25. Rüdiger HW, Schwartz U, Serrand E, *et al.* Reduced O⁶-methylguanine repair in fibroblast cultures from patients with lung cancer. *Cancer Res* 1989, **49**, 5623–5626.
26. Domoradzki J, Pegg AE, Dolan ME, Maher VM, McCormick JJ. Depletion of O⁶-methylguanine-DNA-methyltransferase in human fibroblasts increases the mutagenic response to *n*-methyl-*n'*-nitro-*n*-nitrosoguanidine. *Carcinogenesis (Lond.)* 1984, **6**, 1823–1826.
27. Meister A, Anderson ME, Glutathione. *Ann Rev Biochem* 1983, **52**, 711–760.
28. Moldeus P, Jernstrom B. Interaction of glutathione with reactive intermediates. In: Larsoon A, Orrenius S, Holmgren A, Mannervik B, eds. *Functions of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects*. New York, Raven Press, 1983, 99–108.
29. Vos RME,Van Bladeren PJ. Glutathione S-transferases in relation to their role in the biotransformation of xenobiotics. *Chem Biol Interactions* 1990, **75**, 241–255.
30. Reed DJ. Regulation of reductive processes by glutathione. *Biochem Pharmacol* 1986, **35**, 7–13.
31. Wolf CR, Lewis AD, Carmichael J, Adams DJ, Allan SG, Ansell DJ. The role of glutathione in determining the response of normal and tumour cells to anticancer drugs. *Biochem Soc Trans* 1987, **45**, 728–730.
32. Wanamarta AH, Van Rijn J, Blank LECM, Haveman J, Van Zandwijk N, Joenje H. Effect of *N*-acetylcysteine on the antiproliferative action of X-rays or bleomycin in cultured lung tumour cells. *J Cancer Res Clin Oncol* 1989, **115**, 340–344.
33. Di Re F, Böhm S, Oriana S, Spatti G, Zunino F. Efficacy and safety of highdose cisplatin and cyclophosphamide with glutathione protection in the treatment of bulky advanced epithelial ovarian cancer. *Cancer Chemother Pharmacol* 1990, **25**, 355–360.
34. Boyer ThD. The glutathione S-transferases: an update. *Hepatology* 1989, **9**, 486–496.
35. Hayes JD, Wolf CR. Review article. Molecular mechanisms of drug resistance. *Biochem J* 1990, **272**, 281–295.
36. Seidegard J, Pero RW, Miller DG, Beattie EJ. A glutathione transferase in human leucocytes as a marker for the susceptibility to lung cancer. *Carcinogenesis* 1986, **7**, 751–753.
37. Seidegard J, Vorachek WR, Pero RW, Pearson WR. Hereditary differences in the expression of the human glutathione transferase active on *trans-stilbene oxide* are due to a gene deletion. *Proc Natl Acad Sci USA* 1988, **85**, 7293–7297.
38. Waxman DJ. Glutathione S-transferases: role in alkylating agent resistance and possible target for modulation chemotherapy. A review. *Cancer Res* 1990, **50**, 6449–6454.
39. Thor H, Hartzel P, Svensson SA, *et al.* On the role of thiol groups in the inhibition of liver microsomal Ca^{2+} sequestration by toxic agents. *Biochem Pharmacol* 1985, **34**, 3717–3723.
40. Ali-Osman F. Quenching of DNA cross-link precursors of chloroethyl-nitrosoureas and attenuation of DNA interstrand cross-linking by glutathione. *Cancer Res* 1989, **49**, 5258–5261.
41. Todorovic V, Ho Y-F, Guenthner TM. Identification of a discrete nuclear glutathion pool that is depleted by adriamycin + carmustine. 82nd Annual Meeting of the AACR, Houston, Texas, 15–18 May 1991.
42. Lemoine A, Lucas C, Ings RMJ. Metabolism of the chloroethylnitrosoureas. *Xenobiotica* 1991, **21**, 775–791.
43. Boutin JA, Norbeck K, Moldeus P, *et al.* Effects of the new nitrosourea derivative, fotemustine, on the glutathione reductase activity in rat tissues *in vivo* and in isolated rat hepatocytes. *Eur J Cancer Clin Oncol* 1989, **25**, 1311–1316.
44. Brakenhoff JPG, Lamorée MH, Lucas C, te Koppele JM, Vermeulen NPE. Glutathione-related metabolism of fotemustine in the rat: urinary excretion of sulfur-containing metabolites. Third International ISSX Meeting on Drug Metabolism. Amsterdam, 24–28 June 1991 (poster abstract).

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The Identification of Informative Parameters in the Flow Cytometric Analysis of Breast Carcinoma

John Lawry, Kenneth Rogers, John L. Duncan and Christopher W. Potter

DNA ploidy and the measurement of proliferation or S-phase fraction are both of prognostic significance in breast cancer, yet clinical use is minimal in the U.K. Immunohistochemistry is, however, used to aid diagnosis, so a panel of antibodies were analysed by flow cytometry to assess their predictive value for prognosis, tumour stage and grade. Of 10 parameters tested on 202 breast tumour samples, tumour cell proliferation and DNA ploidy were the two most informative; cytokeratin staining, natural killer and B-cell infiltration also proved to be of value but there was no prognostic value in measuring tumour infiltrating monocytes, helper/suppressor T-cell ratios, tumour cell reactivity with carcinoembryonic antigen or human milk fat globulin antibodies. For each of the informative parameters, scores numerically weighted towards a poorer prognosis were derived which when combined, correlated with tumour grade, stage and prognosis. Such data interpretation is objective, and can be transposed to other human tumours.
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INTRODUCTION

NUMEROUS REPORTS have suggested that flow cytometric parameters parallel clinicopathological observations in tumours of the breast, and can thus be used as indicators of tumour stage and prognosis; in particular, tumour cell ploidy [1–4] and the percentage of dividing cells [5, 6] have proven to be valuable markers. In contrast, other proposed parameters remain controversial; these include the expression of carcinoembryonic antigen (CEA) [7, 8] and human milk fat globule (HMFG) [9], and the measurement of tumour infiltrating lymphocytes, natural killer (NK) cells and monocytes. Thus, there remains a need to identify and confirm which of the many cytometric parameters proposed are of clinical value, and to devise a method by which these measurements can be interpreted.

In order to identify the cytometric measurements which correlate with histological grade, pathological stage and survival, 202 primary breast carcinoma samples, together with samples of normal tissue, were examined by cytometry using 10 separate

flow cytometric parameters. Informative parameters were then divided into ranges and given a numerical score weighted towards a poorer prognosis. We report that of the 10 parameters tested, five were of value, and the combined scores were predictive of tumour grade, stage and survival.

MATERIALS AND METHODS

Sample group

The patient group comprised women presenting with primary breast carcinoma: tumour samples and, when possible, axillary nodes were obtained at mastectomy. Clinical details were recorded. Ages ranged from 15 to 80 years (mean 59.5 years, S.D. 12.8 years); clinical follow-up was for a maximum of 61 months (median 50 months). Patients on chemotherapy were excluded. Tissue samples were divided to enable histological analysis and flow cytometric tests to be carried out on adjacent pieces. Clinicopathological data for the sample group was designated independently by two pathologists (Dr J.R. Goepel, Department of Pathology, University Medical School, and Dr S. Holt, Department of Pathology, Northern General Hospital).

Tumour analysis

Enzymatic dissociation of the tissue samples was carried out using standard methods [10] with 0.2 mg/ml collagenase type II and 0.02% DNAase (Sigma). Cell suspensions were either fixed (95% ethanol, 5% acetic acid) for cytokeratin (CAM 5.2), HMFG-1 and HMFG-2, CEA staining, or used fresh for anti-leucocyte antibody staining (CD3, CD4, CD8, CD15, CD19, CD57). Single (SPC) and dual parameter correlated (DPC) flow cytometric DNA/monoclonal antibody (Mab) staining techniques were used, as previously reported [11]. Mithramycin (93.6 μg/ml) and ethidium bromide (37.5 μg/ml) were used together for specific DNA G-C base pair staining for DNA measurements alone; whilst propidium iodide (PI, 50 μg/ml with RNase treatment) was used in dual parameter (DNA/Mab) staining to distinguish tumour infiltrating cells from tumour cell populations, and to simultaneously measure proliferation and ploidy.

DNA histograms were analysed using plannimetric methods of Crissman and Tobey [12] and Dean [13], with the measurement of G0/G1 and G2/M-phase cells and an estimation of S-phase cells. Doublet discrimination was not possible, but samples were filtered, and histograms analysed critically for the presence of doublet and triplet peaks: any equivocal samples were retested. Diploidy was defined as a DNA index of 1.0, near diploid as 1.1–1.3, aneuploid as 1.4–1.9 and tetraploidy as > 1.9 (adapted from Beerman *et al.* [1]). Histograms with a G0/G1 peak coefficient of variation (cv) exceeding 10 were discarded. Samples included in the present study had a median diploid G0/G1 cv of 4.5 (n = 202, range 2.2–8.9).

Analysis of results

Control values for Mab staining were produced from the analysis of six samples of normal breast tissue. All Mab tests contained appropriate positive and negative controls. Lymphocytes were used as diploid controls for DNA staining. All results were statistically analysed using Student's t-test, Mann–Whitney U-test or χ^2 analysis for parametric, non-parametric and "count" data, respectively. In addition, data were entered into a data-file for analysis by the LIFETEST and PHGLM procedures of the SAS statistical package. The PHGLM procedure fits the Cox [14] proportional hazards linear regression model to survival data, and computes test χ^2 statistics for each variable.

RESULTS

DNA ploidy

In the present study, tumour samples were designated diploid, comprising 34% of the study group, tetraploid (twice the DNA content) having a frequency of 8.5%, or aneuploid (mid-range) with a frequency of 48.3%; the term multiploid was used for those samples containing three or more populations and was seen at a frequency of 8%. These data are summarised in Table 1, with the division of these results into histological grade, pathological stage or survival groups. The incidence of abnormal ploidy (aneuploid, tetraploid and multiploid) increased from grade 1 (50%), to grade 2 (62%), to grade 3 (75%) [$P < 0.05$ by χ^2 analysis, degrees of freedom (df) = 6]; from stage I (45%), to stage II (68%), to stage III (75%), to stage IV (100%) ($P < 0.01$, df = 9); and from disease-free patients (55%) to those who died (80%) ($P < 0.05$, df = 4). Diploidy was, therefore, more commonly seen in low-grade/low-stage disease. It was interesting to note that multiploidy was characteristic of a high histological grade but not high stage, although the sample group was small. Patient survival, however, was significantly increased if the tumour was diploid. No statistical differences in prognosis were seen between near-diploid, intermediate or near-tetraploid DNA indices within the aneuploid sample group (results not shown). When aneuploidy was seen in both the primary tumour and involved lymph nodes, the ploidy indices correlated with a coefficient of $r = 0.982$ ($n = 21, P < 0.01$).

Tumour cell proliferation

Tumour proliferation data, summarised as the S-phase fraction of the cell cycle, in included in Table 1. Thus, samples classified as high grade or stage correlated to high levels of S-phase cells, compared to low grade or stage samples ($P < 0.05$). In addition, elevated percentages of dividing cells were also associated with recurrent disease and a short survival

Table 1. The analysis of tumour cell DNA ploidy index, and S-phase fraction with division into clinicopathological criteria

		n	Diploid	Tetraploid	Aneuploid	Multi-ploid	Median %S
H. Grade	1	18	9 (50)	1	7 (39)	1	2.8
	2	104	40 (38)	5	52 (50)	7	2.5
	3	81	20 (25)	11 (14)	39 (48)	8	4.3
P. Stage	I	44	24 (55)	4	10 (23)	6	2.7
	II	112	36 (32)	11 (10)	60 (54)	6	2.6
	III	36	9 (25)	5	17 (47)	2	4.2
	IV	7	0	0	6 (86)	0	5.0
Survival							
	D-F	105	47 (45)	6	46 (44)	6	2.5
	Rec	19	8 (42)	1	7 (37)	0	3.5
	Died	41	8 (20)	4	23 (56)	6	5.0

Figures in parentheses are percentages for results along rows. D-F = Disease-free, Rec = recurrent breast disease.

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Table 2. The detection and quantitation of tumour cell populations

	Cytokeratin			HMFG-1			HMFG-2			CEA		
	n	Range	%	*n*	Range	%	*n*	Range	%	*n*	Range	%
H. grade 1	5	7–72	40	2	43–80	66	2	47–84	66	3	17–47	35
2	38	11–86	43	27	6–76	50	22	11–86	46	21	4–86	36
3	76	10–82	46	21	4–67	30	27	4–67	30	37	9–82	36
P. stage I	15	10–70	35	7	13–53	44	6	11–52	46	10	8–82	25
II	44	8–82	42	24	6–89	69	29	8–87	58	22	8–86	31
III	18	10–86	42	19	12–73	27	16	13–38	13	29	4–73	44
IV	5	14–66	56	0			0			0		
Survival												
D-F	57	10–80	33	32	11–85	36	27	9–86	39	24	8–55	27
Rec	10	13–73	37	5	23–70	46	3	27–47	38	1	—	60
Died	22	11–76	38	14	6–76	34	18	11–60	33	16	6–49	23

%, Percentage positivity after background subtraction.

($P < 0.01$). Aneuploid tumours also had the highest levels of proliferating cells (results not shown).

Anti-tumour markers

The results obtained for the four tumour Mabs are shown in Table 2. Staining for cytokeratin was the most informative, and comprised the largest study group. Results show an increase in the percentage of cytokeratin-positive cells from low to high grade, low to high stage, and increasing with reduced survival, although statistical significance was not found. Lower values of cytokeratin-positive cells in a sample may indicate elevated levels of leucocyte infiltration or increased sclerosis. Differential staining such as this was not seen for either of the anti-HMGF Mabs or for CEA.

The CAM 5.2 anti-cytokeratin Mab (k8, 18, 19) was also of value in the study of tumour/lymph node pairs, since it provided a method of detecting secondary tumour deposits down to the micro-metastases level in nodal tissue [11]. Stained tumour cells were clearly distinguishable from non-staining stromal and infiltrative cell populations in these sample pairs.

In the sample group of normal breast tissue, cells of epithelial origin, such as those lining the ducts that connect the breast lobules, were stained with the cytokeratin Mab at levels of 0–10%; and less than 1% in suspensions of normal lymph node tissue. Thus, cytokeratin staining of tumour samples was used to quantitate the number of epithelial tumour cells present, an incidence of more than 10% being indicative of tumour. Normal breast tissue showed positivity with other antibodies at median levels of 20.9% (CD3), 7.8% (CEA), 17.6% (HMFG-1), 15.7% (HMFG-2), and a median S-phase fraction of 0.5%.

Tumour lymphocyte infiltration

Table 3 summarises the data obtained for tumour lymphocyte infiltration, including CD3 (T-cells), CD19 (B-cells), CD57 (NK cells and fc receptor sites) and CD15 (monocytes). CD3 staining was used as a measure of lymphocyte infiltration, and also as a control for non-specific anti-tumour antibody staining. A total of 150 tumour samples were analysed: results showed that whilst the level of CD3 positivity and anti-tumour antibody staining was complimentary (excluding stromal cells, etc.), CD3 staining alone showed no correlation with clinicopathological criteria or survival, but did reflect pathological observations of lymphocyte infiltration. Analysis of B and NK cells indicated that their presence was indicative of low grade/stage, and of improved survival. These comparisons were statistically significant at $P < 0.05$ (grade 1 and 2 vs. 3, stage I vs. III, disease-free vs. died). No statistical significance was obtained from the enumeration of monocytes infiltrating samples of breast tissue, although CD15-positive cells constituted up to 37% of the total.

CD4 and CD8 data was expressed as a ratio, and ranged from 0.5:1 to 2.5:1 (median 1:1, $n = 66$, results not shown); ratios were generally low compared to the normal for circulating cells (1.5:1 to 2:1). Due to the range of results, no correlations could be made with prognosis, grade or stage. Blood differentials revealed no abnormalities in peripheral lymphocyte counts. These results suggest cytotoxic lymphocytes (CD8) are the dominant infiltrating cell, being independent of circulating levels.

Designation of score values

In order to simplify the results of the present study, data was first analysed as percentage staining, or ploidy groups, as shown above; and was then stratified by dividing up ranges of percentage figures, which were then given a numerical score. This procedure used information obtained from normal breast tissue, calculated medians from tumour samples, and correlations to clinical parameters. In each case, high numerical scores were correlated with a poor prognosis (see below).

Scores were established for ploidy by allocating diploid and tetraploid tumours a score of 1, aneuploidy score 2 and multiploidy score 3. S-phase fractions were divided into 0–3% (score 1), 3–5% (score 2) and > 5% (score 3). Cytokeratin staining was divided into 0–10% (score 0), 11–30% (score 1), 31–50% (score 2) and > 50% (score 3). NK and B-cell enumeration was categorised 0–10% (score 1), > 10% (score 0).

Individual χ^2 analysis of data entered into contingency tables showed that ploidy, S-phase, B and NK cell enumeration all gave significance in predicting prognostic groups at $P < 0.05$ ($\chi^2 = 24.99, 71.6, 6.41$, and 14.9, respectively). Ploidy, S-phase fraction and cytokeratin staining were better at predicting pathological stage ($P < 0.01$, $\chi^2 = 63.0$, 117.35 and 29.75, respectively) than NK cells ($P < 0.05$, $\chi^2 = 7.0$). Histological grade was similarly predicted by S-phase fraction ($P < 0.001$,

Table 3. The analysis of tumour lymphocyte infiltration

	CD3			CD19			CD57			CD15		
	n	Range	%	*n*	Range	%	*n*	Range	%	*n*	Range	%
H. Grade 1	16	11–45	23	12	4–44	18	19	2–32	20	19	2–22	10
2	43	6–70	20	21	2–33	14	31	0–22	7	45	0–34	10
3	83	8–66	28	20	3–18	8	21	0–27	5	20	0–33	12
P. Stage I	35	8–51	27	13	6–22	14	17	7–33	14	15	1–22	15
II	75	6–70	21	17	2–34	15	36	4–23	9	35	0–37	11
III	22	10–57	16	18	3–14	5	12	1–27	4	24	1–26	11
IV	7	10–55	26	5	5–18	6	4	5–06	4	5	5–33	12
Survival												
D-F	70	9–49	25	25	1–33	15	23	7–35	17	32	1–34	15
Rec	8	8–50	21	1	—	14	1	—	20	2	8–31	19
Died	20	8–76	23	19	3–18	8	32	1–20	8	26	7–34	14

CD3 (pan T-cell), CD19 (B cell), CD57 (NK cell), CD15 (monocyte) MoAbs. %, Percentage positive cells after background subtraction.

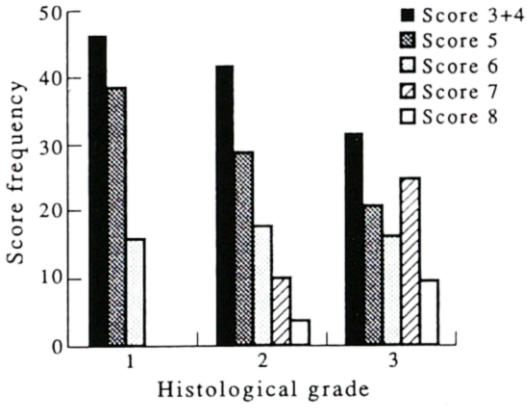

Fig. 1. Frequency distribution for the combined score of DNA ploidy index, S-phase fraction and cytokeratin positivity in predicting histological grade.

$\chi^2 = 130.7$), ploidy ($P < 0.01$, $\chi^2 = 62.56$) and B-cells ($P < 0.05$, $\chi^2 = 6.4$).

Data for each parameter was also analysed stepwise in the Cox proportional hazards model for significance in predicting overall survival. These calculations showed the best "traditional" parameter to be the lymph node status ($P < 0.009$, $\chi^2 = 11.0$) followed by the pathological stage ($P = 0.057$, $\chi^2 = 3.6$), and grade ($P < 0.064$, $\chi^2 = 3.34$) with tumour size being non-significant ($P < 0.5$, $\chi^2 = 0.45$). Flow cytometric parameters gave significance with %S ($P < 0.019$, $\chi^2 = 6.8$), ploidy ($P = 0.05$, $\chi^2 = 3.8$), %NK cells ($P = 0.053$) and %B cells ($P < 0.069$), and %CK $P < 0.08$ ($\chi^2 = 3.01$).

In an attempt to improve on these correlations, parameters were then combined to see if a score could be used to predict tumour grade and stage or survival, and the most informative are shown graphically in Figs 1–3. Figure 1 illustrates the prediction of grade by the combined scores of DNA ploidy index and S-phase fraction, enhanced by the addition of scores for cytokeratin staining. Decreasing frequencies of scores 3, 4 and 5 are seen as the grades increase, with a corresponding emergence of scores 7 and 8 ($\chi^2 = 16.1$ df $= 8$, $P < 0.05$). Figures 2 and 3 show that the best predictors of stage and survival were score combinations of DNA ploidy and S-phase fraction, although it is still not possible to accurately define stages I and II with low scoring samples. The life table analysis illustrated in Fig. 3, has

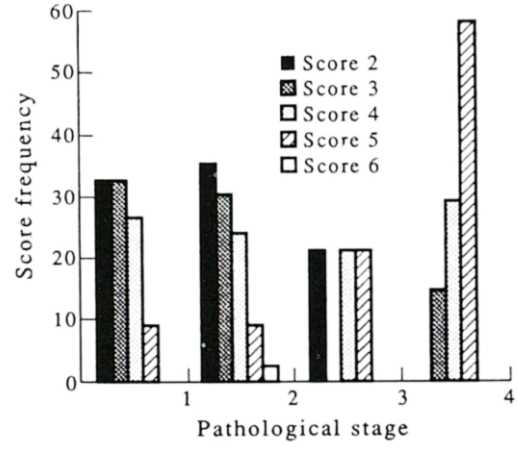

Fig. 2. Frequency distribution for the combined score of DNA ploidy index and S-phase fraction in predicting pathological stage.

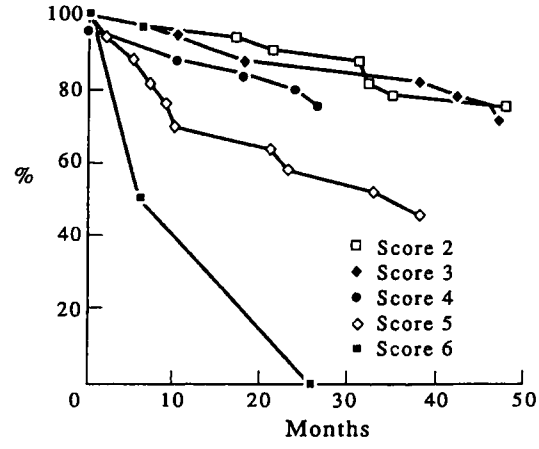

Fig. 3. Survival curves for individual scores produced by the combination of the DNA ploidy index and S-phase fraction. Statistical significance between scores 2, 3, 4 and score 5 = $P < 0.005$, and score 6 = $P < 0.001$. Median survival for score 2 = 28 months, score 3 = 29 months, score 4 = 15 months, score 5 = 16 months, score 6 = 13 months.

a P value < 0.02 ($\chi^2 = 4.9$), compared to $\chi^2 = 4.1$ $P < 0.04$ for %S and CK; $\chi^2 = 3.5$ $P < 0.06$ for %CK and ploidy; and $\chi^2 = 4.2$ $P < 0.04$ for ploidy, %S and %CK combined.

DISCUSSION

The analysis of samples of solid tumour by flow cytometry has been well documented for the analysis of DNA ploidy, and the enumeration of cell cycle, and has shown that these parameters are significant indicators of prognosis [1–4, 6, 15–17]. The flow cytometric, or immunohistochemical analysis of tumour lymphocyte infiltration has not, however, been as extensively reviewed [18, 19]; nor has the application of anti-tumour Mabs, other than by those reports that cite immunohistochemical analysis [20–22]; yet this can be achieved simply using dual parameter flow cytometry. Indeed, this technique is discussed in an extensive review of morphological parameters in breast carcinoma by Visschner *et al.* [23]. In many instances, DNA histogram analysis is difficult when diploid and non-diploid populations are both present. Dual DNA/Mab analysis resolves tumour cell populations from infiltrative and stromal cells, and aids cell cycle measurements. The present study demonstrates that the use of an anti-cytokeratin antibody is of value in identifying tumour cells within a cell suspension, and also in detecting metastatic spread [11]; previously only reported using immunohistochemical techniques [20–22]. The present study has also shown that the prognostic value of DNA ploidy was enhanced when combined with S-phase measurements, and is in agreement with a study by Kallioniemi *et al.* [24].

The analysis of tumour infiltration may provide details of the immunology of the tumour environment, but not of immune function, nor the efficacy of host anti-tumour activity. The results of the present study confirm the value of measuring B and NK cells in correlations with clinical and pathological criteria. This was not found with monocyte infiltration, helper/suppressor cell ratios or the total T-cell content. Helper/suppressor T-cell ratios were either balanced, or dominated by cytotoxic T-cells: this was in partial agreement with Von Kleist *et al.* [19] who either found balanced levels of T-cells or a higher frequency of helper cells.

Whilst cytokeratin staining was of value as a tumour cell marker in breast cancer no additional value was obtained by

the measurement of HMFG or CEA. These findings are in agreement with those of Parham *et al.* [25].

A limiting factor in the multi-parametric analysis of a tumour sample is in the amount of data thus produced. One method of reducing data is to substitute numerical scores for groups of linear data. By combining the scores for each parameter under consideration, a sample can be represented by a single figure. Thus, scores used in the present study identified highly proliferating, aneuploid tumours, with a high tumour cell content, as being of a poor prognosis (survival 12 months); conversely, a non-dividing, diploid (or tetraploid) tumour with few tumour cells, and hence high stromal or infiltrating component, had a good prognosis.

Other multi-parameter indices have been devised: Van der Linden *et al.* [26] combined the mitotic index, tumour size, axillary lymph node status and DNA content to predict prognosis; whilst Haybittle *et al.* [27] derived a clinical prognostic index of lymph node stage, tumour size and pathological grade [28]. The present study indicates that a "combined flow cytometric score" is also of clinical value.

Cox's multivariate analysis identified lymph node status being highly significant in predicting survival; followed by %S-phase, then DNA ploidy, stage and NK enumeration. Comparable findings have been reported in a study of 140 paraffin-embedded tissue samples, in which O'Reilly *et al.* [29] demonstrated a similar survival advantage for patients having low S-phase fractions, using a median of 7.1% as their cut-off level. The present study showed %S-phase and DNA ploidy to be better predictors of survival than histological grade or tumour size. This is contrary to a second report by O'Reilly *et al.* [30] in which the combination of tumour size and S-phase fraction was a highly significant predictor of relapse-free period.

Hence, the summation of parameters, clinical, flow cytometric or both, would seem to be of greater significance than an individual parameter. Flow cytometry has the advantage of speed over many techniques, and the ability to objectively quantitate Mab positivity and antigen density. It is possible that linear grading of percentage data may replace the grouping illustrated in the present study, and that further parameters such as growth factor receptors and oncogene expression [31] be included to improve the "combined score", and consolidate the clinical value of flow cytometry.

1. Beerman H, Kluin M, Hermans J, Van De Velde CJH, Cornelisse CJ. Prognostic significance of DNA-ploidy in a series of 690 primary breastcancer patients. *Int J Cancer* 1990, **45**, 34–39.
2. Dressler LG, Seamer LC, Owens MA, Clark GM, McGuire WL. DNA flow cytometry and prognostic factors in 1331 frozen breast cancer specimens. *Cancer* 1988, **61**, 420–427.
3. Ewers S, Langstrom E, Baldetorp B, Killander D. Flow cytometric DNA analysis in primary breast carcinomas and clinicopathological correlations. *Cytometry* 1984, **5**, 408–419.
4. Fallenius AG, Auer GU, Cartensen JM. Prognostic significance of DNA measurement in 409 consecutive breast cancer patients. *Cancer* 1988, **62**, 331–341.
5. Feichter GE, Kaufmann M, Muller A, Haag D, Eckhardt R, Goerttler K. DNA index and cell cycle analysis of primary breast cancer and synchronous axillary lymph node metastases. *Br Cancer Res Treat* 1989, **13**, 17–22.
6. Lawry J, Giri D, Rogers K, Duncan JL. The value of assessing cell proliferation in breast cancer. *J Microscopy* 1990, **159**, 267–275.
7. Beard DB, Haskell CM. Carcinoembryonic antigen in breast cancer. *Am J Med* 1986, **80**, 241–245.
8. Bravo AI, Sorin I, Guman N, Mordoh J. Carcinoembryonic antigen and differentiation in human breast cancer. *J Exp Clin Cancer Res* 1985, **4**, 317–324.
9. Wilkinson MJS, Howell A, Harris M, Taylor-Papadimitriou J, Swindell R, Sellwood RA. The prognostic significance of two epithelial membrane antigens expressed by human mammary carcinomas. *Int J Cancer* 1984, **33**, 299–304.
10. Owainati AAR, Robins RA, Hinton C, *et al.* Tumour aneuploidy, prognostic parameters and survival in primary breast cancer. *Br J Cancer* 1987, **55**, 449–454.
11. Lawry J, Rogers K, Duncan JL. Simultaneous measurement of DNA cell cycle and monoclonal antibody binding of breast carcinoma by flow cytometry. *Surg Res Comm* 1988, **3**, 61–70.
12. Crissman HA, Tobey RA. Cell cycle analysis in 20 minutes. *Science* 1974, **184**, 1297–1298.
13. Dean PN. A simplified method of DNA distribution analysis. *Cell Tissue Kinetics* 1980, **13**, 299–308.
14. Cox DR. Regression models and life tables. *J Royal Statistical Soc* B1972, **34**, 187–220.
15. Friedlander ML, Hedley DW, Taylor IW. Clinical and biological significance of aneuploidy in human tumours. *J Clin Pathol* 1984, **37**, 961–974.
16. Seckinger D, Sugarbaker E, Frankfurt O. DNA content and human cancer. *Arch Pathol Lab Med* 1989, **113**, 619–626.
17. Giaretti W, Santi L. Tumour progression by DNA flow cytometry in human colorectal cancer. *Int J Cancer* 1990, **45**, 597–603.
18. Steele RJC, Eremin O, Brown M, Hawkins RA. A high macrophage content in human breast cancer is not associated with favourable prognosis. *Br J Surg* 1984, **71**, 456–458.
19. Von Kleist S, Berling J, Bohle W, Wittekind C. Immunohistological analysis of lymphocyte subpopulations infiltrating breast carcinomas and benign lesions. *Int J Cancer* 1987, **40**, 18–23.
20. Bussolati G, Gugliotta P, Morra I, Pietribiasi F, Berardengo E. The immunohistochemical detection of lymph node metastases from infiltrating lobular carcinoma of the breast. *Br J Cancer* 1986, **54**, 631–636.
21. Bryne J, Waldron R, McAvinchey D, Dervan P. The use of monoclonal antibodies for the histopathological detection of mammary axillary metastases. *Eur J Surg Oncol* 1987, **13**, 409–411.
22. Sedmak DD, Meineke TA, Knechtges DS. Detection of metastatic breast carcinoma with monoclonal antibodies to cytokeratins. *Arch Pathol Lab Med* 1989, **113**, 786–789.
23. Visschner DW, Zarbo RJ, Greenawald KA, Crissman JD. Prognostic significance of morphological parameters and flow cytometric DNA analysis in carcinoma of the breast. *Pathol Annual* 1990, **25**, 171–210.
24. Kallioniemi O-P, Blanco G, Alavaikko M, *et al.* Improving the prognostic value of DNA flow cytometry in breast cancer by combining DNA index and S-phase fraction. *Cancer* 1988, **62**, 2183–2190.
25. Parham DM, Coghill G, Robertson AJ. Critical evaluation of monoclonal antibody staining in breast carcinoma. *J Clin Pathol* 1989, **42**, 810–813.
26. Van der Linden JC, Lindeman J, Baak JPA, Meijer CJLM, Herman CJ. The multivariate prognostic index and nuclear DNA content are independent prognostic factors in primary breast cancer patients. *Cytometry* 1989, **10**, 56–61.
27. Haybittle JL, Blamey RW, Elston CW, *et al.* A prognostic index in primary breast cancer. *Br J Cancer* 1982, **45**, 361–366.
28. Todd JH, Dowle C, Williams MR, *et al.* Confirmation of a prognostic index in primary breast cancer. *Br Cancer* 1987, **56**, 489–492.
29. O'Reilly SM, Camplejohn RS, Barnes DM, *et al.* DNA index, S-phase fraction, histological grade and prognosis in breast cancer. *Br J Cancer* 1990, **61**, 671–674.
30. O'Reilly SM, Camplejohn RS, Barnes DM, Rubens RD, Richards MA. Node-negative breast cancer: prognostic subgroups defined by tumour size and flow cytometry. *J Clin Oncol* 1990, **8**, 2040–2046.
31. Dent GA, Leglise MC, Pryzwansky KB, Ross DW. Simultaneous paired analysis by flow cytometry of surface markers, cytoplasmic antigens, or oncogene expression with DNA content. *Cytometry* 1989, **10**, 192–198.

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