DNA content analysis of colorectal cancer defines a distinct ‘microsatellite and chromosome stable’ group but does not predict response to radiotherapy

Wakkas Fadhil*, Karin Kindle*, Darryl Jackson*, Abed Zaitoun†, Nina Lane‡, Adrian Robins‡ and Mohammad Ilyas*†

*Division of Pathology, School of Molecular Medical Sciences, Queen’s Medical Centre, University of Nottingham, Nottingham, UK, †Department of Histopathology, Queen’s Medical Centre, Nottingham University Hospitals NHS Trust, Nottingham, UK and ‡Division of Immunology, School of Molecular Medical Sciences, Queen’s Medical Centre, University of Nottingham, Nottingham, UK

SUMMARY

Colorectal cancers (CRC) are thought to have genetic instability in the form of either microsatellite instability (MSI) or chromosomal instability (CIN). Recently, tumours have been described without either MSI or CIN, that is, microsatellite and chromosome stable (MACS) CRCs. We investigated the (i) frequency of the MACS-CRCs and (ii) whether this genotype predicted responsiveness to neoadjuvant chemoradiotherapy. To examine the frequency of MACS-CRCs, DNA content (ploidy) was examined in 89 sporadic microsatellite-stable CRCs using flow cytometry. The tumours were also screened for mutations in KRAS/BRAF/TP53/PIK3CA by QMPCR. To examine the value of tumour ploidy in predicting response to chemoradiotherapy, DNA content was tested in a separate group of 62 rectal cancers treated with neoadjuvant chemoradiotherapy. Fifty-one of 89 CRCs (57%) were aneuploid and 38 (43%) were diploid. There was no significant association between mutations in TP53/KRAS/BRAF/PIK3CA and ploidy. Testing of association between mutations revealed only mutual exclusivity of KRAS/BRAF mutation (P < 0.001). Of the 62 rectal cancers treated with neoadjuvant chemoradiotherapy, 22 had responded (Mandard tumour regression grade 1/2) and 40 failed to respond (Grade 3–5). Twenty-five of 62 (40%) tumours were diploid, but there was no association between ploidy and response to therapy. We conclude that MACS-CRCs form a significant proportion of microsatellite-stable CRCs with a mutation profile overlapping that of CRCs with CIN. A diploid genotype does not, however, predict the responsiveness to radiotherapy.

Keywords
colorectal cancer, genomic instability, tumour ploidy, radiation

The role of genomic instability in the development of cancer is a contentious issue. Some have argued that onset of genomic instability, through mutation of ‘caretaker’ genes which are responsible for the maintenance of genomic integrity, results in an increased mutation rate, and this is essential to the carcinogenetic process (Levitt & Hickson 2002). Others, however, have argued that tumour evolution through waves of mutation-driven clonal expansion is sufficient for tumour development without the requirement of genomic instability (Sieber et al. 2003). Irrespective of its precise role in tumour development, genomic instability is very commonly seen in many different cancers and it accelerates the rate of tumour development.

Sporadic colorectal cancers (CRCs) are usually considered to have two main types of genomic instability, that is, microsatellite instability (MSI) or chromosomal instability (CIN) (Ilyas et al. 1999; Jass 2007). Colorectal cancers acquire MSI as a result of loss of mismatch repair function.
arising from mutational or epigenetic inactivation of any one of four genes – MLH1, MSH2, PMS2 and MSH6 (Thibodeau et al. 1993; Karlan 1995; Lothe 1997; Abdel-Rahman et al. 2006). Loss of mismatch repair function results in a failure to repair base-pair mismatches, and small scale insertion/deletion mutations resulting in genome-wide mutations of these types. Karyotypically, however, CRCs with MSI (MSI-CRCs) have a diploid or near-diploid DNA content (Kouri et al. 1990; Frei 1992; Curtis et al. 2000).

CIN is more or less mutually exclusive with MSI, and it is characterised by large-scale genomic changes including deletions, amplifications and gains/losses of whole chromosomes. The cause of CIN in CRCs is not known although a number of candidates such as APC, AXIN2, TP53, AURORA A and FBXW7 have been proposed (Williams et al. 1997; Fodde et al. 2001; Rajagopalan et al. 2004; Hadjipanayis et al. 2006; Baba et al. 2009). Those CRCs with CIN (CIN-CRCs) characteristically show aneuploidy on examination of DNA content (Pino & Chung 2010).

Around 10–15% of sporadic CRCs show MSI whilst the remainder are microsatellite stable and are usually considered to show CIN. However, prior to the discovery of mismatch repair deficiency and MSI, tumour DNA content was commonly evaluated and a review of these published studies shows that the proportion of diploid tumours reported varied from 11% to 64% (Heimann et al. 1990; Armitage et al. 1991; Tang et al. 1995; Flyger et al. 1999). A recent meta-analysis of these studies estimated that, of 5478 patients, 42% showed a diploid genotype (Araujo et al. 2007). If the cases with probable MSI are removed, then the data would suggest that a significant proportion of all CRCs have neither MSI nor CIN (henceforth referred to as microsatellite and chromosomal stable, MACS). This point is reinforced in studies which have exclusively tested rectal cancers. Of the tumours located at this site, only 2–3% will have MSI (Hutchins et al. 2011) but the reported frequency of diploid rectal tumours is 30–64% (Fisher et al. 1989; Heimann et al. 1990; Michelassi et al. 1992). There have been some reports of MACS-CRCs although these are relatively few (Georgiades et al. 1999; Hawkins et al. 2001; Silver et al. 2011). Despite these reports and the circumstantial evidence from earlier studies, a dogma has emerged that CRCs must have one or other form of genomic instability and recent reviews have cited the frequency of CIN as around 85% (Fearon 2011; Pritchard & Grady 2011).

The MACS-CRCs therefore may form a third group which is distinct from CRC with MSI and CIN. As well as having implications for cancer theory, this may have clinical implications. MSI-CRCs have distinct clinicopathological features including, amongst others, right-sided location and a comparatively good clinical outcome. Conversely, CIN-CRCs have a left-sided predominance and a generally poorer outcome than MSI-CRCs. As MACS-CRCs are a relatively poorly described group, their clinicopathological correlates are also poorly described.

As well as providing prognostic information, genotypic analysis of tumours may predict responsiveness to specific therapies. For example, MSI-CRCs reportedly have poor response to 5-fluoro-uracil but better response to irinotecan than their CIN counterparts (Ribic et al. 2003; Bertagnolli et al. 2009). In recent years, neoadjuvant (chemo)radiotherapy has become commonplace for rectal CRCs. Approximately 15–25% of these tumours undergo marked regression (Mandard grade 1 or 2) although currently it is impossible to predict which tumours will respond (Vecchio et al. 2005; Suarez et al. 2008; Dhadda et al. 2009, 2011). If responsiveness to radiation could be predicted with certainty, it would allow a significant proportion to patients to avoid major surgery. We hypothesised that MACS-CRCs may respond differently to radiation than CIN-CRCs. If so, tumour ploidy could be used as a predictive test for radiation responsiveness.

Given the relative paucity of knowledge about the MACS-CRCs, we firstly evaluated the DNA content and mutation profile in an unselected series of CRCs which were shown to be microsatellite stable. To test our hypothesis that MACS-CRCs and CIN-CRCs may have different responses to radiation, we then evaluated the DNA content of preradiotherapy biopsies from a series of rectal cancers and correlated tumour regression with ploidy status.

**Materials and methods**

**Sample collection**

Two sample sets were tested. Sample set 1 was used for the study to evaluate the DNA content and mutation profiles of CRCs. A total of 100 anonymised cases of sporadic CRC who underwent surgery between 2004 and 2005 were selected from the archives of the Nottingham University Hospitals Department of Histopathology. Only cases in which clinic-pathological data were available were selected, and one tumour block (containing at least 50% tumour) from each case was chosen for testing. Of these, 11 cases were excluded due to either the presence of microsatellite instability (see below) or PCR failure. The remaining 89 cases underwent DNA content analysis and mutation profiling.

Sample set 2 was used only for the study to evaluate the association between DNA content (ploidy) and regression following neoadjuvant chemoradiotherapy (CRT). A series of 62 cases were selected from a previously reported series (Dhadda et al. 2011). Of these, 22 cases had shown a marked response (i.e. Mandard tumour regression grade 1 or 2) whilst 40 cases had shown little or no response (Mandard tumour regression grade 3–5). If ploidy is associated with responsiveness to CRT, one would expect a non-random distribution of aneuploid and diploid genotypes between the responders and non-responders. The pre-CRT biopsies from these cases were retrieved from the archives of the Nottingham University Hospitals Department of Histopathology and were tested for DNA content analysis. All experimental work was undertaken with local institutional approval.
Molecular analysis of tumour samples

DNA extraction. DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue from all cases of sample set 1 as previously described (Fadhil et al. 2010). Four 10-μm-thick sections were de-waxed in xylene and underwent standard proteinase K digestion before extraction through DNeasy columns (QIAGEN, Hilden, Germany) in accordance with the manufacturer’s instructions. Template DNA was diluted to a final concentration of 20 ng/μl.

Testing for microsatellite instability. All cases in sample set 1 were screened for the presence of microsatellite instability (MSI) which was performed by PCR followed by high-resolution melting (HRM) analysis as previously described (Fadhil et al. 2012). A panel of six markers (BAT25, BAT26, NR21, NR22, NR24 and B-CAT25) was used to evaluate instability at mononucleotide repeat sequences. PCR was carried out in 10 μl reaction containing 1X Sensi- Mix™ HRM Kit (Bioline, London, UK), 0.25 μM concentration of each primer, 1X LC green® Plus+ dye (Idaho Technology, Salt Lake City, UT, USA) and 20 ng DNA template. HRM was performed immediately after PCR and consisted of one cycle of ([95 °C for 15 s]/[55 °C for 1 min]) followed by slow ramping up (at 0.03 degrees/s) to 95 °C during which fluorescent data were captured. Thermal cycling and HRM was performed on the AB 7500 fast PCR machine (ABI Biosystems, Paisely, UK) as previously described. Data were analysed using the Applied Biosystems High-Resolution Melting software v2.0. Samples were only regarded as showing MSI if there was instability at two or more of six markers.

Mutation analysis. All cases in sample set 1 were screened for mutation in the hotspots of KRAS (Codons 12/13, 61 and 146), BRAF (Codon 600 and exon 11) and PIK3CA (exon 1, exon 9 and exon 20) using the quick multiple consensus (QMC) protocols followed by HRM analysis as previously described (Fadhil et al. 2010). Samples were screened for mutation in the TP53 mutation hotspot (exon 5–8) using standard PCR followed by HRM as previously described (Fadhil et al. 2012). Thermal cycling, HRM and data analysis were performed on the AB 7500 fast PCR machine using the Applied Biosystems High-Resolution Melting software v2.0. All primer sequences are given in Supplementary Table S1.

Ploidy analysis

Isolation of nuclei. The DNA content of tumour cells was measured by flow cytometry using FFPE tumour tissue. All tumours (in both sample sets) were tested, and whole nuclei were extracted using a modification of the method of Hedley et al. (1983). Following pathology review (to choose the block with the highest proportion of tumour cells), a single 30-μm-thick section was cut. Sections were de-waxed in xylene and rehydrated in graded alcohols. The sections were washed in distilled water (twice) and then digested at 37 °C in 1 ml of 1.5% pepsin (p-7000; Sigma Aldrich, Gillingham, UK) in 0.9% NaCl (pH 1.5) for 2 h with intermittent vortex mixing. Following digestion, the sections were vigorously vortexed for 60 s to release the nuclei. The suspended nuclei were counted using a Neubauer chamber to ensure that there were more than 106/ml nuclei present. The tissues were then passed through a 70-μm nylon filter (Becton Dickinson) and centrifuged at 239 g for 5 min. The supernatant was removed and the pellet was re-suspended and washed twice in PBA (0.1% BSA in PBS) to remove residual pepsin. The cell pellet then was re-suspended in the staining solution in the flow cytometer tubes.

Flow cytometry. Prior to analysis by flow cytometry, the suspended nuclei were stained by incubation for 30 min with propidium iodide and Rnase (both sigma) at a respective final concentration of 1 and 10 mg/ml in PBA. Flow cytometry was performed on a Beckman Coulter FC500 flow cytometer, and experiments were performed following the guidelines of Ormerod et al. (1998), using doublet discrimination on a DNA fluorescence area vs. peak plot. Fluorescence from a minimum of 30,000 events per sample was captured over 1024 channels, and samples were deemed unacceptable if background aggregates and debris (i.e. sub-G1 fraction and clumps) comprised >20% of the total events.

Each tumour sample consists of tumour cells together with stromal cells and lymphocytes. The non-tumour cells are diploid and were used to set internal parameters and as internal controls. The photomultiplier tube (PMT) voltage of the flow cytometer was adjusted so that the G0/G1 peak of the non-tumour cells was captured at a channel number >200. The G0/G1(2c) and G2/M (4c) peaks of the internal controls were assigned to the channel with the maximum fluorescence for each, and a sample was acceptable if there was linearity between the G0/G1 and G2/M peaks (i.e. the ratio was between 1.95 and 2.05), the G2/M peak did not exceed 10% of the total events, the proportion of events at >6c (i.e. clumps) was not >1% and the co-efficient of variation of the G0/G1 peak for the internal controls was <6%.

Data analysis. Data analysis was performed using the WEASEL v2.7 software (Walter and Eliza Hall Institute, Parkville, Australia; www.wehi.edu.au). Every single sample had easily identifiable 2c and 4c peaks for the internal non-tumour cells. The DNA index (DI) for additional peaks was calculated as the ratio of the channel position of the tumour G0/G1 peak to the control G0/G1 peak. Tumour samples were considered diploid/near diploid when the DI was between 0.9 and 1.1 and aneuploid when the DI was outside these ranges. If the G2/M (4c) peak of the controls comprised more than 10% of the total and there was a corresponding 8c peak, the tumour was deemed as tetraploid.

Statistical analyses

Statistical analysis was performed using the GRAPHPAD PRISM software package (GraphPad Software Inc., La Jolla, CA, United States).
USA). Categorical data were tested for association using a two-sided Fisher’s exact test. Nonparametric continuous data were analysed using the Mann–Whitney test. In all cases, $P$-values < 0.05 was considered as statistically significant.

**Results**

**Screening cases for microsatellite instability**

A total of 100 cases were included in sample set 1, and these were tested for the presence of microsatellite instability. Each tumour was tested using a panel of six mononucleotide markers. Of the tumours tested, eight showed instability in over two of the markers (Figure 1a) and were excluded from sample set 1.

**Flow cytometry**

Of the 92 tumours tested for flow cytometry, reliable data could not be obtained for three of these and these were excluded from further analysis. Reliable data were obtained from the remaining 89 tumours, and there were three distinct patterns seen. Firstly, there were samples in which a single G0/G1 peak was seen with a smaller G2/M peak (Figure 1b) and this was deemed to be a diploid tumour. A second pattern was that of two peaks at G1/G0 that lay very

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**Figure 1** Evaluation of the DNA content of colorectal tumours. Firstly, the tumours were screened for the presence of microsatellite instability (MSI) by PCR/high-resolution melting (HRM) for six separate mononucleotide markers. (a) shows data for BAT26 and BCAT25 markers. The microsatellite-stable cases show similar patterns of melting (circled) whilst two cases are shown (each in duplicate and denoted by the arrows) in which there is instability at these markers resulting in aberrant melting. (b)–(d) show the three patterns seen when evaluating microsatellite-stable cases by flow cytometry. (b) shows a diploid pattern in which the tumour cells and stromal cells lie within a single dominant diploid peak. The arrow indicates the G2/M peak (representing 4c DNA content). (c) shows a ‘near-diploid’ pattern in which a separate peak (single arrow) is clearly discernible from the dominant G0/G1 peak, but the DNA index of this is >1.1. The G2/M peaks are shown by the double arrow. (d) the arrow shows a clearly aneuploid peak which, in this case, has a DNA index of 1.6.
close to each other with a ratio of 0.9:1.1 seen between the two peaks. There were also two closely associated G2/M peaks, and this pattern was deemed a ‘near-diploid’ tumour (Figure 1c). The third pattern was that of an aneuploid population in which extra peaks were seen between the G0/G1 and G2/M peaks of the internal diploid controls. There was usually just one extra peak although occasionally multiple extra peaks were seen. These represented the G0/G1 peak of an aneuploid population (Figure 1d).

Of the 89 microsatellite-stable CRCs tested, a total of 51 (57%) were aneuploid (i.e. CIN-CRCs) whilst 38 (43%) were diploid or near diploid (i.e. MACS-CRCs). Thus, a high proportion of the tumours tested were of the MACS genotype, but there were no significant clinico-pathological differences seen between CIN-CRCs and MACS-CRCs (Table 1).

The mutation profile of CIN-CRCs and MACS-CRCs

All 89 tumours in the first series were screened for the presence of mutation in the hotspots of KRAS, BRAF, PIK3CA and TP53 (Table 1). The frequency of mutations of the total sample set was as follows: KRAS: 46%, BRAF: 10%, PIK3CA: 15% and TP53: 61%. The frequency of mutation is in line with what would be expected for an unselected series of CRCs. Subgroup analysis and comparison of CIN-CRCs with MACS-CRCs showed that generally there was a similar mutation frequency within the two groups with no significant difference emerging. Of interest, however, was that 69% of CIN-CRCs contained TP53 mutation whilst this was seen in 50% of the MACS-CRCs. This was no statistically significant (P = 0.08) but raises the possibility that TP53 mutation may be permissive for the aneuploid state as has been previously suggested and consistent with its proposed role as a ‘caretaker’ gene. Similarly, 39% of CIN-CRCs were mutant for KRAS compared to 55% of MACS-CRCs, suggesting a preference of the latter for development along a KRAS-dependent pathway. Evaluation of the total mutation burden showed that 5/51 (10%) CIN tumours did not have mutation in any of these genes whilst, for the MACS group, 8/38 (27%) tumours were wild type for these genes. The difference was not statistically significant (P = 0.22).

The association of mutations with each other was also tested. In the group overall, there was the expected negative association between mutation of KRAS and BRAF (P = 0.001). Apart from this, there was no significant association seen in either the overall group or either of the subgroups (Table 2).

The association of DNA content with radiation responsiveness

We had hypothesised that tumour responsiveness to radiation may be predicted by DNA content. To test this, we evaluated the DNA content of diagnostic biopsies from a separate series of 62 rectal cancers. Of these, 22 had responded (Mandard tumour regression grade 1 & 2) and 40 had shown little response (Mandard tumour regression grade 3–5) following neoadjuvant chemoradiotherapy. Overall, 35/62 (60%) cases were aneuploid whilst 25/60 (40%) cases were diploid. Of the responders, 12/22 (54%) were CIN-CRCs and 10/22 (46%) were MACS-CRCs. Of the non-responders, 25/40 (62%) were CIN-CRCs and 15/40 (38%) were MACS-CRCs. Subgroup analysis, however, failed to show any association between tumour DNA content and response to the radiotherapy.

Discussion

In this study, we evaluated the DNA content of microsatellite-stable CRCs to evaluate the proportion of aneuploid and diploid tumours. The aneuploid group were deemed to show chromosomal instability (CIN) and were therefore designated as CIN-CRCs. The diploid group, because of their stable karyotype, were considered to be both microsatellite and chromosomal stable (MACS) and were designated as MACS-CRCs. Two independent sets of tumours were
analysed – a series of 89 colorectal tumours was tested to test the association between ploidy and clinicopathological/molecular features, and a second series of 62 rectal cancers which had received neoadjuvant CRT was tested to ascertain whether ploidy could predict responsiveness to radiation.

Using flow cytometry to quantify the tumour DNA content, we found that MACS-CRCs comprised 43% of the first sample set and 45% of the second sample set. Archival studies have reported the proportion of diploid tumours as ranging from 20% to 60%, but it has always been greater than the proportion of expected MSI-CRCs. Both of our sample sets are consistent with the published literature and with each other. All of our cases were reviewed to ensure a minimum of 50% tumour cells within the samples tested. We chose to use propidium iodide to stain the DNA rather than DAPI as, in our hands at least, both gave identical results during the optimisation of the flow cytometry protocol (data not shown). We are therefore confident that our proportion of MACS-CRCs is not due to false-negative flow cytometry results. Our data strongly challenge the dogma that CRCs can all be dichotomised into tumours which progress along either the MSI or CIN pathway (Fearon 2011; Pritchard & Grady 2011). We have shown that a significant proportion of CRCs belong to a third group which involves neither CIN nor MSI. These tumours may not have any genomic instability or they may have a third form of genomic instability [such as that caused by \textit{POLE} mutations (Palles et al. 2013; The Cancer Genome Atlas Network 2012)].

In sample set one, we sought to evaluate whether there were any clinico-pathological or molecular differences between MACS-CRCs and CIN-CRCs. Firstly, comparison of the clinico-pathological features of MACS-CRCs with the CIN-CRCs did not show any significant differences. Old studies of tumour ploidy have found a significant association of diploid tumours with the right side of the colon and with better prognosis although these would have been obviously confounded by the inclusion of tumours with MSI. Studies that have evaluated MACS-CRCs specifically have published mixed data with some reporting a left-sided predominance of the MACS-CRCs or a poor prognosis whilst others have not found these features (Hawkins et al. 2001; Silver et al. 2011).

Screening for mutation in four genes commonly mutated in CRCs demonstrated no significant differences apart from a slightly greater frequency of \textit{TP53} mutation in the

| Table 2 Association of gene mutations in CIN-CRCs and MACS-CRCs |
|---------------------|---------------------|---------------------|---------------------|
|                     | \textbf{BRAF}       | \textbf{PIK3CA}     | \textbf{TP53}       |
|                     | Mutant   | Wild type | \textit{P}-value | Mutant | Wild type | \textit{P}-value | Mutant | Wild type | \textit{P}-value |
| All cases           |          |          |                  |        |          |                  |        |          |                  |
| KRAS                | Mutant   | 0        | 41                | 0.003  |
|                     | Wild type| 9        | 39                |        |
| BRAF                | Mutant   | 1        | 8                 | 1.00   |
|                     | Wild type| 12       | 68                |        |
| PIK3CA              | Mutant   | 6        | 7                 | 0.36   |
|                     | Wild type| 48       | 28                |        |
| CIN-CRCs            |          |          |                  |        |          |                  |        |          |                  |
| KRAS                | Mutant   | 0        | 20                | 0.14   |
|                     | Wild type| 5        | 26                |        |
| BRAF                | Mutant   | 1        | 4                 | 1.00   |
|                     | Wild type| 8        | 38                |        |
| PIK3CA              | Mutant   | 4        | 5                 | 0.12   |
|                     | Wild type| 31       | 11                |        |
| MACS-CRC            |          |          |                  |        |          |                  |        |          |                  |
| KRAS                | Mutant   | 0        | 21                | 0.03   |
|                     | Wild type| 4        | 13                |        |
| BRAF                | Mutant   | 0        | 4                 | 1.00   |
|                     | Wild type| 4        | 30                |        |
| PIK3CA              | Mutant   | 2        | 2                 | 1.00   |
|                     | Wild type| 17       | 17                |        |

CIN, chromosomal instability; CRCs, colorectal cancers; MACS, microsatellite and chromosome stable.
CIN-CRCs. This series of cases is of insufficient size to pick up small or moderate differences in mutation frequency between the two groups. Our data do show that, for the genes tested, the differences between the two groups are not stark and that whilst TP53 mutation may be permissive for aneuploidy, it is not a major cause of aneuploidy. The similarity in the mutation profiles of the CIN-CRCs and the MACS-CRCs also suggests that the events which lead to aneuploidy probably occur late in the carcinogenic process after these mutations are likely to have occurred. This is consistent with data showing that aneuploid clones rarely occur in early or intermediate stage adenomas but can be identified in the late stage adenomas (van den Ingh et al. 1985; Goh & Jass 1986; Quirke et al. 1986).

We also investigated whether the MACS phenotype was associated with responsiveness to neoadjuvant radiation therapy in a separate set of rectal cancers. Around 15–25% of rectal CRCs show a dramatic response to neoadjuvant radiation therapy, and identification of the features predicting responsiveness would be a major step forward in the management of patients with rectal cancer. We hypothesised that the DNA content may be associated with responsiveness, and thus, pre-radiotherapy diagnostic biopsies from cases of responsive and non-responsive tumours were tested. There was no difference in the rate of response between CIN-CRCs and MACS-CRCs, leading us to conclude that tumour ploidy is not associated with tumour response to radiation.

In summary, we have demonstrated that MACS-CRCs form a significant group in the microsatellite-stable CRCs. The Fearon and Vogelstein model of colorectal tumorigenesis was postulated over 30 years ago and did not feature tumour ploidy. This should be revisited and should include three distinct groups, that is, MSI-CRC, CIN-CRC and MACS-CRC within the classification. Whilst these groupings may be associated with the biology of CRCs, they do not influence the response to radiation therapy.

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Contributors
Study concept and design: MI. Data acquisition: WF, KK and NL. Data interpretation: WF, MI and AR. Review of tumour regression: AZ. Preparation of manuscript: MI and WF. Approval of manuscript: all authors.

Conflict of interest
It is declared that the named authors on this manuscript have no conflicts of interest regarding the contents of the manuscript.

Ethics
Permission for the study and access to anonymised data and samples was granted by the Nottingham Research Biobank (Ref: ACP000007).

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