Relationship between Chromosome 18q Status and Colorectal Cancer Prognosis: A Prospective, Blinded Analysis of 280 Patients

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Abstract. *Background: The relationship between chromosome 18q allelic imbalance (AI) and survival in colorectal cancer (CRC) is unclear, and study design may have contributed to inconsistent results previously reported. Patients and Methods: Two hundred and eighty tumours from CRC patients participating in a molecular sub-study from a single multi-centre trial of adjuvant intra-portal 5-fluorouracil were genotyped at 5 chromosome 18q microsatellite markers, blinded to clinical data and prospective to follow-up. The relationship between overall survival and AI was examined. Results: Two hundred and fifty-five tumours were informative for AI. The overall rate of AI was 49%. AI was not associated with age, tumour site or size. There was no difference in five-year survival rate between patients with (60.0% SE 5.2%) and without AI (61.4% SE 5.0%), even after correcting for covariates (HR=1.17, 95%CI:0.79-1.74, p=0.4). Conclusion: Our data does not support chromosome 18q AI as an important marker of survival in the adjuvant setting. It should not, therefore, be used outside clinical trials.*

Colorectal cancer (CRC) is one of the commonest causes of cancer death in the world, accounting for approximately 60,000 deaths annually in North America (1, 2). For patients with early CRC, surgery still remains the most effective therapeutic option (3). Approximately 60% of patients present with potentially curable stage II or III disease (4), but unfortunately a high proportion of patients relapse, even following adjuvant chemotherapy (5). While adjuvant 5-fluorouracil (5-FU) treatment was shown to significantly improve overall survival (OS) in patients with stage III CRC (6-9), its value in stage II disease seems to be limited mainly to patients at high risk of relapse, based on adverse histological features (10).

Molecular analyses have demonstrated genetic heterogeneity in CRCs (11, 12), and somatic genotype may contribute to the inter-individual differences in clinical outcome (13, 14). Identifying robust molecular markers to supplement standard clinicopathological staging systems is of particular relevance in helping select patients who are most likely to benefit from adjuvant chemotherapy, thereby enabling targeted adjuvant strategies based on probable outcome or drug efficacy. This is likely to become increasingly important with the introduction of new drugs showing efficacy against CRC (9, 15).

One of the potentially promising markers of CRC prognosis studied to date is allelic imbalance (AI) at chromosome 18q. A number of key genes involved in colorectal carcinogenesis including *DCC* (16), *SMAD2* (17), and *SMAD4* (18), map to this region and chromosome 18q AI is one of the most frequently observed chromosomal changes, detectable in up to 70% of tumours (19). A number of studies have investigated the relationship between chromosome 18q AI and OS (13, 14, 20-22), but results have been inconsistent, with some showing a poorer survival in tumours displaying AI (13, 14, 22), whereas others found no such relationship (20, 21, 23). A number of factors may account for these differences in findings, including relatively small sample sizes investigated, retrospectively collected and analysed clinical datasets, varying methodology and non-blindness in the assessment of results.

To help assess the value of chromosome 18q AI as a prognostic index of long-term survival, we prospectively investigated the relationship between this genotype and OS

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in a well-defined cohort of stage I-III CRC patients accrued to a single multi-centre randomised trial of adjuvant chemotherapy, with molecular investigators blinded to clinical data. The pre-specified hypothesis tested was that chromosome 18q AI is a marker of poorer overall survival in potentially curatively resected CRC. Our results are reported according to the REMARK guidelines (24).

Patients and Methods

Patients and samples. All patients in the present study had previously been accrued to a large randomised trial of the effects of seven-day continuous intra-portal vein infusion of 5-FU immediately after potentially curative tumour resection, involving over 10,000 patients from 200 hospitals in China (25, 26). Study recruitment took place during 1994-98 and the current cycle of follow-up on survival continued to the middle of 2003, with median follow-up of 6 years in survivors. Of the trial-collaborating hospitals, 39 agreed to participate in a sub-study of molecular prognostics that commenced in 1996 and involved the prospective collection of a pair of paraffin-embedded, formalin fixed tissue blocks (one tumour tissue and one normal tissue) from a random subset of randomised patients at time of potentially curative surgery. All required approval for the main trial and the molecular sub-study was obtained from relevant government organisations and ethics committees in China and informed consent was obtained from individual patients prior to the investigation. Overall, a total of 967 sets of samples were collected, and following official approval by the Chinese Administration for Human Genetic Materials, all specimens were subsequently sent to the UK Clinical Trial Service Unit (CTSU), where they were anonymised prior to forwarding to a separate research laboratory for molecular analysis.

Genotyping. Fifteen-micron sections of tumours from formalin-fixed paraffin-embedded samples were cut onto double-sided clear adhesive tape on glass slides. Regions of at least 70% confluent tumour were microdissected after light staining with toluidine blue. Ten-micron whole sections of normal bowel mucosa from a separate paired block of normal colorectal mucosa from each patient were used as a source of germline DNA. Standard commercial methods were used to extract DNA (QIAmp DNA mini-kit, Qiagen, West Sussex, UK). Dinucleotide microsatellite loci D18S69, D18S64, D18S55, D18S61, D18S58, were chosen for genotyping as *per* Jen *et al.* (13). Markers mapped to a 13.6 Mbp region telomeric to *DCC* and primers were designed from sequences reported (www.gdb.org). Target DNA sequences were amplified using ^{32}P end-labelled primers. Mismatch repair status was assessed on basis of BAT26 genotype and tumours were assigned as microsatellite unstable (MSI) or microsatellite stable (MSS) by the presence or absence of novel alleles at autoradiography. Tumours showing MSI at BAT26 or at any of the five chromosome 18q microsatellites were arbitrarily excluded from survival analysis due to non-informativity for AI. Genotyping was performed at least twice per sample. Allelic imbalance was defined as complete loss or reduction in somatic allelic intensity, scored by direct visual comparison of the relative allelic ratios of somatic and germline genotypes. Homozygotes were considered non-informative. Allele loss was considered present if heterozygous somatic allelic relative intensity differed to that in the germline by a factor of at least 1.5 at any of the markers genotyped. Only tumours with unambiguous genotypes were assigned either MSI or AI status. All genotyping and result scoring was performed blinded to clinical outcome, and recorded on a separate database prior to merging with the main database kept in Oxford CTSU for the final data analysis.

Statistical analysis. Differences in demographic data and between different categories of markers were tested for statistical differences using the χ^2 test for categorical variables and the Student's *t*-test or Mann Whitney non-parametric test for continuous variables. A Cox regression model was used with individual markers as the exposure variables and OS (from time of surgery to time of death or end of current follow-up) as the outcome, adjusting simultaneously for gender, age, tumour size, grade, stage and sites as well as use of post-operative adjuvant therapies. The cumulative probability of survival was calculated by the Kaplan-Meier method and groups compared by the stratified logrank test. Information on progression-free survival was not available for all randomised patients, nor were many histopathological features *e.g.* T-stage, and number of lymph nodes resected at time of surgery. All *p*-values were two-sided, with values less than 0.05 regarded as conventionally statistically significant. Assuming a control survival rate of 60% and 50% of patients with chromosome 18q AI (23), analysis of tissue samples from 280 patients has 80% power to detect 16% difference in overall survival associated with 18q AI.

Results

Patient demographics and genotype. Of the 967 samples collected for genotyping, 687 (71%) were either refractory to PCR amplification, had significant quantities (>40%) of normal mucosa interspersed between tumour, or had un-representative specimens, resulting in genotyping data available from 280 cases. Twenty-five (9%) tumours had MSI and were considered non-informative for AI. Data from these individuals was therefore excluded from further analysis, resulting in 255 samples available for AI assessment. The overall characteristics of these patients were similar to those in the main trial (Table I). Thirty-four percent of the patients had colon cancer, 58% had stage II disease, and 42% were aged <55. Eighty-two percent of the patients had received post-operative 5-FU-based chemotherapy at physician discretion in addition to randomly allocated treatment. The 5-year survival rate for informative patients was 60.7% (SE 3.6%) and was similar to that observed in the main clinical trial (59.5% SE 0.5%).

The relationship between chromosome 18q status and patient characteristics is provided in Table II. There were no statistical differences in clinicopathological variables between patients with and those without AI. Overall, about half (124, 49%) of the tumours genotyped demonstrated AI at any of the chromosome 18q loci (Figure 1). AI was not associated with age, site of disease, or tumour size at resection (Table II).

Table I. *Principal characteristics of patients with informative chromosome 18q genotypes and those in whom chromosome 18q genotype was unknown.*

Characteristics	Tumours with informative genotypes (%)		Tumours without genotype data (%)	
Gender				
Male	125	(49)	5,742	(54)
Female	130	(51)	4,929	(46)
Age at diagnosis (years)				
<45	66	(26)	2,724	(26)
45-54	41	(16)	2,316	(22)
55-64	86	(34)	3,327	(31)
>65	62	(24)	2,304	(22)
Site				
Colon	86	(34)	3,837	(36)
Rectum	164	(64)	6,702	(63)
Other	5	(2)	132	(1)
Tumour size				
<4.0cm	67	(26)	2,107	(20)
4.0-5.9cm	101	(40)	4,881	(46)
6.0-7.9cm	45	(18)	2,249	(21)
>8.0cm	42	(16)	1,434	(13)
Stage				
I	13	(5)	946	(9)
II	149	(58)	5,851	(55)
III	93	(36)	3,874	(36)
Tumour differentiation				
Well/moderate	230	(91)	466	(86)
Poor	24	(9)	79	(14)
Neoadjuvant radiotherapy administered				
Yes	3	(1)	146	(1)
No	252	(99)	10,525	(99)
Non-trial adjuvant chemotherapy given				
Yes	210	(82)	8,545	(80)
No	45	(18)	2,126	(20)
Randomised treatment				
Portal 5-FU	115	(45)	5,335	(50)
Control	140	(55)	5,336	(50)

5-FU, 5-fluorouracil.

Table II. *Patient characteristics according to chromosome 18q genotype.*

Characteristics	Chromosome 18q genotype			
	AI (%)		No AI (%)	
Gender				
Male	58	(47)	67	(51)
Female	66	(53)	64	(49)
Age at diagnosis (years)				
<45	35	(28)	31	(24)
45-54	23	(19)	18	(14)
55-64	32	(26)	54	(41)
>65	34	(27)	28	(21)
Site				
Colon	41	(33)	45	(34)
Rectum	80	(65)	84	(64)
Other	3	(2)	2	(2)
Tumour size				
<4.0cm	32	(26)	35	(27)
4.0-5.9cm	53	(43)	48	(37)
6.0-7.9cm	20	(16)	25	(19)
>8.0cm	19	(15)	23	(18)
Stage				
I	7	(6)	6	(5)
II	72	(58)	77	(59)
III	45	(36)	48	(37)
Tumour differentiation				
Well/moderate	110	(89)	120	(92)
Poor	13	(11)	11	(8)
Neoadjuvant radiotherapy administered				
Yes	3	(2)	0	(0)
No	121	(98)	131	(100)
Non-trial adjuvant chemotherapy given				
Yes	99	(80)	111	(85)
No	25	(20)	20	(15)
Randomised treatment				
Portal 5-FU	53	(43)	62	(47)
Control	71	(57)	69	(53)

5-FU, 5-fluorouracil; AI, allelic imbalance.

Marker status and survival. Of the 255 patients informative for AI, 101 (40%) died during the follow-up period, mostly from disease recurrence, with a median follow-up of 51 months (0-100). There was a significant relationship between CRC stage and OS (HR=1.99, 95%CI: 1.35-2.93, p=0.005), but no apparent difference in OS was observed between tumours with or without chromosome 18q AI, with a 5-year survival rate of 60.0% (SE 5.2%) and 61.4% (SE 5.0%), respectively (HR=1.08, 95%CI: 0.73-1.60, p=0.7) (Figure 2). When all standard prognostic clinical variables were included as co-variables in a Cox proportional hazards model, there was again no evidence that chromosome 18q AI was significantly associated with OS (HR=1.17, 95%CI: 0.79-1.74, p=0.4).

Discussion

The present study is one of the largest molecular studies reported to date of the possible prognostic role of chromosome 18q AI in CRC. We adopted a prospective study design, together with blinded molecular methods. The study results indicate that the proportion of tumours with AI in the Chinese CRC patient population was generally consistent with that reported in previous studies in Western populations (13, 14, 22, 27-31), suggesting that regardless of differing rates of CRC incidence in China and Western countries, a similar proportion of patients seem to develop CRC though the chromosomal instability

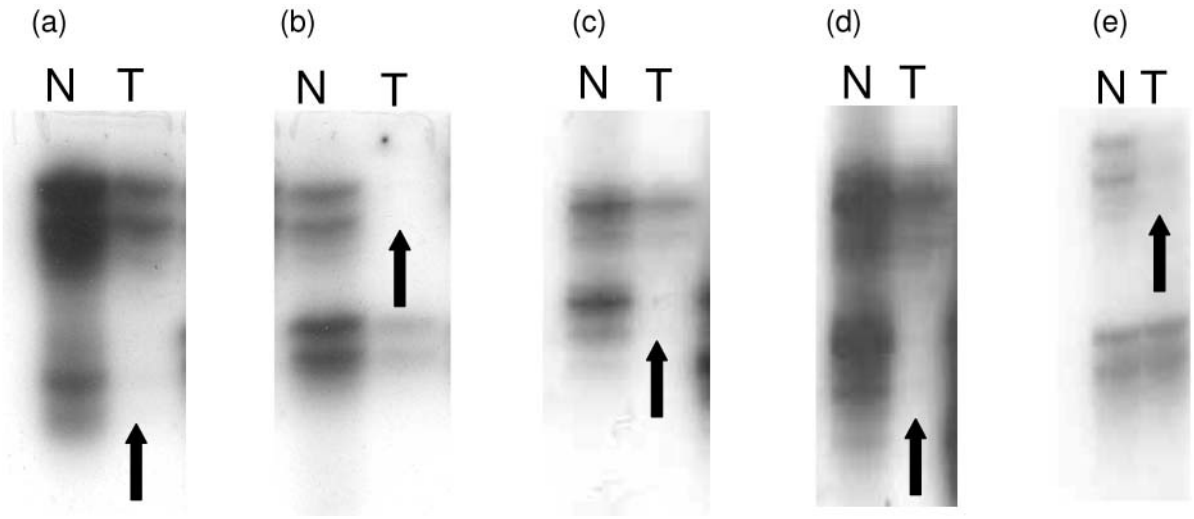

Figure 1. *Representative examples of somatic (T lanes) and paired germline (N lanes) autoradiographs at the microsatellite markers (a) D18S69, (b) D18S64, (c) D18S55, (d) D18S61, (e) D18S58. Somatic alleles (lanes T) were lost, compared to heterozygous genotypes (lanes N), as indicated by the arrows.*

pathway. However, there is no clear evidence that the presence of AI at chromosome 18q significantly influences long-term survival from the disease. These results, though non-conclusive due to the relatively small sample size involved, appeared to be broadly consistent with a number of similar-sized studies in the adjuvant CRC setting (20, 21, 28, 32, 33).

The notion that AI, at chromosome 18q and chromosome 18q21 in particular, is a determinant of CRC prognosis is highly plausible given that a number of tumour suppressor genes implicated in CRC development map to this region: *DCC, SMAD2* and *SMAD4*. *DCC* is most frequently included in regions of allele loss (16, 34) and its loss correlates with both reduced RNA levels (34, 35) and DCC expression (36, 37). *DCC* has recently been shown to act as a conditional tumour suppressor by binding netrin-1 (38) and rates of allelic losses at this region have correlated with both adenoma formation and CRC stage, implying a role for the gene in both colorectal carcinogenesis and tumour progression (19, 39).

A number of studies found that patients whose tumours displayed chromosome 18q AI had poorer outcomes (23). However, this finding was not supported by other studies (20, 21). A number of methodological factors may contribute to the discrepancies between studies, including the use of retrospectively retrieved tissue samples and clinical data, as well as unblinded molecular analyses. Although these factors might bias any molecular marker correlative study, several of these issues are particularly relevant to analyses of chromosome 18q AI and CRC prognosis. Of particular concern are the varied laboratory methods used in the analysis of 18q AI, ranging from karyotyping, comparative genomic hybridisation (CGH), Southern blot analysis, microsatellite genotyping, to assessment of DCC or SMAD4 expression, or assessment of inconsistent genomic regions (23). Moreover, most studies typically involved less than 100 patients, the smallest including 29 patients (40) and the

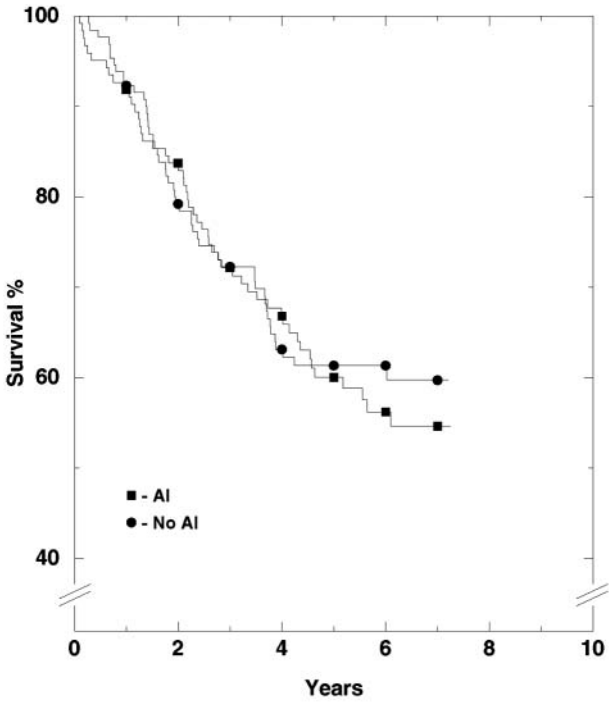

Figure 2. *Overall survival in all patients stratified by chromosome 18q genotype.*

largest with 386 patients (20). Consequently, these were too small to detect a modest but clinically meaningful effect, with many being only sufficiently powered to detect at least a 30% poorer prognosis, assuming an AI rate of around 50%. The current study, though still not sufficiently large accounted for most of these potential methodological problems commonly seen in many previous studies by adopting a prospective study design, assessing trial patients, employing blinded molecular investigators, and using microsatellite genotyping, the most common method of assessing the prognostic impact of AI (23).

The biological effect of AI at chromosome 18q has primarily been thought to be mediated through abrogation of DCC function. Although our markers were chosen on the basis of their use in previously published work (13), they map slightly telomeric to *DCC* (May 2004, hg17, human genome assembly, http://genome.ucsc.edu/cgi-bin/hgGateway) and hence may not fully reflect DCC status in cancer (12). This is, however, unlikely to have significantly affected findings since the most centromeric maker (D18S16) is only 2.6 Mbp telomeric to *DCC* and generally *DCC* deletion is a consequence of large-scale loss of chromosome material (12, 40-43). Moreover, the markers we employed overlapped with the minimally deleted chromosome 18q region previously reported (34). It is possible that the functional effect of AI at 18q is not solely a consequence of DCC loss and may involve other genes such as *SMAD4* or *SMAD2*, which map centromeric to DCC.

It is also possible that the lack of association between 18q AI and OS may be related to the potential impact of mismatch repair deficiency in tumours. Whilst most CRCs develop through the chromosomal instability pathway and are characterised by high levels of anueploidy, a smaller proportion develop as a consequence of deficiency in mismatch repair, detectable as MSI in tumours. Such tumours tend to retain the native diploid state (44). Assignment of AI status to samples with MSI is impossible by genotyping microsatellites due to the presence of novel alleles in these tumours. We have been conservative in our assumptions by excluding such samples from survival analysis. The handling of samples with MSI in previous studies based on microsatellite genotyping has, however, been varied, with some studies excluding these samples from survival analysis (20, 21, 31, 32, 45), some taking no account for this (28, 33, 46-48) and several recognising this potential confounder but assuming samples with MSI had no AI (13, 14, 29). However, even when excluding MSI data and using raw genotypes, no significant survival correlate associated with AI either by univariate or multivariate analyses was observed, indicating that MSI status did not impact on our results. In the present study, tissue samples without satisfactory PCR or amplification refractory were excluded from the main analysis. The relatively high proportion of such samples can be accounted for by the widespread use of unbuffered formalin in China, resulting in degraded DNA refractory to amplification (49). It is, however, unlikely that exclusion of these samples could have produced any biased results, as the samples eventually genotyped were generally representative of those from the main trial.

Although the current study is one of the largest molecular studies of chromosome 18q AI and demonstrated no prognostic utility in CRC, it is not sufficiently powered to detect a more moderate difference in prognosis associated with chromosome 18q AI of less than 20%. Accepting this caveat, our results provide little evidence that chromosome 18q AI is a robust marker of CRC prognosis and should not be used in routine clinical decision-making.

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Conflict of Interest: The authors declare no conflict of interest.

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