ORIGINAL ARTICLE

Metastatic recurrence of early-stage colorectal cancer is linked to loss of heterozygosity on chromosomes 4 and 14q

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J Clin Pathol 2006;**59**:624–630. doi: 10.1136/jcp.2005.033167

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Accepted for publication 1 January 2006

Objective: To investigate the prognostic value for loss of heterozygosity (LOH) of chromosomes 4 and 14q in early-stage colorectal cancer (CRC).

Methods: A total of 70, largely microsatellite stable, tumours and their corresponding normal mucosa were subjected to microdissection and analysed for LOH at chromosomes 4 and 14q by using 13 highly polymorphic microsatellite markers. LOH was correlated with the survival of the patients, using univariate, multivariate and Kaplan–Meier's survival curves.

Result: LOH at D4S2935, D4S1579 and D4S1595 on chromosome 4 was significantly associated with metastatic recurrence of early-stage CRC. For chromosome arm 14q, two minimal regions of deletion were associated with metastatic recurrence and mapped to neighbouring markers D14S275/D14S49 at 14q12–13 and D14S65/D14S250 at 14q32. High-level loss (loss of five to eight of the informative microsatellite markers) on both chromosomes 4 and 14q, to be an independent prognostic indicator in early-stage CRC was shown by multivariate analysis.

Conclusion: Determining the LOH of chromosomes 4 and 14q and their extent in primary tumours of patients with early-stage CRC may constitute a molecular signature of metastatic recurrence. This may be achieved if new finding sheds light on the treatment of this subgroup of patients that have been largely ignored.

Early-stage colorectal cancer (CRC) defined by the lack of microscopic and clinical metastases represents a considerable challenge to the treating physicians. Although the prognosis of untreated early-stage CRC is generally better than more advanced stages, 10–45% of patients with early-stage CRC relapse from metastasis after curative surgery.[1–4] The use of standard clinicopathological parameters to identify this subset of patients proved unreliable.[1 5] In addition, the predictive value of several metastasis suppressor genes has been disappointing.[6–9] Treating all patients having early-stage CRC with chemotherapy is not a feasible or economical option, especially with the knowledge of the consequential morbidity and mortality associated with this type of treatment. Moreover, administering adjuvant treatment to patients with early-stage colon cancer has yielded controversial results.[10 11] A plausible solution to this dilemma is to interrogate the genome, transcriptome or proteome of the primary tumours in an attempt to predict their behaviour and select patients who show the signature of advanced or metastatic CRCs for treatment. This is not an unprecedented scenario because it has been shown earlier that the characteristics of primary tumours govern their metastatic behaviour.[12] Moreover, recent studies have shown that the metastatic signature can be identified from the transcriptome of primary tumours.[13 14] More recently, the expression of 23 genes from 74 patients has been associated with metastasis in early-stage CRC.[15] Currently, limited data on the genomic aberrations in early-stage CRC exist in the literature. We have previously identified several genomic aberrations capable of predicting the behaviour of early-stage CRC by using conventional and microarray-based comparative genomic hybridisation.[16] Loss of chromosomal regions at 4p14–16, 4q24–28 and 4q32–35 on chromosome 4 and 14q11.2–13.3 or 14q24.1–32.3 on chromosome arm 14q seemed to have a major metastasis predictive power. Here, we utilised microsatellite allelotyping to define minimal regions of LOH on both chromosomes, which may contribute to potential localisation of metastasis suppressor genes.

MATERIALS AND METHODS

Patients

A total of 70 patients with early forms of sporadic CRC were examined in this study (49 patients were from Glasgow and 21 were from Kuwait): 27 patients with early-stage CRC who had no evidence of metastatic disease at the time of surgery (thus they are considered to have early-stage cancers at diagnosis), but who subsequently relapsed with metastasis; 43 age-matched and stage-matched (at diagnosis) patients, who remained disease-free postoperatively. All patients received no treatment other than surgery, except for 16 patients with Dukes' B2 stage from Kuwait who were treated surgically and with six cycles of standard chemotherapy and were followed up prospectively. Patients were followed up at regular intervals for a minimum period of 2 years (range 2–9 years), with a median of 5.5 years of follow up for survivors and 84% of survivors followed up for more than 3.5 years, and were clinically assessed for symptoms and signs of recurrence. Metastatic recurrences were confirmed radiologically and/or histologically, or at postmortem examination. For assessing disease-free survival, patients who died of causes unrelated to cancer but had no evidence of metastatic recurrence at the time of death were censored (seven patients). Five patients were lost to follow-up and 10 patients had no recurrence time recorded, but died from metastatic disease. For these patients the date of death was used to calculate the disease-free survival.

DNA extraction

Two pathologists examined the sections stained with haematoxylin-eosin and scored them for various histopathological parameters. About 6 μm sections of formalin-fixed paraffin wax-embedded tissue blocks of primary carcinomas and distant normal tissues from the same patients were dried on plain glass slides, dewaxed, dehydrated and stained with 0.1% toluidine blue. They were dissected by using a Leitz

Abbreviations: CRC, colorectal cancer; LOH, loss of heterozygosity

Table 1 Microsatellite markers used for the analysis of LOH on chromosomes 4 and 14q in early-stage CRC

Marker name	Location (NCBI decode cM)	Informative cases (%)	LOH (%)	No of disease-free patients		No of patients with metastatic recurrence		p Value†
				Het	LOH*	Het	LOH	
D4S2935	4p16 (NA)	31 (44)	12 (39)	13	3	5	9	0.024
D4S2986	4q23 (104.3)	22 (31)	8 (36)	7	2	7	6	NS
D4S1579	4q31.1 (136.82)	47 (67)	25 (53)	16	7	4	18	0.001
D4S1586	4q31.2 (141.21)	33 (47)	15 (45.5)	9	3	9	10	NS
D4S1595	4q33 (168.07)	26 (37)	13 (50)	10	2	2	10	0.003
D4S2920	4q35 (186)	36 (51)	11 (31)	15	4	10	6	NS
D14S283	14q11.2 (14.7)	40 (57)	13 (32.5)	16	5	11	6	NS
D14S275	14q12 (22.21)	33 (47)	11 (33)	16	3	4	8	0.007
D14S49	14q13 (NA)	21 (30)	8 (38)	11	2	2	6	0.018
D14S63	14q23 (63.5)	31 (44)	10 (32)	12	6	7	3	NS
D14S267	14q31 (NA)	38 (54)	19 (50)	10	5	8	12	NS
D14S65	14q32 (106.52)	44 (63)	14 (32)	21	4	8	9	0.018
D14S250	14q32 (114.7)	32 (46)	10 (31)	15	1	6	8	0.004

cM, centimorgans; CRC, colorectal cancer; Het, heterozygous; LOH, loss of heterozygosity; NA, not applicable; NCBI, National Center for Biotechnology Information; NS, not significant.
Markers in bold represent microsatellites that map to minimal deleted areas found by using comparative genomic hybridisation.[16]
*Five patients were lost to follow-up. Thus, their recurrence status cannot be assumed.
†p Values were calculated using two-sided χ^2 test or Fisher's exact test.

model M micromanipulator (Leica Microsystems (UK) Ltd, Milton Keynes, UK). The proportion of carcinoma cells in each neoplastic tissue sample was estimated and recorded. Tissue samples (0.1–1 mm^2) were digested with proteinase K at 37°C for 18 h. After inactivation of the proteinase K at 90°C for 10 min, the material was used directly for amplification of microsatellites by PCR. The median estimated percentage of carcinoma cells from the complete series of 70 patients was 75–90%.

Multiplex touchdown PCR and LOH analyses

Microsatellite markers obtained from ABI Human Linkage Mapping sets V2.5 panels (Applied Biosystems, Foster City, California, USA) for chromosomes 4 and 14 were used in

Table 2 Clinical characteristics of patients in relation to disease-recurrence status

Clinical characteristics	No of patients (n = 70)	Metastatic recurrence (n = 27)	Cancer did not recur (n = 38)*	p Values†
Sex				
Male	42	14	25	0.26
Female	28	13	13	
Age				
Mean (years)	65	68	63	0.12
Site‡				
Right sided	17	5	10	0.54
Left sided	48	21	24	
Site‡				
Rectum	18	12	6	0.017
Colon	47	14	28	
Differentiation§				
Well	26	13	11	0.14
Moderate	35	10	23	
Poor	6	3	2	
Mean counts (10 high-power fields)				
Mitosis	5.7	5.97	5.5	0.64
Apoptosis	11.4	10.5	11.9	0.646
Dukes' stage and treatment				
A (surgery only)	7	0	7	
B1 (surgery only)	47	24	22	
B2 (surgery and chemotherapy)	16	3	9	
pT stage¶				
pT1 and pT2	28	10	16	0.8
pT3 and pT4	38	15	20	
Lymphatic invasion				
Yes	12	3	8	0.34
No	58	24	30	
Vascular invasion				
Yes	12	2	9	
No	58	25	29	

*Five patients were lost to follow-up.
†p Values were calculated using two-sided χ^2 test or Fisher's exact test. Mann–Whitney U test was used to compare means.
‡Cancer site was colonic, but the side affected was unknown in five cases. Right-sided cancers include caecum and ascending colon. Left-sided cancers include transverse, descending, sigmoid colon and rectum.
§Differentiation was undetermined in three cases.
¶T stage could not be assessed in four cases.

LOH analyses performed in this study. On chromosome 4 these were D4S2935, D4S2986, D4S1579, D4S1586, D4S1595 and D4S2920. On chromosome 14 these were D14S283, D14S275, D14S49, D14S63, D14S267, D14S65 and D14S250. The physical order and genetic map location of these microsatellite markers were obtained from the National Library of Medicine at the National Institute of Health websites Genome database at http://www.ncbi.nlm.nih.gov, with additional centimorgan mapping detail obtained from the Marshfield genetic map (table 1). Microsatellite markers were also selected on the basis of size, with an upper limit not greater than about 233 bp to minimise amplification difficulties inherent to DNA extracted from paraffin wax-embedded tissues and to facilitate vigorous amplification of the product.

All multiplex PCR reactions were performed using 1 µl from each 10 µM primer mix for a total of three microsatellite markers per reaction on the basis of predetermined marker groups, 100 ng of genomic DNA and 45 µl of ABgene Reddymix 1.1 X PCR Master Mix (3.0 mM $MgCl_2$; ABgene House, UK) in each reaction. A touchdown PCR approach spanning annealing temperature of the markers was then initiated with a preliminary denaturation step at 95℃ for 3 min, 10 cycles of 95℃ for 15 s, 63℃ (0.5℃ decrease for each cycle) for 30 s and 72℃ for 40 s. This was followed by a second round of 40 cycles at 95℃ for 30 s, 55℃ for 30 s and 72℃ for 40 s, with a final extension step of 72℃ for 8 min. The resultant product then underwent electrophoresis using the ABI Prism 3100 Genetic Analyzer, with generated data analysed by the ABI Genescan software.

Allelic status (LOH) was then assessed by comparing constitutionally informative (heterozygous) alleles with their corresponding tumour alleles (supplementary figs A,B). Non-informative cases (homozygous) and those exhibiting microsatellite instability in three or more markers were excluded from consideration. Microsatellite loci were regarded as having undergone LOH if either the entire upper or lower tumour allele of informative cases were visually absent, or were calculated to have a 50% or greater diminishment of the allele.

Microsatellite instability analysis

Microsatellite stability was assessed in all 70 patients by using the 13 dinucleotide markers mentioned above. Microsatellite instability in ⩾30% of the analysed loci was classified as highly microsatellite instable (one patient who was excluded from the survival analysis); allelic instability in one or two of the 13 microsatellites or <30% of the loci analysed was considered to be microsatellite low (13 patients). Samples with no microsatellite instability were classified as stable (50 patients). In six patients, microsatellite stability could not be assessed.

Statistical analysis

Disease-free survival distribution was calculated for all generated LOH data by using the Kaplan–Meier method and evaluated by the log rank test. A p value of <0.05 was considered to be significant. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS V.12.0, Chicago, Illinois, USA) software.

RESULTS AND DISCUSSION

Table 2 shows the clinicopathological characteristics of the cohort in relation to metastatic recurrence. Only rectal location (hazard ratio (HR) 4.6, 95% confidence interval (CI) 1.7 to 12.4, $p = 0.003$) and T stage 3/4 (HR 4.5, 95% CI 1.6 to 12.8, $p = 0.005$) could predict survival independently. These results confirm the limited usefulness of current clinicopathological parameters in identifying patients at risk from metastatic relapse in early-stage CRC.

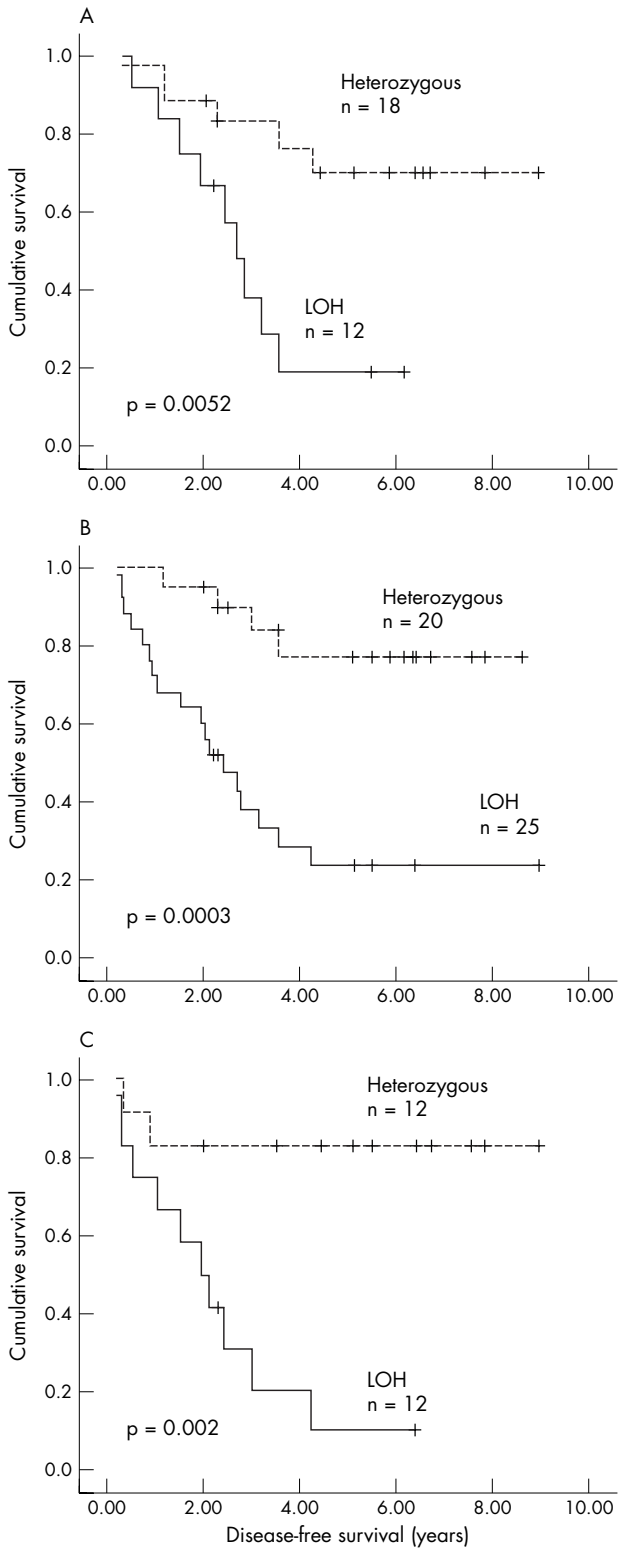

Figure 1 Kaplan–Meier plots of disease-free survival of the patients with early-stage colorectal cancers in relation to loss of heterozygosity (LOH) at microsatellites (A) D4S2935, (B) D4S1579 and (C) D4S1595. Solid black lines represent patients with LOH at the corresponding micosatellite and dashed black lines are patients whose tumours were heterozygous. p Values represent the log rank test.

For chromosome 4 markers, informative cases ranged between 31% and 67% and incidence of LOH was observed in 31–53% of tumours among the six loci examined (table 1 and

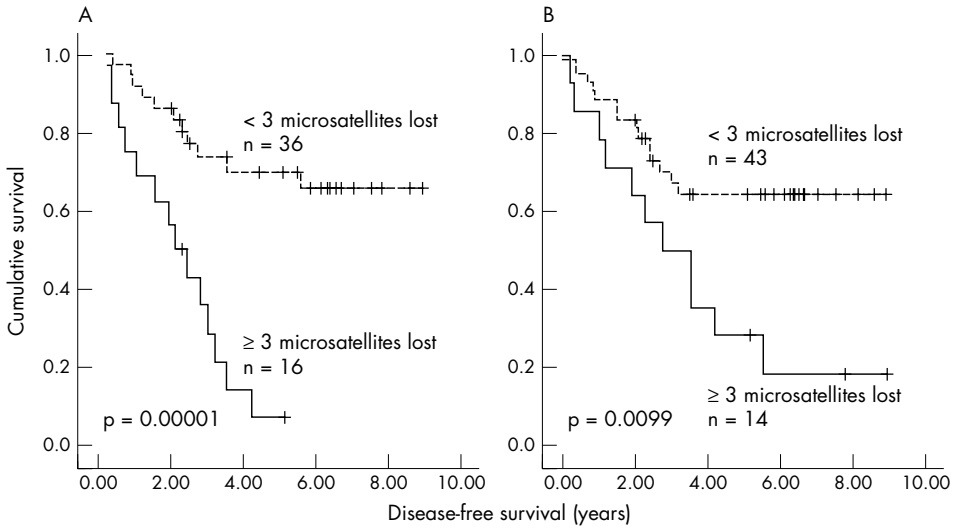

Figure 2 Kaplan–Meier plots of disease-free survival of the patients with early-stage colorectal cancers in relation to the number of loss of heterozygosity (LOH): (A) chromosome 4 and (B) chromosome arm 14q. Solid black lines represent patients with LOH at three or more of the corresponding markers and dashed black lines are patients whose tumours harboured losses in less than three markers. p Values represent the log rank test.

supplementary fig A). The highest LOH was noted at D4S1579. LOH at D4S2935, D4S1579 and D4S1595 was notably associated with early-stage CRC, which subsequently metastasised (table 1). No relevant association was found between LOH at any of the six microsatellite markers and clinicopathological features, including age, sex, site (left or right, colon or rectum), differentiation, extracellular mucin deposits, mitotic or apoptotic indexes, Dukes' stage (A *v* B1 or B2) and lymphatic or vascular invasion. A non-significant trend was found for LOH at D4S2935 and D4S1579 and higher invasive stages, namely tumour-node-metastasis stages 3 and 4 ($p = 0.05$ and 0.069, respectively, by using two-sided χ^2 test or Fisher's exact tests).

Univariate analysis showed that LOH at D4S2935, D4S1579 and D4S1595 were distinctively associated with shorter disease-free survival (table 3). This association was also observed using Kaplan–Meier's survival curves (fig 1). This finding was maintained after stratification for Dukes' stage, treatment and tumour site (data not shown). Our data are consistent with previously published work indicating the association of 4p14–16 locus in tumour suppression and CRC aggressiveness.[17]

We then examined the extent of LOH by counting the number of microsatellites lost of the six examined on chromosome 4 in each patient. The mean number of LOH in primary tumours that metastasised was 2.4, whereas non-metastatic CRC had a mean of 0.64 ($p = 0.0001$ using Student's t test). Moreover, LOH in three or more microsatellites was significantly associated with reduced 5-year survival compared with patients who lost less than three microsatellites (fig 2A). This association was maintained even after varying the cut-off category of high LOH to four or more, indicating the robustness of the data (data not shown). Rectal tumours showed a significant association with high LOH compared with colon cancers, consistent with their aggressive nature (data not shown). Our data are consistent with the notion that loss of chromosome 4 and therefore the decision for metastasis may be made early in cancer development. In this context, Shivapurkar *et al*[18] showed that LOH within 4p16.3, 4q33–34 (which are the same chromosomal regions identified here) and 4q25–26 occurred as early as adenomas.

For chromosome 14 markers, informative cases ranged between 30% and 63% and incidence of LOH was observed in 31–50% of tumours among the seven loci examined (table 1 and supplementary fig B). The highest LOH was noted at D14S67. This is consistent with previously published LOH

Table 3 Hazard analysis in relation to chromosomal aberrations in early-stage colorectal cancer

Marker name	Hazard ratio	95% CI	p Value
D4S2935	4.37	1.42 to 13.5	0.01
D4S2986	0.525	0.18 to 1.57	0.25
D4S1579	5.94	1.99 to 17.69	0.001
D4S1586	1.72	0.69 to 4.28	0.24
D4S1595	8.61	1.85 to 40.12	0.006
D4S2920	1.78	0.64 to 4.93	0.27
D14S283	1.79	0.66 to 4.9	0.25
D14S275	5.4	1.6 to 18.17	0.007
D14S49	6.39	1.28 to 39.93	0.024
D14S63	1.29	0.33 to 5.01	0.72
D14S267	1.87	0.76 to 4.59	0.17
D14S65	3.8	1.45 to 9.95	0.007
D14S250	3.55	1.23 to 10.25	0.019

Markers in bold represent microsatellites that map to minimal deleted areas found using comparative genomic hybridisation.
*Hazard ratio <1 indicates a survival benefit, whereas hazard ratio >1 represents an increased risk of metastatic relapse.

Table 4 Independent prognostic factors based on various categories of variables in patients with early-stage colorectal cancer

Model	Hazard ratio*	95% CI	p Value
I. Clinicopathological parameters alone			
Tumour site (rectum *v* colon)	4.6	1.7 to 12.4	0.003
pT stage (T3-T4 *v* T1-T2)	4.5	1.6 to 12.8	0.005
Extracellular mucin production (pools *v* low)	0.1	0.018 to 1.005	0.051
II. LOH alone			
High level (5–8 markers) *v* moderate or low loss	6.7	2.63 to 17.1	0.0001
III. All variables			
High level (5–8 markers) *v* moderate or low loss	7.3	2.5 to 21.1	0.0001

Hazard ratios were estimated in a multivariate analysis using forward stepwise procedure.
*Hazard ratio <1 indicates a survival benefit, whereas hazard ratio >1 represents an increased risk of metastatic relapse.

data on chromosome 14q from CRC.[19–22] Two minimal regions of deletion associated with metastatic recurrence were mapped to neighbouring markers D14S275/D14S49 at 14q12–13 and D14S65/D14S250 at 14q32. Previously, Bando *et al*[21] mapped the minimal region of LOH between markers D14S65 and D14S250 in their CRC cohort. Unfortunately, they presented no follow-up data to illuminate the effect of these deletions on the survival of the patients. Moreover, Thorstensen *et al*[23] mapped two areas on chromosome 14, namely 14q13–21 and 14q24–31, which were frequent in metastases and in primary CRCs. The 14q24–31 area agrees with our results, but we did not probe the 14q13–21 area.

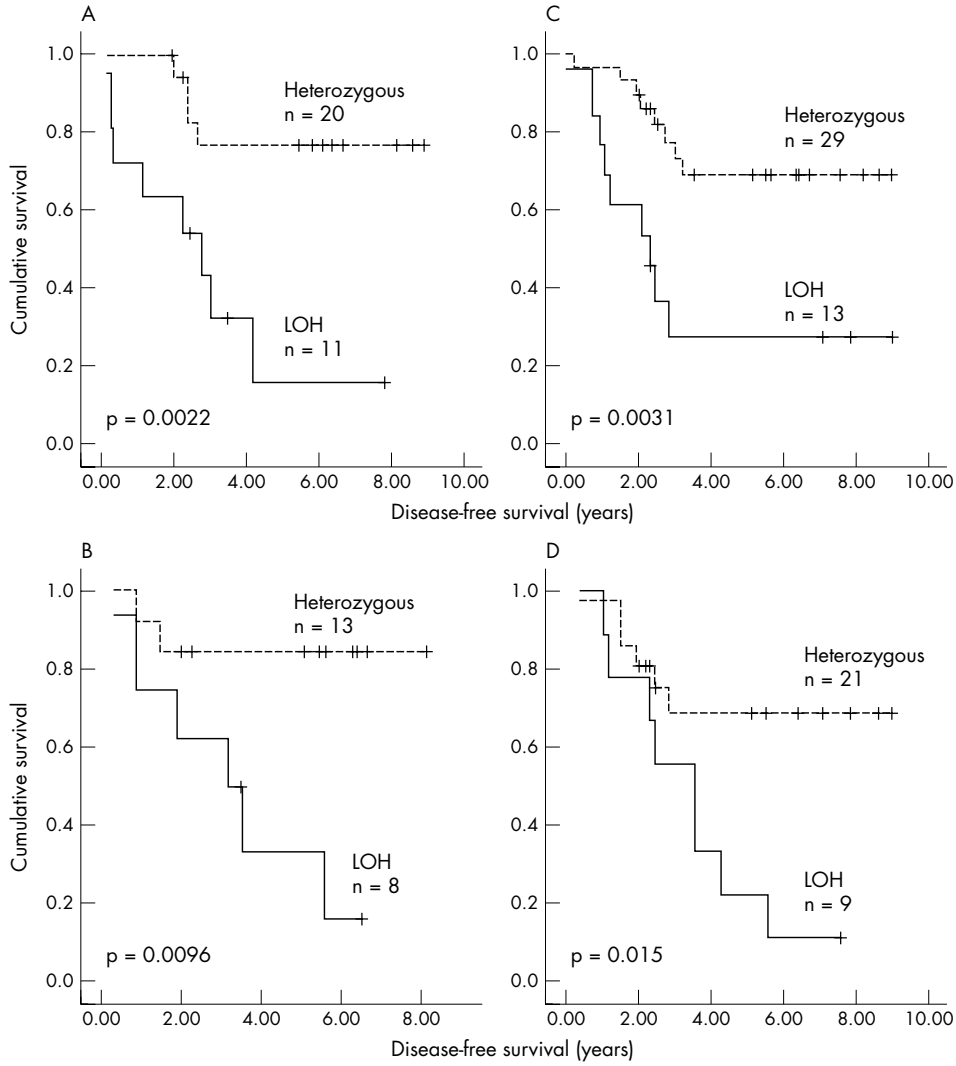

Figure 3 Kaplan–Meier plots of disease-free survival of the patients with early-stage colorectal cancers in relation to loss of hererozygosity (LOH) at microsatellites (A) D14S275, (B) D14S49, (C) D14S65 and (D) D14S250. Solid black lines represent patients with LOH at the corresponding micosatellite and dashed black lines represent patients whose tumours were heterozygous. p Values represent the log rank test.

Nevertheless, here we have shown that LOH at the two regions was markedly associated with metastatic behaviour of early-stage CRCs (table 1).

No significant association was found between LOH at any of the seven microsatellite markers and clinicopathological features, including age, sex, site, tumour-node-metastasis stage, differentiation, extracellular mucin deposits, mitotic or apoptotic indexes, Dukes' stage (A *v* B1 or B2) and lymphatic or vascular invasion.

Univariate analysis using Cox's regression showed that LOH at D14S275, D14S49, D14S65 and D14S250 was significantly associated with shorter disease-free survival (table 3). This association was also observed using Kaplan–Meier's survival curves (fig 3). The observed results were maintained after stratification for Dukes' stage, treatment and site (data not shown). The extent of LOH at the seven microsatellites examined on chromosome 14 in each patient showed that a major number of patients with metastatic relapse had higher number of allelic loss than patients who remained disease free. The mean number of LOH in tumours that metastasised was 2.08, whereas non-metastatic tumours had a mean of 0.81 ($p = 0.0001$ using Student's t test). LOH in three or more microsatellites was significantly associated with reduced 5-years survival compared with patients who lost less than three microsatellites on chromosome arm 14q (fig 2B).

We showed that the mean number of microsatellite markers lost in metastatic tumours was 4.3 (95% CI 3.5 to 5.1) compared with 1.3 (95% CI 0.7 to 1.75) for tumours that did not metastasise ($p = 0.0001$). Using the number of microsatellites lost of the 13 examined on both chromosomes 4 and 14q as a guide, we categorised the extent of LOH detected in informative patients into high level, with the loss of five to eight markers; moderate loss, with the loss of four markers; and low loss, with the loss of zero to three markers from the combined LOH data on both chromosomes. Figure 4 shows that patients with high-level loss had significantly reduced 5-year survival compared with patients with moderate or low loss, which was independent of Dukes' stage and treatment. Agreeably, univariate (table 3) and multivariate analyses (table 4) showed high-level loss including both chromosomes 4 and 14q to be an independent prognostic indicator in early-stage CRC. Our data are consistent with the concept that advanced CRCs are genetically more complex than less advanced primary tumours.[24] In this connection, primary tumours with a propensity to metastasise had higher numbers of LOH on chromosomes 4 or 14q. Similarly, high-level LOH on both chromosomes from the combined microsatellite data showed the most remarkable association with disease-free survival in a multivariate Cox's proportional hazards model in these patients. This is consistent with the notion that aneuploidy in itself correlates with a poor prognosis in patients with CRC.[25] The fact that monosomy of chromosomes 4 and 14 was not infrequent in some of our patients, as shown by the extensive LOH, could indicate that chromosomal instability is a favoured mechanism for the initiation of metastasis because it would allow loss of several metastasis suppressor genes at once. Thus, the association between chromosome 4 and 14q loss and reduced disease-free survival could be a reflection of such aneuploidy. Nevertheless, localised LOH on either chromosome 4 or 14 was associated with metastatic relapse, probably indicating a selective advantage for tumours harbouring these losses. Accordingly, this may indicate the presence of several metastasis suppressor genes on these chromosomal regions and that their loss may be responsible for the metastatic behaviour in early-stage CRC. The precise molecular and cellular mechanisms behind such selection remain to be elucidated and the identification of these genes is awaited. Recently, a meta-analysis of 30 reports on

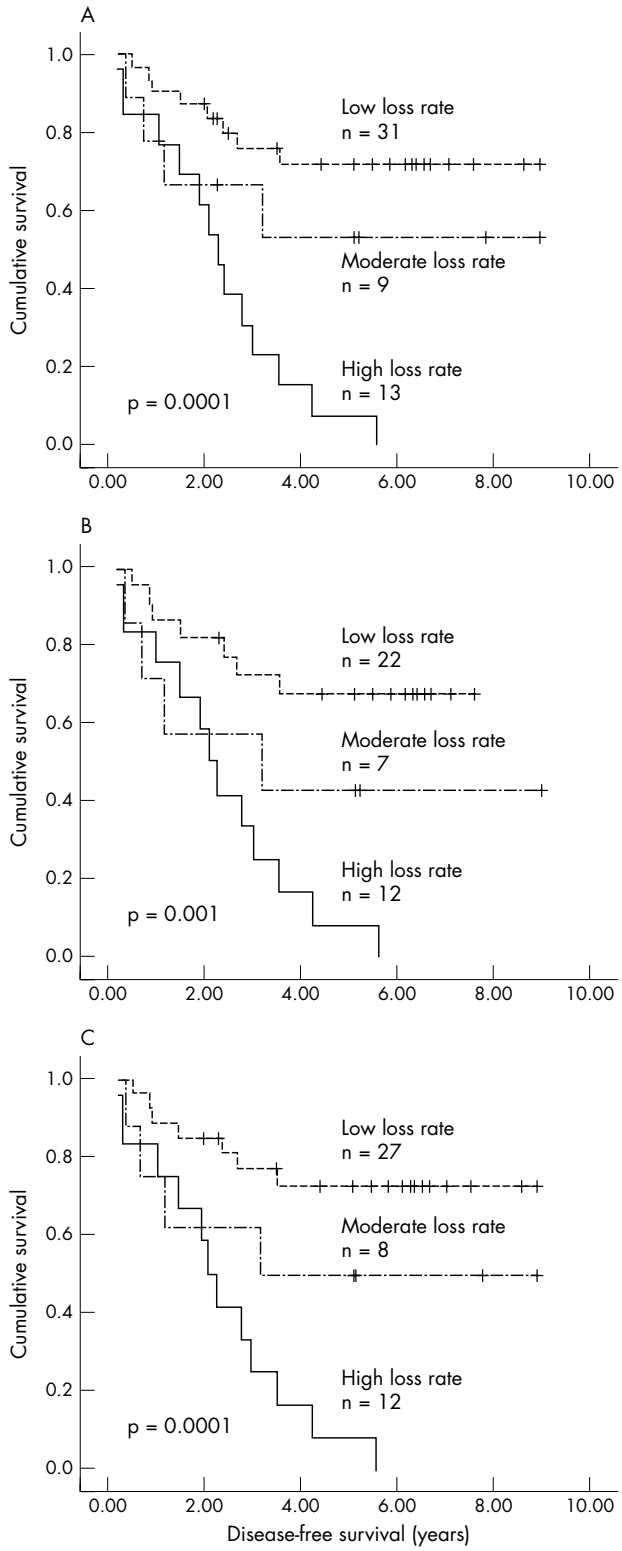

Figure 4 Kaplan–Meier plots of disease-free survival of the patients with early-stage colorectal cancers in relation to the number of LOH on chromosomes 4 and 14. The analysis included three levels of chromosomal loss— that is, low (dashed lines), moderate (grey lines) and high (solid black lines) levels. (A) Effect of loss rates on disease-free survival in patients without stratification. (B) Data limited to Dukes' stage B1. (C) Data limited to patients treated with surgery alone. p Values represent the log rank test.

Take-home messages

- Early-stage colorectal cancer (CRC) defined by the lack of microscopic and clinical metastases represents a considerable challenge to doctors. Patients with early-stage CRC are not usually offered treatment beyond surgical intervention, although 10–30% relapse. This paper discusses the value of molecular profiling of primary tumours for identifying these patients.
- We used microsatellite allelotyping to define minimal regions of loss of heterozygosity (LOH) on chromosomes 4 and 14, which may contribute to potential localisation of metastasis suppressor genes.
- Determining the LOH on chromosomes 4 and 14q and their extent in primary tumours of patients with early-stage CRC may constitute a molecular signature of metastatic recurrence and could aid in identifying the 10–30% of patients who have been erroneously diagnosed with early-stage CRC, where in fact they could have harboured occult metastases beyond the resolution of current imaging techniques.
- These novel findings, which have been largely ignored, may permit a more precisely targeted treatment of this subgroup of patients. In practice, allelotyping can be applied to DNA extracted from formalin-fixed and paraffin wax-embedded tissues, and can be easily integrated into the mainstream investigation on patients in surgical pathology departments.

comparative genomic hybridisation analysis of CRC has shown a notable association between loss of chromosome 4 and transition to advanced Dukes' stage and frequent loss of chromosome 14q in liver metastases. Also, the authors have shown that the detected aberrations were present equally in primary tumours and metastases, suggesting that the primary carcinomas could already harbour genetic changes at the time of metastasis.[26] Regardless of the mechanism, the fact that the molecular signature of metastasis could be identified from primary tumours of patients with early-stage CRC, using a simple technique applied to a more stable molecule like DNA rather than RNA, may enable a more precisely targeted treatment of this subgroup of patients, who have been largely ignored. In practice, allelotyping can be applied to DNA extracted from formalin-fixed and paraffin wax-embedded tissues, and can be easily integrated in the mainstream treatment of patients in Surgical Pathology Departments. A larger prospective study on the identity of the relevant metastasis-suppressor genes and their influence on prognosis and targeted treatment is now indicated.

ACKNOWLEDGEMENTS

This study was supported by the Kuwait Foundation for the Advancement of Sciences grant number 99-07-07. We thank Dr Anita Mathew for the follow-up data of patients from Kuwait and Professor David Hole for the follow-up data of patients from Scotland. We also thank Govindarajulu Varadharaj for his help with optimising the multiplex LOH PCR.

Additional figures can be viewed online at http://www.jclinpath.com/supplemental

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Competing interests: None.

The procedures followed were in accordance with the ethical standards implemented at the Universities of Glasgow and Kuwait and with the Helsinki Declaration of 1975, as revised in 1983.

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