ORIGINAL ARTICLE

Microsatellite instability and gastric non-invasive neoplasia in a high risk population in Cesena, Italy

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Background/Aims: In the natural history of gastric cancer, non-invasive neoplasia (NiN) precedes invasive carcinoma. A histological classification of gastric NiN has recently been proposed by a World Health Organisation international panel of experts. Genetic instability is known to be among the molecular pathways involved in gastric oncogenesis. In this retrospective cross sectional study, microsatellite instability (MSI) was analysed in a consecutive series of NiN and NiN related histological alterations from a northern Italian region at high risk for gastric cancer.

Patients/Methods: Fifty five consecutive cases (indefinite for NiN, 29 cases; low grade NiN, 17 cases; high grade NiN, nine cases) were analysed by radioactive polymerase chain reaction and electrophoresis for microsatellite alterations at six loci (BAT25, BAT26, D2S123, D5S346, D17S250, and D3S1317). MSI was defined according to the Bethesda criteria distinguishing: (1) no instability in the analysed loci; (2) low frequency MSI (MSI-L); and (3) high frequency MSI (MSI-H). Immunohistochemical expression of MLH1 and MSH2 proteins was also analysed in all cases.

Results: Overall, MSI was found in 11 of 55 cases (indefinite for NiN, five of 29 (MSI-L, four; MSI-H, one); low grade NiN, three of 17 (MSI-L, one; MSI-H, two); high grade NiN, three of nine (MSI-L, one; MSI-H, two)).

Conclusions: In an Italian high risk area for gastric cancer, MSI is part of the spectrum of genetic alterations in gastric non-invasive neoplasia. In European populations at high risk of gastric cancer, DNA repair system alterations are thought to be among the early molecular events in gastric carcinogenesis.

Gastric cancer (GC) may coexist with alterations of the adjacent glands, featuring cyto-architectural (de)differentiation somewhere between that of the native mucosa and that of the concomitant carcinoma.^{1,2} Such phenotypic alterations, considered the precursor of malignant transformation, have been defined as epithelial dysplasia. Whereas in the Western literature the histological category of dysplasia rules out spreading of neoplastic epithelia into the lamina propria, the Eastern literature allows for the coexistence of dysplasia with intramucosal cancer cells.^{3–5} The World Health Organisation (WHO) has recently re-defined dysplasia as non-invasive neoplasia (NiN) and longterm follow up studies have shown that, in the natural history of GC, NiN precedes invasive adenocarcinoma.^{6,7}

“Mismatch repair defects lead to high frequency microsatellite instability”

Genetic instability resulting from the inactivation of mismatch repair system genes (mostly MLH1, MSH2, and MSH6) is known to be one of the molecular pathways involved in gastric oncogenesis.^{8–18} Mismatch repair defects lead to high frequency microsatellite instability (MSI). Germline defects in mismatch repair have been associated with hereditary non-polyposis colorectal cancer and GC is listed among the hereditary non-polyposis colorectal cancer related extracolonic cancers.^{19,20}

In Asian high risk populations, genetic instability is one of the possible pathways of gastric oncogenesis; however, all but one of the published studies include in the same histological category both non-invasive and early invasive neoplastic lesions.^{8,21–25}

In Italy, Cesena is one of the geographical areas at greatest risk of GC, with an incidence (age standardised rates for the years 1993–1997) of 34.0 for women and 47.2 for men (<http://www.registri-tumori.it/incidenza/gruppi.html>).

Our study was designed to assess the prevalence of genetic instability in histological lesions coming within the spectrum of gastric NiN. In a consecutive series of retrospectively selected gastric biopsy samples obtained from northern Italian outpatients, genomic instability was tested using molecular methods; the immunohistochemical expression of the products of two mismatch repair genes (MLH1 and MSH2) was also tested.

PATIENTS AND METHODS

Patients

Our retrospective cross sectional study comprised 55 consecutive white patients (M/F, 34/21; mean age, 59 years; range, 46–78), born and living in the Italian area of Cesena. All patients underwent upper gastrointestinal endoscopy for dyspepsia between 1997 and 2000, and one or more of the gastric biopsies (which included at least four samples: two from the antrum and two from the corpus) showed morphological lesions belonging to the spectrum of gastric NiN.

On the basis of their own (colon cancer confirmed before or after the gastric biopsy was obtained) and their family's cancer history, four subjects fulfilled the Amsterdam II criteria for hereditary non-polyposis colorectal cancer.^{19,20}

Abbreviations: GC, gastric cancer; MSI, microsatellite instability; MSI-H/L, high/low frequency of microsatellite instability; MSS, microsatellite stable; NiN, non-invasive neoplasia; PCR, polymerase chain reaction; WHO, World Health Organisation

history of cancer was recorded in at least one first degree relative of six patients. Family history was negative for cancer in 40 patients and unknown in five subjects.

After the histological diagnosis of NiN (or indefinite for NiN), 40 of 55 patients were followed up with upper gastrointestinal endoscopy for at least six months (mean follow up, 18 months; range, six to 18). Among these patients, six invasive GCs were documented histologically: one adenocarcinoma was detected in a patient enrolled with indefinite for NiN lesions, one patient entered with low grade NiN, and the initial biopsy of the four remaining patients had documented high grade NiN. WHO/UICC postsurgical cancer histotyping and staging were available in two of six patients (one enrolled with indefinite for NiN lesions and one with high grade NiN); both cancers had the glandular phenotype and were pTNM stage I.⁶

METHODS

Pathological study and histological assessment

Gastric biopsy samples were fixed in 5% formalin and embedded in paraffin wax. For the histological assessment, serial histological sections (5 µm thick) were stained with haematoxylin and eosin. *Helicobacter pylori* infection was assessed histologically (modified Giemsa stain) on the whole set of biopsy samples obtained during endoscopy.

Histological lesions were jointly assessed by two pathologists according to the international Padova classification and WHO criteria, distinguishing: (1) indefinite for NiN, 29 cases; (2) low grade NiN, 17 cases; and (3) high grade NiN, nine cases.^{6,7} Cases of NiN coexisting with "suspected invasive neoplasia" were excluded.

Immunohistochemistry for MLH1 and MSH2 gene products

The expression of MLH1 and MSH2 protein was analysed by immunohistochemistry. Tissue sections were incubated with a 1/30 dilution of anti-MLH1 antibody (PharMingen International, San Diego, California, USA) or a 1/100 dilution

of monoclonal anti-MSH2 (clone AB-2; Oncogene Research Products, San Diego, California, USA). Cases with less than 10% nuclear reactivity in the target epithelia were considered negative. Normal epithelia were analysed as internal positive controls.

Molecular assessment of microsatellite status

Six different loci were considered for MSI assessment, including all those recommended by the Bethesda panel for colon cancer (BAT25, BAT26, D5S346, D2S123, and D17S250).¹⁹ Because allelic loss and/or instability in the region encompassing the VHL gene had previously been described in a series of gastric cancers selected from the same geographical area, an additional marker (D3S1317) was also included at locus 3p26.^{13,26}

Polymerase chain reaction (PCR) was performed using specific primers in a total volume of 25 µl using 200µM dNTPs, 2.0mM MgCl₂, 1 U Amplitaq Gold (Applied Biosystems, Foster City, California, USA), 15 pmol of each primer, 0.7 µg/µl bovine serum albumin, and 1 µCi ³³P-dATP. PCR products were then diluted 1/2 with a 95% formamide dye solution, heated to 95°C for five minutes, and electrophoresed in a denaturing 5% acrylamide gel containing 8% urea.^{27,28} The gel was dried and exposed to x ray film at -80°C for 24–48 hours. MSI was scored according to the presence of a shifted mobility pattern in the DNA PCR products obtained from the target lesions compared with those obtained from normal gastric mucosa (biopsy samples with no NiN or intestinal metaplasia) (fig 1). Samples showing a shifted mobility pattern were double checked. When more than one microsatellite locus was altered (MSI in ≥ 30% of the tested markers), cases were defined as highly unstable (high frequency MSI; MSI-H); cases showing MSI at only one locus (MSI in < 30% of the tested markers) were categorised as low frequency MSI (MSI-L); cases with no MSI were considered stable (MSS).¹⁹

Statistical tests

Frequency tables were analysed using Fisher's exact test and the χ^2 test for linear trend was used as appropriate. For all calculations a p value < 0.05 was considered significant.

RESULTS

Prevalence of *H pylori* infection

Helicobacter pylori was detected histologically in 39 of the 55 cases: 18 of 29 cases indefinite for NiN, 13 of 17 low grade NiN, and eight of nine high grade NiN. No association was detected between MSI phenotype (both MSI-L and MSI-H) and *H pylori* infection (Fisher's exact test, p = 0.173).

Microsatellite instability

We investigated the amplification of six microsatellite loci (BAT25, BAT26, D2S123, D17S250, D5S346, and D3S1317). In 44 of 55 cases, all six loci were successfully amplified. Five and four loci were amplified in five and two cases, respectively. In four cases, only three markers could be amplified.

Overall, the prevalence of the MSI phenotype was 11 of 55 (table 1). In six of 11 cases, only one microsatellite locus was unstable (MSI-L); in the other five cases, two to five loci were unstable (MSI-H) (table 1).

Among indefinite for NiN lesions, the prevalence of MSI was five of 29 (MSI-L, four; MSI-H, one); in low grade NiN, it was three of 17 and two of these were MSI-H. Of the three of nine cases of MSI detected in high grade NiN, two were MSI-H (tables 1, 2). The increasing prevalence of MSI-H in indefinite for NiN lesions, low grade NiN, and high grade NiN was marginally significant (χ^2 test for linear trend, p = 0.08).

Figure 1 Electrophoretic pattern of two cases showing high frequency microsatellite instability (C12, high grade non-invasive neoplasia; C26, low grade non-invasive neoplasia). Mobility shifts in five of six of the loci analysed are apparent in the tumours when compared with the normal tissue. N, normal biopsy sample; T, tumour biopsy sample.

Table 1 Pathology, MSI, and MSH2/MLH1 protein expression (by immunohistochemistry) in the 11 MSI cases

Case	Pathology	Microsatellite markers							Immunohistochemistry	
		BAT25	BAT26	D5S346	D2S123	D17S250	D3S1317	MSI	MSH2	MLH1
C5	Indefinite for NiN	–	–	MSI	–	MSI	MSI	MSI-H	Positive	Negative
C14	Indefinite for NiN	–	–	–	–	–	MSI	MSI-L	Positive	Positive
C190	Indefinite for NiN	–	–	–	MSI	–	–	MSI-L	Positive	Positive
C20	Indefinite for NiN	–	–	MSI	NA	–	NA	MSI-L	Positive	Positive
C31	Indefinite for NiN	–	–	–	–	MSI	–	MSI-L	Positive	Positive
C18	Low grade NiN	–	–	NA	MSI	–	NA	MSI-L	Negative	Positive
C7	Low grade NiN	–	NA	NA	MSI	MSI	NA	MSI-H	Positive	Negative
C26	Low grade NiN	MSI	MSI	MSI	–	MSI	MSI	MSI-H	Negative	Positive
C9	High grade NiN	–	–	NA	NA	MSI	NA	MSI-L	Positive	Positive
C6*	High grade NiN	–	–	MSI	MSI	–	–	MSI-H	Negative	Positive
C12*	High grade NiN	MSI	MSI	MSI	–	MSI	MSI	MSI-H	Positive	Negative

*MSI cases in which invasive adenocarcinoma was histologically diagnosed during short term follow up.

MSI, microsatellite instability; MSI-H/L, high/low frequency of microsatellite instability; MSS, microsatellite stable; NA, not assessable; NiN, non-invasive neoplasia.

Immunohistochemistry for MLH1 and MSH2 gene products

None of the 55 cases lacked both MLH1 and MSH2 signals. Overall, no MLH1 and MSH2 immunostaining was found in nine cases (table 2): six of the nine were associated with MSI (one of five cases indefinite for NiN; three of three cases of low grade NiN; and two of three cases of high grade NiN; tables 1, 2). No immunostaining for MLH1 or MSH2 was found in three of the 44 MSS cases (one indefinite for NiN; two high grade NiN; table 2). A significant association was found between microsatellite status (MSI *v* MSS) and immunophenotype (negative *v* positive immunostaining for MLH1 or MSH2) (Fisher's exact test, *p* = 0.00001).

All five MSI-H cases were associated with loss of MLH1 or MSH2 expression (table 1), whereas only one of the six MSI-L cases showed no immunoreactivity for one of the target proteins (MSH2) (Fisher's exact test, *p* = 0.01298).

GCs detected during short term follow up and microsatellite status at initial biopsy

Six GCs were detected in the 44 patients who underwent endoscopic follow up. Two of these six patients were enrolled with high grade NiN, associated with MSI-H (table 1). The other four patients had the MSS phenotype.

DISCUSSION

A relation between gastric NiN and adenocarcinoma has been demonstrated by clinicopathological follow up studies.²⁹ In gastric oncogenesis, both environmental and host related gene silencing/activation have been considered.^{30–32}

In our present series of gastric precancerous lesions, the prevalence of *H. pylori* infection was higher than 80%, and no association was found between infection and MSI phenotype.^{29, 33}

MSI has been considered among the molecular pathways leading to the development of hereditary (gastrointestinal) cancers, and clinical criteria (Amsterdam II) have been

proposed to identify patients at high risk of such cancer syndromes.^{10, 19, 20, 34, 35} Available information on MSI in both precancerous and advanced gastric neoplasia is inconsistent (tables 3, 4). In our present study, four patients fulfilled the Amsterdam II criteria, but none revealed an MSI positive phenotype.³⁵

In comparing the results of our study with existing data, two main factors must be taken into account, namely: (1) most of the available information is based on the genotyping of Asian patients with cancer and the ethnic setting is considered a major source of heterogeneity; (2) in all but one study, precancerous lesions have been histologically classified according to Japanese criteria, which include both non-invasive and early invasive neoplastic lesions in the same histological category.^{4, 12, 42–44} Moreover, variability in: (a) the molecular assessment of MSI status (radioactive *v* non-radioactive methods), (b) the number/location of considered loci, (c) the definition of mutator phenotype, and (d) the clinical setting from which samples are obtained (co-existence/absence of precancerous lesions with invasive neoplasia) all make it difficult to compare available data with each other, and with the results of our present study.^{22–24, 36, 45–49} Consequently, it seems reasonable, once again, to subscribe to the recommendation of a standardised approach to both the method(s) of assessing MSI and the strict use of internationally validated histological classifications.^{50, 51}

In advanced GC, the prevalence of MSI ranges between 5% and 46%, with significant differences between different ethnic groups.^{15, 52} It is noteworthy, however, that some Eastern series significantly associate the MSI phenotype with the foveolar type of GC, which theoretically occurs via a carcinogenic pathway different from that of gastric mucosa intestinalisation.²¹ In our present series, all cases showed extensive intestinal metaplasia (NiN arising in intestinalised glands).

The relations between intestinal metaplasia (both with and without GC) and microsatellite status have been investigated

Table 2 MSI and loss of MSH2/MLH1 expression in lesions indefinite for gastric NiN, low grade NiN, and high grade NiN

MSI and MSH2/MLH1 expression (IHC)	Indefinite for NiN 29 cases (%)	Low grade NiN 17 cases (%)	High grade NiN 9 cases (%)	Total 55 cases
MSI (MSI-L and MSI-H)	5 (17.2)	3 (17.6)	3 (33.3)	11
MSI-L	4 (13.8)	1 (5.9)	1 (11.1)	6
MSI-H	1 (3.4)	2 (11.8)	2 (22.2)	5
Loss of MSH2 protein (IHC)	1 (3.4) (MSS)	2 (11.8) (1 MSI-H; 1 MSI-L)	1 (11.1) (MSI-H)	4
Loss of MLH1 protein (IHC)	1 (3.4) (MSI-H)	1 (5.9) (MSI-H)	3 (33.3) (1 MSI-H; 2 MSS)	5

IHC, immunohistochemistry; MSI, microsatellite instability; MSI-H/L, high/low frequency microsatellite instability; MSS, microsatellite stability; NiN, non-invasive neoplasia.

Table 3 MSI in gastric IM

First author	IM		MSI		Microsatellite markers
	Without coexisting GC	With coexisting GC	MSI-L (%)	MSI-H (%)	
Garay (2004) ³⁶	58		0		BAT26, D3S1067, D3S1262, D5S346, D12S347, D12S351
Roa (2003) ³⁷	22		13 (59)	0	BAT25, BAT26, BAT40, D2S123, D3S1067, D3S1262, D3S3640, D5S346, P53CA, D17S250, C17S588, D18S55
Kim (2002) ³⁸		15	0		BAT25, BAT26, D2S123, D5S346, D17S250, D13S170, TP53
Jin (2001) ²⁵		17	0		BAT25, BAT26, D2S123, D5S346, D17S250
Leung (2000) ³⁹	45		17 (37.8)	3 (6.7)	BAT25, BAT26, BAT40, D2S123, D5S346, D13S170, D17S250, TP53
		30	12 (40)	4 (13.3)	
Kobayashi (2000) ⁴⁰		33	11 (33)	0	BAT25, BAT40, D2S123, D1S103, D3S1067, D5S107, D13S153, D17S261, D18S34
Hamamoto (1997) ⁴¹		15	4 (26.7)		BAT25, BAT26, BAT40, D1S191, D7S486, D11S29, TP53, D17S855
Semba (1996) ⁸		9	3 (33)		D2S123, D2S110, D2S136, D3S1067, D5S5O5, D7S486, D11S29, TP53, D17S855

MSI-L and MSI-H are distinguished according to the criteria adopted by each author.

GC, gastric cancer; IM, intestinal metaplasia; MSI, microsatellite instability; MSI-H/L, high/low frequency microsatellite instability.

extensively, with divergent results (table 3). Because only NiNs arising in intestinalised glands were studied here, it is worth briefly summarising the results of other studies in which MSI was tested in intestinal metaplasia (table 3). When MSI-H was distinguished from MSI-L, the prevalence of MSI-H ranged from 0% to 13% (table 3). The fact that when intestinal metaplasia coexists with GC, the same MSI pattern is detectable in both lesions, biologically supports the precancerous significance of metaplastic transformation. Such an observation is consistent with the hypothesis that the “field cancerisation process” in the stomach is mucosal intestinalisation.³³

“The prevalence of a high frequency of microsatellite instability increased from indefinite for non-invasive neoplasia (NiN) lesions, to low grade NiN, to high grade NiN, is consistent with the hypothesis that the prevalence of genotype alterations increases with the dedifferentiation of the histological phenotype”

Data pertaining to MSI in advanced precancerous lesions must be considered with caution. Western and Eastern publications give different names to the same histological

lesion, or include different histological alterations under the same histological label; as a result, the available data on the genotyping of advanced gastric precancerous lesions are bewildering.

When the spectrum of gastric precancerous alterations is considered as a whole (adenoma or dysplasia or non-invasive neoplasia of both low and high grade), the prevalence of MSI ranges from 0% to 42% (table 4), and it could be said that the prevalence of the mutator phenotype is higher the larger the number of microsatellites tested. When MSI-H is defined according to the criteria recommended for colorectal cancer, the prevalence of MSI in low grade lesions is consistently reported to be lower than 10%. Applying the current internationally validated classification of gastric precancerous lesions to a series of Japanese patients, Jin *et al* found MSI-H in 5% and 19% of low grade and high grade NiNs, respectively. Similarly, our present study detected a prevalence of MSI-H that increased from indefinite for NiN lesions (3.4%) to low grade NiN (11.8%), to high grade NiN (22.2%), which is consistent with the hypothesis that the prevalence of genotype alterations increases with the dedifferentiation of the histological phenotype. In line with the histological classification adopted, our results provide the first evidence of MSI being involved in non-invasive

Table 4 MSI in gastric advanced precancerous lesions

First author	Adenoma/Dysplasia/NiN		MSI		Microsatellite markers
	Without coexisting GC	With coexisting GC	MSI-L (%)	MSI-H (%)	
Abraham (2003) ²¹	12 LG-GED		0	1 (8)	BAT25, BAT26, D2S123, D5S346, D17S250
	4 HG-GED	2 HG-GED	0	2 (33)	
Jin (2001) ²⁵	20 LG-NiN		0%	1 (5)	BAT26, D2S115, D4S404, D5S178, IL9, D6S265, D7S490, D11S900, MYH6, TP53, D17S1176, D18S46, D21S1407
	32 HG-NiN		4 (12.5)	6 (18.7)	
Lee (2002) ²²	75 Ad		7 (9)	2 (3)	BAT26, D2S123, D5S346, D17S250, BAT25
		35 Ad	3 (9)	6 (17)	
Chang (2002) ²³	67 Ad		7 (10.4)		BAT26
		65 Ad	8 (12.3)		
Endoh (2000) ¹⁴	24		8 (33)		BAT26
Ohmura (2000) ⁴²	13 LG		?	1 (7.7)	D2S115, D4S404, D5S178, IL9, D6S265, D7S490, D11S900, MYH6, TP53, D17S1176, D18S46, D21S1407
	20 HG		?	8 (40)	50 loci
Kim (2000) ¹⁶		41 LG-Ad	9 (22)		
		22 HG-Ad	4 (18)		
Isogaki (1999) ²⁴	13		1 (7.6)		D1S116, D2S136, D6S87, D10S197, TP53
		37	6 (16.2)		
Semba (1996) ⁸		12	5 (42)		D2S123, D2S110, D2S136, D3S1067, D5S5O5, D7S486, D11S29, TP53, D17S855

The different histology/nomenclature categories applied by the authors are reported. MSI-L and MSI-H are distinguished according to the criteria adopted by each author.

Ad, adenoma; GC, gastric cancer; GED, gastric epithelial dysplasia; HG, high grade; LG, low grade; MSI, microsatellite instability; MSI-H/L, high/low frequency microsatellite instability; NiN, non-invasive neoplasia.

Take home messages

- We have provided the first evidence that microsatellite instability does occur in gastric non-invasive neoplasia (NiN) in white populations from high risk areas
- In European populations at high risk of gastric cancer, DNA repair system alterations are early molecular events in gastric carcinogenesis
- Our results support the hypothesis that low and high grade gastric NiN are different phenotypes of the same biological disease

neoplastic alterations originating from gastric intestinalised glands in a white population at high risk for GC.

Loss of immunohistochemical expression of DNA repair system gene products has been considered a marker of genetic instability, and loss of one of the target proteins has been demonstrated in more than 85% of MSI cases.⁵⁴ In our present series of biopsy tissue samples, the significant correlation found between MSI phenotype (all MSI cases and the MSI-H subgroup) and MLH1/MSH2 protein loss suggests that immunohistochemistry should be considered as a suitable method for MSI assessment in gastric precancerous lesions.

In conclusion, the results of our study provide the first evidence that MSI does occur in gastric NiN in white populations, also supporting the hypothesis that the two grades (low and high) of gastric NiN may represent different phenotypes of the same biological disease.

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