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

M Rugge, G Bersani, R Bertorelle, G Pennelli, V M Russo, F Farinati, D Bartolini, M Cassaro, V Alvisi

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.

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 and GC is listed among the hereditary non-polyposis colorectal cancer related extracolonic cancers. 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.

Abbreviations: GC, gastric cancer; MSI, microsatellite instability; MSI-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/UITC 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.

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. 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). 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.

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 32P-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.

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).

Statistical tests
Frequency tables were analysed using Fisher’s exact test and the χ² 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 (χ² test for linear trend, p = 0.08).

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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 between microsatellite status (MSI) and immunophenotype (negative or positive) (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. In gastric oncogenesis, both environmental and host related gene silencing/activation have been considered. 30–32

MSI has been considered among the molecular pathways proposed to identify patients at high risk of such cancer syndromes. 30–32 36–38 In our present study, four patients fulfilled the Amsterdam II criteria, but none revealed an MSI positive phenotype. 39

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. 40 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 (coexistence/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. 41–45–49

In advanced GC, the prevalence of MSI ranges between 5% and 46%, with significant differences between different ethnic groups. 45–52 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. 49 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 | Low grade NiN | High grade NiN | Total 55 cases |
| 29 cases (%) | 17 cases (%) | 9 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) | 1 (11.1) | 4 |
| Loss of MSH2 protein (IHC) | 1 (3.4) (MSI-H) | 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 stable; NA, not assessable; NiN, non-invasive neoplasia.
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 MSI-L and MSI-H are distinguished according to the criteria adopted by each author.

When MSI-H was distinguished from MSI-L, the prevalence of MSI in gastric IM ranged from 0% to 13% (table 3). The fact that MSI-L and MSI-H are distinguished according to the criteria adopted by each author.

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

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**Table 3** MSI in gastric IM

<table>
<thead>
<tr>
<th>First author</th>
<th>IM Without coexisting GC</th>
<th>IM With coexisting GC</th>
<th>MSI-L (%)</th>
<th>MSI-H (%)</th>
<th>Microsatellite markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garay (2004)</td>
<td>58</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>BAT26, D2S1067, D3S1262, D5S346, D17S347, D12S351</td>
</tr>
<tr>
<td>Rao (2003)</td>
<td>22</td>
<td>13 (59)</td>
<td>1 (3)</td>
<td>0</td>
<td>BAT25, BAT40, D2S123, D3S1067, D3S1262, D5S346, D17S250, C17S588, D18S555</td>
</tr>
<tr>
<td>Kim (2002)</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>BAT25, BAT26, D2S123, D5S346, D17S250, D13S170, TP53</td>
</tr>
<tr>
<td>Leung (2000)</td>
<td>45</td>
<td>30</td>
<td>12 (40)</td>
<td>4 (13.3)</td>
<td>BAT25, BAT26, D2S123, D3S1262, D5S346, D17S250</td>
</tr>
<tr>
<td>Kobayashi (2000)</td>
<td>33</td>
<td>11 (33)</td>
<td>0</td>
<td>0</td>
<td>BAT25, BAT40, D2S1067, D3S1067, D15S107, D13S153, D17S261, D18S54</td>
</tr>
<tr>
<td>Hamamoto (1997)</td>
<td>15</td>
<td>4</td>
<td>2 (6.7)</td>
<td>0</td>
<td>BAT25, BAT26, D2S1067, D3S1067, D15S107, D13S153, D17S261, D18S54</td>
</tr>
<tr>
<td>Semba (1996)</td>
<td>9</td>
<td>3 (33)</td>
<td>0</td>
<td>0</td>
<td>BAT25, D2S1067, D3S1262, D5S346, D17S347, D12S351</td>
</tr>
</tbody>
</table>

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.

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**Table 4** MSI in gastric advanced precancerous lesions

<table>
<thead>
<tr>
<th>First author</th>
<th>Adenoma/Dysplasia/NIN Without coexisting GC</th>
<th>Adenoma/Dysplasia/NIN With coexisting GC</th>
<th>MSI-L (%)</th>
<th>MSI-H (%)</th>
<th>Microsatellite markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abraham (2003)</td>
<td>12 LG-GED</td>
<td>0</td>
<td>1 (8)</td>
<td>5 (42)</td>
<td>BAT25, BAT26, D2S123, D5S346, D17S250</td>
</tr>
<tr>
<td>Jin (2001)</td>
<td>4 HG-GED</td>
<td>0</td>
<td>2 (33)</td>
<td>0</td>
<td>BAT25, BAT26, D2S123, D5S346, D17S250, D15S107, TP53</td>
</tr>
<tr>
<td>Lee (2002)</td>
<td>32 HG-NIN</td>
<td>4 (12.5)</td>
<td>6 (18.7)</td>
<td>0</td>
<td>BAT26, D2S123, D5S346, D17S250, D15S107, TP53</td>
</tr>
<tr>
<td>Chang (2002)</td>
<td>75 Ad</td>
<td>7 (9)</td>
<td>2 (3)</td>
<td>0</td>
<td>BAT26, D2S123, D5S346, D17S250, D15S107, TP53</td>
</tr>
<tr>
<td>Endoh (2000)</td>
<td>67 Ad</td>
<td>7 (10.4)</td>
<td>6 (17)</td>
<td>0</td>
<td>BAT26, D2S123, D5S346, D17S250, D15S107, TP53</td>
</tr>
<tr>
<td>Ohmura (2000)</td>
<td>65 Ad</td>
<td>8 (13.2)</td>
<td>2 (3)</td>
<td>0</td>
<td>BAT26, D2S123, D5S346, D17S250, D15S107, TP53</td>
</tr>
<tr>
<td>Kim (2000)</td>
<td>13 LG</td>
<td>7</td>
<td>1 (7.7)</td>
<td>8 (40)</td>
<td>D15S116, D2S136, D6S87, D10S197, TP53</td>
</tr>
<tr>
<td>Isogaki (1999)</td>
<td>22 HG-Ad</td>
<td>7</td>
<td>1 (7.6)</td>
<td>8 (40)</td>
<td>D2S123, D2S110, D2S136, D5S1067, D5S505, D7S486, D11S29, TP53, D17S585</td>
</tr>
</tbody>
</table>

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.
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. 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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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.

Authors’ affiliations

M Rugge, B Bertoppi, G Pennelli, M Cassaro, Department of Oncology and Surgical Sciences, University of Padova, I-35121 Padova, Italy
G Bersani, Gastroenterology Unit, Malatesta-Novello Hospital, I-47023 Cesena, Italy
V M Russo, Department of Pathology, San Luigi Hospital, I-95100 Catania, Italy
F Farinatti, Department of Gastroenterology and Surgical Sciences, University of Padova, I-35128 Padova, Italy
D Bartolini, Department of Pathology, Maurizio Bufalino Hospital, I-47023 Cesena, Italy
V Alvisi, School of Gastroenterology, University of Ferrara, I-47023 Cesena, Italy

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