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Diploid Predominance and Prognostic Significance of S-phase Cells in Malignant Mesothelioma

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70 histologically verified, malignant mesotheliomas were analysed by flow cytometry for DNA content and S-phase fraction (SPF) of tumour cells. 60% (42/70) were DNA diploid. 18 of the 28 aneuploid tumours were near-diploid with DNA indices of 1.3 or less. SPF could be calculated in 51 cases. SPF was significantly higher in aneuploid (median 16.0%) than in diploid tumours (median 5.6%). DNA ploidy was not a prognostic determinant; survival was the same for both aneuploid and diploid tumours. SPF, however, was significantly correlated ($P = 0.039$) with prognosis. Patients who had tumours with a low SPF survived almost twice as long as those with a high SPF. Thus malignant mesothelioma has a peculiar DNA ploidy pattern compared with many other solid tumours, with a predominance of diploid or near-diploid type cells. As in many other tumours, SPF may be used as a clinically relevant prognostic indicator.
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INTRODUCTION

DNA FLOW CYTOMETRY (FCM) analysis of malignant tumours provides information on DNA ploidy and the proliferative activity of tumour cells, which may be correlated with clinical characteristics to identify prognostic factors and to increase knowledge of tumour biology. Malignant mesothelioma is increasing in frequency in most countries, with the use of asbestos between 1940–1970 [1–5]. However, little is known of the biology of mesothelioma, and only limited information is available from DNA FCM [6, 7].

Our group has examined systematically various biological characteristics of malignant mesotheliomas such as chromosomes [8], *in vitro* growth ability [9] and asbestos fibre content [10], as well as clinical aspects of this disease [11]. We use thoracotomy for routine staging in the management of mesothelioma. This provides samples for histological verification as well as for other methods of tissue examination, such as FCM.

For this report we have analysed DNA ploidy profiles and the proliferative activity of 70 histologically verified malignant mesotheliomas by measuring the S-phase fraction (SPF) of tumour cells. We have also correlated FCM parameters with prognosis, with the specific aim of testing the clinical application of FCM in the examination panel for malignant mesothelioma.

PATIENTS AND METHODS

Patients

Tumour samples were obtained from 70 patients with malignant mesothelioma all diagnosed and treated at the Helsinki University Central Hospital between 1978 and 1989. Histological diagnosis and subtyping was done by the panel of the Lung Cancer Cooperative Group of the European Organization for Research and Treatment of Cancer. The patients participated in clinical trials of multimodality therapy [11] consisting of debulking surgery, chemotherapy and hemithorax irradiation (Table 1).

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Table 1. Patients' characteristics

No.	70
Mean age (yr) (range)	57 (24–80)
M/F	54/16
Pleural mesotheliomas	66
I	13
IIA	40
IIB	1
IIIA	1
IIIB	9
IV	2
Peritoneal mesothelioma	4
Histological subtype	
Epithelial	25
Fibromatous	5
Mixed	39
Unknown	1*

*Insufficient sample for detailed subtyping.

DNA analysis

For the DNA analysis only one fresh-frozen biopsy specimen was available. The analysis in this single case was done as described [12]. DNA measurement from paraffin blocks was done as described by Hedley *et al.* [13] with slight modifications [14] as follows. One 50 μm section cut by microtome was deparaffinised, rehydrated and digested overnight with 0.25% trypsin (Orion Diagnostica, Helsinki), in a 10 mmol/l Tris-HCl buffer pH 7.5 containing 1 mmol/l Na-EDTA and 0.3% non-ionic detergent (Nonidet P40, BDH). The cells were stained with propidium iodide 50 mg/l (Sigma) in Tris-HCl containing 1 mg/ml RNAse I (Sigma). Immediately before analysis the samples were filtered through a 30 μm nylon mesh.

DNA was analysed with an EPICS C flow cytometer (Coulter Electronics, Hialeah, Florida) equipped with a 2 W argon-ion laser. Excitation of propidium iodide occurred at 488 nm, and the fluorescent emission was measured above 590 nm. A minimum of 15 000 nuclei from each specimen were analysed.

Tumours were classified as aneuploid if there was a second G1 peak in addition to the diploid G1 peak. The DNA index was calculated as the ratio of the aneuploid stem line G1 DNA peak channel to the diploid stem line G1 DNA peak channel. The peak with least DNA content was considered to represent diploid cells in paraffin material. In the fresh-frozen sample chicken and trout red blood cells were used as internal DNA standards. The coefficient of variation (half-width method) was determined by the Statpack program (Coulter). The mean coefficient of variation for diploid G_0/G_1 peaks was 4.8 (S.D. 1.3, range 1.9–9.38). SPF was calculated as described [14], based on the assumption that SPF is a rectangle between the G_0/G_1 and G_2/M phases of the cell cycle [15]. Counts per channel in the mid S-phase (10 channels) were calculated and multiplied by the number of channels between the G_0/G_1- and G_2/M-peaks to obtain the total SPF. In aneuploid tumours, where clear separation of diploid and aneuploid cell cycle phases were detected, counts per channel within 10 channels near the aneuploid G_2/M phase were calculated and multiplied by the number of channels between the aneuploid G_0/G_1- and G_2/M-peaks. When multiple samples were available the highest SPF value was used. Samples with a coefficient of variation greater than 8.0%, a large amount of debris or with near-diploid aneuploidy were excluded from the cell cycle analysis.

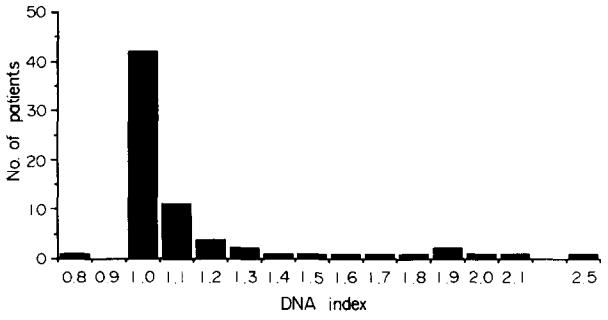

Fig. 1. Distribution of DNA indices in mesotheliomas.

Statistical methods

All statistical analyses were performed with the BMDP. Differences between groups were analysed by the likelihood ratio χ^2 (G^2) test or analysis of variance (ANOVA). Survival curves were constructed by the product-limit method; groups were compared with Mantel–Cox's test. Brookmeyer–Crowley 95% confidence intervals for median survival time are reported.

RESULTS

60% (42/70) of the tumour samples were DNA diploid and 40% were aneuploid. DNA indices ranged from 0.81 to 2.52 (Fig. 1). The fresh tumour sample had a hypodiploid clone. 18 of the 28 aneuploid tumours (paraffin-embedded) were classified as near-hyperdiploid, having DNA indices of 1.3 or less. If diploid and near-diploid tumours were grouped together, 86% (60/70) of the tumours were in this category. Tetraploid tumours with DNA indices of 1.90–2.10 were observed in 3 cases (4%). The SPF could be calculated in all but 1 of the diploid tumours, but in only 9 out of the 28 aneuploid tumours. Median SPF was 5.6% (range 1.2–19.9) in DNA diploid and 16.0% (1.9–35.0) in aneuploid tumours (Table 2). This difference was significant ($P = 0.026$).

The survival data for the patients were compared with the FCM results. DNA ploidy did not correlate with prognosis. The median survival of patients with DNA diploid tumours was 12.8 months and for those with DNA aneuploid tumours 12.7 months. In contrast, SPF correlated with the course of the disease. Median survival for patients with low SPF tumours was nearly twice as long as that for the patients with high SPF tumours. Patients with low (at median or below median) and high (above median) SPF diploid tumours survived for 14.0 and 7.6 months, respectively, and those with aneuploid tumours for

Table 2. Proportions of S-phase cells in diploid and aneuploid mesotheliomas

Ploidy	No. measured	Median (%)	Mean (%)	95% CI
Diploid ($n = 42$)	41	5.6	6.8	5.4–8.2
Aneuploid ($n = 28$)	9	16.0	16.1	8.2–24.0

Table 3. Median survival of mesothelioma patients correlated with proportion of S-phase cells

Ploidy	S-phase cells (%)	Median survival (mo)	*P*
Diploid			
A	≤ 5.6 (median)	14.0	0.099
B	> 5.6	7.6	
Aneuploid			
C	≤ 16 (median)	17.6	0.358
D	> 16	8.9	
Combining groups			
A + C		14.0	0.039
B + D		8.9	

17.6 and 8.9 months, respectively (Table 3). Combining the results from diploid and aneuploid tumours and with the median SPF as a cut-point, patients with SPF above the median survived significantly less time than those with SPF at or below the median (Table 3, Fig. 2).

DISCUSSION

Flow cytometric DNA analysis is rapid, objective, reproducible and feasible for routine examinations not only of fresh tissue but also of fixed paraffin-embedded samples [13]. We did FCM on 70 histologically confirmed cases of malignant mesothelioma, to establish DNA ploidy and SPF. We found a predominance of diploid and near-diploid tumours; only 14% had a DNA index greater than 1.3.

Although the frequency of malignant mesothelioma is increasing rapidly, there is little FCM information on this malignancy. Frierson *et al.* examined ploidy profile from 19 formalin-fixed, paraffin-embedded mesothelioma specimens [6]. 9 (47%) were diploid, 7 (37%) were clearly aneuploid and the remaining 3 tumours were probably near-diploid with DNA index of hyperdiploid type. They further analysed the DNA content of 28 fresh pleural effusion specimens containing abundant proliferating mesothelial cells. In a study by Burmer *et al.* [7] of paraffin-embedded specimens from 46 cases of malignant mesothelioma, 65% were diploid. This is consistent with our observations on a larger group of patients. Consequently, the conclusion by Frierson *et al.* that DNA aneuploidy in an effusion specimen containing atypical mesothelial cells would strongly support a diagnosis of mesothelioma may not be justified. Aneuploidy cannot be considered a good diagnostic marker for malignant mesothelioma.

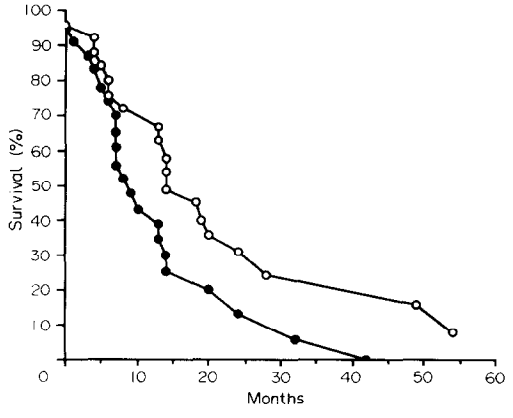

Fig. 2. Survival of patients with malignant mesotheliomas related to SPF. ●——● = patients with SPF higher than median (in diploid tumours, ~ 5.6; in aneuploid tumours ~ 16.0). ○——○ = patients with SPF at median or below median.

Unlike many other solid tumour types, diploid or near-diploid predominance seems to be a peculiar feature of malignant mesotheliomas. In almost all the other malignant solid tumours (except malignant lymphomas) aneuploidy is predominant and DNA indices are frequently above 1.3—i.e. not in the near-diploid range [16, 17]. This is particularly true for lung cancer, where the predominance of aneuploidy has been reported, according to most studies, in 80–96% of tumours [18–21]. We have no explanation for the similarity between mesothelioma and lymphoma, or the dissimilarity between mesothelioma and other non-lymphoid solid tumours. Recently we have detected among colorectal tumours a subgroup with family history of fairly similar DNA ploidy distribution [14]. Two other reports have proposed a familial history in malignant mesotheliomas [22, 23].

The prognostic significance of FCM DNA parameters has been evaluated in most of the major human malignancies. To date, no such knowledge has been available for malignant mesothelioma. In many solid tumours some disagreement exists on the prognostic value of DNA ploidy [14, 17–20]. Frequently, DNA aneuploid tumours have been more readily associated with unfavourable prognosis than have diploid tumours. This has not, however, been a consistent finding. In some cases, no difference between diploid and aneuploid tumours was observed [17], and this was the finding in our study of malignant mesothelioma. Sometimes the natural differences in clinical behaviour of diploid and aneuploid tumours may be masked by a greater sensitivity to therapy of aneuploid tumours. Aneuploid neuroblastomas [24] have been reported to be more sensitive to chemotherapy than diploid neuroblastomas. Also, in microcellular lung cancer, near-diploid type tumours might be more resistant to chemotherapy than hyperdiploid tumours [25].

Interestingly, similar findings have been observed in radiosensitivity of certain malignancies. Aneuploid laryngeal cancers [26], carcinomas of the oral cavities [27] and the cervix uteri [28] as well as bladder carcinomas [29] have been reported to be more radiosensitive than their diploid counterparts. This has probably contributed to the better prognosis of patients with aneuploid tumours after curative radiotherapy in these tumour groups [26, 27]. On the other hand, in most of the studies of tumours treated primarily by surgery, such as breast cancer and colorectal malignancies, aneuploidy indicates a less favourable prognosis [17]. In malignant mesotheliomas the significance of aneuploidy compared with diploidy in different treatment modalities is unknown and cannot be estimated from our study because of the limited number of patients. The degree of significance will, however, be an interesting future aspect of this research. However, our finding of predominant diploid or near-diploid DNA pattern may reflect the general resistance of mesotheliomas to current therapies [30, 31] as reported in other malignancies [24–29].

SPF could be measured in our study in all except 1 of the diploid tumours but in only one third of the aneuploid tumours. In two thirds of the aneuploid tumours, the aneuploid peak invariably made analysis unreliable due to the near-diploid type of the histograms. In the tumours where SPF could be

determined, median SPF was almost three times higher in aneuploid than in diploid tumours. Similar findings have also been observed in other malignancies [17].

As with ploidy pattern, SPF has prognostic value in solid tumours [17]. In the present study, low SPF indicated good prognosis. Patients with SPF at or below median SPF had almost twice as long median survival as those with high SPF. This makes FCM analysis a useful tool in the clinical research of malignant mesothelioma. It still remains to be seen whether this FCM variable can be used as a guideline for selecting a treatment strategy for each patient. Further expansion of this study, and multivariate analysis of other tumour indices as well as treatment results, will hopefully provide more information.

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