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Prognostic impact of genomic instability in colorectal cancer

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Background: The prognostic impact of an indication of chromosomal instability (CIN) is evaluated in a consecutive series of 952 colorectal cancer patients treated at Aker University Hospital, Norway, during 1993–2003. Microsatellite instability (MSI) in this case series has recently been reported and made it possible to find the co-occurrence and compare the prognostic significance of CIN and MSI.

Methods: Data sets for overall survival (OS; $n=855$) and time to recurrence (TTR; $n=579$) were studied. To reveal CIN we used automated image cytometry (ICM). Non-diploid histograms were taken as indicative of the presence of CIN. PCR-based measures of MSI in this material have already been described.

Results: As with MSI, CIN was found to be an independent predictor of early relapse and death among stage II patients (TTR: $n=278$: HR 2.19 (95% CI: 1.35–3.55), $P=0.002$). Of the MSI tumours (16%), 71% were found to be DNA diploid, 21% were DNA tetraploid and 8% were DNA aneuploid. Among microsatellite stable tumours, 24% were DNA diploid, 15% were DNA tetraploid and 61% were DNA aneuploid.

Conclusion: For patients presenting with stage II disease, genomic instability as detected by DNA image cytometry has the potential to provide a useful biomarker for relapse and cancer-related death following surgery with curative intent.

For primary adenocarcinomas of the colon or rectum, tumour stage is still the best predictor of survival after resection. However, among patients diagnosed with UICC stage II (pT3–pT4, N0, M0) colorectal cancer (CRC), up to 35% will experience recurrence (Staib *et al*, 2002; Jemal *et al*, 2004). Several molecular and gene-expression profiling biomarkers have been proposed as markers of poor prognosis in stage II patients, but none has yet been validated in a large-scale prospective clinical trial (Barrier *et al*, 2007; Gangadhar and Schilsky, 2010; Ågesen *et al*, 2012). We report on the stage-dependent prognostic significance of chromosomal instability (CIN) and microsatellite instability (MSI) in a large consecutive series of colorectal carcinomas treated in one hospital with a defined catchment area.

DNA aneuploidy, an accepted marker for CIN, is found in the majority of sporadic CRC and has been linked to poor prognosis (Lengauer *et al*, 1998; Mouradov *et al*, 2013). Two recent meta-analyses of 67 separate studies demonstrated poorer overall survival (OS) for patients with stage II–III colorectal tumours showing CIN (HR 1.45 (1.33–1.55)) (Araujo *et al*, 2007; Walther *et al*, 2008).

Microsatellite instability is a marker for relatively good prognosis in CRC, also supported by meta-analysis (Guastadisegni *et al*, 2010).

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In this study we have had access to data from a recent PCR study of the occurrence of MSI in the present material (Merok *et al*, 2013).

Traditionally, CIN and MSI have been seen as different and complementary pathways to CRC. However, studies including other biomarkers have shown that the picture is more complicated (Toyota *et al*, 1999; Hawkins *et al*, 2001; Domingo *et al*, 2013). This is the first study to examine the stage-related prognostic impacts of CIN and MSI together in one large consecutive patient series.

MATERIAL AND METHODS

Patient data. This study is based on consecutive primary CRCs referred to Aker University Hospital in Oslo, Norway, from 1993 to 2003. The series comprised 1274 patients out of which 952 underwent major resection. Median age at presentation was 74 (19–96) years. Details of preoperative investigations, operative procedures, histopathological examinations, patient follow-up and mortality data have been reported previously (Nesbakken *et al*, 2002; Sjo *et al*, 2008).

Exclusions. We excluded 44 patients who died of post-operative complications and 53 cases where the nuclear monolayer or DNA content histograms were unsatisfactory, resulting in a data set containing crude OS, clinical data and DNA content histograms for 855 colorectal adenocarcinomas at stages I–IV.

Recurrence analysis. For time to recurrence (TTR) analysis, a further restricted data set ($n = 579$) was prepared by eliminating patients presenting with metastatic disease, residual cancer ($R > 0$), unknown cause of death or synchronous lesions (Punt *et al*, 2007). Median patient age at surgery was 73 years (30–94).

Tumour location. Tumours located proximal to the splenic flexure were considered as proximal (right), while tumours located at or distal to the splenic flexure were considered to be distal (left). Supplementary Table S1 shows the anatomical distribution of tumours in the TTR data set.

Tissue processing and DNA ploidy measurement. Automated image-based DNA cytometry was performed as previously described (Kristensen *et al*, 2003). Nuclear DNA content histograms were classified as DNA diploid, DNA tetraploid or DNA aneuploid. Non-diploid histograms were taken as indicative of the presence of CIN.

Microsatellite instability assessment. Details of PCR MSI determination in this material were recently described (Merok *et al*, 2013).

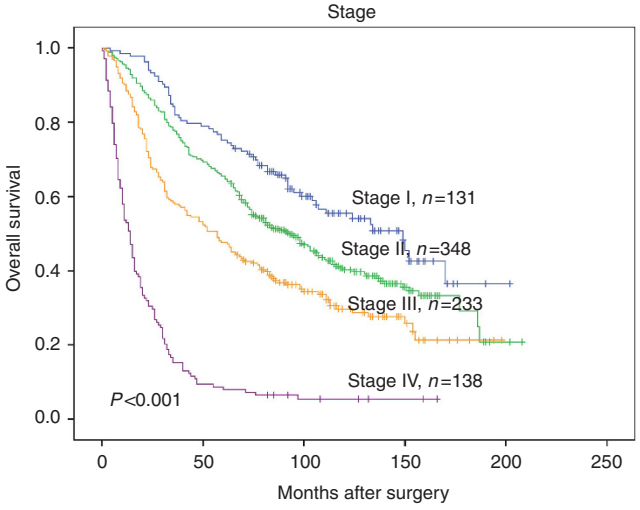


Figure 1. Kaplan–Meier overall survival for patients with colorectal cancer stage I-IV.

Statistical analysis. Overall survival was computed in months from the date of surgery until death or May 2010. Death from any cause was registered as an event (data from the Norwegian Death Registry). Living patients were censored at the end of the study (mean follow-up: 69 months). In analyses of TTR, local or distant recurrence or death from CRC were registered as events, and patients were censored at death from other causes or at study closure.

Significance of associations between categorical variables was evaluated using Fisher's exact test. The Mantel–Cox log-rank test was used for univariate analysis. For multivariate evaluation of

Table 1. Clinicopathological data for the time to recurrence data set

	All tumours		DNA diploid		DNA non-diploid	
Variables	***n***	**%**	***n***	**%**	***n***	**%**
Relapse	220	38.0	55	30.1	165	41.7
No relapse	359	62.0	128	69.9	231	58.3
MSI status						
MSS	452	83.9	108	63.5	344	93.2
MSI	87	16.1	62	36.5	25	6.8
Age at surgery (years)						
⩽72	289	49.9	92	50.3	197	49.7
>72	290	50.1	91	49.7	199	50.3
Gender						
Male	289	49.9	75	41.0	214	54.0
Female	290	50.1	108	59.0	182	46.0
Stage						
I	112	19.3	37	20.2	75	18.9
II	278	48.0	96	52.5	182	46.0
III	189	32.6	50	27.3	139	35.1
pT status						
pT1	27	4.7	10	5.5	17	4.3
pT2	103	17.8	32	17.5	71	17.9
pT3	415	71.7	135	73.8	280	70.7
pT4	34	5.9	6	3.3	28	7.1
pN status						
N0	389	67.3	133	72.7	256	64.8
N1	152	26.3	41	22.4	111	28.1
N2	37	6.4	9	4.9	28	7.1
Grade						
H (well diff.)	58	10.0	19	10.4	39	9.8
M (moderately diff.)	453	78.2	127	69.4	326	82.3
L (poorly diff.)	63	10.9	34	18.6	29	7.3
Mucinous diff.	5	0.9	3	1.6	2	0.5
Location						
Proximal colon	227	39.2	90	49.2	137	34.6
Distal colon	179	30.9	48	26.2	131	33.1
Rectum	173	29.9	45	24.6	128	32.3
Type of surgery						
Elective	519	89.6	171	93.4	348	87.9
Acute	60	10.4	12	6.6	48	12.1

Abbreviations: diff. = differentiated; H = high; L = low; M = moderate.

prognostic impact we used a Cox proportional hazards regression model. In multivariate models including CIN status we excluded MSI due to the high correlation between the two variables. Separate analysis of the prognostic impact of CIN in the first and second 5-year periods supported validity of the proportional hazard assumption. All statistics were generated using SPSS 20.0 (IBM Corporation, Armonk, NY, USA). Statistical significance was defined as two-sided $P<0.05$.

The study was performed according to the Helsinki Declaration and approved by the Norwegian Regional Committees for Medical Research (REK; #1.2005.1629).

RESULTS

Overall survival. Of 855 patients, 291 were alive at the end of follow-up (mean 69 months). Figure 1 shows Kaplan–Meier OS curves for all stages.

Chromosomal instability and MSI status were strongly negatively associated ($P<0.001$) such that individual tumours were likely to show either MSI or CIN.

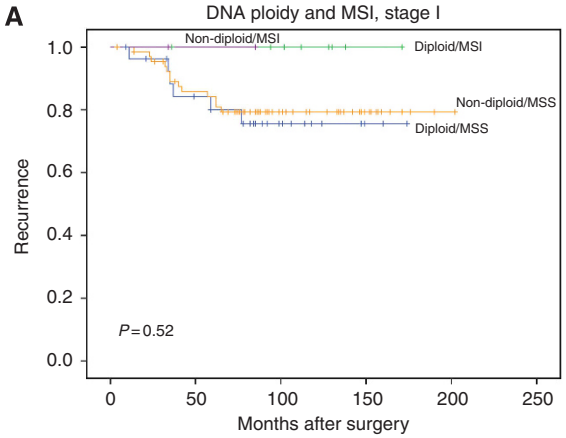
Supplementary Table S2 shows the results of univariate and multivariate analyses on prognostic factors and OS. Chromosomal instability was a significant prognostic marker in patients with stage II disease in a Cox regression model that included age, grade, R-status and type of surgery ($n=348$; HR 1.46 (1.06–1.99), $P=0.019$). Probably because of the high percentage of stage II patients overall, CIN also emerged as a significant predictor of survival in multivariate Cox regression analysis for the entire patient cohort ($n=855$; HR 1.24 (1.02–1.52, $P=0.031$).

Microsatellite instability was borderline significant as predictor of univariate improved OS in stage II ($P=0.055$), but not in stage III. Microsatellite instability was a significant predictor of improved OS in the entire stage I–IV cohort ($P=0.007$) (Supplementary Table S2). In line with previous reports, MSI tumours were predominantly DNA diploid and located proximally,

Table 2. Time to recurrence related to prognostic factors for stage I–III and stage II colorectal cancer patients

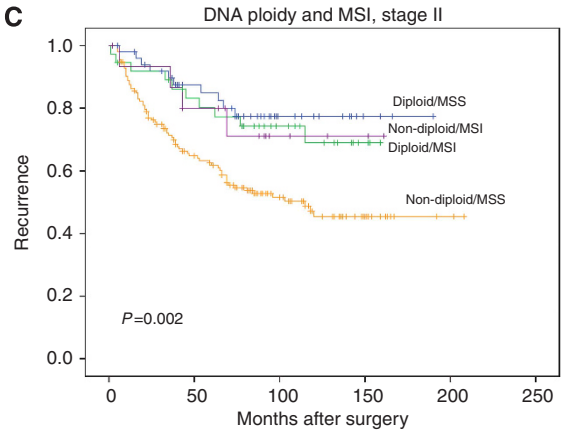
	Univariate analysis all				Univariate analysis stage II				Cox multivariate analysis all			Cox multivariate analysis stage II		
Variables	No of pts	5-y TTR	10-y TTR	*P*-value	No of pts	5-y TTR	10-y TTR	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age at surgery (years)				0.003				0.060			0.003			
⩽72	289	0.73	0.64		130	0.75	0.65		Ref					
>72	290	0.62	0.49		148	0.67	0.50		1.51	1.15–1.98				
DNA ploidy				0.006				0.001			0.007			0.002
Diploid	183	0.74	0.66		96	0.83	0.74		Ref			Ref		
Non-diploid	396	0.64	0.53		182	0.65	0.49		1.56	1.13–2.15		2.19	1.35–3.55	
MSI status				0.097				0.049						
MSS	452	0.65	0.54		205	0.67	0.53							
MSI	87	0.72	0.64		53	0.80	0.69							
Stage				<0.001							<0.001			
I	112	0.85	0.81						Ref					
II	278	0.71	0.58						2.01	1.22–3.32				
III	189	0.52	0.41						3.88	2.36–6.39				
Grade				0.002				0.986			0.001			
H (well diff.)	58	0.78	0.74		16	0.69	0.69		Ref					
M (moderately diff.)	453	0.69	0.58		227	0.71	0.58		1.45	0.84–2.52				
L (poorly diff.)	63	0.52	0.40		31	0.73	0.56		2.76	1.46–5.22				
pT stage				<0.001				0.275						
pT1	27	0.88	0.88											
pT2	103	0.82	0.77											
pT3	415	0.64	0.52		261	0.71	0.59							
pT4	34	0.51	0.34		17	0.65	0.49							
Gender				0.953				0.770						
Male	289	0.67	0.57		138	0.72	0.58							
Female	290	0.68	0.57		140	0.70	0.58							
Location				0.723				0.483						
Proximal colon	227	0.68	0.54		129	0.77	0.58							
Distal colon	179	0.65	0.56		82	0.67	0.58							
Rectum	173	0.70	0.62		67	0.66	0.60							
Type of surgery				<0.001				0.001			0.001			0.006
Elective	519	0.70	0.59		244	0.73	0.61		Ref			Ref		
Acute	60	0.48	0.36		34	0.53	0.38		1.84	1.27–2.69		2.04	1.23–3.38	

Abbreviations: CI = confidence interval; diff. = differentiated; H = high; HR = hazard ratio; L = low; M = moderate; No = number; pts = patients; Ref = reference; TTR = time to recurrence; y = year.



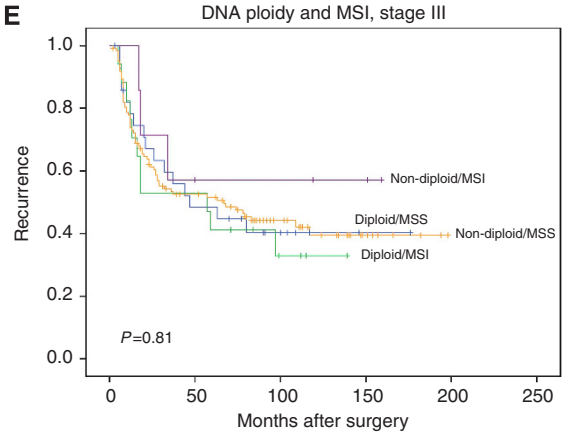
B

	Diploid	Tetraploid	Aneuploid
MSS	28	13	54
MSI	8	1	1



D

	Diploid	Tetraploid	Aneuploid
MSS	51	35	119
MSI	37	13	3



F

	Diploid	Tetraploid	Aneuploid
MSS	29	22	101
MSI	17	4	3

G

Stage	Phenotype	HR	95% CI
I	MSS/non-diploid	1	
	MSS/diploid	1.18	0.45–3.1
	MSI/non-diploid	NA	NA
	MSI/diploid	NA	NA
II	MSS/non-diploid	1	
	MSS/diploid	0.36	0.18–0.69
	MSI/non-diploid	0.48	0.17–1.30
	MSI/diploid	0.47	0.24–0.90
III	MSS/non-diploid	1	
	MSS/diploid	1.00	0.58–1.72
	MSI/non-diploid	0.63	0.20–2.01
	MSI/diploid	1.18	0.62–2.23

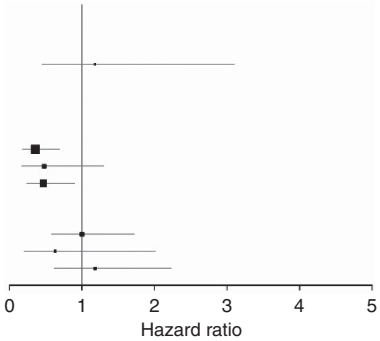


Figure 2. Kaplan–Meier plots of time to recurrence among patients presenting with stage I (**A**), stage II (**C**) and stage III (**E**) tumours. Each panel shows relapses for four separate categories of tumour phenotype: diploid MSI (green line), non-diploid MSI (purple line), diploid MSS (blue line) and non-diploid MSS (orange line). To the right of each panel is a table (**B**, **D** and **F**) showing the distribution of DNA ploidy status categories among MSI and MSS tumours in that tumour stage. A forest plot (**G**) summarises the effect of MSI/CIN in the different stages.

whereas the microsatellite stable (MSS) tumours were predominantly DNA non-diploid and distal.

Recurrence. Of the 579 patients in the TTR data set, 220 (38%) experienced recurrence (153) or died of CRC (67) after a median of 23 months (range 1–120). In stage II disease the 10-year estimated recurrence rate was 42% (Table 2). Clinicopathological features of the TTR data set are summarised in Table 1.

CIN and recurrence. Analysis for prognostic factors in stage II cases alone and in the entire TTR data set are shown in Table 2. As in the OS analysis, CIN status was found to be significant in univariate ($P=0.001$) and multivariate analysis in stage II that included age, three levels of histology grade and emergency *vs* elective surgery, (HR 2.19 (1.35–3.55), $P=0.002$). In the stage II TTR cohort, the independent prognostic factors for recurrence were CIN and urgency of surgery. Among the stage II patients with CIN ($n=182$) 43% experienced recurrence, compared with 22% among DNA diploid stage II patients ($n=96$).

In stage I tumours, we observed a similar trend as for stage II, but this difference failed to reach statistical significance, due to small patient numbers. Among stage III CRCs, no prognostic impact of CIN was found (Figure 2). This pattern was similar for both colon and rectal stage III cancers (Figure 3).

Taking stages I–III together, CIN continued to be a significant prognostic factor in univariate analysis ($P=0.006$) and it retained significance as an independent prognostic factor in Cox multivariate analysis (HR 1.56 (1.13–2.15), $P=0.007$).

In order to investigate the effect of heterogeneity for CIN between the three stage groups we included the interaction term CIN*stage in the Cox regression model and found that both the interaction term and stage were significant in the model, while CIN was not. We may interpret this as stage having an effect regardless of CIN status, while the effect of CIN is different in the three stage groups. The prognostic effect of CIN in the three stage groups is illustrated in Figure 4.

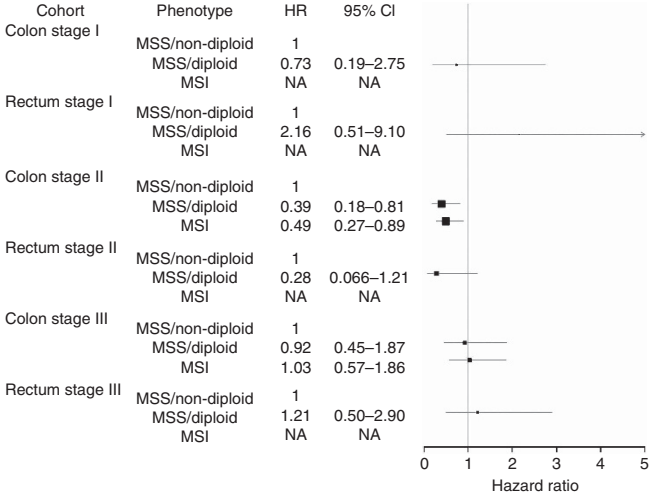


Figure 3. Forest plot of effect of CIN for colon and rectal tumour stages I–III.

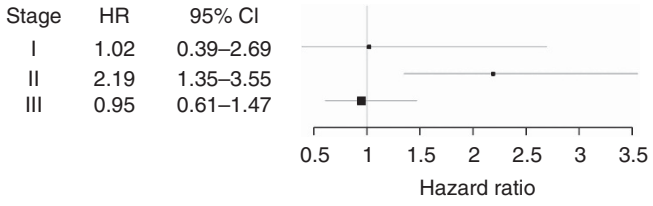


Figure 4. Forest plot of effect of CIN in stages I–III colorectal tumours.

MSI and recurrence. The positive prognostic impact of MSI among stage II, but not stage III, colorectal tumours from the present material has already been reported (Merok *et al*, 2013). In our TTR data set, MSI was borderline significant ($P=0.049$) in univariate analysis, but not significant ($P=0.094$) in multivariate analysis in stage II (Supplementary Table S3).

Figure 2 shows Kaplan–Meier plots for relapse of the different combinations of MSI and CIN status as stratified by stage. Tables in the same figure show how the relationship between MSI/MSS and DNA ploidy status was dependent on stage. There were no relapses among the 10 patients with stage I MSI tumours. Fifty-three patients presented with stage II MSI tumours, of which 37 were diploid, 3 were aneuploid and 13 were tetraploid. The relatively good prognosis of the 104 MSI and diploid MSS stage II lesions contrasted with the poor prognosis of 154 patients with the CIN-MSS phenotype. Patients with stage III tumours had a uniformly high level of relapse that was independent of both MSI and CIN.

Figure 3 compares the stage-dependent prognostic impact of MSI with that of CIN in the colon and rectum. Among stage II lesions in both locations non-diploid MSS tumours show a significantly increased probability of relapse. As reported elsewhere, MSI tumours were rare in the rectum (5 of 173 tumours). In the colon, diploid MSS tumours had similar relapse rates to MSI tumours.

In total, 133 (25%) of the patients did not follow the diploid/MSI–non-diploid/MSS pattern.

DISCUSSION

This study is the first to report survival data in relation to CIN/MSI after stratifying according to tumour stage. In fact few survival studies have measured CIN and MSI in the same resection specimens. Supplementary Table S4 summarises some relevant findings from eight studies as well as from the present report. Despite the often quoted 'typical incidence' figures of 85% CIN and 15% MSI in CRC, the incidence of CIN varied between 35–70% in these studies, while MSI varied from 5–23% and tumours showing neither CIN nor MSI were common (19–52%). In the light of the present finding of a marked stage-specific difference in the prognostic impact of both CIN and MSI, the variability between results in Supplementary Table S4 can be seen as following inclusion of different proportions of stage I–IV material in the different studies.

Twenty-five per cent of the stage II cancers with MSI in this study were DNA tetraploid, and these carried good prognosis equal to that of the other MSI tumours (Figure 2). The biological basis of this difference from MSS tetraploidy remains to be clarified.

There are advantages and disadvantages with all end points. For OS, non-cancer-related deaths add variability. This is to some degree taken into account by censoring at 5 years after surgery. With TTR, inclusion of cancer-related deaths is controversial as this is often hard to demonstrate. However, the main conclusions regarding CIN and MSI remain the same regardless of end point, although *P*-values and hazard ratios change to some degree. Supplementary Figure S1 illustrates this for CIN in the data sets representing both OS and TTR end points. For direct comparison with the previously published work on MSI status in this material we include figures in Supplementary Figure S1 for the prognostic impact of CIN using the 5-year relapse free survival (RFS) model employed by Merok *et al* (2013). Interestingly, the 5-year RFS *P*-values for CIN status are higher than the corresponding *P*-values for OS and TTR. A possible explanation is that CIN has a

continuing prognostic impact after 5 years, which is not taken into account with the 5-year end point.

We report that stage II tumours with MSI have very similar prognosis to diploid MSS lesions. CIN occurred more often than MSI, and both CIN and MSI were confirmed in our TTR analysis as independent predictors of recurrence in stage II CRC. Although rarely occurring outside of the proximal colon, MSI is currently seen as a clinically useful predictor of good outcome for patients with stage II CRC. This is supported by positive results from retrospective trials such as the present study in which multivariate analysis shows MSI to have significant predictive value. But from the point of view of an individual patient, the clinical picture is less than ideal – we found MSI in only 51 of the stage II resections in this study, and 13 of these patients went on to experience recurrence. As for CIN, our results support the present consensus that CIN is also an independent prognostic marker in stage II CRC, and possibly a more useful marker than MSI (Sinicrope *et al*, 2006; Mouradov *et al*, 2013). But again, from the individual patient's point of view CIN is less than ideal. Of the 182 stage II resections with CIN in the TTR cohort, 75 were associated with relapse, while there were 23 recurrences among the 96 patients with diploid resections. Adding CIN to the MSI results produced only a small improvement in prediction for relapse. Can the clinical usefulness of DNA cytometry be improved? This remains a very promising area for research. Here we have used the most robust binary DNA histogram classification (CIN or DNA diploid), but it is clear that a great deal more information still remains to be extracted from the Feulgen images of tumour nuclei (Dunn *et al*, 2011).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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