

DNA ploidy in bronchopulmonary carcinoid tumours

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ABSTRACT Fifty three bronchopulmonary carcinoid tumours were studied to assess the significance of DNA ploidy, determined by flow cytometry of paraffin embedded tissue. Twenty eight were typical carcinoid tumours and 25 well differentiated neuroendocrine carcinomas. Twenty seven were DNA diploid and 26 DNA aneuploid. DNA aneuploidy was significantly associated with histological features of increased malignant potential. Survival data were available for 43 patients. Of the 19 with DNA diploid tumours, 16 survived five years, compared with 14 of 24 with DNA aneuploid tumours—the difference being at the borderline of statistical significance. In a Cox multivariate regression analysis with other histological variables, DNA ploidy did not confer independent prognostic information. It is concluded that, although DNA aneuploidy as determined by flow cytometry is an indicator of increased malignant potential in bronchopulmonary carcinoid tumours, it does not provide clinically useful information additional to the results of routine histological examination.

Carcinoid tumours are relatively uncommon, accounting for only 1–6% of all lung tumours.¹ At one time they were regarded as benign but they are now recognised as tumours of low grade malignant potential, forming part of a histological spectrum ranging from typical carcinoids to small cell neuroendocrine carcinomas.² They have a more favourable prognosis than other malignant lung tumours, but their behaviour may be difficult to predict from the histological pattern. Factors that have been associated with a worse prognosis in bronchial carcinoid tumours are increasing age, higher T stage or N stage, mitotic count, necrosis, nuclear pleomorphism, vascular and lymphatic invasion, and an undifferentiated growth pattern.³

Cytogenetic changes are a recognised feature of many human tumours and may be related to clinical behaviour.⁴ The number of chromosomes may be either increased (hyperdiploid) or, less frequently, decreased (hypodiploid), and structurally abnormal “marker” chromosomes may appear. Quantitative changes in chromosome content can be estimated indirectly by measuring cellular DNA content by static or flow cytometry.^{5,6} Two groups of tumours are

distinguished: those with a normal or near normal, diploid DNA content, and those with an abnormal, aneuploid DNA content.

The measurement of cellular DNA content has been increasingly facilitated by flow cytometry, and techniques are now sufficiently refined to be clinically useful. Methods for the analysis of paraffin embedded tissue⁷ have allowed material from uncommon tumours such as bronchial carcinoids to be re-examined.

The aim of the present study was to determine whether the measurement of tumour DNA content by flow cytometry could enhance the pathological assessment and help to predict the prognosis of bronchopulmonary carcinoid tumours.

Methods

The study group consisted of 53 patients presenting with bronchopulmonary carcinoid tumours to the North West Regional Cardiothoracic Centre (Wythenshawe Hospital) during 1961–85. Survival information was obtained for 43 patients from clinical records and from the Cancer Registry at the Christie Hospital and Holt Radium Institute.

Slides were reviewed to confirm the diagnosis and to determine the depth of invasion, growth pattern, presence or absence of lymphatic and vascular invasion, mitotic count and presence or absence of affected lymph nodes. The growth pattern was

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classified as differentiated (insular, trabecular, and glandular) or undifferentiated. Tumours were classified, by the criteria of Gould *et al.*,² as typical carcinomas or well differentiated neuroendocrine carcinomas, the latter group consisting of tumours with a pattern similar to that of typical carcinoids but showing more cellular pleomorphism and increased mitotic activity. Sections were also stained by the Grimelius technique to demonstrate argyrophilia and immunohistochemically for neurone specific enolase. Histological features were analysed independently of ploidy determination by one observer (PH).

Samples for flow cytometry were prepared by the method of Hedley *et al.*⁷ Sections 30 μm thick were cut from paraffin blocks, dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed twice in distilled water. Nuclei were released by cytoplasmic digestion with 0.5% pepsin in NaCl (pH 1.5) at 37°C for 30 minutes. Samples were centrifuged and nuclear pellets resuspended in 1 μg of 4',6-diamidino-2-phenylindol-dihydrochloride (DAPI) per millilitre of Roswell Park Memorial Institute tissue culture medium. DAPI is a fluorescent dye that binds stoichiometrically to DNA. Samples were analysed on a Coulter EPICS V flow cytometer by means of ultraviolet excitation, which induces blue fluorescent emission from DNA bound DAPI. Fluorescence intensity is directly proportional to nuclear DNA content. A total of 20 000 nuclei were analysed in each sample and the full peak coefficient of variance of the diploid peak was calculated with the incorporated software. If a coefficient of variance greater than 10 was obtained the process was repeated, but the result was considered valid if it was still greater than 10. Tumour samples invariably contained normal diploid stromal cells giving a DNA diploid peak in all histograms, which served as an internal control. DNA aneuploidy was defined as the presence of any other G0/G1 peak which comprised at least 10% of the total cells analysed. The DNA content of aneuploid nuclei was estimated by calculating the ratio of the fluorescence intensity of the aneuploid population to that of the diploid population—the DNA index.

DNA ploidy and histological variables were compared by analysis with Yates's continuity correction for small numbers. Survival curves were constructed by the life table method and compared by means of the log rank test. Cox regression analysis was used to identify variables of independent significance.

Results

Forty of the 53 tumours were centrally situated and 13 were peripheral. Twenty eight were typical bronchopulmonary carcinoids and 25 were well differentiated neuroendocrine carcinomas. Argyro-

philia was seen in 41 and 50 stained positively for neurone specific enolase.

Interpretable DNA histograms were obtained for all tumours and the mean coefficient of variance was 7.0 (SE 2.0). Twenty seven of the 53 were DNA diploid and 26 DNA aneuploid. Twenty four of the DNA aneuploid tumours were hyperdiploid and two were hypodiploid with a DNA index of 0.6 (fig 1). In both hypodiploid cases there were two G0/G1 peaks, but the second peaks were in the channel range for normal diploid peaks (about channel 50). The presence of a DNA hypodiploid population was confirmed by repeating the analysis of the samples and including normal lung tissue from a different block from the same specimen. This enriched the second G0/G1 peak, indicating that the first peak in a low channel number represented a hypodiploid population (fig 2).

Nine of the 28 typical carcinoids were DNA aneuploid, compared with 17 of the 25 well differentiated neuroendocrine carcinomas ($\chi^2 = 5.44$, $p < 0.02$). DNA aneuploidy was present in a significantly higher proportion of undifferentiated tumours (15 out of 21) than of tumours with a differentiated growth pattern (11 out of 32; $\chi^2 = 5.6$, $p < 0.02$). Thirteen of 18 tumours with necrosis were DNA aneuploid, compared with 13 of the 35 without necrosis ($\chi^2 = 4.5$, $p < 0.05$). Twenty one of 26 tumours with pleomorphic nuclei were DNA aneuploid, compared with only five of the 27 with more normal nuclei ($\chi^2 = 18.1$, $p = 0.001$). In 12 tumours the mitotic count was greater than 1 per high power field: three were DNA diploid and nine DNA aneuploid. The depth of invasion and the presence of vascular or lymphatic invasion were independent of DNA ploidy status. The incidence of DNA aneuploidy was similar in central and peripheral tumours.

Lymph nodes accompanied the resection specimens of 40 tumours, and there was metastatic disease in two cases of typical carcinoid and 11 of well differentiated neuroendocrine carcinoma. The incidence of DNA

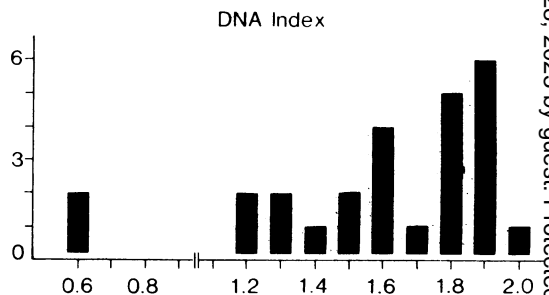


Fig 1 Distribution of DNA aneuploid tumours according to DNA index.

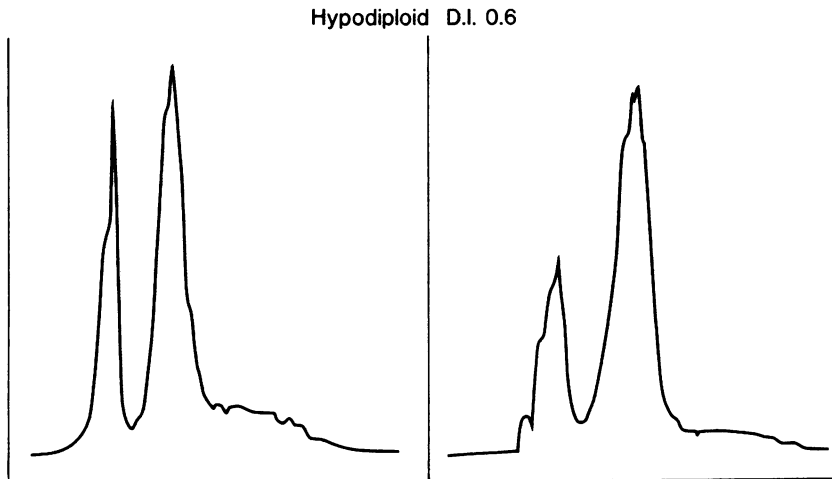


Fig 2 DNA hypodiploid tumour. (a) Tumour only, with two distinct G₀/G₁ peaks. (b) Tumour mixed with normal lung tissue from the same specimen. The right peak is enriched and corresponds to the DNA diploid population; the left peak therefore represents a DNA hypodiploid population with a DNA index (DI) of 0.6.

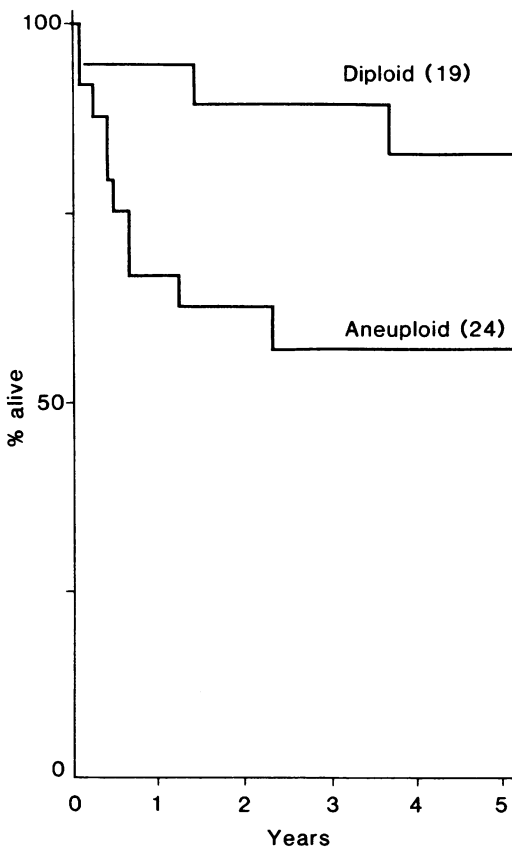


Fig 3 Comparison of survival for patients with DNA diploid and DNA aneuploid tumours ($\chi^2 = 3.77$, 1 df, $p = 0.052$).

aneuploidy in tumours with metastases (11/13: 85%) was significantly higher than in those without affected lymph nodes (12/36: 33%; $\chi^2 = 8.1$, $p < 0.02$). Both of the typical tumours with lymph node metastases were DNA aneuploid.

Information on survival was available for 43 patients. Sixteen (84%) of the 19 patients with DNA diploid tumours survived five years, compared with 14 (58%) of the 24 with DNA aneuploid tumours (fig 3). This difference was at the borderline of significance ($p = 0.052$). In a Cox multivariate regression analysis, the most powerful predictor of prognosis was the histological growth pattern. The only other variables of independent prognostic significance were the presence of affected lymph nodes and the presence of nuclear pleomorphism. Determination of DNA ploidy did not confer independent prognostic information. The duration of survival of those with DNA aneuploid tumours was independent of the DNA index and the size of the aneuploid peak.

Discussion

The measurement of cellular DNA content by flow cytometry is emerging as a potential aid to the pathological assessment of various human tumours. In the present study DNA aneuploidy was detected both in typical bronchopulmonary carcinoid tumours and in well differentiated neuroendocrine carcinomas, although the incidence in the latter was significantly higher. Blondal *et al*⁸ studied 15 bronchopulmonary carcinoids using static cytophotometric techniques; 13 were DNA diploid and two DNA hypodiploid. It was suggested that DNA ploidy determination may help to

discriminate carcinoids from other malignant lung tumours, which are more commonly DNA aneuploid. In our study, however, DNA aneuploidy was detected in one third of typical carcinoids and two thirds of well differentiated neuroendocrine carcinomas, indicating that DNA ploidy does not distinguish different types of lung tumours.

DNA aneuploidy was associated with decreased survival, but did not have independent prognostic significance in a Cox multivariate regression analysis. The survival of patients with typical carcinoids was good, irrespective of ploidy, although the only patient in this subgroup who died had a DNA aneuploid tumour.

These results suggest that although DNA aneuploidy is an indicator of increased malignant potential, its value is limited by comparison with established histological criteria. DNA ploidy determination did not identify tumours with a malignant clinical course that were not also identified histologically.

There is a high incidence of DNA aneuploidy in small cell and non-small cell carcinoma of the lung (over 80% in most series), although conflicting reports have emerged regarding its prognostic significance.⁹⁻¹⁵ DNA aneuploid tumours arising at other sites may be classified according to DNA index, and a more favourable prognosis is suggested for patients with DNA tetraploid breast^{16,17} and colorectal carcinomas.¹⁸ In the present study, however, there were no discernible aneuploid subgroups of prognostic significance.

Two of the tumours were DNA hypodiploid with a DNA index of 0.6, and both were well differentiated neuroendocrine carcinomas. It is unusual to detect DNA hypodiploidy by flow cytometry. In both cases there were two G0/G1 peaks, but the second peak corresponded to the anticipated diploid channel range. Fluorescence intensities vary between different paraffin embedded specimens, making reference to external standards unreliable. When the measurements were repeated to include normal lung tissue from other blocks of the same specimen¹⁹ the second peaks were enriched, confirming that the first peaks represented true DNA hypodiploid populations. Blondal *et al*⁸ also found two DNA hypodiploid tumours in their series, and two other carcinoids with reduced chromosome content have been detected by cytogenetic techniques.^{20,21}

The biological relevance of DNA aneuploidy to malignant transformation and progression is uncertain. In the present study DNA aneuploidy was not a prerequisite for malignant behaviour and did not invariably confer features of increased malignant potential. It has also been detected in premalignant dysplastic lesions in chronic ulcerative colitis,^{22,23} Barrett's oesophagus,²⁴ and benign colorectal polyps.²⁵

The generation of an aneuploid population is probably an epiphenomenon, reflecting increased genetic instability associated with malignant transformation. If chromosomal rearrangements are implicated in the pathogenesis of neoplasia, they are presumably sub-chromosomal or limited to specific genes. Measurement of cellular DNA content by flow cytometry is not sufficiently sensitive to detect such changes.

In conclusion, DNA aneuploidy is an indicator of increased malignant potential in bronchopulmonary carcinoid tumours. The determination of DNA ploidy by flow cytometry, however, is of limited clinical value by comparison with conventional pathological assessment.

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References

- 1 Anonymous. Bronchial adenomas [editorial]. *Br Med J* 1982;**282**:252.
- 2 Gould VE, Linnoila RF, Memoli VA, Warren WH. Neuroendocrine cells and neuroendocrine neoplasms of the lung. *Pathol Annu* 1983;**18**:287-330.
- 3 Hasleton PS, Gomm S, Blair V, Thatcher N. Pulmonary carcinoid tumours: a clinico-pathological study of 35 cases. *Br J Cancer* 1986;**54**:963-7.
- 4 Sandberg AA. *The chromosomes in human cancer and leukemia*. New York: Elsevier, 1980.
- 5 Friedlander ML, Hedley DW, Taylor IW. Clinical and biological significance of aneuploidy in human tumours. *J Clin Pathol* 1984;**37**:961-74.
- 6 Quirke P, Dyson JED. Flow cytometry: methodology and applications in pathology. *J Pathol* 1986;**149**:79-87.
- 7 Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrove EA. A method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. *J Histochem Cytochem* 1983;**31**:1333-5.
- 8 Blondal T, Arnorsson T, Bengtsson A, Wilander E. Nuclear DNA in carcinoid tumours of the lung. *Eur J Respir Dis* 1983;**64**:298-305.
- 9 Abe S, Makimura S, Itabashi K, Nagai T, Tsuneta Y, Kawakami Y. Prognostic significance of nuclear DNA content in small cell carcinoma of the lung. *Cancer* 1985;**56**:2025-30.
- 10 Volm M, Drings P, Mattern J, Sonka J, Vogt-Moykopf I, Wayss K. Prognostic significance of DNA patterns and resistance predictive tests in non-small-cell lung carcinoma. *Cancer* 1985;**56**:1396-403.
- 11 Volm M, Mattern J, Sonka J, Vogt-Schaden M, Wayss K. DNA distribution in non-small-cell lung carcinoma and its relationship to clinical behaviour. *Cytometry*

- 1985;6:348–56.
- 12 Moran RE, Melamed MR. Flow cytometric analysis of human lung cancer. Correlation with histologic type and stage. *Anal Quant Cytol* 1984;6:99–104.
- 13 Bunn PA, Carney DN, Gazdar AF, Whang-Peng J, Matthews MJ. Diagnostic and biological implications of flow cytometric DNA content analysis in lung cancer. *Cancer Res* 1983;43:5026–32.
- 14 Tirindelli-Danes D, Teodori L, Mauro F, *et al.* Prognostic significance of flow cytometry in lung cancer. *Cancer* 1987;60:844–51.
- 15 Zimmerman PV, Hawson GAT, Bint MH, Parsons PG. Ploidy as a prognostic determinant in surgically treated lung cancer. *Lancet* 1987;ii:530–3.
- 16 Ewers SB, Langstrom E, Baldetorp B, Killander D. Flow cytometric DNA analysis in primary breast carcinomas and clinicopathological correlations. *Cytometry* 1984;5:408–19.
- 17 Baildam AD, Zaloudik J, Howell A, Barnes DM, Moore M, Sellwood RA. DNA analysis by flow cytometry, response to endocrine treatment and prognosis in advanced carcinoma of the breast. *Br J Cancer* 1987;55:553–9.
- 18 Quirke P, Dixon MF, Clayden AD, *et al.* Prognostic significance of DNA aneuploidy and cell proliferation in rectal adenocarcinomas. *J Pathol* 1987;151:285–91.
- 19 Schutte B, Reynards MMJ, Bosman FT, Blijham GH. Flow cytometric determination of DNA ploidy level in nuclei isolated from paraffin-embedded tissue. *Cytometry* 1985;6:26–30.
- 20 Fisher ER, Palekar A, Paulson J. Comparative histopathologic electron microscopic and tissue culture studies of bronchial carcinoids and oat cell carcinomas of lung. *Am J Clin Pathol* 1978;69:165–72.
- 21 Falor WH. Chromosomes in bronchial adenomas and in bronchogenic carcinomas. *Am Rev Respir Dis* 1971;104:198–205.
- 22 Hammarberg C, Slezak P, Tribukait B. Early detection of malignancy in ulcerative colitis: a flow cytometric study. *Cancer* 1984;53:291–5.
- 23 Fozard JBJ, Quirke P, Dixon MF, Giles GR, Bird CC. DNA aneuploidy in ulcerative colitis. *Gut* 1986;27:1414–8.
- 24 Reid BJ, Haggitt RC, Rubin CE, Rabinovitch PS. Barrett's esophagus: correlation between flow cytometry and histology in detection of patients at risk for adenocarcinoma. *Gastroenterology* 1987;93:1–11.
- 25 Goh HS, Jass JR. DNA content and the adenoma carcinoma sequence in the colorectum. *J Clin Pathol* 1986;39:387–92.