Journal of Pathology
J Pathol 2007; **213**: 249–256
Published online 24 September 2007 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/path.2234

Original Paper

Analysis of copy number changes suggests chromosomal instability in a minority of large colorectal adenomas

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No conflicts of interest were declared.

Received: 28 April 2007
Revised: 2 July 2007
Accepted: 26 July 2007

Abstract

We have examined chromosomal-scale mutations in 34 large colorectal adenomas (CRAs). A small number of changes (median = 2, IQR = 0–4) were found by array-comparative genomic hybridization (aCGH) in most tumours. The most common changes were deletions of chromosomes 1p, 9q, 17, 19, and 22, and gains of chromosomes 13 and 21. SNP-LOH analysis and pseudo-digital SNP-PCR analysis detected occasional copy-neutral LOH. Some aCGH changes found frequently in colorectal carcinomas, such as deletions of chromosomes 4q and 18q, were very infrequent in the adenomas. Almost all copy number changes were of small magnitude, far below the predicted levels even for single copy gain/loss; investigation suggested that these changes were either artefactual or occurred in sub-clones within the tumours. In some cases, these sub-clones may have represented progression towards carcinoma, but comparison with aCGH data from carcinomas showed this to be unlikely in most cases. In two adenomas, there was evidence of a large, outlying number of copy number changes, mostly resulting from part-chromosome deletions. Overall, moreover, there was evidence of a tendency towards part-chromosome deletions — consistent with chromosomal instability (CIN) — in about one-sixth of all tumours. However, there was no evidence of CIN in the form of whole-chromosome copy number changes. Our data did not support previous contentions that CRAs tend to show chromosome breakage at fragile sites owing to CIN associated with an elevated DNA damage response. Chromosomal-scale mutations occur in some CRAs; although CIN is not the norm in these lesions, it probably affects a minority of cases.

Keywords: **colorectal tumours; early lesions; copy number changes; genomic instability**

Introduction

The role of genomic instability — in other words, an intrinsically elevated mutation rate — in carcinogenesis remains controversial, despite this being a long-standing and important issue for cancer biologists. In essence, the chief problem is the inability to measure directly rates of mutation in human tumours, closely followed by an imprecise conception of the most common form of hypermutation, chromosomal instability (CIN).

Colorectal carcinoma (CRC) is probably the malignancy that has been most intensively studied in the quest to understand CIN and other forms of hypermutation such as microsatellite instability (MSI). Despite this, the timing and form of CIN in CRC have not yet been determined. Although it is beyond dispute that gross karyotypic abnormalities occur in some CRCs, these genetic changes take many forms and a specific tendency towards chromosome mis-segregation, or breakage, or telomere dysfunction, or mitotic checkpoint failure has not been shown; the changes might all be accounted for by an alternative mechanism, such as initial tetraploidization followed by loss at a 'normal' rate of all or parts of chromosomes.

A similar difficulty exists with the stage at which CIN — if it is truly CIN — occurs during tumourigenesis, with some arguing that CIN initiates colorectal tumorigenesis [1], others suggesting that it occurs early in adenoma growth [2], and yet others suggesting that it occurs after the adenoma–carcinoma transition [2–4]. This debate has been enlivened by findings from mouse ES cells that were homozygous

for the Apc^{1638T} mutation [5]. Although this mutation does not cause tumours *in vivo*, the *Apc*-mutant cells exhibited CIN, manifest as multiple numerical and structural chromosomal abnormalities. Since *APC* mutations occur in about 75% of human CRCs and about 70% of these are aneuploid/polyploid, there is therefore a strong case that APC deficiency causes CIN (with the proviso that it is not sufficient, because some near-diploid, but *APC*-mutant, CRCs exist).

We have previously used flow cytometry to show that sporadic and familial colorectal adenomas (CRAs) are usually near-diploid, especially when small and of low grade [4], and these findings form the basis of the analysis described below. Others have criticized our findings on the grounds that our method was not sufficiently sensitive to detect more subtle manifestations of CIN, such as occasional aneusomy [6]. Indeed, methods such as digital-SNP PCR (dS-PCR) and array-comparative genomic hybridization (aCGH) have since been used by other groups to demonstrate aneusomies in CRAs [2,7]. dS-PCR relies on dilution of DNA molecules and PCR of a single such molecule. The method is technically extremely demanding and cannot distinguish between gains, deletions, and loss of heterozygosity (LOH) without copy number change. The recent aCGH studies on CRAs have analysed multiple small regions (about 0.6 mm^2) of microdissected tumour, with the aim of identifying aneusomic subclones within the tumour [6]. A mean of about three aCGH changes was found in familial adenomatous polyposis (FAP) adenomas, and about six changes in MYH-associated polyposis (MAP) adenomas. Common sites of change included chromosomes 1p, 13, 17p, 19, and 22. The aCGH studies relied, however, on whole-genome amplification of the tumour DNA, a potential source of artefact. Moreover, copy number changes were scored for any smoothed data point that lay outside the 15% and 85% quantile bounds, a choice of thresholds that might lead to high sensitivity at the expense of specificity. Indeed, many samples of normal colorectum were found to show aCGH changes and these samples were not separated from the tumour samples by cluster analysis [6]. In addition, previous studies using aCGH have had the limitation of not being able to identify copy number-neutral LOH events that may contribute to tumourigenesis.

In this study, our aims were to examine the evidence for underlying tendencies towards chromosomal-scale changes in CRAs using multiple methods, including aCGH, a variant of dS-PCR, SNP-LOH analysis, and conventional LOH analysis using microsatellites. We deliberately analysed lesions that had grown and/or progressed from the very small, mildly dysplastic stage, so as to increase the chances of detecting genetic changes. Our results are consistent with most CRAs having a near-diploid karyotype, but show that chromosomal-scale changes are present within a subset of these lesions.

Materials and methods

For aCGH analysis, anonymised, single CRAs from nine patients with familial adenomatous polyposis (FAP), 11 patients with MAP, and 14 isolated adenoma cases were snap-frozen for analysis, together with paired blood or morphologically normal tissue. The clinico-pathological features of these tumours are shown in Table 1, all having been selected to be among the largest in our collection. An additional 22 adenomas of varying sizes and morphologies from five FAP patients were used for pseudo-dS-PCR analysis. All samples were collected according to local research ethics committee-approved protocols. Germline mutations in *APC* and *MYH* had already been detected in the FAP and MAP cases. After histological review to confirm at least 65% dysplastic tissue in each tumour, DNA was extracted by standard proteinase K digestion at 55 °C, followed by phenol–chloroform extraction. All tumours had been found to be microsatellite-stable using markers BAT25 and BAT26 (details not shown). A set of 60 unselected CRCs with aCGH data was available from a previous study [8].

For copy number assessment, aCGH analysis was undertaken using the method described by Fiegler *et al* [9]. We have shown previously that this platform can readily detect a single copy gain or loss in a tumour composed of less than 50% 'normal' cells [10]. In brief, the CGH array comprised spotted DNA from 3452 large insert genomic clones at an average spacing of about 1 Mb throughout the genome. Cy5- and Cy3-labelled test and paired normal DNAs were hybridized to the arrays together with herring sperm and Cot1 DNA. After scanning, rejecting poorly hybridized arrays, and correcting for background, the ratios of the fluorescence intensities of test to control (T : N ratios) were calculated, following normalization to the remainder of the genome in that tumour. The determination of copy number changes was performed using the program DNAcopy (http://www.bioconductor.org/repository/release1.5/package/html/DNAcopy.html), supplemented by a formal paired t-test comparing tumour : normal and normal : normal hybridizations to determine regions of significant copy number change using a threshold of $p < 0.001$. Only autosomes were analysed.

For increased resolution and the ability to detect copy-neutral LOH, SNP-LOH analysis was carried out on a sub-set of CRAs with plentiful DNA using the Illumina Sentrix HumanHap300 genotyping Beadchip arrays (317K TagSNP phase 1) according to the manufacturer's specifications (Illumina, San Diego, CA, USA). Briefly, 750 ng of polyp DNA was amplified overnight at 37 °C using WG-AMM and WG-MP1. Following amplification, the DNA was fragmented and precipitated with isopropanol. The dried pellet was then resuspended in WG-RA1 and hybridized to the Beadchip along with formamide overnight at 48 °C. Following hybridization, the samples underwent single-base extension and the Beadchips were then

stained, dried, and scanned using a Beadarray reader (Illumina). Image data were analysed and processed using Beadstudio 3 (Illumina).

For microsatellite LOH analysis, whole tumours were initially analysed. Subsequently, for analysis of potential spatially distributed sub-clones within the tumour, frozen sections of CRA were cut onto slides and 12 samples of epithelium (each about the size of a white needle tip) were removed with the aid of the dissecting microscope. DNA extraction was performed by overnight incubation in proteinase K solution (20 mg/ml proteinase K and 10 µl of ATL lysis buffer). Paired normal tissue was digested for 48 h (45 µl of ATL and 5 µl of 10 mg/ml proteinase K). DNA was purified using the DNeasy kit (Qiagen) and eluted in 20 µl of water. Whole tumours, each of the 12 dissected samples, and paired normal tissue were genotyped at 17 microsatellite markers (D1S201, D1S2729, D1S2749, D1S470, D9S1818, D9S1826, D9S1829, D9S1830, D17S786, D17S1832, D17S1353, D17S1844, D19S886, D19S878, D19S215, D19S565, and D19S591) with standard reaction conditions. The products were analysed with the ABI3130 sequencer and Genotyper software. At each marker, LOH was considered present if the area under one allelic peak in the adenoma was greater than twice that of the other allele, after correcting for the relative allelic areas using constitutional DNA.

For assessment of dS-PCR, we initially tested constitutional DNA to check for heterozygosity at five SNPs, on chromosomes 1p (CGAP-C-51 904 and CGAP-C-53 916), 5q/*APC* (CGAP-C-1960 and CGAP-C-1756), and 18q (CGAP-C-1468), that had previously been studied by Zhou *et al* [11] using dS-PCR in CRAs. In informative samples, we counted alleles by PCR-amplifying DNA from adenomas and paired normal tissue across each SNP. We cloned the resulting PCR product into pGEM T-easy and following transfection into JM109 cells, we directly sequenced about 25–100 clones derived from each PCR product. We scored the numbers of each allele at each SNP in each polyp. We refer to this method as pseudo-dS-PCR below.

Results

Overall aCGH analysis

Nine FAP adenomas were analysed by aCGH. Six of these tumours showed copy number changes by aCGH; four tumours had one to three changes, one had seven changes, and one had nine changes (Table 1 and Figure 1). Of 24 changes in total, nine involved whole chromosomes. Of 11 MAP adenomas analysed, eight had changes; four tumours had one to three changes, four had six to seven changes, and one had 17 changes (Table 1). Of 45 changes in total, 11 were of a whole chromosome. In the 14 sporadic adenomas, aCGH-detectable changes were present in ten cases (Table 1). Of these tumours, six had one to three changes, two had four changes, one had 12 changes, and one had 13 changes. Of 44 changes in total, 12 involved a whole chromosome.

The overall frequency of genetic changes detectable by aCGH (Table 1), the proportion of gains versus

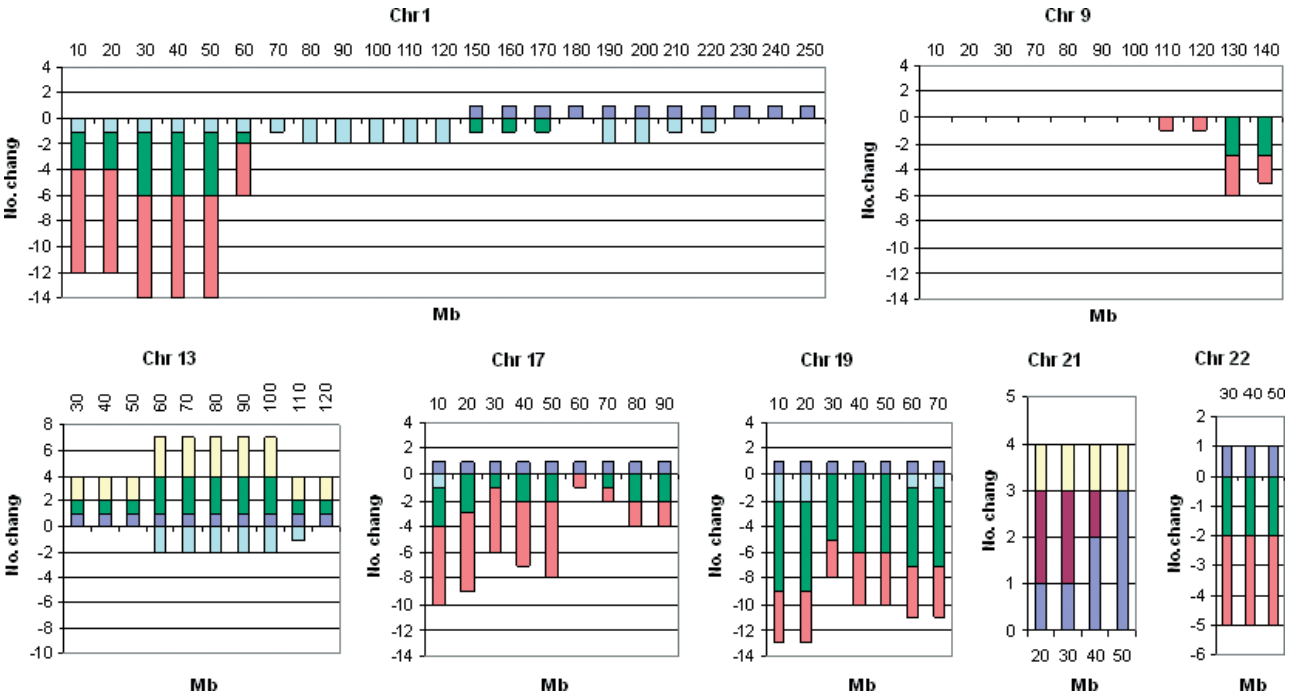

Figure 1. Summary of aCGH data in adenomas. Frequencies of gains and deletions are illustrated for all chromosomes that showed any changes in more than four of the 34 CRAs studied. Blue = gains in FAP adenomas, purple = gains in MAP adenomas, and yellow = gains in sporadic adenomas. Turquoise = deletions in FAP adenomas, green = deletions in MAP adenomas, and red = deletions in sporadic adenomas

Table 1. Summary of polyps and aCGH changes. The table shows chromosome number, region of change (in Mb from 2006 Human Genome Build), and gain or deletion of material. Changes were all of small magnitude — that is, a significant but small deviation from a $\log_2$ T : N ratio of zero — except for those higher magnitude changes shown in bold. Histology is shown as diameter (in cm), morphology [tubular (TA), tubulo-villous (TVA), and villous (VA)], together with the degree of dysplasia [mild (MiD), moderate (MoD) or severe (SeD)] where reported

Patient	Polyp	Histology	aCGH changes
FAP	F1	2.1, TA, MiD	
	F2	3.0, TA, MiD	17, 0–8, del; 19, 0–18, del; 19, 51-ter, del
	F3	2.6, TA, MoD	
	F4	1.5, TA, MiD	1, 76–112, del; 1, 188–218, del; 13, 53–96, del; 16, all, gain; 17, all, gain; 18, 24–39, del; 19, all, gain; 21, 33-ter, gain; 22, all, gain
	F5	1.0, TA, MiD	1, 183–196, del; 13, 54–104, del; 21, all, gain
	F6	2.6, TA, MiD	21, 41-ter, gain
	F7	1.8, TA, MoD	1, 0–118, del; 1, 143–244, gain; 5, 55–165, del; 6, all, gain; 13, all, gain; 18, all, del; 20, all, gain
	F8	3.5, TA, MoD	19, 0–18, del
	F9	2.3, TA, MiD	
MAP	M1	3.5, TA, MiD	**7, all, gain**; 19, all, del; 20, all, gain
	M2	1.2, VA, SeD	17, 0–10, del; 19, all, del
	M3	1.4, VA, SeD	1, 27–46, del; **8, 0–31, del; 17, 0–20, del; 17, 65-ter, del; 18, all, del**; 19, ?all, ?del; **22, all, del**
	M4	0.8, TA, MiD	1, 0–41, del; 1, 145–162, del; 3, 49–58, del; 4, 0–8, del; 4, 14-ter, gain; 6, 33–37, del; 7, 78–127, gain; 9, 121-ter, del; 12, 111-ter, del; 13, 56–96, gain; 14, 88-ter, del; 17, 18–50, del; 17, 71-ter, del; 19, all, del; 20, 29-ter, del; 21, 13–31, gain
	M5	0.9, TA, MiD	**13, all, gain**
	M6	2.1, TA, MiD	1, 0–46, del; 9, 121-ter, del; 13, 59–96, gain; 17, 0–10, del; 17, 35–46, del; 19, 0–17, del; 19, 38-ter, del
	M7	3.3, TA, MiD	1, 0–51, del; 17, 0–12, del; 19, 0–18, del
	M8	0.6, TA, MoD	
	M9	4.1, TA, MoD	1, 28–46, del; 3, 19–36, gain; 9, 121-ter, del; 12, 111-ter, del; 17, 0–20, del; 19, all, del; 21, 0–29, gain
	M10	0.6, TA, MiD	
	M11	0.7, TA, MoD	
Sporadic	S1	1, TA, MiD	1, 0–54, del; 6, 33–47, del; 8, all, gain; 9, 122-ter, del; 13, all, gain; 16, 0–31, del; 16, 67-ter, del; 17, 0–50, del; 19, all, del; 20, all, gain; 21, all, gain; 22, all, del
	S2	13, TVA, MoD	1, 0–51, del; 2, 123–152, del; 17, all, del; 19, all, del
	S3	1.6, TA, MiD	1, 0–57, del; 9, 104–111, del; 17, 0–50, del
	S4	3, TA, MiD	1, 0–47, del; 17, 0–50, del; 22, all, del
	S5	1, TA, MiD	
	S6	1.5, TA, MiD	1, 0–46, del; 17, 44–50, del
	S7	1, TVA, MiD	1, 0–46, del; 10, 91–103, del; 18, 51–64, del; 19, ?all, ?del
	S8	6, VA, MoD	1, 0–46, del; 7, 62–74, del; 9, 123-ter, del; 12, 111-ter, del; 13, 53–96, gain; 16, 0–31, del; 16, 66-ter, del; 17, 0–50, del; 17, 72-ter, del; 19, 0–18, del; 19, 38-ter, del; 20, 31–36, del; 22, all, del
	S9	7, TVA, MoD	
	S10	3.8, TVA, MoD	1, 0–51, del
	S11	2.4, TVA, MoD	13, all, gain
	S12	1.5, TVA, MiD	
	S13	3.5, TVA, MoD	17, 0–14, del
	S14	1, TA, MiD	

deletions, and the proportion of whole-chromosome versus part-chromosome changes did not differ significantly among the three groups of patients ($p > 0.45$ in all cases, Kruskal–Wallis test) or vary with adenoma size, morphology, or degree of dysplasia ($p > 0.48$ in all cases, Kruskal–Wallis test). In the whole sample set, gains were less common than deletions [medians of 0 (range 0–5) and 1.5 (0–13), respectively]. Whole-chromosome changes were less common than part-chromosome changes [medians of 0 (range 0–6) and 1 (0–15), respectively]. Adenomas with larger numbers of whole-chromosome changes tended to have more part-chromosome changes (linear regression analysis, $t = 2.23$, $p = 0.033$), and tumours with higher numbers of gains tended also to have higher numbers of deletions (linear regression analysis, $t = 3.17$, $p = 0.003$).

In order to search for evidence of a set of tumours with qualitatively higher numbers of chromosomal-scale changes, we examined the frequency distributions of aCGH changes in the full adenoma set. The numbers of whole-chromosome changes in each adenoma fitted a Poisson distribution well, with a mean of 0.94 ($z = 0.34$, $p = 0.73$). For numbers of part-chromosome changes, there were two outlying adenomas (Table 1), one with 12 changes (S1) and the other with 15 (M4). However, even after excluding these two tumours, the numbers of part-chromosome changes, most of which were deletions, still failed to fit the Poisson distribution ($z = 4.02$, $p < 0.001$).

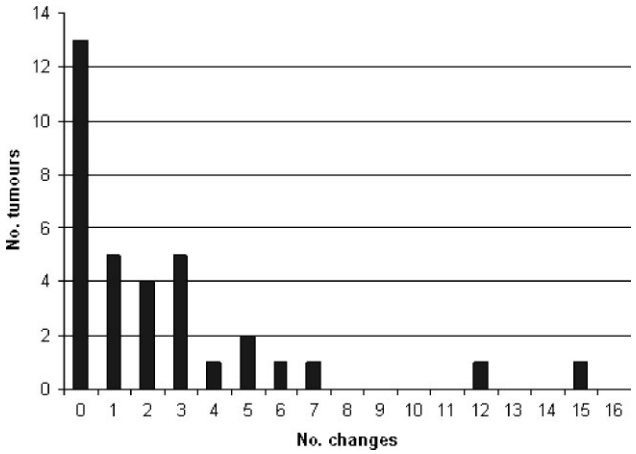

Figure 2. Typical aCGH profiles. Bar chart showing the frequencies of total numbers of part-chromosome copy number changes in the 34 adenomas studied

Inspection (Figure 2) and further analysis showed that the six tumours with five or more changes provided the bulk of the evidence for non-random distribution of part-chromosome copy number changes. The fit to the Poisson distribution was good for the remaining 28 tumours ($z = 0.76$, $p = 0.45$).

Genomic locations of aCGH changes

Figure 1 shows the frequency of deletions and gains in the whole sample set for all chromosomes for which more than four adenomas harboured changes. Particularly frequent sites of change were deletion of chromosome 1p (about 41% of adenomas), gain of chromosome 13 (~21%), deletion of 17p (~29%), deletion of chromosome 19 (~38%), gain of chromosome 21 (~12%), and deletion of chromosome 22 (~15%). There were no significant differences between the three patient types or any relationship to adenoma morphology when the changes at these locations were considered (details not shown). Comparison of the frequencies of change among whole chromosomes showed a clear tendency for deletion or gain of the smaller chromosomes (linear regression analysis, $t = 3.18$, $p = 0.005$). With the exception of chromosome 1, which showed frequent deletion involving its short arm, a similar tendency was found for part-chromosome changes (linear regression analysis, $t = -3.11$, $p = 0.006$).

Magnitude of copy number changes and the possibility of artefacts and genetic heterogeneity

In general, we have estimated from sequencing that mutations are rarely detectable if they comprise less than 25% of tumour DNA (making allowance for contaminating normal tissue). However, the great majority of copy number changes in our samples were of magnitudes estimated to be far below this level. Indeed, only seven changes, all in MAP polyps, demonstrated $\log_2$ T : N ratios close to ± 0.5 (Table 1 and Figure 3). Most ratios were, by contrast, closer to ± 0.1 (theoretically corresponding to a gain or loss of only 7% of DNA in each tumour sample). Such low-magnitude changes were, moreover, found in the MAP polyps that also harboured changes with $\log_2$ T : N ratios close to ± 0.5; examples are shown in Figure 3. In such adenomas, a plausible explanation was that the copy number changes detected were present in tumour sub-clones.

We attempted to confirm the copy number changes using microsatellite-based LOH analysis on the same DNA extracted from the whole tumours that had been used for the aCGH analysis. Using markers on chromosomes 1, 9, 17, and 19 (see the Materials and methods section), no LOH was found. We then examined LOH using the same microsatellites in a single adenoma (M9) that had shown low-level deletion by aCGH in these regions and from which frozen sections were available. After dissection to separate 12 different regions of tumour epithelium, we found no chromosome 9 or 19 LOH, but we found LOH in two of 12 tumour regions using chromosome 1 markers (Figure 4) and in two different regions using chromosome 17 markers. In neither case were the regions that showed LOH contiguous within the tumour.

We also examined the possibility that some of the low-magnitude changes were artefacts of the aCGH technique, since some studies have reported these in

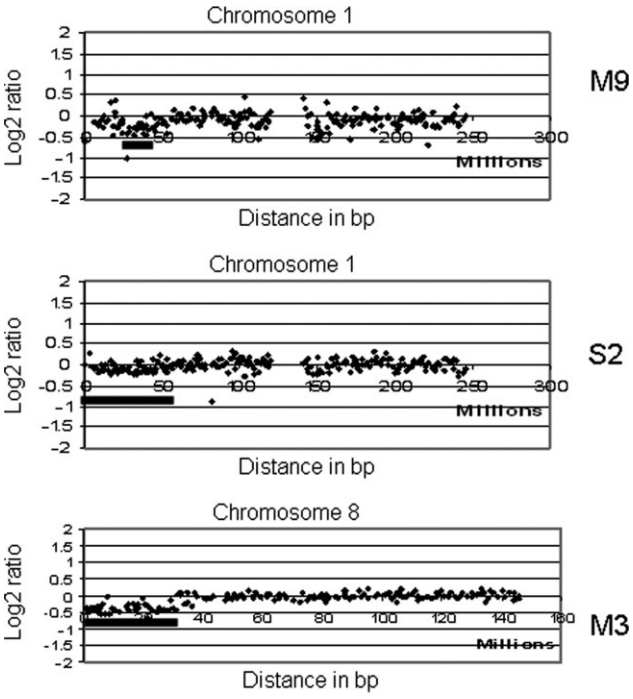

Figure 3. Typical aCGH profiles. The plots show estimated $\log_2$ gene dosage (T : N ratios) along each chromosome, such that a ratio of 1 corresponds to a doubling of copy number in the tumour relative to the normal tissue and a ratio of -1 corresponds to a halving of copy number. Copy number changes are shown by bars. The low magnitude changes typical of this study are illustrated by chromosome 1 (adenomas M9 and S2) and the rarer changes of higher magnitude more typically found in carcinomas are shown by chromosome 8 (M3)

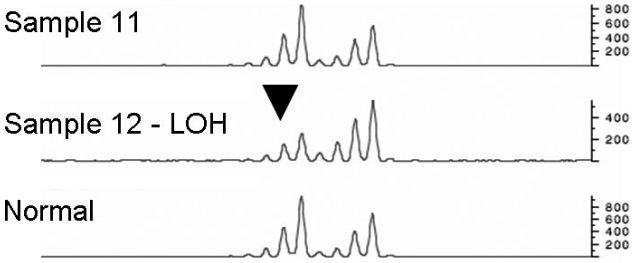

Figure 4. LOH on chromosome 1 in adenoma M9 that had shown low-magnitude deletion by aCGH. The area of the tumour corresponding to sample 11 shows no LOH compared with the normal tissue, but sample 12 shows clear loss (arrowed)

gene- and GC-rich regions such as chromosome 19 (N Carter, personal communication). It has frequently been speculated, moreover, that aCGH data-smoothing algorithms might not be effective at removing regional differences in hybridization efficiency that resulted in systematic over- or under-reporting of Cy3 : Cy5 ratios. In order to test whether this effect might have led to any of the changes detected in our samples (and even though poor quality hybridizations were excluded from our analysis), we calculated for each tumour the variance in copy number in regions with no copy number change. In the whole sample set, we then regressed the total number of copy number changes against that variance. There was a significant positive correlation ($t = 2.47$, $p = 0.019$), suggesting that some changes were indeed artefactual.

Comparison of aCGH changes in adenomas and carcinomas

We then asked whether the aCGH changes in CRAs were a sub-set of those found in CRCs. Although some changes (notably 18q deletions) essentially occurred only in CRCs, other changes were less frequent in adenomas than in cancers and yet others (notably 1p deletions) were similarly frequent in adenomas and carcinomas (Table 2). We were surprised to find that deletions involving chromosome 19 were actually more frequent in the adenomas than in the CRCs ($\chi^2_1 = 10.7$, $p < 0.01$, Table 2), but chromosome 19 is a technically difficult chromosome to analyse and some of these findings may have been artefactual. This contention was supported by the absence of chromosome 19 LOH in polyp M9 using microsatellite analysis, even though this tumour showed deletion of the whole chromosome on aCGH (see above).

Given that deletion of 1p occurred in about 40% of adenomas, albeit probably in sub-clones in all cases, we asked whether the sub-clones were likely to have formed the cancer had our adenomas been left *in situ* to progress. The majority of 1p deletions (11/16, 69%) in the CRAs extended from the short arm telomere to a breakpoint between 41 and 57 Mb, and only one polyp had deleted the whole chromosome arm. In CRCs, by contrast, significantly fewer deletions (2/23, 9%) extended from pter to 41–57 Mb ($\chi^2_1 = 15.3$) and

Table 2. Comparison between the frequencies of aCGH changes in adenomas and carcinomas. The table shows (i) changes that are found relatively frequently in CRAs and CRCs and (ii) changes found rarely or never in CRAs, but present in CRCs at frequencies of more than 20%. The CRC data ($N = 60$) are from our published findings [8]. The data for the changes found rarely in adenomas are shown by the presence of any copy number change on that chromosome arm, since discrete regions have not been defined

	Chromosome	Region (Mb)	Change	Adenoma frequency	Carcinoma frequency
(i)	1	0–60	Del	0.4	0.27
	9	90–110	Del	0.18	0.1
	13	All	Gain	0.21	0.57
	17	0–50	Del	0.29	0.53
	17	70–90	Del	0.12	0.10
	19	All	Del	0.38	0.10
	21	40–60	Gain	0.12	0.08
	22	40–60	Del	0.15	0.37
(ii)	4p		Del		0.20
	4q		Del		0.27
	5p		Gain		0.23
	7p		Gain		0.28
	8p		Del		0.42
	8q		Gain		0.35
	14q		Del		0.32
	15q		Del		0.23
	18p		Del		0.52
	18q		Del		0.63
	20p		Gain		0.32
	20q		Gain		0.67
	21q		Del		0.20

7/23 involved the whole chromosome arm (Fisher's exact test, $p < 0.001$). These findings suggest that not all of the 1p deletions in adenomas occur in clones that are progressing on the pathway to malignancy.

SNP-LOH analysis

We analysed seven adenomas studied by aCGH (F2, F4, F5, F7, F8, M8, and M10) and one additional adenoma (M13) with the Infinium II assay. One MAP polyp (M10), a relatively small tubular adenoma without any aCGH changes, showed copy number-neutral LOH involving the whole of chromosomes 7 and 12 (Figure 5). This tumour showed no changes by aCGH analysis.

Pseudo-digital SNP-PCR

dS-PCR and our pseudo-dS-PCR variant can, in principle, detect both LOH and/or copy number changes, because these methods 'count' alleles, but cannot distinguish between them. We analysed a total of 31 CRAs, all from FAP patients, using pseudo-dS-PCR. A shortage of tissue meant that not all adenomas could be analysed at each SNP and we were only able to analyse CRAs from three of the patients (F4, F7, and F8) whose tumours had also been analysed using aCGH. At the APC SNPs, 3 of 13 (23%) CRAs showed significant deviation (at $p < 0.05$) from the allelic ratios in normal tissue. One of these tumours had already

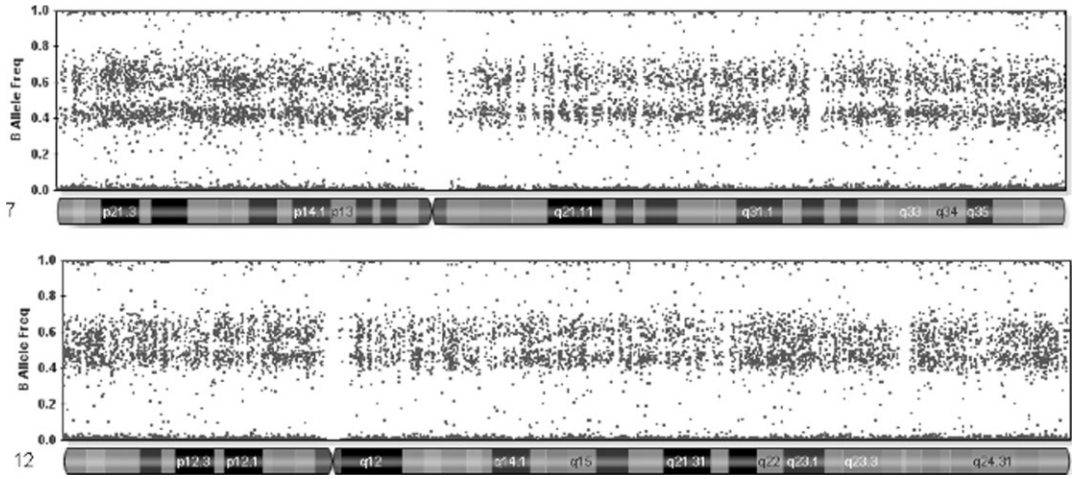

Figure 5. Copy-neutral LOH in adenoma M10 involving the whole of chromosomes 7 and 12. The split allele frequency call around the 0.5 level is indicative of LOH

been analysed by aCGH and showed no deletion of 5q, suggesting that LOH was copy-neutral, as is generally the case for APC [4]. On chromosome 1, 8/26 (31%) CRAs showed LOH by pseudo-dS-PCR; both of the tumours that also had aCGH data showed deletion of chromosome 1p. Only one of 18 (5.5%) CRAs showed pseudo-dS-PCR LOH on chromosome 18q.

Discussion

We have found that a small number of large-scale genetic changes (median = 2, IQR = 0–4) can be found by aCGH in the majority of adenomas from FAP and MAP patients and from sporadic cases. Our aCGH data were supported by our findings using pseudo-dS-PCR. Unlike others, we found no significant differences in terms of frequencies or locations of large-scale changes between lesions from these three origins [6], although our data were consistent with a small excess of changes in MAP, compared with FAP, and all the relatively high-level copy number changes occurred in the MAP polyps. Whilst our adenomas were not 'early' lesions based on their size, almost all would almost certainly be scored as near-diploid by flow cytometry, based on previous data [4,12] and on the relatively small proportion of the genome and/or of the tumour clone that was affected by copy number changes in this study.

The most frequent regions of change that we have found in our adenomas are similar to those found by Cardoso *et al* [6], with the exception that chromosome 7 was not commonly gained in our series. We have also shown, however, that some changes, particularly those involving chromosome 19, are probably artefactual. Chromosome 19 is gene-rich and has been strongly suspected for some time to be prone to aberrant behaviour in conventional- and array-CGH experiments; it should only be included in such studies with caution. Other regions, such as the proximal part of chromosome 1p, are also thought by some groups (N Carter, personal communication) to be prone to erroneous reporting, and it remains possible that some of our changes identified in these regions are artefactual.

Given our findings that almost all copy number changes in our tumours involved quantitatively small changes in gene dosage and our failure to show that these changes resulted in LOH, our data fit best with a scenario in which those deletions and gains that are non-artefactual affect adenoma sub-clones rather than the bulk of the tumour. Our LOH data on the microdissected tumour support this possibility. Our results therefore complement those of Cardoso *et al*, whose strategy was to focus initially on tumour sub-clones [6]. It is tempting to speculate that these sub-clones are derived from the hypothetical tumour stem cells and represent early progression towards carcinoma. However we urge caution. First, some changes — such as the deletion of chromosome 18q — that are thought to be present in early carcinomas [3] were almost absent in the adenomas. Second, whilst there was overlap between some of the regions commonly affected in carcinomas and adenomas, closer inspection showed that there were differences in the regions targeted. Third, most aCGH changes involved the smaller chromosomes, suggesting that these may have been tolerated as 'background' changes that produced no great selective advantage or disadvantage.

Do the changes in our adenomas result from CIN, in the sense of an elevated frequency of large-scale chromosomal mutations resulting from a specific cause? Clues as to the existence of CIN may come from one or more of the following: (i) a tendency for an individual tumour to harbour changes of a specific type; (ii) deviation of the number of changes in tumours from the expected Poisson or normal distribution; and (iii) changes in excess of the 'expected' number compared with normal tissue (very difficult to assess). With the exception of a few tumours with high levels of part-chromosome changes, the number of aCGH changes followed Poisson distributions for whole-chromosome

and part-chromosome changes; these levels are not *a priori* indicative of CIN. Some evidence for CIN, perhaps from a raised level of unrepaired double-strand breaks, came from the two adenomas that were striking outliers in terms of their large numbers of their part-chromosome changes and from the six tumours in total that caused deviation from the expected Poisson distribution. These tumours may have been further along the pathway to carcinoma than those with very few changes, although they showed no special morphological features (Table 1). No adenoma showed evidence of an outlying number of whole-chromosome copy number changes. Overall, there was an association between whole- and part-chromosome changes. We conclude that CIN, by part-chromosome deletion, appears to occur in some adenomas, but only in relatively few cases.

It is still possible that some of the copy number changes in our CRAs resulted from a different type of CIN, such as a generally increased rate of chromosomal-scale changes compared with normal tissues, resulting from features inherent to tumourigenesis. Bartkova *et al* [13] have raised the possibility that most early tumours have an elevated DNA damage response, perhaps as a result of oncogene activation. They suggested that CIN was present in these tumours, including those of the colorectum, and was manifest in an elevated rate of breakage at fragile sites, specifically at chromosomes 8p23.1/8p21.3, 9q32, and 11p15.1. None of the changes in our adenomas appeared to arise from chromosome breakage at these sites. Our data therefore provide no evidence in support of the hypothesis of Bartkova *et al* [13].

We conclude that although most colorectal adenomas are classed as near-diploid by flow cytometry, a small number of large-scale copy number changes occur in most — but not all — adenomas of relatively large size and/or grade. In addition, a smaller number of copy-neutral LOH changes are found. In some cases, such as chromosome 19, the detected changes may be artefacts of the aCGH method. Most copy number changes appear to affect only tumour subclones and there is doubt that these sub-clones would have been the ones that formed the carcinoma had the adenomas been allowed to progress. In a similar vein, we have previously shown that the spectrum of p53 mutations in CRAs differs markedly from that in CRCs [14]. In conclusion, our data suggest that CIN in the sense of large-scale chromosomal changes is present in many CRAs; CIN in the sense of an increased, specific tendency towards such changes is not a universal feature of CRAs, but this sort of 'true CIN' is likely to exist in a proportion of these lesions.

Acknowledgements

We are grateful to the Equipment Park, Cancer Research UK London Research Institute, Maesha Deheragada for histological input, and to Olivia Will for collection of some of the clinical data.

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