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ORIGINAL ARTICLE

Mismatch repair, p53 and chromosomal aberrations in primary colorectal carcinomas

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Abstract

Colorectal carcinoma progresses via at least two genetic pathways. Microsatellite instability, due to defective mismatch repair genes, characterizes one pathway and gross chromosomal instability another. The involvement of p53 and mismatch repair gene abnormalities within these pathways has not been fully explored. We aimed to investigate the relationships of p53 and mismatch repair gene defects on gross chromosomal aberrations detected by comparative genomic hybridization in 49 colorectal carcinomas. Tumours demonstrating loss of expression for hMLH1 or hMSH2 proteins demonstrated a highly significant attenuation in the number of gross chromosomal aberrations ($p = 0.007$) and were less likely to show p53 overexpression ($p = 0.02$). Within the mismatch repair normal tumours, p53 status did not affect the total number of chromosomal aberrations but p53 overexpression was significantly associated with a higher frequency of amplifications at 8q22-ter and at 13q21-22. Colorectal cancer demonstrates distinct molecular phenotypes and should be sub-classified accordingly.

The genetic alterations fundamental to sporadic colorectal cancer appear to follow at least two distinct pathways [1]. Multiple chromosomal aberrations including deletions and/or point mutations of tumour suppressor genes and amplifications and/or mutations of oncogenes are characteristic of sporadic tumours following the classical "Vogelstein" stepwise pathway of chromosomal instability (CIN) [2]. In the alternative sporadic microsatellite instability (MIN) pathway, epigenetic silencing of one of the mismatch repair (MMR) genes leads to a rapid accrual of mutations with increasing risk of carcinogenesis. The hallmark of MMR deficiency is high frequency microsatellite instability (MSI-H) and it has been demonstrated that 95% of MSI-H tumours show loss of expression of the hMLH1 or hMSH2 proteins [3] and commonly lack p53 gene alterations [4]. The gold standard for identification of this phenotype is a polymerase chain reaction (PCR) based assay but the immunohistochemical detection of these tumours by observing loss of expression of the MMR proteins hMLH1 and hMSH2 is highly predictive [5,6]. Aberrant overexpression of p53 detected by widespread immunohistochemical staining of tumour cell nuclei ($>20\%$ of cells) strongly concurs with the presence of p53 gene mutation determined by single strand conformational polymorphism (SSCP) and mutational analysis [7].

A functioning p53 protein is postulated to maintain genetic stability [8]. However, mutation of p53 is felt to be a late, rather than an initiating step, in colorectal cancer [9] and therefore, defective p53 may not be the driving force behind chromosomal instability in sporadic colorectal tumours.

In this study we aimed to explore the correlation of MMR and p53 status with chromosomal aberrations using comparative genomic hybridisation in sporadic primary colorectal carcinomas. Comparative genomic hybridisation (CGH) is a powerful cytogenetic tool that allows copy number change across the entire genome to be assessed in a single hybridization. The allocation of colorectal tumours into

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distinct genetic pathways may facilitate the individualization of patient treatment.

Materials and methods

Tumour samples

Forty-nine sporadic colorectal cancer specimens were collected from patients undergoing surgical resection at Castle Hill Hospital between 1995 and 1998. Representative specimens from the tumour were excised, snap frozen in liquid nitrogen and stored at -80°C. Pathological assessment of the remaining tumour specimen was performed by a consultant colorectal histopathologist (AM) according to standard UICC criteria. In order to ensure that CGH was being carried out on tumour DNA, a cryostat-sectioning and histological confirmation technique was employed. Sections deemed by an independent pathologist to contain $>90\%$ neoplastic cells were used for analysis. Genomic DNA was isolated using a proteinase K/phenol chloroform extraction procedure according to standard protocols [10].

Comparative Genomic Hybridisation

CGH analysis was performed as previously described by Ashman et al. [10]. All results were analysed independently by two workers (BJM & JNEA). The gain and loss thresholds used in this study were 1.15 and 0.85; these thresholds were used based on control hybridisations of cytogenetically normal DNA. The control experiments never exceeded these threshold values. As the tumour specimens and normal reference DNA were not sex matched the X and Y chromosomes were omitted from the analysis.

Immunohistochemical staining of hMSH2, hMLH1 and p53.

All 49 tumours were immunohistochemically stained according to the technique previously described [5]. Briefly, antigenic sites were retrieved by boiling slides in 1500 ml distilled water with 15 ml Antigen Unmasking Solution (Vector Laboratories Ltd, Burlingame, CA) in a pressure cooker for 3 minutes at 15 psi. Non-specific protein was blocked with $1\times$ casein (Vector Laboratories Ltd) and endogeneous avidin and biotin were blocked using the Avidin Biotin Blocking Kit (Vector Laboratories Ltd). Sections were incubated at room temperature for 2 hours with primary antibody. A final dilution of 1:100 was used for antibodies against p53 (#554293, BD Biosciences, Oxford, UK) and MSH2 (#NA27, Merck Biosciences Ltd, Nottingham, UK); 1:50 was used for MLH1 (#554073, BD Biosciences). A negative control was also included in each batch of slides, in which the primary antibody was omitted. All results were scored without the knowledge of CGH results. Loss of expression of hMLH1 and hMSH2 was recorded when nuclear staining was seen in normal tissue but not in the majority of adjacent malignant tissue. Overexpression of the p53 protein was recorded when strong nuclear staining was seen in the majority of malignant cells.

Statistical analysis

All statistical analyses were undertaken using the SPSS statistical computer software package (SPSS Inc., Chicago Il, USA). Statistical analysis of differences in prevalence of the most common gains and deletions between groups was performed using Fisher's exact test. Analysis of the number of aberrations (scored as a sum of the number of chromosomal arms in each tumour which contained at least one gain or loss) in different groups was performed using the Mann-Whitney U test.

Results

Genetic aberrations detected by CGH

Forty-nine primary colorectal carcinomas were studied and the results are summarized in Figure 1 and Table I. A large number of aberrations were identified with a median of 12 (range 0–36) aberrations per tumour. The most common deletion was 1p35-ter, which was identified in 27 of 49 (55%) tumours. Other common deletions included 18q21 in 24 of 49 (48%), 17p13-ter in 21 of 49 (42%), 8p22-ter in 17 of 49 (34%), 9q33-ter in 15 of 49 (30%) and 1q25-ter in 12 of 49 (24%) cases.

The most common amplification was 13q21-22, which was present in 28 of 49 (57%) samples. Other common amplification included 8q22 in 23 of 49 (46%), 12q13-15 in 13 of 49 (26%) and 8q21 in 20 of 49 (40%). Gain of the short arm of chromosome 20 was seen in 21 of 49 (42%) tumours. Three tumours demonstrated no aberrations by CGH, 2 of which demonstrated a MMR defect.

MMR and p53 status

MMR status, p53 status, age, tumour site, UICC stage and number of aberrations are shown in Table I. Twenty-three of 49 (46%) tumours demonstrated p53 over-expression by immunohistochemistry. A total of 6 of 49 (12%) tumours demonstrated loss of expression of hMLH1 (5 tumours) or hMSH2 (1 tumour) and were classified as MMR

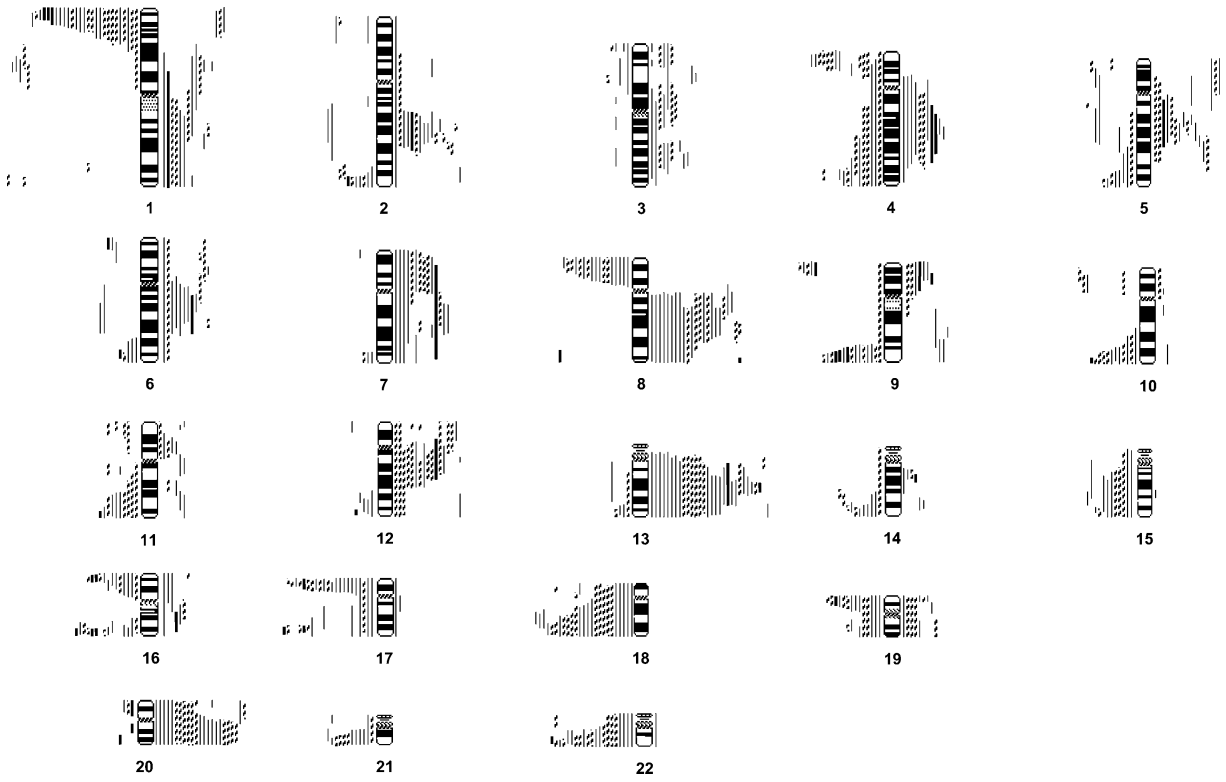

Figure 1. Karyogram depicting the chromosomal locations of all regions of DNA copy number imbalance in 49 sporadic colorectal carcinomas. Regions of deletion are represented by lines to the left of the chromosome ideograms and regions of gain by lines to the right. Thin black lines represent Group 1 tumours (p53 overexpression/MMR normal) shaded lines represent Group 2 tumours (p53 normal/MMR normal) and thick black lines represent Group 3 tumours (MMR defective/p53 normal).

defective. All of these tumours were right sided ($p < 0.002$, Fisher's exact) and none of these tumours demonstrated p53 over-expression ($p = 0.02$, Fisher's exact).

Table II shows statistical evaluation of CGH abnormalities for tumours classified into subgroups according to their p53 and MMR status. Initially, tumours were classified by their p53 protein status only. Tumours demonstrating p53 over-expression ($n = 23$) had a median of 15 (range 1–36) chromosomal aberrations as compared to 8.5 (range 0–30) in p53 normal ($n = 26$) tumours ($p = 0.03$, Mann-Whitney U test). However, the p53 normal group included 6 tumours with a MMR defect, which demonstrated a median of only 4 (range 0–11) aberrations per tumour ($p = 0.0072$ vs MMR normal tumours, Mann-Whitney). Therefore, for subsequent analysis we removed the 6 MMR defective tumours from the p53 normal group and considered 3 different groups (Table II). Group 1 contained 23 tumours which demonstrated p53 over-expression and normal MMR protein expression. Group 2 contained 20 tumours which demonstrated normal expression of both p53 and MMR proteins. Group 3 consisted of 6 MMR defective (p53 normal) tumours.

Analysing the number of aberrations per tumour in this manner demonstrated that the p53 over-expression/MMR normal tumours (Group 1) had a median of 15 (range 1–36) aberrations per tumour ($p = 0.17$, Mann-Whitney), which is not statistically different to the p53 normal/MMR normal tumours (Group 2) with a median of 12 (range 0–30) aberrations per tumour. The p53 normal/MMR defective tumours (Group 3) had a median of 4 (range 0–11) aberrations per tumour, which is significantly less than both the p53 overexpression/MMR normal tumours in Group 1 ($p = 0.003$, Mann-Whitney) and the p53 normal/MMR normal tumours in Group 2 ($p = 0.03$, Mann-Whitney; Table II).

Chromosomal aberrations by group

The MMR defective tumours (Group 3, $n = 6$) demonstrated very few chromosomal aberrations using CGH. Alterations commonly seen in the MMR normal groups, such as 18q21 deletion, 17p deletion, 20q gain and 8p22-ter deletion were not seen at all in the MMR defective group ($p = 0.02$, $p = 0.03$, $p = 0.03$ & $p = 0.08$ respectively, Fisher's exact). The small number of MMR defective

Table I. Clinicopathological and molecular data for the 49 primary colorectal tumours.

Tumour	Side*	Age	UICC Stage	p53 status	MMR status	No. of Gains	No. of Deletions	Total no. of Aberrations
1	Left	71	T3N0M0	Normal	Normal	0	0	0
2	Right	43	T4N0M1	Normal	**hMSH2§**	0	0	0
3	Right	55	T2N0M0	Normal	**hMLH1**	0	0	0
4	Left	76	T3N1M0	Defective	Normal	1	0	1
5	Left	77	T3N0M0	Defective	Normal	1	0	1
6	Right	72	T3N1M0	Normal	**hMLH1**	1	0	1
7	Right	63	T3N0M0	Normal	Normal	2	1	3
8	Right	73	T3N0M0	Defective	Normal	4	0	4
9	Right	51	T3N1M0	Normal	Normal	4	0	4
10	Left	63	T3N1M0	Normal	Normal	0	4	4
11	Left	87	T3N0M0	Normal	Normal	6	0	6
12	Left	62	T3N1M0	Defective	Normal	1	5	6
13	Right	81	T3N1M0	Normal	**hMLH1**	5	2	7
14	Left	75	T3N1M0	Normal	Normal	2	5	7
15	Left	71	T4N0M0	Defective	Normal	4	4	8
16	Left	61	T2N0M0	Normal	Normal	4	4	8
17	Right	75	T2N0M0	Normal	**hMLH1**	4	4	8
18	Left	52	T2N0M0	Normal	Normal	3	5	8
19	Left	59	T4N1M0	Defective	Normal	5	4	9
20	Right	73	T3N0M0	Normal	Normal	4	5	9
21	Left	70	T3N3M0	Defective	Normal	1	8	9
22	Left	79	T3N0M0	Defective	Normal	5	6	11
23	Right	68	T3N0M1	Normal	**hMLH1**	5	6	11
24	Right	71	T3N3M0	Normal	Normal	3	8	11
25	Left	76	T3N0M0	Defective	Normal	5	7	12
26	Right	64	T3N1M0	Defective	Normal	8	5	13
27	Right	52	T4N0M0	Normal	Normal	5	8	13
28	Right	61	T4N1M1	Defective	Normal	6	8	14
29	Left	76	T3N1M0	Normal	Normal	5	9	14
30	Left	61	T2N0M0	Normal	Normal	5	9	14
31	Left	67	T4N0M0	Normal	Normal	4	10	14
32	Left	62	T3N0M0	Defective	Normal	11	4	15
33	Left	67	T3N0M0	Defective	Normal	6	9	15
34	Left	76	T3N1M0	Defective	Normal	8	8	16
35	Right	77	T3N0M0	Normal	Normal	7	9	16
36	Left	68	T3N1M0	Normal	Normal	6	11	17
37	Left	65	T2N0M0	Normal	Normal	6	11	17
38	Left	62	T3N0M0	Defective	Normal	6	12	18
39	Left	70	T3N0M0	Defective	Normal	12	7	19
40	Left	74	T2N1M0	Defective	Normal	7	12	19
41	Left	44	T3N1M0	Normal	Normal	12	11	23
42	Right	42	T3N2M0	Defective	Normal	12	13	25
43	Left	69	T3N0M0	Defective	Normal	10	15	25
44	Right	63	T4N1M0	Normal	Normal	12	14	26
45	Left	73	T3N2M0	Defective	Normal	19	11	30
46	Right	71	T4N2M0	Normal	Normal	12	18	30
47	Right	81	T4N0M0	Defective	Normal	14	17	31
48	Left	74	T4N1M0	Defective	Normal	15	18	33
49	Left	86	T3N0M0	Defective	Normal	17	19	36

*Right and Left of splenic flexure. §Denotes loss of expression of (hMSH2 or hMLH1).

tumours precludes further meaningful analysis of aberration frequency in this group.

Segregation of MMR normal tumours according to p53 status (Groups 1 and 2) revealed a number of significant differences in chromosomal aberration frequency. Amplification at 8q22-ter was demonstrated in 15 of 23 (65%) Group 1 (p53 overexpression/MMR normal) tumours compared with 5 of 20 (25%) Group 2 (p53 normal/MMR normal) tumours ($p = 0.01$, Fisher's exact). Amplification of 12q13-15 was demonstrated in 3 of 23 (13%) Group 1 tumours compared with 9 of 20 (45%) Group 2 tumours ($p = 0.04$, Fisher's exact). Amplification at 13q21-22 was demonstrated in 18 of 23 (78%) Group 1 tumours compared with 8 of 20 (40%) Group 2 tumours ($p = 0.01$, Fisher's exact).

There was no significant difference between the groups in the frequencies of certain common aberra-

Table II. Statistical analysis of median number of aberrations in colorectal tumours catagorised by p53 and MMR status.

	n	Number of Aberrations median (range)	p
Group by p53 status only			
p53 overexpression	23	15 (1–36)	
p53 normal	26	8.5 (0–30)	0.03†
Group by p53 & MMR status			
GROUP 1 p53 overexpression /MMR normal	23	15 (1–36)	
GROUP 2 p53 normal/MMR normal	20	12 (0–30)	0.17†
GROUP 3 p53 normal/MMR defective	6	4 (0–11)	<0.03 vs both groups†

† Mann-Whitney U test.

tions. The 18q21-ter deletion was seen in 13 of 23 (56%) Group 1 and 11 of 20 (55%) Group 2 tumours. Deletions of 17p13 were demonstrated in 13 of 23 (56%) Group 1 tumours and in 8 of 20 (40%) Group 2 tumours. The 1p35-ter deletion was seen in 13 of 23 (56%) Group 1 tumours and 11 of 20 (55%) Group 2 tumours. A gain at 20q was demonstrated in 11 of 23 (47%) Group 1 tumours and 10 of 20 (50%) Group 2 tumours.

Discussion

Forty-nine primary colorectal carcinomas were studied and a large number of aberrations were identified with a median of 12 (range 0–36) aberrations per tumour. The aberrations frequently identified in this study include well-described chromosomal aberrations in colorectal cancer such as 18q and 17p loss, which have been extensively demonstrated previously [2]. These losses correspond to the DCC region on 18q and the p53 gene on 17p.

Following stratification of the samples into 3 groups according to p53 and MMR protein expression status, further observations were made. The MMR defective tumours (Group 3) all demonstrated normal p53 expression, which is a recognized association of the mutator pathway [4,11]. This group of tumours demonstrated very few chromosomal aberrations with a median of 4 (range 0–11) gross alterations, as described previously [12,13] and did not exhibit common deletions on 18q and 17p. This finding is consistent with a similar study performed by Curtis et al. [12] who also demonstrated a reduced frequency of 8p and 18q deletions in MMR defective sporadic colorectal tumours. Li et al. [13] analysed 39 MMR defective colorectal tumours by CGH and identified that the most frequent gains were on 4q and 8q and the most frequent losses were on 9q, 1p and 11q. Although only 6 MMR defective tumours were included in our study, some of these aberrations were seen. These findings support the concept that tumours arising via the MIN pathway are driven by small mutations within critical oncogenes and tumour suppressor genes, which would be below the resolution of CGH, as opposed to gross chromosomal aberrations. The MMR defective group was significantly different from the MMR normal groups (Groups 1 and 2), which is in agreement with the recent microarray-based CGH study by Nakao et al. [14], and these tumours should be segregated in any colorectal cancer series which aims to assess the frequency of gross chromosomal alterations.

The p53 protein expression status of MMR normal tumours did not affect the total number of chromosomal aberrations. Group 1 (p53 overexpression) demonstrated a median of 15 (range 1–36) alterations and Group 2 (p53 normal) exhibited a median of 12 (range 0–30) alterations per tumour. This finding suggests that, although p53 has some impact on chromosomal aberrations, other causative factors exist which are as yet undefined. The 18q deletion (DCC region) was identified in over 55% of tumours in both groups. The 17p13 deletion (p53) was exhibited in over 40% of tumours in both groups. Alterations at 1p35-ter and 20q were also seen at high frequencies in both groups. However, p53 protein status was significantly correlated with aberrations in certain chromosome regions. Group 1 tumours (p53 overexpression) exhibited a significantly higher frequency of amplifications at 8q22-ter and at 13q21-22, confirming previous reports [15]. Amplification of 12q13-15 was seen at a higher frequency in the Group 2 tumours, suggesting that this locus may be associated with the cause of chromosomal instability in the p53 normal tumours. Interestingly the MDM2 oncogene, which is a known inhibitor of p53 [16] is located within this region at 12q14.3-q15.

There were a number of tumours which had very few gross chromosomal aberrations and were not mismatch repair defective, which suggests that there is a further subgroup of colorectal cancers [17]. This MIN(−)CIN(−) group has recently been shown to display clinicopathological differences to MIN(−)CIN(+) tumours [18].

In summary, this study has demonstrated that MMR defective colorectal cancers demonstrate significantly different patterns of chromosomal aberrations. There appears to be a p53 independent mechanism, which is responsible for gross chromosomal instability in tumours which do not demonstrate p53 overexpression. It is generally accepted that colorectal cancer does have distinct molecular variants that can affect the prognosis of the patient [19] and this study reinforces the concept that colorectal cancer should not be treated as one disease [20]. The recent systematic review of p53 research in colorectal cancer by Munro et al., which outlines a valiant attempt to definitively report the importance of p53 in these tumours, also underlines the problem of heterogeneity of such studies in the literature [21].

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