European Journal of Surgical Oncology 1996; **22**: 508–515

The flow cytometric analysis of total p53 protein content and proliferation indices in colorectal cancer, in relation to clinical outcome

D. A. Rew*, R. Karkera†, M. A. Mullee, S. A. Julious‡ and G. D. Wilson§

**University of Leicester, UK,* †*The University Surgical Unit, Southampton General Hospital, UK,* ‡*Medical Statistics and Computing, University of Southampton, UK,* §*The Gray Laboratory, Northwood, UK*

This study was undertaken to assess the value of flow cytometric measurements of total p53 protein content and proliferation indices derived from *in vivo* halogenated pyrimidine labelling. Two series of colorectal cancer specimens were studied for which clinical outcome data were recorded. A series of 84 archival, ethanol-fixed, bromodeoxyuridine (BrdUrd) labelled colorectal tumours were analysed by flow cytometry for their total and cell cycle phase p53 protein content using the pAb1801 monoclonal antibody. A second series of 33 freshly obtained tumours was used for assay evaluation and for comparison with the archival material. In the archival series ($n=84$), the median p53-pAb1801 LI was 81.9% (range: 11.1–99.8%). In only three tumours could significant amounts of p53 protein not be detected. The median phase specific p53-pAb1801 LI in G0/G1 was 71.6%, in S was 95.5%, and in G2/M was 98.5%. In the series of fresh tumours ($n=33$), the median p53-pAb1801 labelling index (LI) was 94.6% (range: 17.9–99.9%). Only two tumours failed to express significant amounts of p53 protein. There was no significant difference in the generally high levels of p53 protein content between the fresh and archival series. Life-table analysis of the patients in the archival series failed to demonstrate a statistical difference in life expectancy in relation to Dukes' stage when tumours were stratified by the median total p53 labelling index. In this study, p53 content and proliferative indices measured by flow cytometry do not have independent predictive value over Dukes' grading in determining the outcome of colorectal cancer. Flow cytometry is confirmed as a practical tool for multi-parametric and cell cycle analysis of oncoprotein expression in human tumour biopsies.

Key words: **flow cytometry; p53; proliferation indices; colorectal cancer.**

Introduction

Key nucleoproteins regulate the growth and behaviour of human tumour cells, and hence are likely to influence the biological characterisitics of the tumour itself. Among the most intensively studied is the 393 amino acid phosphoprotein product of the p53 gene, which may have a regulatory role in the cell cycle[1] and in programmed cell death, or apoptosis.[2] Early theories of p53 gene function, which categorized p53 as an oncogene with normal (wild type) and mutant variants,[3,4] have been complicated by the recognition of a large number (>2600) of sequence variations and point mutations in the protein.[5–7] In general terms only, normal p53 appears to prevent progression through the cell cycle of genetically damaged cells (as after cytotoxic drug treatment or irradiation), while mutant p53 allows uncontrolled cell cycle progression and impairs apoptosis, both of which processes lead to the accumulation of malignant cells.

In colorectal cancer, the gene is an important component of the cascade of molecular changes leading to frank carcinoma.[9–12] Histochemical studies have demonstrated the presence of p53 in colorectal tumours[13] and have suggested that p53 may have prognostic significance in colorectal cancer.[14,15] The measurement of total p53 protein content in tumour cells may thus be hypothesized to be a marker of tumour cell biological behaviour, including proliferative activity, which can in turn be quantified by *in vivo* labelling with a halogenated pyrimidine.

While histochemistry allows qualitative static measurements of p53 content in tissues,[16] multiparameter flow cytometry (MFCM) can be used to quantify the expression and cell cycle distribution of nucleoproteins in large, heterogenous cell populations.[17–23] Remvikos *et al.*[24,25] showed that p53 can be measured by FCM in archival colorectal tumours and suggested a correlation with prognosis in a study of 78 tumours using the p421, pAb240 and pAb1801 antibodies.

There are as yet no widely adopted standards for the flow

Correspondence to: Mr D. A. Rew, Senior Lecturer in Surgery, The University Surgical Unit, The Glenfield Hospital, Groby Road, Leicester LE3 9PQ, UK.

0748-7983/96/050508+08 $12.00/0

cytometric analysis of nucleoprotein expression in human tumours. This hinders the adoption of the technology for clinical assays. In this study, we apply a model of multiparametric flow cytometric analysis of nucleoprotein expression in clinico-pathological material in order to relate p53 protein expression and the BrdUrd-derived proliferation indices to clinical outcome. We here report the expression of the p53 protein in archival samples of human colorectal tumours ($n=84$) labelled *in vivo* with bromodeoxyuridine. We have previously reported the use of this model[26] in the measurement of proliferating cell nuclear antigen. A series of freshly obtained tumours ($n=33$) was also studied for comparative and evaluative purposes. We test the nul hypothesis that the expression of the normal and total (i.e. p1801 detected) p53 protein content detected by multiparameter flow cytometric assay in ethanol-stored, archival primary tumour biopsies has no bearing on clinical outcome measures.

Materials and methods

Flow cytometry studies of fresh tumours

A series of specimens from freshly excised colorectal adenocarcinomas ($n=33$) were collected. Optimization of tissue disaggregation, nuclear antigen preservation, antibody titres, controls and data analysis was first undertaken in a subgroup of these samples. No follow-up or survival data were collected for patients whose tumours were included in this part of the study.

Randomly selected tumour samples were minced by scalpel and incubated in 0.1 M HCl and 0.4 mg/ml porcine pepsin for 30 min at 37°C and filtered through a 35-micron mesh to obtain suspensions of single nuclei.

pAb1801 staining. The concentrations of nuclei were adjusted using a haemocytometer to yield a total of 1×10^6 per vial. The suspensions were spun at 2000 rpm for 5 min to remove methanol, were washed and spun again. The pellet was resuspended in 0.25 ml of PBS containing 0.25% bovine serum albumin and 0.5% Tween 20 (Sigma), and the appropriate dilution of pAb1801-FITC conjugated antibody. pAb1801 fluorescence tagging was achieved using a second, FITC conjugated rabbit anti-mouse IgG2A (Dako, High Wycombe, UK, catalogue number F313) at the same equivalent antibody concentration as the pAb1801. The samples were incubated in the dark for 1 h at room temperature. After washing with PBS the samples were resuspended in 2 ml of PBS containing 1 mg/ml ribonuclease (Sigma, Poole, UK; R 5503) and 20 ugm/ml propidium iodide (Sigma; P4170).

A dilution series was performed for each batch to determine the optimum antibody concentration to be used for further studies. The optimum dilution was defined as the point at which pAb1801 labelling did not increase further with increasing concentration of applied primary antibody, and was standardized at 1/25 that of the pure pAb1801. Secondary antibody was diluted to 1 in 10.

Preservation of the p53–1801 epitope in pepsin was confirmed in a separate series of experiments.

Flow cytometric analysis

All analyses were performed on a standard FACScan flow cytometer (Becton Dickinson, Oxford, UK). Excitation was performed using a 15 mW argon laser at 488 nm. Green light was collected in channel FL1 with gains of 525 or 681. Red light was collected in channel FL3 so there was no fluorescence overlap. FL3 gains were set between 298 and 319 depending on the drift of the G0/G1 peak. All data were collected in list mode. Debris, doublets and clumps were excluded using the doublet discrimination function. pAb1801 stained and control cells/nuclei were all run at the same gains. Either chick erythrocytes or normal human colorectal mucosa were used as ploidy controls.

A small additional series of samples labelled with the pAb240 antibody (Professor D. P. Lane, Dundee), which detects a mutant variant of p53 protein, were also analysed in a pilot study. Cell cycle associated patterns of protein expression were similar to those obtained when pAb1801 were, and are used for illustrative purposes only in Figs 1–3. Quantitative pab240 data are not discussed further in this paper.

Data analysis. This was performed using the Lysys 2 and Cellfit software packages (Becton Dickinson, San Jose, CA). Gating was performed on the FL1 (fluorescein) width vs FL2-A (propidium iodide) signals. The DNA index and S-phase fractions were calculated for each sample separately using Cellfit.

Control gates. All paired control and p53 antibody labelled specimens were run sequestially, and the resulting FL1-H (p53) versus FL2-A (DNA) dot plots were displayed side by side for further analysis (Figs 1 and 2). The trapezoid (polygonal) gates or regions were adjusted on the control dot plots to select 98% or 99% of fluorescent events, so as reproducibly to distinguish the p53-pAb1801-positive cells from the control fluorescence.

A profile was judged to be satisfactory for p53 analysis if the DNA profile (FL3 histogram) was clearly defined, regardless of any difference in the FL1-H profile of the p53-stained population when compared with the control.

The parameters measured in association with p53-positive fluorescence were as follows: (1) The p53 total labelling index, which is the proportion of labelled cells above the (98%) gate from the total population; (2) the p53 aneuploid labelling index, which is the proportion of labelled cells above the (98%) gate when gating on the aneuploid population (G0/G1.An + S + G2/M.An); (3) the percentage of cells in G0/G1, S and G2/M. These values were only calculated in the diploid tumours, because of the errors introduced by overlapping populations in the aneuploid tumours.

Archival, BrdUrd labelled tumours

Biopsies from 84 previously resected primary colorectal adenocarcinomas were fixed in 70% ethanol and refrigerated at −20°C immediately following surgical resection. Each patient had consented to receive a single bolus dose of BRdUrd between 2.4 and 16.0 h before tumour excision.

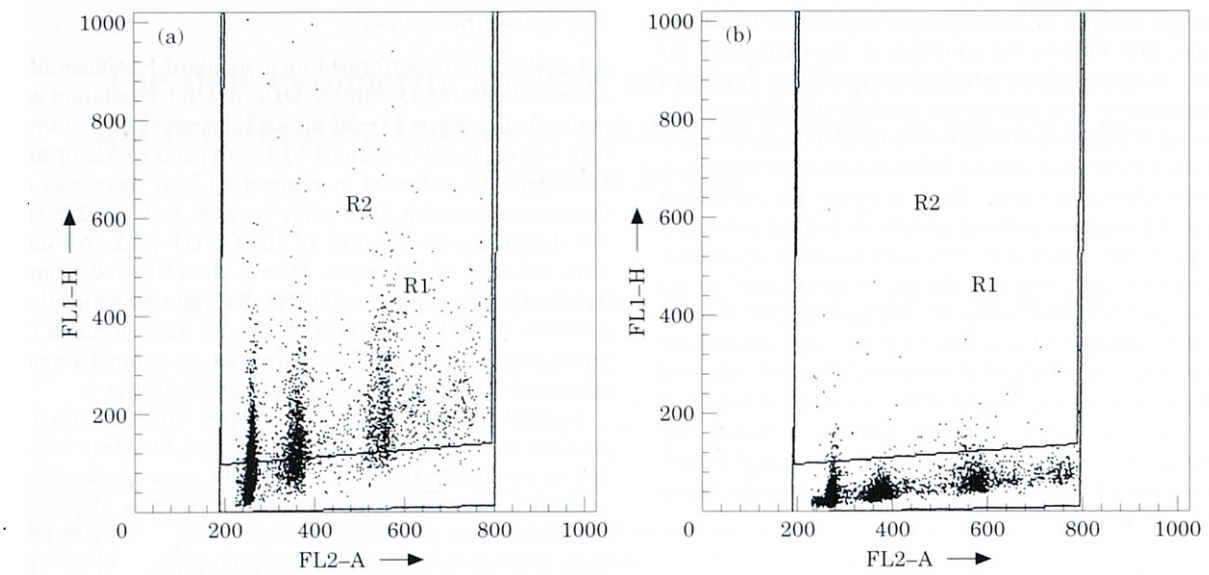

Fig. 1. This dot plot of FL1–H (FITC) vs FL2–A (PI) illustrates the gating profiles of samples labelled with p53–pAb240 (a), paired with their non-specific fluorescence controls (b). Both analyses have been performed with the same instrument gains and settings. All R2 gates are set on the control profile to include 98 +/− 0.3% of fluorescent events. R1 is the total population. This is an aneuploid tumour with good discrimination between p53 and control fluorescence. The p53 LI is 55.1%.

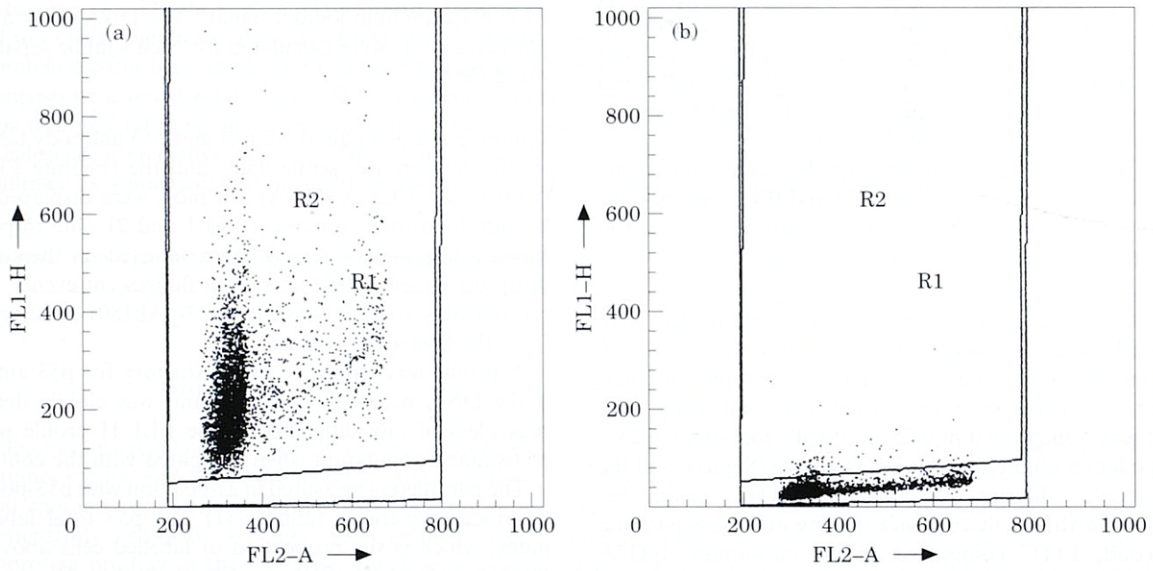

Fig. 2. This is a dot plot (for details see Fig. 1) of a diploid tumour (code nos 03/04) with complete discrimination between control and p53–pAb240 associated fluorescence, i.e. p53 positivity of 99.0%.

The measurement of the kinetic indices of these tumours has been reported elsewhere.[27] Tumours ranged from 2.0 to 10.0 cm in diameter, and biopsies were excised from a variety of sites at the periphery and centre of the tumours. Disaggregation to nuclei was achieved by incubation of minced samples in porcine pepsin, 0.3 mg/ml (Sigma, Poole, UK) in dilute hydrochloric acid for 30 min at 37°C.

pAb1801 analyses. The quantitative measurement of p53 expression in this series of 84 tumours was undertaken as described above. The percentage of p53 labelled cells (above the 98th centile on the control population) was calculated for each phase of the cell cycle, that is G0/G1, S and G2/M, in the diploid tumours. In aneuploid tumours, the overlapping phases of the diploid and aneuploid populations introduce substantial inaccuracies into this calculation. Each phase was defined from the DNA profile and gates drawn around the appropriate p53 population as shown in Fig. 3.

Heterogeneity studies

Five separate samples were excised at random from each of five fresh and five archival tumours. Each sample was

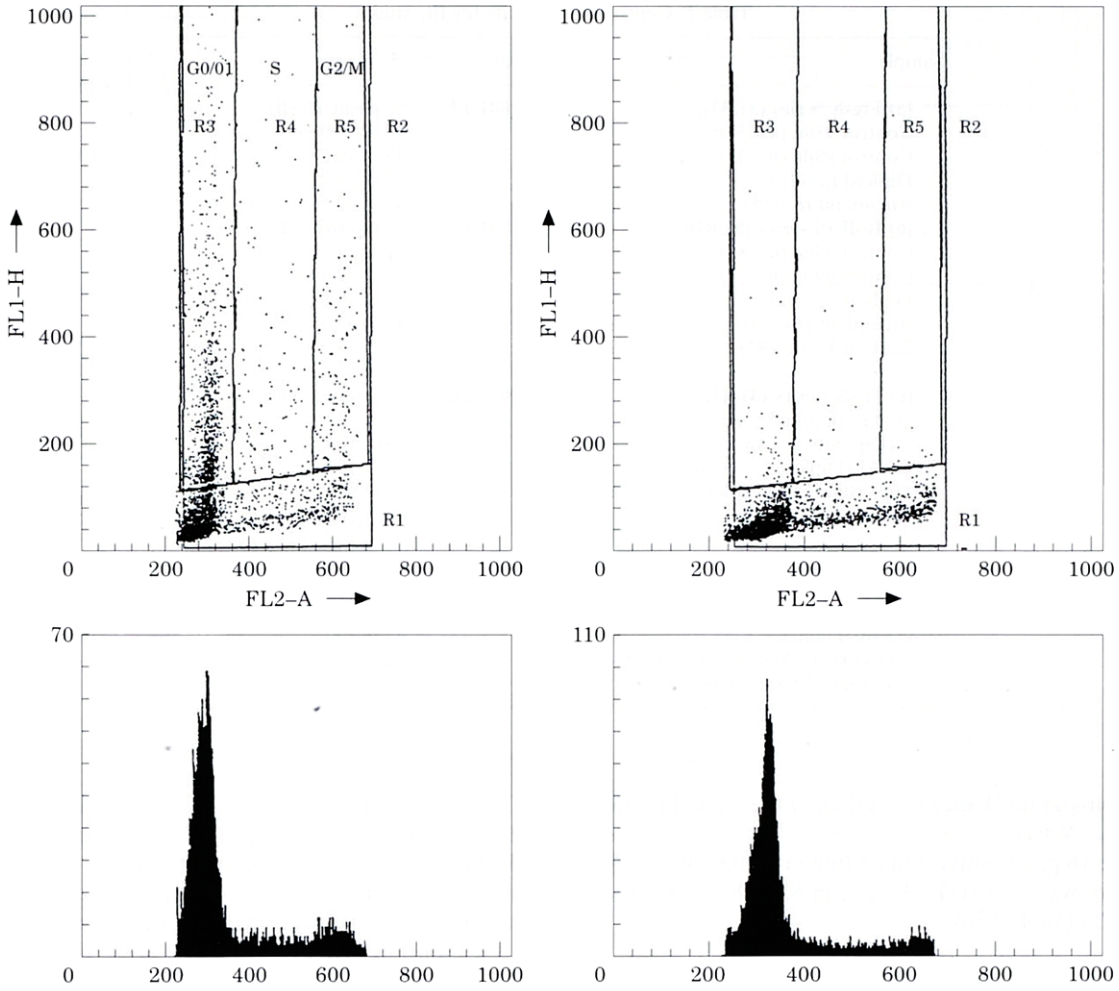

Fig. 3. In order to calculate the cell cycle phase p53 content, gates are drawn on the dot plots which correspond to the G0/G1, S and G2/M phases on the DNA histogram. The numbers and proportions of p53-positive cells in each phase can thus be calculated.

prepared and analysed in identical fashion, and the pAb1801–53 labelling index was calculated. For each tumour, the coefficient of variation of the p53 LI was calculated, and was used as an index of heterogeneity for the p53 moiety within each tumour.

Statistical methods

Descriptive statistics, parametric and non-parametric analyses, and correlations dervied from simple linear regression analysis were performed using the Statgraphics analytical software package (Manugistics Inc, Rockville, Maryland, USA).

Life table analyses were performed upon the follow-up data for patients who had participated in the bromodoexyuridine labelling study ($n=96$), for the large majority of whom ($n=84$) p53 analysis data were also available. Stratification of the tumours was by Dukes' stage, median p53 protein labelling index, ploidy, median BrdUrd labelling index and median p62cmyc labelling index. These analyses were undertaken using the SAS package (SAS Institute, Cary, USA), taking death as the end-point. Significance was assessed using the log rank test.

Results

Fresh tumour series

Samples were collected from 33 freshly resected primary adenocarcinomas of the colorectum. Dukes' stage and other clinico-pathological correlates were not analysed in this series. Seventeen tumours were diploid and 16 were aneuploid, with DNA indices ranging from 1.16 to 2.00. One aneuploid and one diploid tumour expressed less than 10% positivity throughout the cell cycle than the controls. These are regarded as p53-negative for analytical purposes. The median p53 labelling index (LI) of the other 31 p53-positive tumours was 94.6% at the 98th centile for the control gate, and was 82.9% at the 99th centile for the control gate (Table 1(a)). Lower p53 labelling was seen in

Table 1. Consolidated results for the study

Sample	Median	Range
(a) Fresh Series (31/33):	**p53-pAb1801 LI:**	**% positive cells**
Control 98ile ($n=31$)	94.6	17.9–99.9
Control 98ile ($n=31$)	82.9	17.3–99.8
Diploid ($n=16$)	99.4	17.9–99.9
Aneuploid ($n=15$)	81.5	41.9–99.8
(b) BrdUrd series (81/84):	**p53-pAb1801 LI:**	**% positive cells**
Control 98ile ($n=81$)	81.9	11.1–99.8
Control 99ile ($n=81$)	67.6	2.2–99.4
Diploid ($n=42$)	79.1	15.6–99.8
Aneuploid ($n=39$)	85.4	11.1–99.3
BrdUrd LI ($n=81$)	9.2	1.0–22.2
(c) Fresh Series (31/33):	**p53-pab1801 LI:**	**% positive cells**
G0/G1 (Diploids, $n=16$)	99.2	10.9–99.8
S (Diploids, $n=16$)	99.8	35.1–100
G2/M (Diploids, $n=16$)	99.7	56.7–100
Dip. G0/G1 (Aneuploids, $n=15$)	55.3	22.8–99.9
Anp G0/G1 (Aneuploids, $n=15$)	94.9	43.5–100
(d) BrdUrd series (81/84):	**p53-pab1801 LI:**	**% positive cells**
G0/G1 (Diploids, $n=42$)	71.6	6.2–100
S (Diploids, $n=42$)	95.5	26.5–100
G2/M (Diploids, $n=42$)	98.5	41.0–100
Dip G0/G1 (Aneuploids, $n=39$)	72.2	2.2–98.9
Anp G0/G1 (Aneuploids, $n=39$)	96.4	15.0–100

the aneuploid (median: 81.5%) than in the diploid tumours (median: 99.4%).

In the 16 p53-positive diploid tumours, the cell cycle p53 positivity was: in G0/G1, 99.2%; in S, 99.8%, and in G2/M, 99.7% (Table 1(b)).

BrdUrd labelled tumours

pAb1801 analysis. For this experiment, 44 colonic tumours (26 diploid), and 40 rectal tumours (18 diploid) were available. There were five Dukes' A, 32 Dukes' B and 45 Dukes' C tumours. Node status was not assessed in two tumours because of transanal resections.

Satisfactory DNA profiles were obtained for each of the 84 tumours. Two colonic tumours and one rectal tumour expressed less than 10% positivity throughout the profile compared with controls in the samples studied, and are regarded as p53-negative for analytical purposes. The median p53 LI of the other 81 tumours was 81.9% at the 98th centile for the control gate, and was 67.6% at the 99th centile for the control gate. Thus, a 1% change in the setting of the control gate produced a 14.3% change in the median p53 LI (Table 1(c)).

In archival diploid tumours ($n=42$) the median p53 LI in the G0/G1 phase was 71.6%, in the S phase was 95.5%, and in the G2/M phase was 98.5%. Cell cycle progression in p53 content was also noted between the diploid (dip) and aneuploid (anp) G0.G1 peaks on the aneuploid tumour profiles (Table 1d).

There were no associations between p53–pAb1801 expression and Dukes' staging.

Assessment of heterogeneity of the p53 labelling index. Of the 10 tumours studied, six exhibited coefficients of variation (CVs) of less than 0.03, with all p53 labelling indices between 92% and 100%. Three CVs were less than 0.50, where one p53 LI value in each case was less than 50%, and one CV was 0.86, in which case two of the five biopsies failed to demonstrate p53. With a small number of exceptions, there was a uniformly high p53 LI (>90%) in the samples studied.

Survival analysis

At the time of analysis, 54 of 96 patients with BrdUrd-labelled tumours had died either in hospital or in the community. Post mortems were not performed in any case. Three tumours were synchronous. The Dukes' stage was not known for three patients (peranal excision biopsies) of whom two died, and the p53 status was not known for 15 patients (inadequate supply of stored samples), of whom 11 died. There were 17 patients for whom either the p53 status or the Dukes' stage were unknown, of whom 12 died. Follow-up varied between 36 and 53 months. All analyses, both p53-positive and those judged to be p53-negative (pan-cycle p53 LI<10%) are included.

There was a highly significant difference in survival when tumours were stratified by Dukes' stages (A and B against C), ($\chi^2=22.04$, df. = 1, $P=0.0001$, relative risk (RR) = 4.03, 95% confidence interval (CI) = 2.14 to 7.59), thus indicating that this is a representative clinical series by conventional criteria. When the analysis was stratified by the median p53 labelling index, there was a trend towards poorer survival in those tumours displaying a p53 labelling index above the median value, but this did not achieve statistical significance ($\chi^2=3.09$, df. = 1, $P=0.079$, RR = 1.70, 95% CI = 0.93 to 3.13), as seen in Fig. 4. This trend was maintained when the Dukes' A–B tumours ($\chi^2=3.83$, df. = 1, $P=0.05$, RR = 4.21, 95% CI = 0.87 to 20.31) and Dukes' C tumours ($\chi^2=$

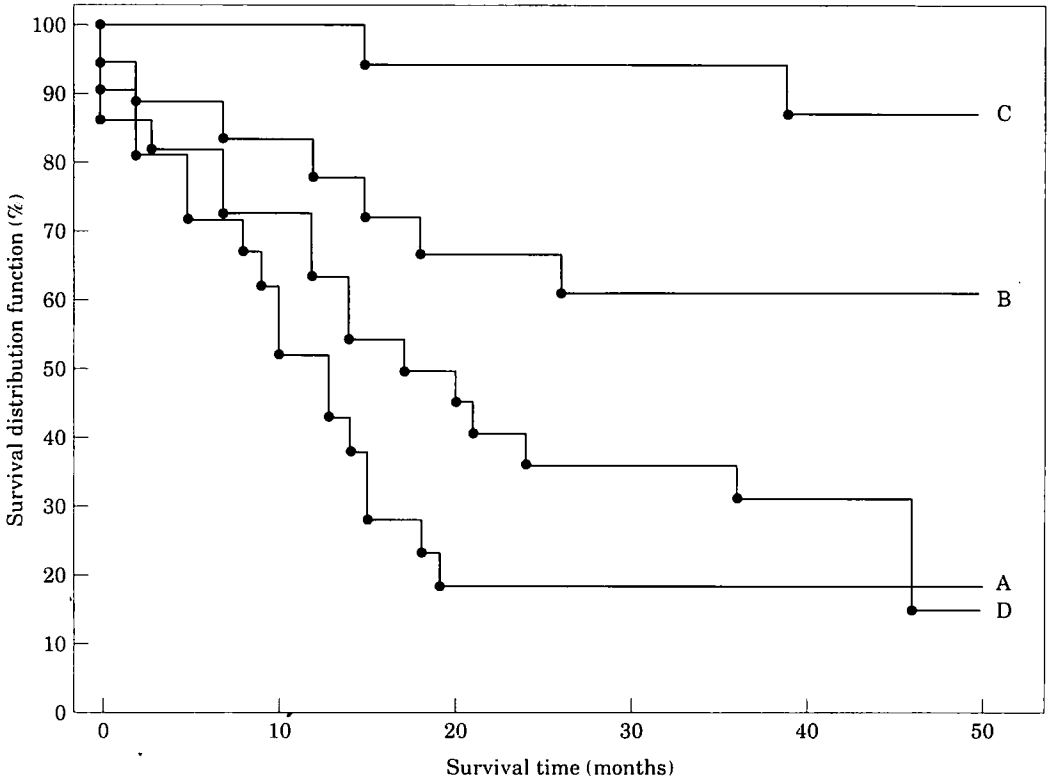

Fig. 4. This shows the survival data stratified by median p53 content and Dukes' stage. (A) Metastatic tumours (Dukes' C) with above median p53 LI; (B) Non-metastatic tumours (Dukes' A, B) with above median p53 LI; (C) Non-metastatic tumours (Dukes' A, B) with below median p53 LI; (D) Metastatic tumours (Dukes' C) with below median p53 LI.

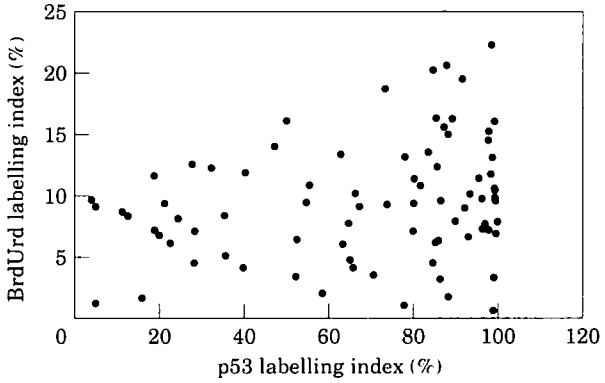

Fig. 5. A plot of all data points for p53 labelling (%) and BrdUrd labelling (%). There was no significant correlation between the values.

1.67, df. = 1, $P = 0.20$, RR = 1.57, 95% CI = 0.78 to 3.16) were analysed independently for p53 content.

Correlations with BrdUrd data. There was no correlation between BrdUrd labelling index in the 81 archival tumours and p53 protein content ($r = 0.18$) (Fig. 5), or p53-positive when the tumours were stratified by diploidy ($r = 0.07$, $n = 41$) or aneuploidy ($r = 0.29$, $n = 38$).

Discussion

Technical issues

Flow cytometry (FCM) is a rapid and quantitative tool for the study of large (>10,000 cells) and heterogenous clinical samples such as tumour biopsies. Single parameter FCM analysis of tumour samples is well established as a clinical research tool in the study of DNA content, and its technical limitations are clearly understood.[28-30] A major constraint is imposed by the need to disaggregate solid tumours into cell or nuclear suspensions, which can produce subcellular debris thus degrading the analysis.

FCM offers both advantages and limitations in studies of nucleoproteins in clinical material when compared with other technologies in cell biology. Molecular biology techniques such as blotting allow quantitation of p53 DNA, mRNA or protein in cells, and have been fundamental to the dramatic advances in the understanding of the p53 system. However, they are laborious when applied to clinical materials and are limited to small and possibly unrepresentative tissue samples.

Fresh colorectal tumour biopsies yield adequate quantities of cells on vigorous mincing or grating. Archival tissues preserved in ethanol readily fragment to nuclei in pepsin, with the loss of cytoplasmic protein. The preservation of protein epitopes during enzymatic extraction varies according to the type and duration of fixation, the method of

disaggregation, and the nature of the epitope. The pAb1801 epitope seems to be robust during mechanical or pepsin extraction, as shown for pAb1801 by the similar results for both the archival and fresh samples.

Problems inherent in the preparation and analysis of the DNA profile by single parameter flow cytometry (ploidy analysis) are compounded when another dimension of data (p53 protein content) is added. The use of multiparameter FCM thus requires the rigorous evaluation of each new antigen, antibody and preparative procedure before it can be used for reliable and reproducible clinical assays.[31] We have addressed a number of technical issues in this and a previous study, to specify criteria for reproducible dual parameter FCM studies of regulatory nucleoproteins such as p53 and PCNA, particularly in relation to the selection and analysis of controls.

The ability of FCM to measure the cell cycle distribution of the protein under study may aid the understanding of its biological function. We have found the distribution of p53 and other nucleoproteins (PCNA, cmyc) to be broad and not phase-specific.[20,27] The problems of precisely defining the G0/G1, S and G2 populations on the DNA profile even under optimum conditions have been reported.[30,32] We have adopted a trapezoid gate for each population, setting the margins of the gate on the DNA profile as defined in the Cellfit programe (Fig. 3). In practice, given the high p53-positivity in all phases of the cell cycle in most diploid tumours, small variations in the setting of the gates do not yield significant variations in the measured p53 LI for that gate.

We conclude that on technical grounds, standardized assays of nucleoproteins such as p53 by monoclonal antibody labelling and flow cytometry are now possible in human tumour biopsies. However, the analytical conditions must be carefully defined and controlled. This technology may have further applications in the study of the biology human solid tumours for prognostic and therapeutic purposes, but it now is necessary for the flow cytometry community to address with rigour the standardization of multiparametric 'DNA plus' assays as has been undertaken for monoparametric DNA assays. Inter-group and inter-institutional standardization is also essential. We propose that the pragmatic model which we have adopted is a basis for the development of urgently needed standard analytical protocols.

Biological and clinical issues

Our studies indicate that pAB1801-p53 is detectable in a large fraction of cells in all phases of the cell cycle in both diploid and aneuploid cells. The proportion of tumour cells expressing pAb1801-p53 in this series is somewhat higher than that reported elsewhere.[24,25] This raises the question of which technique for the expression of p53 protein clinico-pathological material, histochemistry or flow cytometry, yields the most reliable and biologically informative data. It is possible that the choice of a 98% or any other artificial gating threshold to distinguish positively from negatively stained cells may obscure biologically significant data. However, in practice, in the majority of tumours the p53-positive population is clearly defined when compared with controls.

The sensitivity of protein detection of flow cytometric and histochemical studies may also differ, thus producing seemingly divergent results between the two techniques. This is important, as various hypotheses of p53 function have been constructed upon such measurements.[15] It might be surprising if a protein which has a key cell regulatory role were not present in all normal and malignant cells, and the higher p53 labelling indices seen in flow cytometric studies *may* thus be more biologically accurate. In this case, the uniformly high levels of p53 expression may paradoxically reduce the power of the assay in prognostication.

p53 is believed to have a role in the regulation of cell proliferation and apoptosis. It is interesting to speculate that such measurements of key apoptosis-associated nucleoproteins as reported here may allow the development of an 'apoptotic index' (e.g. of p53 or p62cmyc), much as markers such as PCNA, Ki67 and PS1 have been used to develop proliferation indices in clinico-pathological material.

Our data correlating the Dukes' staging of these tumours with survival indicate that our series was representative. There was no association between p53 expression and clinical outcome, ploidy, BrdUrd labelling index, S-phase duration, potential doubling time or p62 cmyc expression[20] in this series.

Tumour heterogeneity confounds the ability of sample analyses to represent events of biological significance throughout the tumour. There are as yet no satisfactory algorithms with which to address heterogeneity, and in an ideal case, numerous measurements of each index in each tumour sample would be studied. This is often impractical. The heterogeneity study here indicates that there is a considerable degree of homogeneity for pab1801-p53 labelling within individual tumours in this series.

The lack of correlation between the p53 labelling index and clinical outcome is not unexpected. The normal p53 protein as detected by pAb1801 is a key regulatory nucleoprotein which would be expected to be widely expressed in normal and transformed living cells regardless of their clonal origin or differentiation. More importantly, biological aggressiveness (and hence survival time) is usually determined by metastases, for which 53 measurements were not undertaken in this study.

In conclusion, Dukes' staging and the presence of metastases in the regional nodes or the liver remain the best predictor of survival in colorectal cancer. While dramatic advances in laboratory technology have allowed the detailed study of a broad spectrum of molecular variables in colorectal tumour cells, measurements of nucleo-protein markers such as p53 or halogenated pyrimidine derived proliferation indices remain as the research techniques for the present.

Acknowledgements

We acknowledge the help of Dr Andrew Lane, formerly of the Tenovus Institute, Southampton, and of Dr A. J. Cooper.

Financial support was generously provided by the Wessex Medical Trust and by the Cancer Research Campaign. Dr J. V. Watson (Cambridge) kindly provided additional technical advice and support.

References

1. Donehower LA, Bradley A. The tumour suppressor p53. *Biochim Biophys Acta* 1993; **1155:** 181–205.
2. Marx J. Cell death studies yield cancer clues. *Science* 1993; **259:** 760–1.
3. Baker SJ, Markowitz S, Fearon E, Willson JKV, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* 1990; **249:** 912–5.
4. Lane DP, Benchimol S. p53: oncogene or anti-oncogene? *Genes and Development*, **4:** 1–8. Cold Spring Harbor Laboratory Press, 1990.
5. Harris CC. p53: at the crossroads of molecular carcinogenesis and risk assessment. *Science* 1993; **262:** 1980–1.
6. Vogelstein B, Kinzler KW. Xrays strike p53 again. *Nature* 1994; **370:** 174–5.
7. Vile RG. p53: a gene for all tumours? *Br Med J* 1993; **307:** 1226–7.
8. Iggo R, Gatter K, Bartek J, Lane DP, Harris AL. Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet* 1990; **335:** 675–9.
9. Baker SJ, Fearon ER, Nigro JM, *et al.* Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* 1989; **244:** 217–21.
10. Kerr IB. Molecular genetics of colorectal carcinoma. *Br Med J* 1989; **299:** 637–8.
11. Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV, Lane DP. p53 mutations in colorectal cancer. *Proc Natl Acad Sci USA* 1990; **87:** 7555–9.
12. Shirasawa S, Urabe, K, Yanagawa Y, Toshitani K, Iwama T, Sasazuki T. p53 gene mutations in colorectal tumours from patients with familial polyposis coli. *Cancer Res* 1991; **51:** 2874–8.
13. Van den Berg FM, Tigges AJ, Schipper MEJ, den Hartog-Jager FCA, Kroes WGM, Walboomers JMM. Expression of hte nuclear oncogene p53 in colon tumours. *J Pathol* 1989; **157:** 193–9.
14. Scott N, Sagar P, Stewart J, Blair GE, Dixon MF, Quirke P. p53 in colorectal cancer: clinicopathological correlation and prognostic significance. *Br J Cancer* 1991; **63:** 317–9.
15. Sun X-F, Carstensen JM, Zhang H, *et al.* Prognostic significance of cytoplasmic p53 oncoprotein in colorectal carcinoma. *Lancet* 1992; **340:** 1369–73.
16. Wynford-Thomas D. p53 in tumour pathology: can we trust immunocytochemistry? *J Pathol* 1992; **166:** 329–30.
17. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res* 1991; **51:** 6304–11.
18. Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild type p53 is a cell cycle determinant following irradiation. *Proc Natl Acad Sci USA* 1992; **89:** 7491–5.
19. Khochbin S, Chabanas A, Albert P, Lawrence JJ. Flow cytometric determination of protein distribution through the cell cycle. *Cytometry* 1989; **10:** 484–9.
20. Rew DA, Taylor I, Watson JV, Wilson GD. The c-myc protein product is a marker of DNA synthesis but not of malignancy in gastrointestinal tissues and tumours. *Br J Surg* 1991; **78:** 1080–3.
21. Watson JV, Stewart J, Cox C, Sikora K, Evan GI. Flow cytometric quantitation of the c-myc oncoprotein in archival neoplastic biopsies of the colon. *Mol Cell Probes* 1987; **1:** 151–7.
22. Watson JV. *An Introduction to Flow Cytometry*. Cambridge: Cambridge University Press, 1992.
23. Morkve O, Laerum OD. Flow cytometric measurement of p53 protein expression and DNA content in paraffin embedded tissue from bronchial carcinomas. *Cytometry* 1991; **12:** 438–44.
24. Remvikos Y, Laurent-Puig P, Salmon RJ, Frelat G, Dutrillaux B, Thomas G. Simultaneous monitoring of p53 protein and DNA content of colorectal adenocarcinomas by flow cytometry. *Int J Cancer* 1990; **45:** 450–6.
25. Remvikos Y, Tominaga O, Hammel P, Laurent-Puig P, Salmon RJ, Dutrillaux B, Thomas G. Increased p53 protein content of colorectal tumours correlates with poor survival. *Br J Cancer* 1992; **66:** 758–64.
26. Sawtell R, Rew DA, Stradling R, Wilson GD. A comparative flow and histometric study of proliferating cell nuclear antigen (PCNA) expression and bromodeoxyuridine labelling in colorectal cancer. *Clin Comm Cytomet* 1995; **22:** 190–9.
27. Rew DA, Taylor I, Weaver PC, Wilson GD. The in vivo proliferation of human colorectal carcinomas. *Br J Surg* 1991; **78:** 61–6.

Accepted for publication 12 June 1996