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Original Paper

Prognostic Significance of Microsatellite Instability in Patients with Gastric Carcinoma

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A proportion of gastric adenocarcinomas exhibit replication errors manifested as microsatellite instability. The clinicopathological and prognostic significance of this abnormality remains uncertain. This study aimed to determine the importance of microsatellite instability by analysing a large series of gastric carcinomas from an English population. Using a novel fluorescent polymerase chain reaction technique, we amplified 11 microsatellite sequences from paired normal and carcinoma DNA from 101 patients who underwent a potentially curative resection for gastric carcinoma. Overall, 21% of cases demonstrated microsatellite instability in at least one locus. At least four loci were examined in each case. A replication error positive phenotype (minimum of 29% of loci affected) was detected in 9% of cases. There was no statistically significant association between the presence of microsatellite instability or replication error positive phenotype and the patient's age, sex, tumour site, stage, node status, histological subtype or grade. Carcinomas confined to the mucosa or submucosa (T1) showed a significantly higher frequency of instability and replication error positive phenotypes than T3 lesions ($P = 0.03$ and $P = 0.05$, respectively). A larger proportion of patients who were microsatellite instability or replication error positive were alive at 5 years compared with those who were negative but this did not reach statistical significance ($P = 0.15$ and $P = 0.16$, respectively). We identified a subset of gastric carcinomas from a relatively low-risk population which showed evidence of microsatellite instability. There were no statistically significant 5-year survival advantages in cases demonstrating microsatellite instability or replication error positive phenotypes. The detection of microsatellite instability is of limited prognostic value in gastric carcinoma. © **1997 Elsevier Science Ltd.**

Key words: microsatellite instability, replication errors, gastric carcinoma, prognosis
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INTRODUCTION

MICROSATELLITE INSTABILITY (MI) is a manifestation of defective DNA mismatch repair [1–4] and is a phenotypic marker for the hereditary non-polyposis colorectal cancer (HNPCC) syndrome [5–7]. MI is recognised by the identification of novel alleles in microsatellite sequences from carcinoma DNA when compared with constitutionally 'normal' DNA from the same patient. Mutation in *hMLH1*, *hMSH2*, *hPMS1* or *hPMS2* DNA mismatch repair genes leads to a widespread increase in genomic frameshift mutations. This results in the expansion or contraction of repetitive sequences which are usually stably inherited and have a low intrinsic mutation rate [8]. Carcinomas from HNPCC kindreds demonstrate a replication error-positive (RER+) phenotype, in which MI typically occurred in at least 2/7 (29%) microsatellite markers examined [5].

Some 9–16% of sporadic colorectal carcinomas exhibit the RER+ phenotype [5–7, 9] and have similar clinicopathological features to HNPCC-associated colorectal carcinomas including a survival advantage over RER-negative (RER−) cases [7, 10]. Sporadic gastric carcinomas from high-risk populations such as Japan show MI+ at reported frequencies of 33% [11] and 39% [12]. The frequency of MI+ and RER+

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in gastric carcinomas from low-risk populations is less well defined. Recently, a Portuguese study (from a relatively high-risk population) showed that RER+ gastric carcinomas exhibit a particular clinicopathological profile and have a better prognosis than RER− tumours [13].

The aim of this study was to determine the prevalence of MI in gastric carcinomas from a relatively low-risk English population. Using a novel, validated, accurate, non-radioactive fluorescent polymerase chain reaction technique together with Genescan analysis [14], we investigated the frequency of MI+ and RER+ phenotypes in a large series of gastric carcinomas from an English population to determine their clinicopathological and prognostic significance.

PATIENTS AND METHODS

Details of patients

Carcinomas from 101 patients who underwent a potentially curative (R0) resection [15] for gastric adenocarcinoma in the Department of Surgery at the Leeds General Infirmary, Leeds, U.K. were studied. Carcinomas were staged according to International Union against Cancer (UICC) TNM classification system [15]. The site of origin of the carcinoma was recorded. The tumours were classified histologically by one Consultant Histopathologist according to Lauren [16], Ming [17] and Goseki [18] classification and grade [19]. Survival data was obtained from the Yorkshire Cancer Registry.

DNA extraction

Two separate areas were outlined on an H&E section by a gastrointestinal histopathologist: the first area containing normal gastric mucosa or muscularis propria and the second, adenocarcinoma (composed of at least 50% neoplastic cells). Corresponding archival paraffin-embedded sections were cut, dewaxed and the marked areas were microdissected into separate tubes. DNA was extracted and purified using a standard phenol/chloroform method [20] and suspended in molecular biology grade water.

Fluorescent PCR and Genescan analysis

A panel of 11 pairs of microsatellite primers in which one oligonucleotide was end-labelled with a fluorescent dye were analysed. The primers amplified *D2S123*, *D3S966*, *D3S1076*, D5S82, *DP1*, *D10S197*, *D11S904*, *D13S175*, *NM23*, *p53* and *DCC* microsatellite loci [14, 21–23]. With the exception of *DCC* and *p53*, all the loci were dinucleotide $(CA)_n$, repeats. *DCC* and *p53* were $(TA)_n$ and $(AAAAT)_n$, repeat motifs, respectively. Fluorescent and non-fluorescent labelled primers were synthesised on an automated DNA synthesiser (Applied Biosystems model 391A, Foster City, California, U.S.A.) in accordance with the manufacturer's instructions.

Fluorescent PCR was performed as previously described [14, 21], using single microsatellite targets on paired normal and carcinoma DNA extracts (diluted 1:20 with molecular biology grade water). Typically 35 cycles of amplification were required for each assay. Annealing temperatures ranged from 55 to 60°C. PCR products were electrophoresed on a 6% polyacrylamide gel for 6–8 h at 30 W using an automated DNA sequencer, and visualised using Genescan Analysis software (Applied Biosystems).

Microsatellite instability

Normal and carcinoma microsatellite DNA amplification products were compared for each patient. The appearance of novel alleles in the carcinoma sample was classified as MI+. RER+ was recorded when at least 29% of loci tested demonstrated MI+ [5]. Cases showing levels of MI below 29% were termed RER−. Cases showing no evidence of instability in at least four markers were categorised MI−. Positive results were confirmed by a repeat PCR and Genescan analysis.

Clinicopathological correlations and survival analysis

Chi-square, Fisher's exact and Mann–Whitney U-tests, Kaplan–Meier life table survival analysis and log-rank tests were performed using Statistical Package for the Social Sciences (SPSS, Chicago, Illinois, U.S.A.) 6.1 software. A P value of less than 0.05 was considered statistically significant.

RESULTS

Out of 101 carcinomas, 21 (21%) demonstrated the MI+ phenotype (with at least one locus showing instability). An example of an electrophoretogram demonstrating MI is shown in Figure 1. The RER+ phenotype (MI in at least 29% of loci) was observed in 9% (9/101) of carcinomas. The RER+ cases exhibited MI in 29–67% (mean of 40%) of the loci tested. A minimum of four loci were examined in each case. The remaining markers yielded no results due to the observation of allelic imbalance, an uninterpretable electrophoretogram or fluorescent PCR failure.

Table 1 shows the number of MI+ and RER+ cases with respect to different clinicopathological features. There was no statistically significant association between the presence of the MI+ or RER+ and the patient's age, sex, tumour location, Lauren, Ming or Goseki classification, grade, UICC stage or lymph node status. A statistically significant difference was observed between the presence of MI+ and RER+ in T1 versus T3 carcinomas ($P = 0.03$ for MI+, by Chi square and $P = 0.05$ for RER+, by Fisher's exact test).

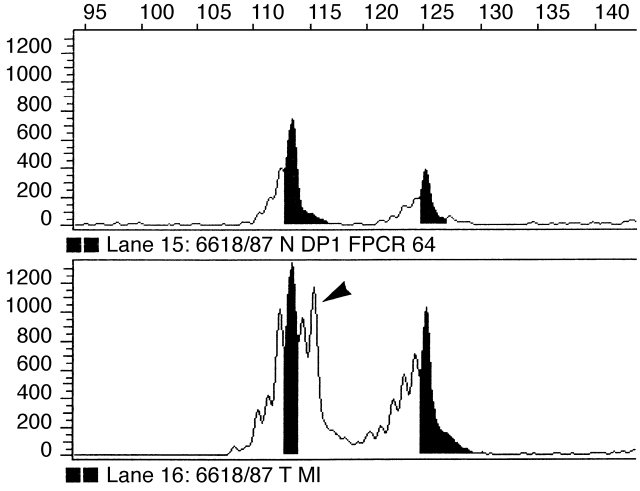

Figure 1. An electrophoretogram showing microsatellite instability at the DP1 locus. Amplification products are shown in cross-section as peaks. The *x*-axis shows the size of the fragment (to the nearest nucleotide) and the *y*-axis shows units of fluorescence. Lanes 15 and 16 show the results from normal and carcinoma DNA, respectively, amplified from the same patient. The shaded peaks correspond to the constitutional (heterozygous) alleles. Microsatellite instability is identified by a novel allele in the carcinoma DNA sample (arrow). Remaining unshaded peaks represent artefactual 'stutter bands' [14].

Table 1. *Relationship between MI+ or RER+ phenotypes with respect to clinicopathological features*

Category	MI+ (%)† $n=21$	RER+ (%)‡ $n=9$	Total $n=101$	P value¶
Age in years				
Range	52–88	58–82	40-88	0.10†
Median	71	75	70	0.39‡
Gender ratio				
Male:female	2.5:1	1.7:1	1.7:1	0.34† 0.90‡
Site				
Upper third*	5 (20)	2 (8)	25	
Middle third	7 (18)	5 (13)	39	
Lower third*	8 (26)	2 (6)	31	0.61†
Remnant	1 (17)	0 (0)	6	0.84‡
Lauren				
Intestinal*	19 (25)	9 (12)	75	
Diffuse*	2 (9)	0 (0)	22	0.17†
Mixed	0 (0)	0 (0)	4	0.18‡
Ming				
Expansile	6 (25)	4 (17)	24	0.56†
Infiltrating	15 (19)	5 (6)	77	0.27‡
Goseki				
I & III (mucin poor)	15 (26)	5 (9)	58	0.14†
II & IV (mucin rich)	6 (14)	4 (9)	43	1.00‡
Grade (differentiation)				
Well or moderate	14 (28)	7 (14)	50	0.08†
Poor	7 (14)	2 (4)	51	0.09‡
UICC stage				
I*	9 (30)	5 (17)	30	
II	5 (16)	2 (6)	32	0.24†
III*	7 (18)	2 (5)	39	0.14‡
TNM 'T' classification				
T1*	6 (40)	4 (27)	15	
T2	8 (22)	3 (8)	37	0.03†
T3*	7 (14)	2 (4)	49	0.05‡
Lymph node status				
Node-negative	10 (24)	4 (10)	42	0.53†
Node-positive	11 (19)	5 (8)	59	0.85‡

*Groups which were compared statistically when there where >2 categories. †MI+ phenotype characterised by the presence of instability in at least one locus examined. ‡ RER+ phenotype characterised by instability in at least 29% of loci examined. ¶P values were calculated for different groups by Mann–Whitney U-test, Chi-square and two-tailed Fisher's exact probability tests where appropriate. A P value of less than 0.05 was considered statistically significant.

Kaplan–Meier life table survival analysis and log-rank testing confirmed a statistically significant relationship between UICC stage ($P<0.0001$), TNM 'T', ($P=0.0002$) and 'N' classifications ($P<0.0001$), Goseki classification ($P=0.01$) and 5-year survival (gastric cancer-specific death). A larger proportion of MI+ (73%) and RER+ (88%) cases survived 5 years compared with MI– (52%) and RER– (53%) carcinomas but these differences did not reach statistical significance ($P=0.15$ for MI and $P=0.16$ for RER status). A Kaplan–Meier survival plot for MI+ versus MI– cases

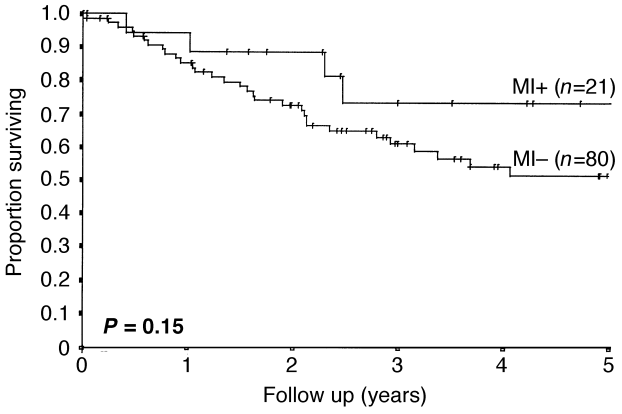

Figure 2. Kaplan–Meier survival plot of 5-year survival (gastric cancer-specific death) with respect to the presence or absence of MI ($P=0.15$, log-rank test). Steps in the line correspond to deaths from gastric cancer, whereas ticks on the line represent censored cases.

is shown in Figure 2. No statistically significant relationship was observed between 5-year survival and tumour site ($P=0.62$), Lauren ($P=0.25$) and Ming ($P=0.48$) classifications and the degree of differentiation ($P=0.57$).

DISCUSSION

There has been conflicting and confusing evidence concerning the role of MI in gastric carcinoma. There are reports of a small series of cases [24–27], studies analysing too few loci [11, 12] and differences in the classification of MI+ and RER+ [28, 29]. We examined a large series of gastric adenocarcinomas from an English (relatively low-risk) population, using a minimum of four markers in each case to identify the clinicopathological and prognostic significance of MI. Using a validated fluorescent PCR technique, with which we have previously demonstrated MI+ (RER+) in 22% (13%) of 54 sporadic colorectal carcinomas (14), we found that 21% (21/101) of gastric carcinomas were MI+ with 9% (9/101) of cases showing RER+ (>29% of loci were affected). Our results are consistent with those found in a smaller German study of 46 gastric carcinomas using autoradiographic methods, which found an incidence of MI+ and RER+ in 24% and 4% of cases, respectively [30].

Attempts have been made to identify clinicopathological features associated with MI in gastric carcinoma. We found levels of MI+ (RER+) in 25% (12%) of intestinal and 9% (0%) of diffuse carcinomas which are similar to those reported in Germany [30]. However, this is lower than the incidence of MI+ or RER+ reported in 41% of intestinal and 32% of diffuse carcinomas from a relatively high-risk population [31]. Japanese reports have demonstrated a statistically significant difference between the incidence of MI+ in poor (64%) versus well (17%) differentiated carcinomas [12]. However, a higher frequency has been reported in well (33%) compared with poorly (18%) differentiated carcinomas by a study which examined a larger number of loci [29]. Our results support the observations of Semba and associates [29].

The site distribution of MI throughout the stomach has showed considerable variation. Instability has been found predominantly in the distal stomach [13, 28, 32], fairly equally distributed in site throughout the stomach [33] and predominantly at the cardia by others [31]. We showed

evidence of MI+ (and RER+) in upper, middle and lower third carcinomas with no statistically significant difference in frequency with respect to tumour location (Table 1).

There have been suggestions that gastric carcinomas with MI+ or RER+ may be associated with a reduction in incidence of nodal metastases [13, 26] and improved survival [13, 28]. This is despite a tendency to observe MI at a higher frequency in advanced carcinomas compared with early lesions [31], and to detect instability in more elderly patients [26]. We found no age differences between our MI+ and RER+ subgroups and no significant relationship with the presence or absence of nodal metastases (Table 1). MI+ and RER+ occurred in stage I, II and III gastric carcinomas. The frequency of MI+ (RER+) was 40% (27%) for T1 tumours compared with 14% (4%) for T3 carcinomas ($P = 0.03$, by Chi square test for MI+ and $P = 0.05$, by Fisher's exact test for RER+). The finding of MI at a higher frequency in early stage carcinomas could either reflect different molecular pathways taken by early and advanced carcinomas or a changing pattern of replication error accumulation which may occur in tumour progression.

Using Kaplan–Meier life-table survival analysis, we found that a larger proportion of patients with MI+ or RER+ gastric carcinomas survived 5 years compared with MI− or RER− cases, respectively. However, this difference did not reach statistical significance, probably due to the small numbers of MI+ and RER+ cases overall. Although we cannot exclude the possibility that the presence of MI may be associated with improved prognosis, the apparent survival advantage may be attributable to the higher frequency of MI+ and RER+ among early stage carcinomas. To demonstrate MI as a significant prognostic factor would entail multivariate analysis on an even larger series of cases with the stage selection bias eliminated. On this sample size, we were able to confirm the important relationship between 5-year survival and UICC stage, TNM 'T' and 'N' classifications and Goseki classification which have been shown to be independent predictors of survival in gastric carcinoma [34].

The genetic basis of RER+ sporadic gastric carcinomas is as yet undetermined. Recently it has been shown that sporadic colorectal carcinomas showing RER+ have a 6% incidence of somatic mutation in *hMLH1* or *hMSH2* DNA mismatch repair genes which most frequently undergo germline and somatic mutation in HNPCC [35]. It is possible that our RER+ gastric carcinomas harbour similar somatic mutations. A proportion of the series demonstrated low levels of MI which cannot be ignored until their genetic basis is understood. Gleeson and associates [36] studied the frequency of instability in 38 upper third gastric carcinomas using 138 microsatellite markers and found 32 cases (84%) had low frequency MI (0.8–11.4% of loci tested were positive) with an RER+ phenotype present in just 3% (1/38) of carcinomas. Low levels of instability may arise due to inactivation of an unknown DNA mismatch repair gene which causes a reduced rate of MI or a poorly penetrant mutation in one of the HNPCC-associated DNA mismatch repair genes. Alternatively, saturation of the mismatch repair machinery due to increased DNA damage may result in 'overflow' MI. The recent observation of MI in inflammatory conditions such as pancreatitis [37] and ulcerative colitis [38] supports the latter suggestion.

We confirmed the presence of MI+ and RER+ phenotypes in a minor subset of gastric carcinomas from an English (low-risk) population. There was a significantly higher prevalence of MI+ and RER+ in carcinomas confined to the mucosa. There was no significant difference in 5-year survival for cases demonstrating MI+ or RER+ phenotypes compared with MI− or RER−, respectively. The detection of MI is therefore of little prognostic value in gastric carcinoma.

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