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Microsatellite Instability in Gastric Cancer Is Associated with Tumor Location and Family History in a High-Risk Population from Tuscany[1]

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ABSTRACT

We studied the presence of microsatellite instability (MSI) in a series of 108 gastric cancers (GCs) previously identified in an epidemiological study carried out in a high-risk area around Florence. To investigate associations between MSI and GC family history, 34 cases (31.5%) who had a GC-affected first-degree relative were included in the series. A family history positive for colorectal cancer was reported quite rarely (5.6%). The analysis of 6 microsatellite loci in DNA from paired normal tissue and tumor samples microdissected from paraffin-embedded specimens revealed varying degrees of instability: 56 cases (51.8%) did not show instability at any of the 6 loci; 19 (17.6%) showed instability at 1 locus; 16 (14.8%) showed instability at 2 loci; 11 (10.2%) showed instability at 3 loci; 4 (3.7%) showed instability at 4 loci; and 2 (1.9%) showed instability at 5 loci. The replication error-positive (RER+) phenotype, defined as the presence of MSI at 2 or more loci, had a frequency of 30.6% (33 of 108) and tended to be positively associated with female sex, intestinal histological type, advanced tumor stage, vascular invasion, positive GC family history, and blood group of A type. No correlation emerged between age at diagnosis and RER+ phenotype, whereas a significant association with the RER+ phenotype was shown by the antral location. A multivariate analysis adjusting for a selected group of potential confounding factors confirmed the strong association of the RER+ phenotype with the antral location ($P = 0.001$) and with a positive GC family history ($P < 0.05$). Survival analyses at 5 and 8 years showed no difference between RER+ and RER− patients, even when corrected for stage distribution.

By the microdissection technique, we also used microsatellite allele patterns to investigate intratumoral heterogeneity and genetic relationships between tumors and adjacent dysplasia and/or intestinal metaplasia. Areas of metaplasia and dysplasia demonstrated MSI only in cases with MSI-positive tumors. In MSI-positive tumors, there was consistent evidence of intratumoral microsatellite allele heterogeneity, indicating the presence of genetically divergent tumor cell clones within the same neoplasm.

INTRODUCTION

Somatic genetic variations consisting in expansions and/or contractions of microsatellite repeats (MSI[3]) have been described in several human cancers. MSI is ascribed to DNA RERs that, at least in tumors associated with HNPCC, are caused by mutations affecting *MMR* genes (1, 2). The mechanism(s) responsible for MSI in sporadic tumors have not yet been elucidated. Most authors agree that a tumor should show MSI at more than 1 locus to be defined as RER+ (3, 4). Several studies reported correlations between RER phenotype and clinicopathological characteristics.

Despite a decreasing trend in incidence and mortality in nearly all developed countries, GC was still recently estimated to represent the second most common cancer in the world and the second leading cause of cancer death (5, 6). The occurrence of GC in HNPCC kindreds suggests that alterations in MMR gene activity play a role in gastric carcinogenesis (7, 8). Actually, genomic instability seems to be an early event in GC progression, and several studies demonstrated the presence of a RER+ phenotype in subsets of sporadic GCs (9–11). In fact, the frequencies of RER+ tumors reported for various GC series range from 15–39%, approximating 30% in most recent studies (12). Different risk factors have been associated with proximal and distal GC; recently, the RER+ phenotype has been reported more frequently in antral GCs (13–15). As in colorectal cancer, in GC the RER+ status has been associated with clinicopathological characteristics suggestive of indolent behavior and better prognosis, including intestinal or poorly differentiated histology, prominent lymphoid infiltration, absence of nodal metastases, and elderly age (15, 16).

Thus, there is ample evidence suggesting that the RER+ status may identify a specific pathway of tumor initiation and/or progression in gastric carcinogenesis. Contrasting results on the presence of an association between familial history and RER+ phenotype have been reported (17–19). A high frequency of RER+ tumors was recently demonstrated in a series of German familial GC patients, one of which harbored a constitutional mutation of the MMR gene *hMLH1* (20). This suggests that unrecognized genetic predisposition could play a role in the pathogenesis of a subset of RER+ GCs that do not occur in the context of HNPCC.

This report investigates the existence of associations between MSI and clinicopathological and individual characteristics in a series of 108 GCs identified in a previous epidemiological case-control study carried out in a high-incidence area around Florence, Italy (21).

PATIENTS AND METHODS

Patients. GC cases were selected from a center participating in a population-based case-control study carried out in several areas of Italy (21). All subjects of the current series were identified in 1985–1987 at the coordinating center of the study in Florence, a high-risk area of central Italy (21). The 108 cases analyzed in the present study were selected among cases less than 75 years old at diagnosis. Criteria for selection of the current series included: (*a*) representation of different histological types and tumor location sites; and (*b*) representation of a sufficient number of cases with familial history of GC to allow statistical comparisons with cases without familial history. Thirty-four unrelated patients with a GC-positive first-degree family history (almost one-third of the series) were included to better evaluate possible associations between MSI and familial GC history.

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[3] The abbreviations used are: MSI, microsatellite instability; RER, replication error; GC, gastric cancer; HNPCC, hereditary nonpolyposis colorectal cancer; MMR, mismatch repair; OR, odds ratio.

Pathology. Formalin-fixed paraffin-embedded samples were retrieved from the archival files of the Pathology Department of the Florence University. Several 5-μm sections were cut from one representative block for each patient; only one section was stained with H&E. All of the cases were originally classified according to Lauren's classification on the basis of all of the slides available from different blocks of GC surgical specimens (22). A panel review was also performed (23).

DNA Extraction. Five-μm paraffin-embedded sections were collected on microscope slides. Areas representative of tumor and of normal tissue (muscularis propria and/or microscopically normal mucosa, with no evidence of intestinal metaplasia or dysplasia) were identified within single unstained sections and microdissected into 1.5-ml polypropilene vials using the H&E-stained slide from the same block as a guide. In some cases, both normal gastric mucosa and unaffected muscularis propria were collected as separate areas for double control. Consideration was also given to selecting metaplastic or dysplastic areas and areas representative of different histological patterns within a tumor. Blood or fresh surgical samples were not available. DNA was extracted as reported previously (24). DNA extractions and set-up of PCR reactions were performed in a laboratory distinct from that in which amplified DNAs were manipulated.

Microsatellite Analysis. The following six microsatellite markers were analyzed: *D2S123*; *D3S1611*; *D5S107*; *ACTC*; *D17S250*; and *D18S34* (25). All these microsatellites are dinucleotide repeats. For microsatellite typing, we used a two-step protocol consisting of a nonradioactive external PCR followed by a radioactive internal PCR that used a 1:10,000 dilution of the primary PCR as a template. Primers, PCR mixture, cycling conditions, electrophoretic separation, and autoradiography were as described previously (24). Paired genotypings of cases positive for microsatellite alterations were confirmed in triplicate experiments. Typings were scored by four independent investigators in a blind fashion, and the final scores were compared to the clinical-epidemiological data. Intensity shifts of microsatellite alleles were observed but not evaluated for the purpose of the present work.

Statistical Analysis. The database available from the previously analyzed case-control study with individual information from the histological review and a questionnaire was merged with the laboratory assay results. The resulting dataset was analyzed on a mainframe computer. A detailed family history reporting the number of first-degree relatives (siblings and parents) affected with GC or other gastrointestinal tumors had been originally obtained during a face-to-face interview; this information had been carefully verified (26, 27) and was available for all cases. Analyses were carried out based on the definition of RER+ phenotype for the cases with MSI at a minimum of 2 loci, one-third of those tested (3, 4). RER+ cases were compared to cases showing no MSI or MSI limited to 1 locus (RER−) using simple descriptive statistics (cross-tabulations) and multivariate methods. Adjusted ORs were determined using logistic models, including terms for potential confounders using the SAS statistical package (28); analyses were also carried out to identify possible effect modifications. Individual information on vital status at the end of the study period (approximately 9 years of follow-up) was available for all cases. Survival analyses were carried out, and Kaplan-Meier curves were estimated.

Because 6 loci were tested for each case and MSI could vary from 0–6 loci, several different definitions of genomic instability were possible beyond that chosen. Additional analyses were carried out to compare results and detect particular patterns; on the basis of a few selected combinations of the number of loci showing MSI, we also classified the study subjects into two (0/1+ loci) or three separate MSI categories (0/1/2+ loci; 0/1–2/3+ loci). Exclusion of cases in the intermediate categories allowed comparisons between extreme groups showing MSI at no loci or multiple loci.

RESULTS

We analyzed 108 cases of primary GC for genetic instability at microsatellite repeats. Markers at 6 dinucleotide repeat loci were compared in paired typings of normal and tumor DNAs obtained by microdissection from a single paraffin-embedded section. Typings with MSI, exemplified in Fig. 1, were identified by random size shifts of alleles in tumor DNA compared to DNA from normal tissue. In 10 cases, it was possible to use both DNA from microscopically normal gastric mucosa and DNA from normal muscularis propria as double controls to determine germ-line microsatellite banding pattern(s). Typings obtained from normal mucosa and muscularis propria were identical. Of 108 cases analyzed, 19 (17.6%) presented MSI(s) at 1 locus, 16 (14.8%) presented MSI(s) at 2 loci, and 17 (15.8%) presented MSI(s) at 3 or more loci. All of the typings showing MSI were confirmed by triplicate experiments using DNA samples derived from independent extractions.

Table 1 shows the distribution of the 108 GC cases according to selected clinicopathological characteristics and classes of MSI status (negative at all loci and positive at 1, 2, and 3 or more loci). The most frequent characteristics were represented by male sex (64.8%), antral location (48.1%), intestinal type according to Lauren's histological classification (50%), advanced tumor and nodal staging (pT3–4, 58.3%; pN+, 75.9%), and low grading (57.4%). The case series had a mean age of 64.2 years. A first-degree family history positive for GC was verified for 34 cases (31.5%), including 5 cases with 2 affected relatives and 1 case with 3 affected relatives. Three familial cases showed MSI at 1 locus, 4 showed MSI at 2 loci, and 10 showed MSI at 3 or more loci. The case who reported three older siblings dead from GC showed MSI at 4 loci. In the whole series, only six cases reported a first-degree relative affected by colorectal cancer, including one case who also had a parent affected by GC. Overall, no specific pattern of MSI was evident in this small subgroup, with three patients positive at 1 or more loci (at 1, 2, and 3 loci, respectively) and three patients negative at all loci (including the case with a family history positive for both GC and colon cancer).

Clinicopathological and epidemiological characteristics of cases with MSI at least at 2 loci (RER+ phenotype) were compared to those of cases showing no MSI or MSI limited to 1 locus (RER−). The RER+ phenotype tended to be positively associated with female sex, intestinal histological type, advanced tumor stage (pT3–4), vascular invasion, positive GC family history, and a blood group of A type (Table 2). A weak inverse association was evident with nodal involvement, whereas no correlation between age at diagnosis and RER phenotype was observed. A positive association with the RER+ phenotype was shown by GCs located in the antrum ($P < 0.01$).

The results of a multivariate analysis adjusting simultaneously for a selected group of potential confounding factors (including age, sex, and histological type) are shown in Table 3. A strong association of the RER+ phenotype with the antral location was confirmed ($P = 0.001$), whereas the association with an advanced pathological tumor staging almost reached the level of statistical significance ($P = 0.06$). At the same time, the RER+ phenotype was significantly associated with a positive GC family history ($P < 0.05$).

These results were not materially modified using a more extreme definition of RER+ phenotype, *i.e.*, excluding cases with MSI at one or even at 2 loci. Actually, the association between RER+ phenotype and positive GC family history was already evident in the univariate analysis. In fact, the prevalence of a positive family history was significantly higher ($P < 0.05$) among cases with MSI at 3 or more loci (10 of 17 cases; 58.8%) than among cases negative at all loci, either considered alone (17 of 56 cases ; 30.4%) or after combination with the cases showing MSI at 1 locus only (20 of 75 cases; 26.7%).

Survival analyses at 5 and 8 years of follow-up showed no substantial difference between RER+ and RER− cases (Table 4). We also estimated survival curves taking into account the distribution of the RER+ and RER− cases in the two combined pT groupings (pT1–2 and pT3–4), but no difference was evident (Fig. 2). Also, no difference was evident when single classes of MSI status (at 0, 1, 2, and 3 or more loci) were tested.

In some cases, exemplified in Fig. 1, it was possible to investigate correlations between MSI status and histological characteristics of the tumor and of the adjacent gastric mucosa. Areas of intestinal meta-

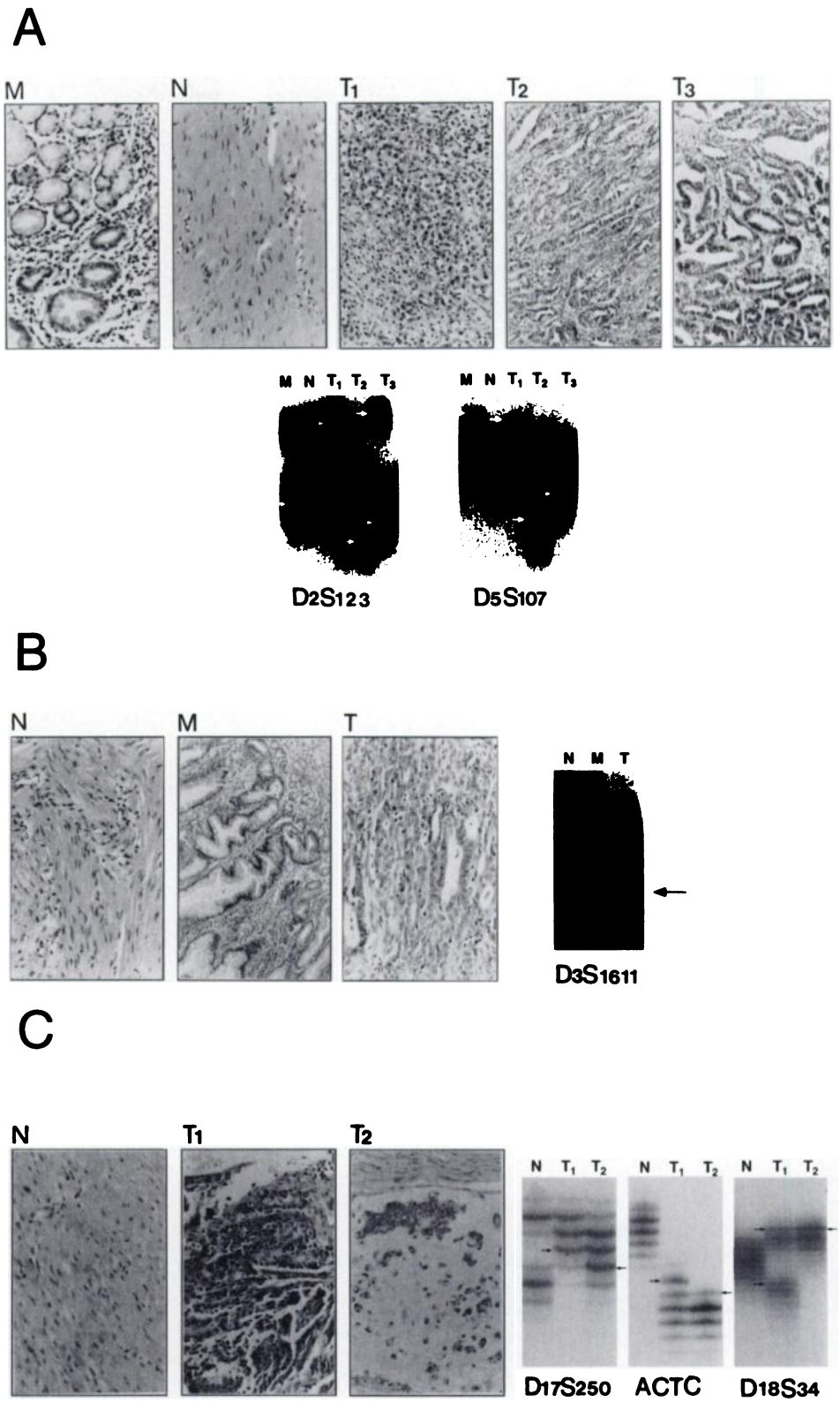

Fig. 1. MSI and histological characteristics of the tumor and the adjacent gastric mucosa. *A* (case 102), relative to normal muscularis propria (*N*), an area of intestinal metaplasia (*M*) and different tumor areas, which partly reflect variations in histological growth pattern [T_1 (diffuse) *versus* T_2 and T_3 (intestinal)], demonstrate distinct MSI banding patterns (*arrows*) at D2S123 and D5S107. *B* (case 88), relative to mucosa with intestinal metaplasia (*M*) and normal muscularis propria (*N*), the tumor (*T*) demonstrates MSI (*arrow*) at D3S1611. *C* (case 113), relative to normal muscularis propria (*N*), different tumor areas, which reflect variations in histological growth patterns (T_1, intestinal; T_2, mucinous), exhibit distinct intratumoral MSI banding patterns (*arrows*) at D17S250, ACTC, and D18S34.

plasia in the unaffected mucosa were sampled in five cases, all of which had tumors positive for MSI (at 1, 1, 2, 3, and 3 loci, respectively). In two cases, the areas of intestinal metaplasia demonstrated MSI respectively at 1 and 2 loci that were also affected in the tumor, but with a distinct banding pattern (see case 102, Fig. 1*A*). In the other two cases, the areas of intestinal metaplasia were MSI-negative (see case 88, Fig. 1*B*). Areas of dysplasia adjacent to carcinomas were sampled in seven cases. In one case, peritumoral dysplasia demonstrated MSI at 1 locus that manifested the same allele shift in the invasive tumor. In the other six cases, both the carcinomas and the areas of dysplasia were MSI negative. Different areas within the same tumor were sampled in 13 cases, 10 of which were MSI positive.

Table 1 *Distribution of the 108 GC cases according to MSI status and clinicopathological characteristics (Florence, 1985–1987)*

Characteristics	MSI − at all 6 loci *n*	MSI + at 1 locus *n*	MSI + at 2 loci *n*	MSI + at ≥3 loci *n*	Total *n*	Total %
Age groups						
<55	10	1	4	1	16	14.8
55–64	16	5	3	6	30	27.8
65+	30	13	9	10	62	57.4
Sex						
Male	37	13	7	13	70	64.8
Female	19	6	9	4	38	35.2
Location						
Antrum	19	10	12	11	52	48.1
Body	22	3	2	3	30	27.7
Cardia	8	3	1	1	13	12.1
Other	7	3	1	2	13	12.1
Lauren classification						
Intestinal	28	8	8	10	54	50.0
Diffuse	20	7	5	5	37	34.3
Mixed/unclassified	8	4	3	2	17	15.7
pT						
1–2	25	7	6	7	45	41.7
3–4	31	12	10	10	63	58.3
pN						
Negative	12	5	5	4	26	24.1
Positive	44	14	11	13	82	75.9
Vascular invasion						
Absent	20	7	5	1	49	45.4
Present	36	12	11	16	59	55.6
Grading[a]						
Low	29	13	7	9	58	57.4
Medium	14	3	4	7	28	27.7
High	9	3	2	1	15	14.9
Blood group[b]						
A	15	8	8	8	39	36.8
B	5	2	2	1	10	9.4
AB	3	1	0	0	4	3.8
O	32	8	5	8	53	50.0
GC family history[c]						
0	39	16	12	7	74	68.5
1	14	2	4	8	28	25.9
2+	3	1	0	2	6	5.6
Total	56	19	16	17	108	

[a] Information was not available for seven cases.
[b] Information was not available for two cases.
[c] Number of first-degree relatives reported as affected with GC.

Distinct intratumoral microsatellite profiles, which partly reflected variations in histological growth patterns, were observed in 8 of these 10 cases (see cases 102 and 113, Fig. 1, *A* and *C*).

DISCUSSION

We investigated the existence of associations between clinicopathological characteristics and MSI using an extended series of GCs that were analyzed at 6 dinucleotide repeat loci and found a significant association with GC family history and tumor location. The GC series analyzed included a high proportion of cases with GC-positive family history.

Because low-level MSI is a common phenomenon, possibly reflecting the inherent instability of microsatellite repeats rather then defects in MMR (29), it has been proposed that evidence of MSI in at least one-third of the loci analyzed should be required to define a tumor as RER+ (4). The overall frequency of RER+ cases observed in the present study (30.6%) is comparable to that found in smaller series of cases (15, 16).

Statistically significant associations between RER+ phenotype and individual clinicopathological characteristics were evident. We found a significant association between RER+ phenotype and antral location of the tumor. A similar association had been suggested by other studies (15, 16). This finding may indicate a role for locally prevalent environmental factors that could induce DNA mutations leading to MSI and/or establish a selective pressure favoring RER-tolerant genotypic variants. Intriguingly, a correlation between tumor location and RER+ phenotype was also observed in colorectal cancer (1, 2).

The association of the RER+ phenotype and family history of GC is currently debated (17–19). In two studies, no correlations between RER+ phenotype and family history were found (17, 18). One of these studies analyzed a series of 76 cases, including 15 cases who had family members from the first to the third degree affected with GC (17). The other study analyzed a series of 39 GCs including 18 first-degree familial cases (18). In contrast, a third study showed correlations between RER+ phenotype and GC-positive family history (19). This latter study included 11 cases with a positive family history up to the second degree of 29 GC cases with available family history information. Considering that the discrepancies in the results of the above-mentioned studies might have been related to the number of familial cases analyzed and to differences in the criteria used in the selection, we enriched the number of familial cases in our series (34 of 108 cases, all with a verified family history) and considered positive for GC family history only cases who had at least a first-degree GC-affected relative. Our analyses showed a significant association between RER+ phenotype and GC-positive family history. Notably, only one case with a positive GC family history also reported a relative affected with colorectal cancer. Thus, it is unlikely that the association between RER+ phenotype and GC family history might be explained by an inadvertent inclusion in our sample of GC cases

Table 2 *Distribution of the 108 GC cases according to RER+ (MSI ≥ 2) versus RER− (MSI ≤ 1) phenotype and selected clinicopathological characteristics (Florence, 1985–1987)*

Characteristics	RER− (MSI ≤ 1) n	(%)	RER+ (MSI ≥ 2) n	(%)	Total n	P[a]
Age groups (yr)						
<55	11	68.7	5	31.3	16	
55–64	21	72.4	9	27.6	30	
65+	43	68.2	19	31.8	62	
						0.86
Sex						
Male	50	71.4	20	28.6	70	
Female	25	65.8	13	34.2	38	
						0.69
Location						
Antrum	29	55.8	23	44.2	52	
Other sites	46	82.1	10	17.9	56	
						0.005
Lauren classification						
Diffuse	27	73.0	10	27.0	37	
Other	48	67.6	23	32.4	71	
						0.72
pT						
1–2	32	71.1	13	28.9	45	
3–4	43	68.2	20	31.8	63	
						0.75
pN						
Negative	17	65.4	9	34.6	26	
Positive	58	70.7	24	29.3	82	
						0.78
Vascular invasion						
Absent	27	81.8	6	18.2	33	
Present	48	64.0	27	36.0	75	
						0.10
GC family history						
Negative	55	74.3	19	25.7	74	
Positive	20	58.8	14	41.2	34	
						0.16
Blood group						
A	23	59.0	16	41.0	39	
Other types[b]	51	76.1	16	23.9	69	
						0.12
Total	75	69.4	33	30.6	108	

[a] *P* values were obtained using the χ^2 test with Yates correction.
[b] Information was not available for two cases that were included in this group.

occurring in large multigeneration HNPCC kindreds. The association between RER+ phenotype and family history might reflect exposure(s) to environmental or dietary factors shared by close relatives (27). On the other hand, the possibility that the RER+ phenotype might be related to a genetically determined GC susceptibility requires additional studies but is supported by the report of a constitutional mutation of the *hMLH1* MMR gene in a patient with RER+ GC and GC-positive family history (20).

The association between MSI status and histological characteristics was analyzed using Lauren's classification and pathological staging. In agreement with previous findings, the RER+ phenotype tended to be more frequent in tumors with intestinal histotype but less frequent in patients with nodal metastases. However, survival analyses at 5 and 8 years of follow-up showed no substantial difference in patients stratified according to RER+ phenotype and RER− phenotype or other classes of MSI status, even when corrected for stage distribution. Considering the current debate on the association between the presence of MSI and better prognosis (15–16), the fact that survival

Table 3 *Multivariate analysis: association between RER+ phenotype (MSI ≥ 2) and selected clinicopathological characteristics; OR and 95% confidence intervals (Florence, 1985–1987)*

Variable	Level	P	OR[a]	95% CI[b]
Sex	Female	0.779	1.2	0.4–3.1
Age	Yr	0.438	1.0	0.9–1.03
Lauren classification	Diffuse	0.311	0.6	0.2–1.7
pT	Advanced (pT3–4)	0.06	2.7	0.9–7.9
Location	Antrum	0.001	5.5	1.97–15.2
GC family history	Positive	0.045	2.8	1.02–7.5

[a] ORs from a logistic regression model including terms for each variable listed.
[b] CI, confidence interval.

Table 4 *Survival analysis of 108 GC cases according to RER phenotype: number of patients at risk, survival percentages, and log rank tests at 5 and 8 years of follow-up (Florence 1985–1987)*

RER phenotype	At start n	At 5 yr n at risk	At 5 yr % survival	At 8 yr n at risk	At 8 yr % survival
Positive (*MSI ≥ 2*)	33	12	36.4	7	24.2
Negative (*MSI ≤ 1*)	75	27	36.0	18	28.9
Log rank test		*P* = 0.88		*P* = 0.60	

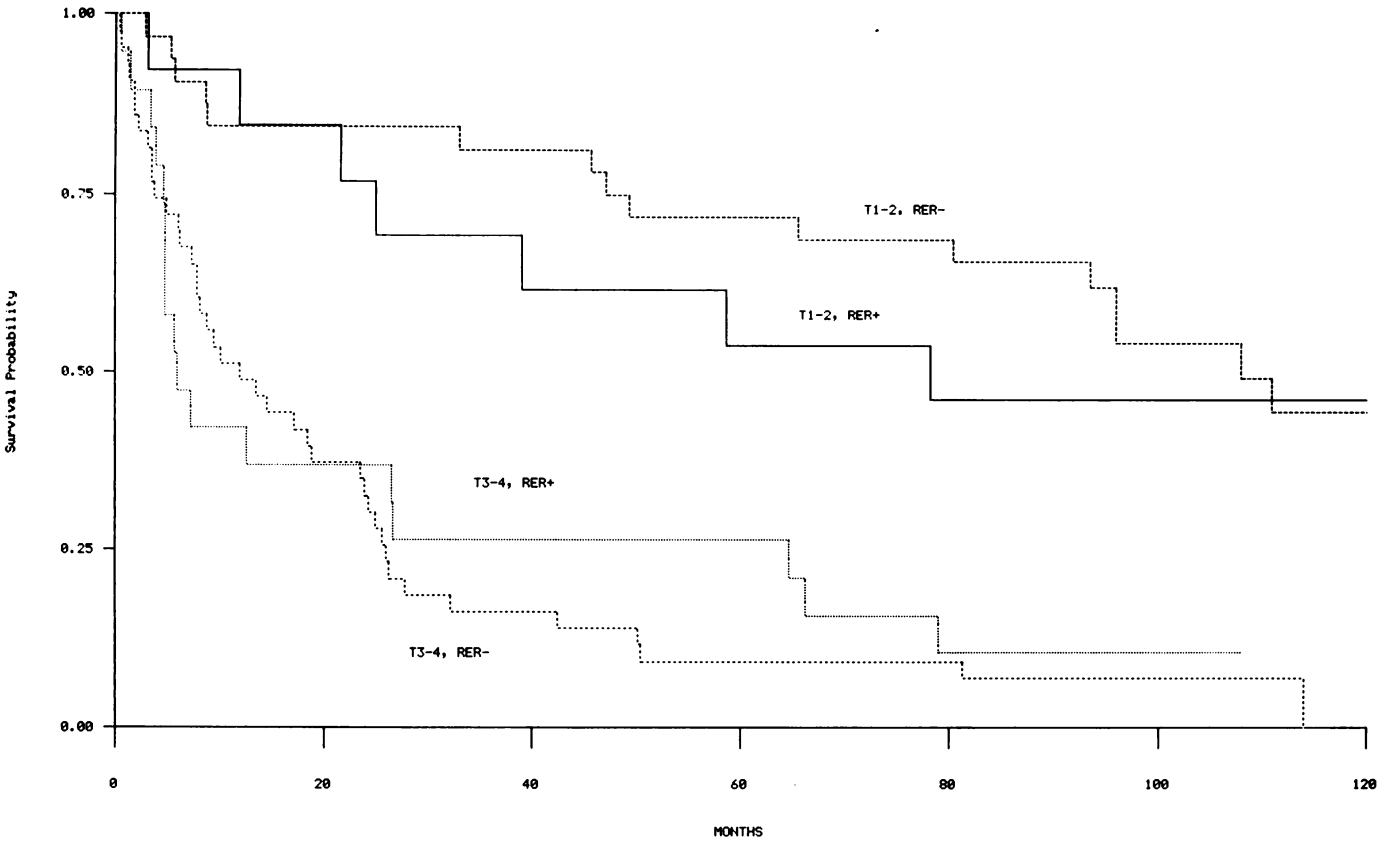

Fig. 2. Kaplan-Meier survival curves of 108 GC cases according to pT (T1–2 *versus* T3–4) and RER+ (MSI ≥ 2) *versus* RER− (MSI 0/1) phenotype (Florence 1985–1987).

analyses showed no difference between RER+ and RER− GCs might be of clinical relevance.

Taking advantage of the microdissection technique, we also used microsatellite allele patterns to investigate genetic relationships between tumors and adjacent areas of dysplasia and/or metaplasia and microsatellite heterogeneity within tumors (18, 29). There was evidence of heterogeneous intratumoral MSI patterns in most of the tumors with multiple sampled areas, suggesting the presence of genetically divergent tumor cell clones within the same neoplasm. Areas of intestinal metaplasia demonstrated MSI at loci that were also affected in the tumor, but with a distinct banding pattern, suggesting the occurrence of independent mutational events in the metaplasia and in the tumor. An area of dysplasia had a novel microsatellite allele in common with the adjacent carcinoma, suggesting a common genetic origin of dysplastic and neoplastic cells as observed by Rhyu *et al.* (30). Interestingly, MSI-negative carcinomas were associated with MSI-negative peritumoral dysplasia.

In conclusion, the present study, conducted on an extensive series of GCs, shows that the RER+ phenotype is significantly associated with two important characteristics: (*a*) GC-positive family history; and (*b*) distal (antral) tumor location. Thus, MSI might identify a subset of gastric tumors sharing a common pathway of carcinogenesis. On the other hand, MSI status was not significantly related to other clinicopathological variables, including survival. The statistical approach used in our study allowed the identification of subsets of GC cases that associate more frequently with the presence of MSI. This approach has the value of revealing correlations between MSI status and clinicopathological variables that may play a role in tumor initiation and progression. However, the occurrence of cases with MSI phenotype that display clinicopathological features more frequently associated with the absence of MSI and *vice versa* indicates that these associations do not have an absolute value. Many questions concerning the causes and significance of MSI in GC, with particular regard to the role of environmental exposure or genetic predisposition, remain open, and additional studies are needed.

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