sensitivity, specificity, and accuracy was 94%, 97%, and 96%, respectively. The AgNOR numbers in two aneuploid malignant patients with negative cytology results were 11·6 (2·56) and 8·93 (2·38). Although the AgNOR num-

Table 3   Comparison of results with flow cytometry and cytology in malignant cases

Flow cytometry	No. of cases	Cytology		
		Negative	Suspicious	Positive
Diploid	16	2	2	12
Aneuploid	17	2	1	14
Total	33	4	3	26

Table 4   Mean (SD) data of flow cytometry S phase fraction

	S phase fraction		
	Range	Mean (SD)	p
Malignant (n = 30)*	20·5– 6·2	12·45 (3·93)	<0·0001
Benign (n = 38)	7·9– 1·6	5·32 (1·67)	
Malignant*			
Aneuploid (n = 14)	20·5–10·0	15·89 (2·36)	<0·0001
Diploid (n = 16)	14·3– 6·2	9·44 (2·09)	

*There were three histograms in which S phase fraction could not be estimated from the histogram.

Table 5   Mean (SD) number of AgNOR dots per nucleus for malignant and benign pleural effusion cells

	AgNOR		
Effusions	Range	Mean (SD)	p
Malignant	18·94–5·67	12·57 (3·64)	<0·001
Benign	8·31–1·92	3·92 (1·39)	
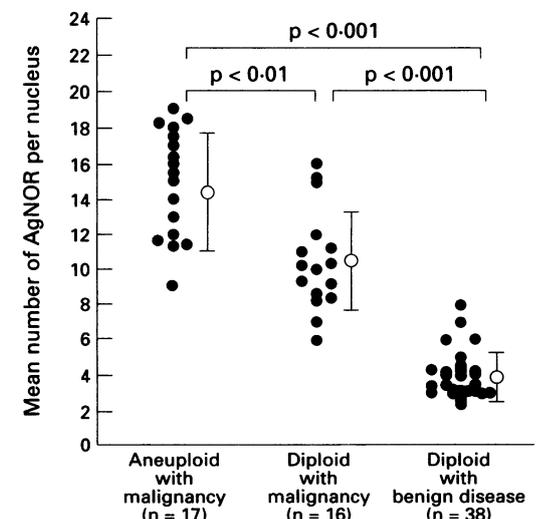
Figure 1   Scattergram to show distribution of the mean AgNOR number per nucleus for specimens examined. There was a significant difference among the three groups.
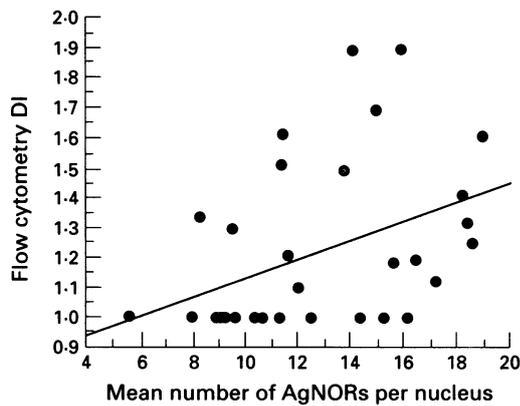
Figure 2 Correlation between the mean number of AgNORs per nucleus and flow cytometry DNA indices (DI) in 33 malignant cases ($\gamma = 0.432$, $p<0.02$).

Figure 3 Correlation between the mean number of AgNORs per nucleus and flow cytometrically determined S phase fraction in 30 malignant cases ($\gamma = 0.605$, $p<0.0005$).

bers of the two diploid malignant cases with negative cytology results showed less than 8 (5·67 (1·98) and 7·97 (0·83)), there were higher S phase fractions (8·5% and 8·8%) than the mean number of S phase fractions of benign cases (5·32%).

COMPARISON OF FLOW CYTOMETRIC DNA INDEX AND AgNOR TECHNIQUES

The mean DNA index of aneuploid tumours (n = 17) was 1·43 (0·25) (range 1·11–1·92). There was no correlation between the flow cytometric DNA index and the mean number of AgNOR dots in aneuploid tumours. However, there was a weak correlation between the DNA index measured by flow cytometry and the mean number of AgNOR dots in malignant cases (n = 33, $\gamma = 0.432$, $p<0.02$; fig 2).

COMPARISON OF S PHASE FRACTION DETERMINED BY FLOW CYTOMETRY AND AgNOR TECHNIQUES

The comparison of S phase fraction determined by flow cytometry and the mean number of AgNOR dots in malignant cases showed a significant correlation (n = 30, $\gamma = 0.605$, $p<0.0005$) (fig 3).

Discussion

Flow cytometry is a process that can be used to determine cellular DNA content, cell cycle distribution kinetics, and chromosomal analysis among cell populations.[18 20–22] In contrast to mitotic karyotyping, the technique allows investigation of interphase cells and is not limited by the proliferative status of the cell population. The S phase fraction measured by flow cytometry provides an important predictor of prognosis in some cancers.[23 24] Clark et al al[25] showed that diploid tumours with a higher S phase fraction had a poorer prognosis than diploid tumours with a lower S phase fraction. The S phase fraction was significantly increased in malignant cases compared with benign controls. Aneuploid tumours have an increased S phase fraction compared with diploid tumours.[23] In our study malignant cases had a significantly higher S phase fraction than the benign cases. The S phase fraction was increased in aneu-

ploid malignant cases compared with diploid malignant cases.
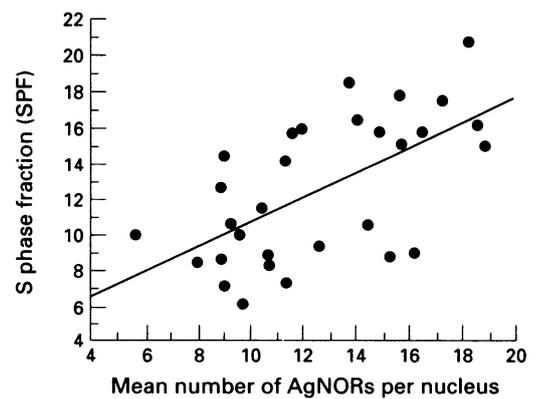
Nearly all malignant neoplasms have chromosomal aberrations. However, the sensitivity of flow cytometric DNA analysis requires a significant quantitative DNA abnormality for detection.[26] In addition, some tumours have chromosomal deletions and duplications that may balance out, resulting in a tumour cell with normal net DNA content by both karyotypic quantitation and flow cytometry but an obviously abnormal karyotype.[26] Moreover, flow cytometric DNA analysis requires sufficient cells with a DNA abnormality to be detected. Malignant tumours with undetectable abnormal genomes are not uncommon.

Hedley et al[9] reported 36% and Weissman et al[27] 43% false negative rates in flow cytometric analysis of the DNA content of pleural effusions. There was a 48% false negative rate by flow cytometry in this study. Obviously flow cytometric DNA ploidy abnormality is not an appropriate diagnostic screening criterion of malignancy and contributes to the significant false negative rate. Our flow cytometric DNA content studies contained no false positive cases and yielded a specificity of 100%. Similar results have been described by others.[8 9 18 27] However, 1–3% false positive rates with flow cytometry have been reported in effusion studies.[28–30]

As the DNA of nucleolar organiser regions is transcribed ultimately into ribosomal RNA and hence leads to protein synthesis, the number or size of NORs might be expected to reflect cellular activity, proliferation, or transformation. Crocker et al[15 31] reported the application of the AgNOR technique to diagnostic tumour pathology. The AgNOR technique has been applied to metaphase chromosome spreads[12] and has been used to identify atypical chromosomes in certain malignancies.[15 31 32] The number of AgNOR dots within nuclei has been shown to differ in many benign and malignant tumours.[13 15 17 31–33] Ayres et al[34] reported that the reactive pleural diseases had significantly higher AgNOR numbers than normal pleura obtained by biopsy. Some studies have described a considerable overlap of AgNOR numbers between benign and malignant conditions.[35] Our study showed that

the interphase NOR count permits significant differences between malignant and benign pleural effusions. Aneuploid malignant patients also had significantly higher AgNOR numbers than diploid malignant patients.

Several methods for assessing cell proliferation and assisting in tumour grading have recently been introduced. Ki-67 immunostaining and BrdU labelling are known to label replicating cells. However, these methods are restricted to frozen samples or are dependent on the intraoperative application of myelotoxic substances. Ki-67 immunostaining and AgNOR staining would appear to yield comparable data in non-Hodgkin's lymphoma,[36] but the AgNOR technique has the advantage of being applicable to fixed and processed paraffin sections.

A recent comparison of NOR counts and flow cytometric DNA analysis of non-Hodgkin's lymphoma showed a very good linear correlation between the mean numbers of AgNOR sites and the percentage of S phase cells. However, the correlation with tumour cell ploidy was poor.[37] Schwarzacher et al[38] showed that aneuploid cells have an increased number of chromosomes and chromosome arms that may bear additional NOR material. Thus, higher AgNOR counts for aneuploid tumours would be expected. Recently, Carbajo et al[39] showed that the AgNOR technique can be considered as a cell proliferation marker. They also confirmed that AgNOR numbers were significantly higher in aneuploid than in diploid malignant pleural effusions.

We conclude that flow cytometry and the AgNOR technique provide comparable measurements in the diagnosis of pleural effusions. However, the AgNOR technique is rapid, simple and inexpensive and may be used routinely in diagnostic cytopathology. The present study has shown that the AgNOR staining technique may be used as a very useful adjunct to flow cytometric DNA content analysis, S phase fraction and conventional cytology in the diagnosis of malignant pleural effusions. Nevertheless, AgNOR staining has the advantage over flow cytometric DNA analysis of being applicable to routinely processed pleural effusions.

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