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Importance of sampling method in DNA analysis of lung cancer

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Abstract

Lung carcinomas are characterised by considerable histological variation within the tumour. The possible effects of this morphological heterogeneity on the estimation of tumour ploidy were investigated. Multiple tissue blocks were systematically taken from 20 lung tumours and analysed by flow cytometry. The routine, archival paraffin wax embedded diagnostic blocks from these cases were also analysed. Nineteen (95%) of the tumours were shown to contain aneuploid stemlines by systematic sampling, but if only one of these systematic tissue blocks had been taken from each case the incidence of DNA aneuploidy could have been as low as 45%. Only 15 (75%) tumours were aneuploid when all the routine archival blocks were analysed, but by specifically selecting tumour areas from the archival material the accuracy of this method was increased to 90%.

It is concluded that tumour sampling methods are of primary importance in assessing the DNA content of lung tumours. Routine paraffin wax embedded archival tissue provides a suitable source of material for this purpose, provided that "turnover" selection is carried out.

Studies of tumour DNA content measured by flow cytometry have shown that determination of ploidy is a promising prognostic tool in non-small cell lung cancer.[1–4] Zimmerman *et al* have shown that the detection of aneuploidy in archival, formalin fixed, paraffin wax embedded tumour tissue indicates a poor prognosis, and these authors have proposed that estimation of tumour DNA content should be part of the clinical work-up of all patients with operable lung cancer.[1]

Lung carcinomas exhibit considerable heterogeneity of histological, ultrastructural, and immunohistochemical features within the tumours.[5–7] In a recent study we showed that this morphological variability is reflected by concomitant variation in DNA content.[8] The effect of heterogeneity is that adequate tissue sampling is of the essence if a lung tumour is to be properly characterised.

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Methods

Twenty consecutive lung tumour resection specimens were included in this study. Following the routine procedure of the Department, lungs were inflated in 10% formol-saline and allowed to fix overnight. The duty pathologist, who was unaware that the case was to be included in the study, then cut the fixed specimens into slices 1 cm thick and selected tissue blocks for diagnosis. The lung specimens were then examined by one of the authors and the tumour was systematically sampled in the following manner (fig 1). Alternate lung slices were selected and blocks of tissue (about 1 × 0·5 × 0·5 cm) were cut from the tumour at 12, 3, 6 and 9 o'clock and from the centre. These blocks excluded macroscopically obvious necrotic tissue. The samples were fixed in formol-saline for a further 12 hours and embedded in paraffin wax in the usual manner.

A section was cut from each of these blocks and stained with haematoxylin and eosin to confirm the presence of tumour tissue. A 50 μm section was then cut from each block and prepared for flow cytometry using a modification of the method described by Hedley *et al*.[9] The tissue was dewaxed and rehydrated, mechanically minced, and digested for one hour at 37°C in a solution of 0·5% pepsin in 0·9% NaCl adjusted to pH 1·5 by adding hydrochloric acid. The suspension obtained was filtered through nylon wool and incubated overnight in propidium iodide. The samples were centrifuged before being analysed on an EPICS CS flow cytometer (Coulter, Hialeah, Florida). Immediately before analysis each sample was rinsed several times through a needle to avoid clumping of nuclei which

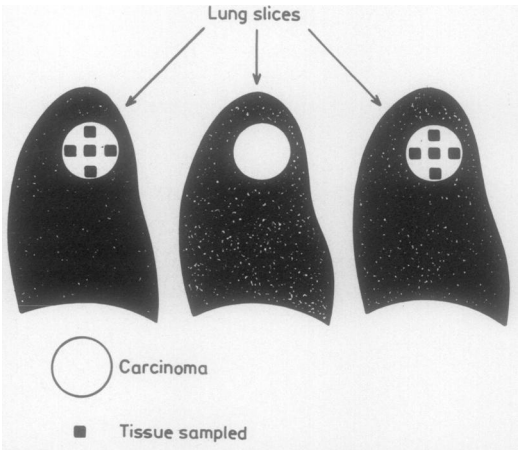

Figure 1 Tissue samples (black squares) are taken from alternate malignant lung slices from the centre of the lesion and from 12, 3, 6 and 9 o'clock (viewed from the hilar aspect with the lung in the upright position).

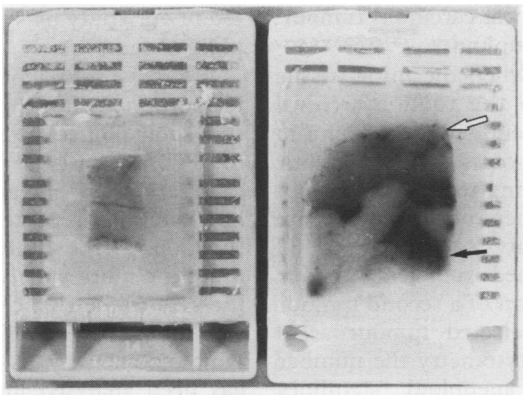

Figure 2 The "systematic" block (left) is smaller than a typical archival block (right). The former consists almost entirely of tumour tissue while the latter also contains normal lung (large arrow) and haemorrhagic and necrotic tissue (small arrow).

could lead to a false impression of tumour tetraploidy. Random samples were examined by fluoresence microscopy to confirm the absence of clumping. The flow cytometer is based on an argon laser tuned to 488 nm with a 570 nm long pass filter fitted. The machine was calibrated with standard fluorescent beads (Coulter UK) before use. A minimum of 15000 nuclei were analysed from each sample.

DNA histograms were interpreted in a standard manner. In each tumour sample stromal and inflammatory cell nuclei were present, providing an internal diploid standard. DNA aneuploidy was defined by the presence of a second G_0/G_1 peak which differed from the diploid G_0/G_1 channel number by at least 10%. Ill defined "shoulders" on the diploid peak were not included as aneuploid peaks. A DNA index (defined as the ratio of the channel number of the aneuploid peak to the channel number of the diploid peak) was calculated for each tumour sample.

The routine archival (diagnostic) tissue blocks from each case in the study were obtained from the departmental file. From each block of malignant tissue a 50 μm section was cut and processed for flow cytometry as described above. Examination by naked eye of the archival blocks showed that the amount of tissue contained in each was variable but, on the whole, was more than that contained in the blocks selected specifically for flow cytometry (fig 2). It was also obvious that many of the routine diagnostic blocks contained non-neoplastic lung tissue, or large areas of necrosis, or both. To prevent the presence of such elements masking the detection of aneuploidy in the tumour tissue, a further 50 μm section was cut at a deeper level from each diagnostic block and a portion of tumour tissue free of these elements and equivalent in size to that selected for systematic sampling, was dissected out using a scalpel blade. The remaining tissue was discarded and the dissected tumour tissue processed for flow cytometry.

Results

The tumours were assigned to conventional histological groups as follows: squamous cell carcinoma (n = 10): adenocarcinomas (n = 4), small cell carcinoma (n = 3); and one each of large cell carcinoma, adenosquamous carcinoma, and atypical carcinoid tumour.

Systematic tumour sampling yielded a total of 208 tissue blocks (a mean of just over 10 per case). Examination of the routinely selected archival material showed that there were 84 blocks containing malignant tissue (a mean of four per case).

Table 1 shows the DNA analysis of the tissue obtained by systematic sampling: DNA aneuploid stemlines were detected in 19 of the 20 (95%) cases. The DNA indices obtained varied from 1·2 to 2·5. DNA indices in the tetraploid range (1·9–2·1) were identified in five cases. In each of these cases, however, other aneuploid stemlines (DNA indices 1·2, 1·6, 1·3, 3·0, 1·5) were detected. The DNA abnormality was thus not merely a nuclear clumping effect. Heterogeneity of DNA content was shown in 18 carcinomas (90% of the total). Of these cases, 12 contained a mixture of diploid and aneuploid areas; in six aneuploid stemlines of different DNA index were identified in separate areas of the tumour.

Table 1 Effect of sampling method in ploidy assessment in lung cancer

		Sampling method					
		Systematic		*Archival I*		*Archival II*	
Case No	*Histology*	*Diploid*	*Aneuploid*	*Diploid*	*Aneuploid*	*Diploid*	*Aneuploid*
1	Squamous	1*	4	0	2	0	2
2	Large cell	0	10	0	3	0	3
3	Squamous	0	10	0	3	0	3
4	Squamous	10	0	4	0	4	0
5	Adeno	0	10	1	5	0	5
6	Squamous	0	10	0	3	0	3
7	Adeno	0	10	0	6	0	6
8	Small cell†	8	2	3	0	1	2
9	Squamous	1	9	5	1	1	5
10	Squamous	1	9	2	1	1	2
11	Atypical carcinoid	0	15	0	5	0	5
12	Adenosquamous†	7	4	7	0	0	5
13	Adeno	0	11	0	3	0	3
14	Squamous†	5	11	5	0	0	4
15	Squamous	9	3	3	1	1	2
16	Squamous	0	10	2	5	1	4
17	Small cell	7	3	0	4	0	4
18	Squamous†	6	2	3	0	3	0
19	Small cell	4	6	3	1	2	2
20	Adeno	0	10	0	3	0	3

*The figures refer to the number of tissue samples in each case.
†Cases whose ploidy state was wrongly designated when initial sections were prepared from routine archival material (archival I). Only case 18 remained wrongly designated when tumour tissue was further selected (archival II).

Only one case, the atypical carcinoid tumour, was homogeneously aneuploid.

On the other hand, when the initial tissue sections prepared from the routine archival blocks was analysed, aneuploidy was found in only 15 of the 20 (75%) cases—that is, in four cases the routine sampling technique did not allow for proper assessment of the ploidy state of the tumour. Seventy seven of the 82 archival blocks contained sufficient tumour tissue to permit dissection of a second tumour sample. When these selected tumour areas were analysed by flow cytometry the number of cases containing aneuploid stemlines increased to 18 (90%). Thus in only one case was the ploidy state of a tumour wrongly designated when this second method of tumour sampling was used. In all cases where aneuploidy was detected in the archival tissue (either by analysing the initial section of the full block or by selecting deeper tumour areas) the DNA index corresponded to that of a stemline detected by systematic sampling of the tumour.

Discussion

Since 1983, when Hedley *et al* first described a technique for recovering nuclei for flow cytometric DNA analysis from archival paraffin wax embedded tissue,[9] there have been many papers published which describe the use of this technique for predicting the behaviour of a wide variety of tumours. Zimmerman *et al* published follow up data on a large series of surgically resected lung carcinomas and found that ploidy, determined by analysis of paraffin wax embedded tissue, was the most important predictor of prognosis on multivariate analysis.[1] In his series 45% of the tumours were aneuploid. Other flow cytometric studies of lung cancer (based on analysis of fresh tissue) have shown variability in the percentage aneuploidy detected (80–96%), although even the lowest of these values is considerably higher than that reported by Zimmerman *et al*.

Table 2 summarises the data from all the major series reported to date. Although there are some differences in tumour stage and histological type among these series, the most important variation that might explain the observed differences is in the tumour sampling technique. The highest percentage aneuploidy reported (96%) was obtained by analysing "multiple samples" (number unspecified) taken from both the tumour core and periphery.[3] Interestingly this figure corresponds to the percentage of aneuploidy detected in our study by systematic (multiple) sampling of tumours.

We have also found that only a small proportion (two out of 20 cases) of lung cancers show no heterogeneity within the tumour of DNA content. It must be assumed, therefore, that the variation in the percentage of aneuploidy reported in different series is a function of the differing sampling methods used; the more widely the tumour is sampled the greater the chances of detecting aneuploid stem lines. Indeed, if only one of the tumour samples taken by us in systematic sampling has been analysed in each case the possible percentage of aneuploidy could have ranged between 45–95%—it is perhaps worth noting that these figures correspond to the range of values reported in the various series published so far.

As the percentage of aneuploidy detected in the different studies has been so variable it might be predicted that attempts to evaluate the prognostic importance of DNA content in lung cancers would yield conflicting results. This, in fact, has been the case. Early work by Bunn *et al* failed to show any association between ploidy and outcome of disease,[10] while Volm *et al*[2] and Zimmerman *et al*[1] reported a strong correlation between the presence of aneuploidy in non-small cell lung cancer and a poor prognosis. Tirindelli-Danesi *et al* found that as only 4% of their tumours were diploid they were unable to make a valid comparison between diploid and aneuploid groups.[3] These authors did, however, find that tumours with DNA stemlines of a DNA index of less than 1·0 or greater than 2·0 had a significantly poorer clinical outcome. More recently Van Bodegom *et al* claimed that the proportion of the total tumour cell population contained within the aneuploid peak was a better predictor of prognosis than the simple presence or absence of an abnormal stemline.[4]

Therefore, it seems likely that in the case of lung cancer, the tumour sampling technique is of cardinal importance in assessing the ploidy state. If flow cytometry is to be of use in predicting clinical outcome standardisation of sampling methods is imperative if relevant comparisons are to be made between different series. In our study wide sampling of tumour resection specimens showed that almost all lung cancers were aneuploid; only 75% of these same cases would have been so designated had routine archival blocks been used for DNA analysis. Dissection of tumour areas free of non-neoplastic or necrotic elements from the same blocks, however, improved the accuracy of sampling to over 90%.[7] This method for avoiding sampling errors has been described in other tissues.[11] We recommend that selection of tumour areas should always be performed when DNA analysis is undertaken on paraffin wax embedded material. Studies are in progress to assess the value of this method in predicting the clinical outcome in a large series of lung cancers.

Table 2 Published reports showing variation in aneuploidy in lung cancer

Report	*Method of sampling*	*Percentage of aneuploidy detected*
Bunn *et al* 1983[9]	Needle aspirates, lymph nodes, bronchial washings	85
Volm *et al* 1985[2]	Not stated	82
Tirindelli-Danesi *et al*[3]	"Multiple samples"	96
Zimmerman *et al* 1987[1]	Archival	45
Van Bodegom *et al* 1989[4]	Archival	56

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1 Zimmerman PV, Hawson GAT, Bint MH, Parsons PG. Ploidy as a prognostic determinant in surgically treated lung cancer. *Lancet* 1987;ii:530–3.
2 Volm M, Drings P, Mattern J, *et al.* Prognostic significance of DNA patterns and resistance-predictive tests in non-small cell lung carcinoma. *Cancer* 1985;**58**:1396–403.
3 Tirindelli-Danesi D, Teodori L, Mauro F, *et al.* Prognostic significance of flow cytometry in lung cancer. *Cancer* 1987;**60**:844–51.
4 Van Bodegom PC, Baak JPA, Stroet-Van Galen C, *et al.* The percentage of aneuploid cells is significantly correlated with survival in accurately staged patients with stage I resected squamous cell lung cancer and long-term follow up. *Cancer* 1989;**63**:143–7.
5 Roggli VL, Vollmer RT, Greenberg SD, McGavran MH, Spjut HJ, Yesner R. Lung cancer heterogeneity: A blinded randomised study of 100 consecutive cases. *Hum Pathol* 1985;**16**:569–78.
6 Dunnill MS, Gatter KC. Cellular heterogeneity in lung cancer. *Histopathol* 1986;**10**:461–75.
7 Broers JLV, Rot MK, Oostendorp T, *et al.* Immunocytochemical detection of human lung cancer heterogeneity using antibodies to epithelial, neuronal and neuroendocrine antigens. *Cancer Res* 1987;**47**:3225–34.
8 Carey FA, Lamb D, Bird CC. Intratumoral heterogeneity of DNA content in lung cancer. *Cancer* 1990;**65**:2266–9.
9 Hedley DW, Friedlander ML, Taylor IW, *et al.* Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. *J Histochem Cytochem* 1983;**31**:1333–5.
10 Bunn PA, Carney DN, Gazdar AF, *et al.* Diagnostic and biological implications of flow cytometric DNA content analysis in lung cancer. *Cancer Res* 1983;**43**:5026–32.
11 Oud PS, Hanselaar TGJM, Reubsaet-Veldhuizen JAM, *et al.* Extraction of nuclei from selected regions in paraffin-embedded tissue. *Cytometry* 1986;7:595–600.