Pergamon

European Journal of Cancer Vol. 32A, No. 4, pp. 612–616, 1996

Printed in Great Britain
0959-8049/96 $15.00+0.00

0959-8049(95)00596-X

Original Paper

An Image Analysis Study of DNA Content in Early Colorectal Cancer

E.W. Kay,[1] H.E. Mulcahy,[2] B. Curran,[1] D.P. O'Donoghue[2] and M. Leader[1]

[1]Department of Pathology, Royal College of Surgeons in Ireland, Dublin 2; and [2]Gatroenterology and Liver Unit, St Vincent's Hospital, Elm Park, Dublin 4, Ireland

The DNA content of 168 consecutive T3,N0,M0 (Dukes' B, Astler-Coller B2) colorectal cancers was studied using image analysis on formalin-fixed paraffin-embedded tissues. 72 cases (43%) were classified as diploid and the remaining 96 (57%) as non-diploid. After a median follow-up period of 6.7 years, a significant survival advantage was found for diploid compared with non-diploid cases (logrank test; $P = 0.008$). The long-term (8 year) survival rate was 70% for diploid and 46% for non-diploid tumours. Subgroup analysis showed that the survival advantage conferred by tumour diploidy was greatest in large (≥5 cm) cancers and was found both in colonic and rectal cancer cases. These data indicate that tumour ploidy status measured by image analysis might be useful in determining risk of colorectal cancer recurrence and death in patients following resection of early colorectal cancer.

Key words: colorectal cancer, ploidy, survival
Eur J Cancer, Vol. 32A, No. 4, pp. 612–616, 1996

INTRODUCTION

QUANTITATIVE ABNORMALITIES of cellular DNA content have been widely studied since flow cytometry and image analysis were first utilised to measure ploidy status of appropriately stained cellular nuclei. Initial studies were performed on freshly obtained tissue specimens, but techniques allowing nuclei to be isolated from paraffin embedded tissue [1] have allowed larger retrospective studies to be performed on patients whose long-term outcome is already known. Abnormal cellular DNA content is associated with a poor prognosis in many neoplastic types, including colorectal cancer [2–13] although this has not been a consistent finding [14, 15].

T3,N0,M0 colorectal cancer patients pose special problems for surgical oncologists. This group accounts for over one third of all colorectal cancer patients [16] of whom approximately 45% suffer disease recurrence and death [16]. Recent results from large randomised trials suggest that the survival benefit for conventional chemotherapeutic agents in T3,N0,M0 cases is marginal [17, 18]. However, the ability to distinguish cases with a poor prognosis by using additional prognostic features would help modify selection procedures for future trials of adjuvant therapy, allowing oncologists to adminster chemotherapy or radiotherapy primarily to patients in whom tumour recurrence may be expected. To test the hypothesis that tumour ploidy status might be useful in this assignment process, we studied a consecutive series of patients with T3,N0,M0 colorectal cancer using image analysis of feulgen-stained nuclei.

Correspondence to E. Kay, Department of Pathology, Beaumont Hospital, Beaumont, Dublin 9, Ireland.
Revised 25 Sep. 1995; accepted 27 Oct. 1995.

PATIENTS AND METHODS

523 patients were admitted to St Vincent's Hospital between 1983 and 1989 of whom 188 (36%) were staged as T3,N0,M0. 20 of these cases (11%) were excluded from the study because of death within the postoperative period ($n = 7$), restaging at the time of histological review ($n = 6$) or insufficient tumour material for analysis ($n = 7$). The final study group thus consisted of 168 patients (93 males, 75 females; mean age 67.8 years; range 26.5–90.0 years). Clinical and pathological data were extracted from a prospective computer database containing details of all colorectal cancer patients admitted since 1983. No study patient received pre- or postoperative adjuvant therapy. Follow-up ended in December 1994 for this study, and median follow-up was 6.7 years (mean 7.1 years; range 3.4–11.5) or until death.

The pathologist and technical investigators were blinded to clinical details and outcome. All tumours were formalin-fixed

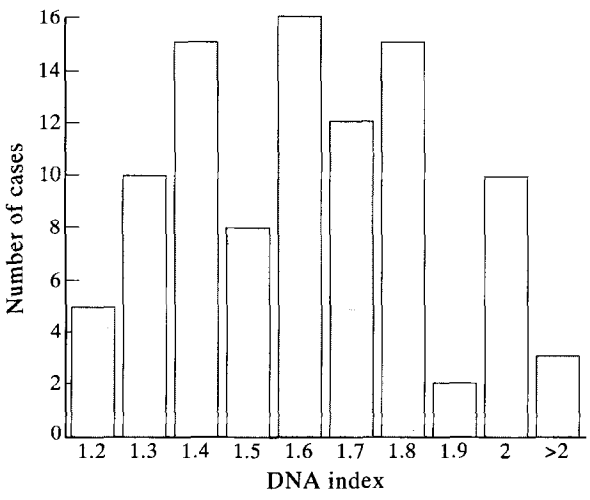
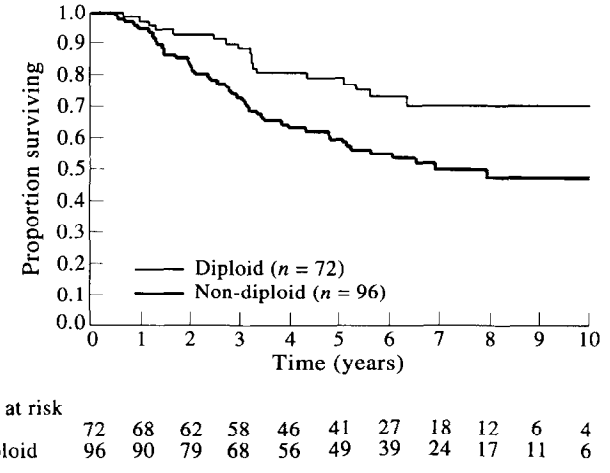


Figure 1. Distribution of non-diploid tumours according to DNA index ($n = 96$).



Number at risk

Diploid	72	68	62	58	46	41	27	18	12	6	4
Non-diploid	96	90	79	68	56	49	39	24	17	11	6

Figure 2. Survival of 168 patients with T3,N0,M0 colorectal cancer stratified by ploidy status.

and paraffin embedded. Tumour blocks consisting of 70% tumour and 30% benign tissue were selected using H&E stained sections where possible. All sections in each case were also evaluated to confirm tumour stage and differentiation and exclude areas of necrosis or haemorrhage. Cell suspensions were prepared according to the modified method of Hedley [1, 19]. Sections (50 μm) were cut, dewaxed in xylene, rehydrated in graded ethanol and washed in distilled water. Nuclei were released by incubation in a pepsin solution at 37°C with intermittent vortexing. Cell suspensions were then prepared for image analysis by cytospinning on to glass slides followed by feulgen staining [20]. Image analysis was performed on a CAS 200 image analyser (Becton Dickinson).

For each case, 20–40 control (non-malignant) cells and 100–200 cancer cells were evaluated, each case taking between 30 and 60 min to analyse. A ploidy index in the range of 0.8–1.2 was taken as diploid; 1.8–2.2 as tetraploid if the peak represented ≥20% of the total cell population and had its own G2M peak; other values were interpreted as aneuploid provided they represented ≥10% of the total cell population

Table 1. Ploidy status and clinical features in 168 patients with T3,N0,M0 colorectal cancer

	Diploid ($n = 72$)	Non-diploid ($n = 96$)	*P* value
Age (years [mean ± S.D.])	69.0 ± 10.1	67.0 ± 12.1	0.25*
Patient sex			
Male	48 (52)	45 (48)	0.01†
Female	24 (32)	51 (68)	
Tumour site			
Rectum	26 (43)	35 (57)	0.96†
Colon	46 (43)	61 (57)	
Tumour size (cm [mean ± S.D.])	5.2 ± 2.0	5.2 ± 2.3	0.99*
Tumour differentiation			
Well	8 (100)	0	
Moderate	60 (40)	90 (60)	0.004†
Poor	4 (40)	6 (60)	

Figures in parentheses represent percentages. *Student's *t*-test. †Chi-square test.

Table 2. Univariate survival analysis of clinical and pathological features in 168 patients with T3,N0,M0 colorectal cancer

	Number of cases	% survival at 8 years	*P* value (logrank test)
Age			
<75 years	121	56.6	0.50
≥75 years	47	53.5	
Sex			
Male	93	58.1	0.95
Female	75	53.2	
Tumour site			
Rectum	61	56.0	0.97
Colon	107	56.2	
Tumour size			
<5 cm	71	54.6	0.91
≥5 cm	97	56.7	
Histological grade			
Well	8	75.0	
Moderate	150	54.2	0.48
Poor	10	60.0	
Ploidy status			
Diploid	72	69.7	0.007
Non-diploid	96	46.4	

and that a corresponding G2M peak could be identified. The coefficient of variation (CV) was recorded in all cases. A CV <10% was required on the tumour cell histogram to include a case in the study. If a low CV (<5%) was achieved, the ranges of ploidy were narrowed as follows: diploid, 0.85–1.15; tetraploid, 1.85—2.15; aneuploid, all other values with the same requirements of percentage population. For the purpose of analysis, aneuploid and tetraploid cases were combined as "non-diploid" tumours.

Statistical analysis

The chi-square test and Student's *t*-test were used to assess categorical and continuous data respectively. Survival curves were generated by the Kaplan–Meier method with cancer-related death as the endpoint. Differences in survival between groups were determined by logrank analysis. The Cox proportional hazards model was used to calculate hazard rate

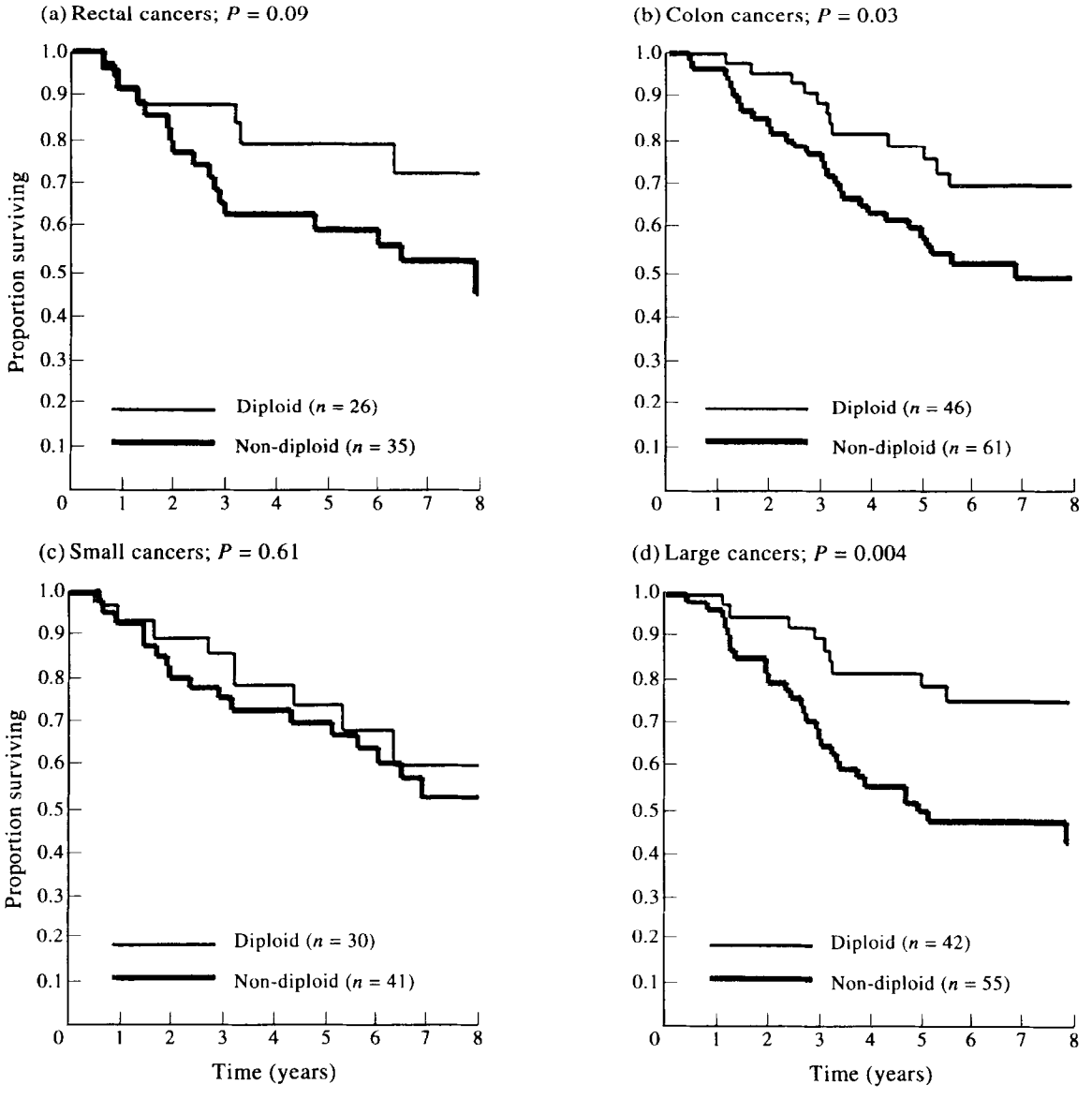


Figure 3. Subgroup survival of 168 patients with colorectal cancer stratified by ploidy status. (a) Rectal cancer (relative risk for aneuploid tumours (RR) 2.21; 95% confidence intervals (95% CI) 0.87–5.62). (b) Colon cancer (RR 1.97; 95% CI 1.01–3.87). (c) Small (< 5 cm) tumours (RR 1.24; 95% CI 0.55–2.79). (d) Large (≥ 5 cm) tumours (RR 2.88; 95% CI 1.36–6.09).

ratios (relative risk of death) and 95% confidence intervals, and to perform regression analyses.

RESULTS

It was possible to assess the DNA index in all 168 tumour specimens. 72 cases (43%) were classified as diploid and the remaining 96 (57%) as non-diploid. The DNA index distribution of non-diploid cases is shown in Figure 1.

Sixty-eight per cent of females had non-diploid tumours compared with 48% of males ($P = 0.01$). All eight well differentiated tumours were diploid in nature ($P = 0.004$) (Table 1). Ploidy status was unrelated to patient age ($P = 0.25$), tumour site (0.96) or tumour size (0.99).

The survival of the entire study population was 67% at 5 years and 56% at 8 years. No patient suffered a cancer-related death after this time. Survival analysis showed that long-term outcome was significantly better for diploid compared with non-diploid cases with 8 year survival rates of 70% and 46%, respectively (Figure 2) (logrank test; $P = 0.008$). Other clinical or pathological features were unrelated to long-term outcome (Table 2). A Cox's regression analysis including all clinical and pathological variables was performed to determine features independently related to outcome. Ploidy status was the only significant variable to emerge from this analysis (relative risk of death for non-diploid cases 2.1; 95% confidence intervals 1.2–3.5). A secondary survival analysis with all causes of mortality was performed to correct for any potential bias in the reporting of deaths. Non-diploidy was again identified as a poor prognostic feature in this secondary analysis (logrank test; $P = 0.02$).

We performed additional survival analysis with the patient population broken down by tumour site and tumour size, and the result is shown in Figure 3. The survival advantage conferred by diploidy was found both in colon ($P = 0.03$) and rectal ($P = 0.09$) cancers and was greatest in large (≥5 cm) tumours ($P = 0.004$). The estimated 8 year survival rate for

patients with large diploid tumours was 76% compared with 44% for those with large non-diploid tumours.

DISCUSSION

Although over 60 years old, the Dukes' system [21] and its many modifications remain of primary importance in colorectal cancer staging. Since the introduction of Dukes' original system, authors have subdivided stage A and C patients into smaller groups, while a stage D has been included for those with unresectable disease or distant metastases. The logical conclusion is the TNM system, in which distinct stages can be derived from a study of tumour depth and the presence or absence of nodal and distant metastases [22]. Even so, the survival of individuals within the different TNM stages tends to be variable, especially in the T3,M0,N0 group and new prognostic indicators would be valuable in identifying patients at high risk of tumour recurrence and death in this group. The results of this study suggest that measurement of tumour DNA content by image analysis might improve our ability to predict outcome within this disease stage.

Some authors have found an almost perfect correlation between DNA indices studied by both image analysis and flow cytometry in different tumour types [23, 24], although assessment of tumour DNA by flow cytometric methods may be difficult when excess diploid stromal elements are present [25]. Flow cytometry has the advantage of allowing the DNA content of large numbers of cells to be rapidly assessed. In contrast, image analysis is a relatively time consuming and labour intensive procedure which does, however, allow one to distinguish neoplastic cells from cellular debris, stromal and inflammatory cells. These can then be excluded from further analysis. We used image analysis in our study to ensure that only tumour cells were examined and because a previous study by Bosari and associates suggested that ploidy status defined by image analysis might be more valuable in predicting outcome in patients with stage II (T3/4,N0,M0) disease than ploidy status determined by flow cytometry [26].

The long-term survival rate for patients with non-diploid tumours measured by flow cytometry in paraffin-embedded tissues is reported to range from 19 to 68%, while comparable survival rates for diploid cases range from 44 to 100% [3–15]. However, the relationship between ploidy status and survival is thought to be tumour stage dependent [27], with little or no association found in patients with residual or metastatic disease [4]. In contrast, a stronger association between ploidy status and outcome is found in curative cases extending beyond the bowel wall with or without nodal metastases (T3–4,N0–3,M0, stage II/III) measured either by flow cytometry [7, 10, 11, 13] or image cytometry [26, 28]. Four studies in addition to ours have examined the relationship between survival and ploidy status of colorectal cancer measured by image analysis. Albe and associates and Bosari and colleagues identified tumour diploidy as a favourable prognostic indicator [26, 28] while Enblad and coworkers, in a study of distal colorectal tumours, also found a trend towards improved survival for patients with diploid cancers [29]. In contrast, Bottger and associates found that outcome was unrelated to DNA content [30], although their analysis did not look specifically at early disease. The results of our study, confined to the T3,N0,M0 group of patients, indicate a significant survival advantage for diploid cases and agree closely with those of Albe and colleagues and Bosari and coworkers who found that image analysis was a particularly valuable prognostic indicator in early disease [26, 27].

We performed subgroup analyses stratified by tumour site and tumour size which showed that DNA content was capable of predicting prognosis in both colon and rectal cancers. It also showed that, although a similar proportion of small and large cancers displayed non-diploidy (58 and 57% respectively), long-term survival was only related to ploidy status in small tumours. This finding has not apparently been previously reported and it is unclear why ploidy status is such an unreliable indicator of outcome in small T3,N0,M0 cancers. These small tumours are clearly capable of penetrating the bowel wall at a relatively early stage in their development, and it is possible that other factors such as the occurrence of specific genetic mutations are more important than abnormal DNA content in their further progression [31]. Sampling error leading to the analysis of non-cancer cells at the periphery of small tumours, with consequent false positive diploidy in these cases, might account for the lack of prognostic information if this study had been performed using flow cytometric methods, but such normal diploid stromal, inflammatory and mucosal cells are excluded from image analysis.

In conclusion, this study was performed to test the hypothesis that ploidy status measured by image analysis might be useful in assigning T3,N0,M0 colorectal cancer patients to low and high-risk subgroups. Overall, the results suggest that useful prognostic information can be gained from image analysis in this group, especially in those cases greater than 5 cm in diameter.

1. Hedley DW, Friedlander ML, Taylor IW, Rukk CA, Musgrove EA. Method for analysis of cellular DNA content of paraffin embedded pathological material using flow cytometry. *J Histochem Cytochem* 1983, **31**, 1333–1335.
2. Wolley RC, Schreiber K, Koss LG, Karas M, Sherman A. DNA distribution in human colorectal carcinomas and its relationship to clinical behaviour. *J Natl Cancer Inst* 1982, **69**, 15–22.
3. Armitage NC, Robins RA, Evans DV, *et al.* The influence of tumour DNA abnormalities in colorectal cancer. *Br J Surg* 1985, 72, 828–830.
4. Kokal W, Sheibani K, Terz J, Harada R. Tumour DNA content in the prognosis of colorectal carcinoma. *JAMA* 1986, **255**, 3123–3127.
5. Quirk P, Dixon M, Clayden A, *et al.* Prognostic significance of DNA aneuploidy and cell proliferation in rectal adenocarcinoma. *J Pathol* 1987, **151**, 285–291.
6. Bauer KD, Lincoln S, Vera-Roman J, *et al.* Prognostic implications of proliferaive activity and DNA aneuploidy in colonic adenocarcinomas. *Lab Invest* 1987, **57**, 329–335.
7. Schutte B, Reynders M, Wiggers T, *et al.* Retrospective analysis of the prognostic significance of DNA content and proliferative activity in large bowel carcinoma. *Cancer Res* 1987, **47**, 329–335.
8. Goh HS, Jass JR, Atkin WS, Cuzick J, Northover JMA. Value of flow cytometric determination of ploidy as a guide to prognosis in rectal cancer: a multivariate analysis. *Int J Colorectal Dis* 1987, **2**, 17–21.
9. Rognum TO, Thorud E, Lund E. Survival of large bowel carcinoma patients with different DNA ploidy. *Br J Cancer* 1987, **56**, 633–636.
10. Jones DJ, Moore M, Schofield PF. Prognostic significance of DNA ploidy in colorectal cancer: a prospective flow cytometric study. *Br J Surg* 1988, 75, 28–33.
11. Visscher DW, Zarbo RJ, Ma CK, Sakr WA, Crissman JD. Flow cytometric DNA and clinicopathologic analysis of Dukes' A and B colonic adenocarcinomas: a retrospective study. *Modern Pathol* 1990, **3**, 709–712.

12. Giaretti W, Danova M, Geido E, *et al.* Flow cytometric DNA index in the prognosis of colorectal cancer. *Cancer* 1991, **67**, 1921–1927.
13. Witzig T, Loprinzi C, Gonchoroff N, *et al.* DNA ploidy and cell kinetic measurements as predictors of recurrence and survival in stages B2 and C colorectal adenocarcinomas. *Cancer* 1991, **68**, 879–888.
14. Wiggers T, Arends J, Schutte B, Volovics L, Bossman F. A multivariate analysis of pathologic prognostic indicators in large bowel cancer. *Cancer* 1988, **61**, 386–395.
15. Fisher E, Siderits R, Sass R, Fisher B. Value of assessment of ploidy in rectal cancers. *Arch Pathol Lab Med* 1989, **113**, 525–528.
16. Chapuis PH, Dent OF, Fisher R, *et al.* A multivariate analysis of clinical and pathological variables in prognosis after resection of large bowel cancer. *Br J Surg* 1985, **72**, 698–702.
17. Swiss Group for Clinical Cancer Research (SAKK). Long-term results of single course of adjuvant intraportal chemotherapy for colorectal cancer. *Lancet* 1995, **345**, 349–353.
18. International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators. Efficacy of adjuvant fluorouracil and folinic acid in colon cancer. *Lancet* 1995, **345**, 939–944.
19. Bauer KD, Clevenger CV, Endow RK, Murad T, Epstein AL, Scarpelli DG. Simultaneous nuclear antigen and DNA content quantification using paraffin embedded tissue and multiparameter cytometry. *Cancer Res* 1986, **46**, 2428–2434.
20. Fausel RE, Burleigh W, Kaminsky DB. DNA quantification in colorectal carcinoma using flow and image analysis cytometry. *Analyt Quant Cyto Histol* 1990 **12**, 21–27.
21. Dukes CE. The classification of cancer of the rectum. *J Pathol Bacteriol* 1932, **35**, 323–332.
22. Sobin LH, Hermanek P, Hutter RVP. TNM classification of malignant tumours. *Cancer* 1988, **61**, 2310–2314.
23. Cope C, Rowe D, Delbridge L, Philips J, Friedlander M. Comparison of image analysis and flow cytometric determination of cellular DNA content. *J Clin Pathol* 1991, **44**, 147–151.
24. Cornelisse CJ, deKoning HR, Moolenaar AJ, *et al.* Image and flow cytometric analysis of DNA content in breast cancer. *Analyt Quant Cytol Histol* 1984, **6**, 9–18.
25. Bose KK, Allison DC, Hruban RH, *et al.* A comparison of flow cytometric and absorption cytometric DNA values as prognostic indicators for pancreatic carcinoma. *Cancer* 1993, **71**, 691–700.
26. Bosari S, Lee AKC, Wiley BD, Heatley GJ, Silverman ML. Flow cytometric and image analyses of colorectal adenocarcinomas: a comparative study with clinical correlations. *Am J Clin Pathol* 1993, **99**, 187–194.
27. Bauer KD, Bagwell CB, Giaretti W, *et al.* Consensus review of the clinical utility of DNA flow cytometry in colorectal cancer. *Cytometry* 1993, **14**, 486–491.
28. Albe X, Vassilakos P, Helfer-Guarnori K, *et al.* Independent prognostic value of ploidy in colorectal cancer: a prospective study using image cytometry. *Cancer* 1990, **66**, 1168–1175.
29. Enblad P, Glimelius B, Bengtsson A, Ponten J, Pahlman L. The prognostic significance of DNA content in carcinoma of the rectum and rectosigmoid. *Acta Chir Scand* 1987, **153**, 453–458.
30. Bottger TC, Potratz D, Stockle M, Wellek S, Klupp J, Junginger T. Prognostic value of DNA analysis in colorectal carcinoma. *Cancer* 1993, **72**, 3579–3587.
31. Laurent-Puig P, Olschwang S, Delattre O, *et al.* Survival and acquired genetic alterations in colorectal cancer. *Gastroenterology* 1992, **102**, 1136–1141.