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Original article

Differences in genomic instability between intestinal- and diffuse-type gastric cancer

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Abstract

Background. **Microsatellite instability (MSI) and loss of heterozygosity (LOH) are lesions in the genome found with different frequencies in gastric carcinomas (GCAs). Despite a great body of studies, no systematic approach to the detailed classification of MSI and LOH in the two major types of GCA has been published.**

Methods. **Thirty-seven advanced GCAs, 25 intestinal-type (IGCAs) and 12 diffuse-type (DGCAs), were assayed with 15 autosomal tetranucleotide markers on 14 chromosomal arms. The observed frequencies and types of microsatellite alterations allowed stratification into subgroups, i.e., high- and low-grade MSI (MSI-H, MSI-L) or microsatellite-stable (MSS), and high- or low-grade, or non-detectable LOH (LOH-H, LOH-L, LOH-N).**

Results. **Collectively, the markers detected MSI-H tumors with sensitivity equal to that of BAT-26 (a single marker highly specific for MSI-H). Likewise, the markers detected LOH at chromosomal arms 5q, 18q, and 21q with a sensitivity equal to markers used previously. Seven (19%) MSI-H and six (16%) LOH-H tumors were found, with a significant association ($P = 0.027$) with IGCA: 92% of MSI-H and LOH-H occurred in IGCA patients only. Conversely, in DGCA, a significantly higher prevalence of a stable (LOH-N/MSS) phenotype was found as compared with IGCA (75.1% vs 28.0%; $P = 0.035$). The MSI-L phenotype was found in 57.9% of non-MSI-H IGCA tumors and was associated significantly ($P = 0.015$) with LOH-H.**

Conclusion. **A clear difference in genomic instability between IGCA and DGCA was found. In IGCA, the MSI and LOH pathways were more commonly involved, whereas in DGCA, a stable phenotype was predominant. As a novel finding, MSI-L as a true phenomenon and its association with LOH was observed in IGCA.**

Key words Stomach cancer · Microsatellite instability · Loss of heterozygosity · Genomic instability

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Introduction

Intestinal gastric carcinoma (IGCA) and diffuse (DGCA) forms are the two main morphological types of gastric carcinoma (GCA) [1–3]. These tumor types diverge in many demographic and clinical characteristics [4,5]. Molecular genetic studies indicate that these types differ in pathogenesis, based on dissimilar errors and expression of various genes. However, no single genomic abnormality is known to be specific to sporadic GCA, or to any of its histological subtypes, as recently reviewed by Tahara [6]. In the initiation and promotion of gastric cancer microsatellite instability (MSI) [7–10], loss of heterozygosity (LOH) [7,11,12] and DNA amplification [13,14] have been described. Determination of the MSI or LOH rate by microsatellites may depend on methodological factors such as the set and number of markers used [15–18], artifacts due to degraded DNA in paraffin-embedded tissue samples [19], or a low number of cancer cells in the tissue sample [20].

The MSI phenotype is characterized by new alleles not present in the normal genotype. Depending on the rate of unstable loci, MSI tumors can be divided into two categories, i.e., MSI-low (MSI-L) and MSI-high (MSI-H) types. MSI-H is a well-established phenotype in gastrointestinal epithelial neoplasias, which results from malfunction of the mismatch repair (MMR) system, caused mainly by hypermethylation of the *hMLH1* promoter [9,21,22]. The target genes for MSI-H-driven carcinogenesis, such as *TGF-β*, *BAX*, *hMSH3*, and *hMSH6*, contain coding repeats in which frameshift mutations produce aberrant protein products [23]. The existence and importance of MSI-L is still unclear, because all gastrointestinal tumors may show a background level of MSI, which has been proposed to be due to clonal expansion at normal mutation rates [16–18].

LOH is considered to be a marker of chromosomal instability, but its mechanisms are poorly understood

[24]. It may indicate a second inactivational hit of a cancer gene and, thus, its frequency may correlate with the level of chromosomal instability, as reviewed earlier [20,25]. The occurrence of LOH and MSI-H phenotypes in tumors is thought to be mutually exclusive [15,25]; however, contradicting data have been recently published [26–30]. Several studies of GCA have focused on LOH at one or a few specific loci on cancer-associated chromosomal arms [7,11,26,27], where no consistent differences in LOH frequency between gastric cancer types have been detected. IGCA has been shown to harbor more LOH and MSI-H than DGCA [12]. In recent studies of colorectal cancer [29,30], overlapping phenotypes, where MSI and LOH coexist, have been found. However, respective data from gastric cancers are lacking.

In our previous studies, we found that MSI and LOH in gastrointestinal tumors could be detected by using tetranucleotide markers, which are highly polymorphic and heterozygous, with minimal stutter artifacts [31]. We also found that, using a set of these markers, they collectively revealed the same MSI-H tumors that were detectable by BAT-26 [9,32]. The aim of the present study was to use these markers in the assessment of differences in the genetic integrity of IGCA and DGCA. Association between the microsatellite phenotypes and clinicopathological parameters of GCA was also studied.

Patients, materials, and methods

Samples

Surgically resected tissue specimens from 37 advanced primary gastric cancers (25 IGCA and 12 DGCA) and their cancer-free adjacent areas were collected during the period 1996–2003 at the Department of Pathology of Jorvi Hospital, Espoo, Finland. After excision, the tissues were snap-frozen in liquid nitrogen, overlayed with Tissue-Tek OCT Compound (Sakura Finetek, Zoetervoude, Netherlands) and stored at −70 °C. Histopathologic diagnosis of the tumor tissues and classification into intestinal and diffuse types was based on the WHO and Laurén classifications [1,2] and was done by one experienced pathologist (P.S.). The samples were verified by microscopy to contain at least 50% cancer tissue as estimated semiquantitatively from the total epithelial tissue volume in the cancer specimen. Staging of the tumor was done according to the International Union Against Cancer (UICC) TNM system. Hereditary nonpolyposis colon cancer (HNPCC) syndrome and familial gastric cancer were excluded by taking patient history from documents.

DNA preparation and microsatellite phenotyping

The DNA was extracted from 200–500 mg of fresh-frozen tissue by digestion with proteinase K and filtration through Qiaquick columns (Qiagen, Hilden, Germany) and was used in subsequent polymerase chain reactions (PCR) reactions in 0.1- to 1-ng aliquots, as described in detail previously [31]. Two commercially available kits, AmpF*l*STR SGM Plus and AmpF*l*STR Profiler (Applied Biosystems, Foster City, CA, USA), together containing 15 autosomal markers, were used to detect the allelic alterations of the tumors in comparison to their healthy adjacent tissues. The PCR amplifications and analysis, carried out by ABI Prism CE310 capillary electrophoresis (Applied Biosystems), were performed as described previously [31]. MSI was identified in a tumor tissue by alleles not present in the control tissue. Using the suggested criteria [15], the tumors were categorized into three MSI phenotypes: MSI-H, with extra alleles at 33% or more of the loci; MSI-L, with extra alleles at fewer than 33% of the loci; and microsatellite-stable (MSS), tumors with no alterations. The MSI-H status was confirmed by amplification of the BAT-26 locus [9,32], where the shortening of monomorphic alleles was observed in the MSI-H tumors. LOH was observed as allele peak ratios below 0.5 in the tumor tissue. Only the non-MSI-H tumors with heterozygote control tissues were considered as informative for LOH. A 25% cutoff level for the proportion of LOH among informative markers was applied to distinguish high- and low-level LOH (LOH-H and LOH-L), as described previously [7]. Samples with no alterations of LOH type were categorized as LOH-non detectable (LOH-N) [7,12].

Statistics

Fisher's exact test, the χ^2 test, the Mann-Whitney *U*-test, and an independent t-test were performed, using SPSS software (SPSS, Chicago, IL, USA). A *P* value of less than 0.05 was considered significant.

Ethics

This study protocol was evaluated and approved by the Institutional Ethics Committee of the University Hospital Helsinki.

Results

Our set of 15 markers and their locations on the 14 chromosomal arms are shown in Table 1. A significantly higher frequency of LOH ($P < 0.001$) was found in IGCA as compared with DGCA tumors. A high

Table 1. Chromosomal location of the microsatellite markers and proportion of LOH at the loci

Locus name	Chromosomal location[a]	LOH at informative cases (%)[b]	
		IGCA[c]	DGCA
TPOX	2p25.3	2/13 (15%)	0/4 (0%)
D2S1338	2q35	4/15 (27%)	0/7 (0%)
D3S1358	3p21.31	0/11 (0%)	0/4 (0%)
FGA	4q31.3	3/13 (23%)	0/7 (0%)
D5S818	5q23.2	2/13 (15%)	0/8 (0%)
CSF1PO	5q33.1	3/15 (20%)	1/7 (14%)
D7S820	7q21.11	3/12 (25%)	0/5 (0%)
D8S1179	8q24.13	0/14 (0%)	0/8 (0%)
TH01	11p15.5	1/13 (8%)	0/8 (0%)
VWA	12p13.31	3/13 (23%)	0/7 (0%)
D13S317	13q31.1	1/15 (7%)	0/5 (0%)
D16S539	16q24.1	3/15 (20%)	0/7 (0%)
D18S51	18q21.33	3/15 (20%)	0/6 (0%)
D19S433	19q12	0/12 (0%)	0/8 (0%)
D21S11	21q21.1	5/18 (28%)	0/7 (0%)

LOH, loss of heterozygosity
[a] Data from the EMBL database (www.ensembl.org)
[b] Percentage of LOH in informative cases
[c] $P < 0.001$ (Mann-Whitney U-test) for difference in LOH frequency between IGCA and DGCA

Table 2. Comparison of rates of LOH at different loci on the indicated chromosomal arms

Reference no.[a]	5q		18q		21q	
Present study	D5S818 (5q23.2)[b] CSFIPO (5q33.1)	15% 27%	D18S51 (18q21.33)	20%	D21S11 (21q21.1)	28%
11	D5S409 (5q21.1)	33%	D18S69 (18q21.1)	32%	D21S1436 (21q21.1)	43%
7	D5S349 (5q13.3) D5S409 (5q21.1) D5S346 (5q22.2) D5S519 (5q33.1) D5S422 (5q34)	29% 31% 37% 33% 34%	D18S67 (18q12.2) D18S57 (18q12.2) D18S474 (18q21.1) D18S58 (18q22.3) D18S70 (18q23)	38% 43% 37% 37% 42%	NA	
26	D5S409 (5q21.1)	50%	D18S69 (18q21.1)	36%	D21S1436 (21q21.1)	40%
27	D5S299 (5q15–13)[c] D5S409 (5q21.1)	32% 27%	D18S70 (18q21.1) DCC (18q21.1) D18S386 (18q22.1)	23% 48% 42%	D21S258 (21q11)[d]	23%

NA, not analyzed
[a] Data represent mostly intestinal (differentiated) gastric adenocarcinomas
[b] Data for chromosomal locations from www.ensembl.org
[c] Chromosomal locations adopted from references 7, 11, 26, 27

variation in the frequency of LOH was also observed between individual markers. Four of the markers used in the present study (D5S818, CSFIPO, D18S51, D21S11) were located in regions which have been shown to be non-randomly lost during the progression of GCA [26] (Table 2). These chromosomal arms, 5q, 18q, and 21q, have been shown to contain the tumor suppressor genes *APC* and *MCC* [33], *DCC* [34], and trefoil factor 1 [35], respectively. Our tetranucleotide markers reported a rate of LOH of about 20% in the corresponding chromosomal region, consistent with previous reports (Table 2). Collectively, the markers were sensitive for MSI, and they found the same MSI-H tumors that were detectable by BAT-26 (data not shown), in agreement with our previous findings [31].

The microsatellite phenotypes of the GCAs and their numbers and proportions found are provided in Tables 3 and 4. MSI-H was found in 24.0% of IGCA tumors and in 8.3% of DGCA tumors. LOH-H was found in 24% of IGCA tumors, whereas in DGCA tumors this phenotype was absent. The IGCAs included significantly more (48.0%; $P = 0.027$) cases with severe genetic lesions (MSI-H and LOH-H) than the DGCAs (8.3%;

Table 3. Tumor specimens analyzed in the study

Sample number	Histologic type	LOH type	MSI type[a]
57	DGCA	LOH-N	MSS
78	DGCA	LOH-N	MSS
90	DGCA	LOH-N	MSS
102	DGCA	LOH-N	MSS
175	DGCA	LOH-N	MSS
177	DGCA	LOH-N	MSS
178	DGCA	LOH-N	MSS
147	DGCA	LOH-N	MSS
50	DGCA	LOH-N	MSS
151	DGCA	LOH-N	MSI-L
97	DGCA	LOH-L	MSS
176	DGCA	ND	MSI-H
1	IGCA	LOH-N	MSS
6	IGCA	LOH-N	MSS
62	IGCA	LOH-N	MSS
114	IGCA	LOH-N	MSS
136	IGCA	LOH-N	MSS
157	IGCA	LOH-N	MSS
2	IGCA	LOH-N	MSS
119	IGCA	LOH-N	MSI-L
150	IGCA	LOH-N	MSI-L
186	IGCA	LOH-N	MSI-L
188	IGCA	LOH-N	MSI-L
72	IGCA	LOH-L	MSI-L
158	IGCA	LOH-L	MSS
154	IGCA	LOH-H	MSI-L
155	IGCA	LOH-H	MSI-L
7	IGCA	LOH-H	MSI-L
189	IGCA	LOH-H	MSI-L
194	IGCA	LOH-H	MSI-L
99	IGCA	LOH-H	MSS
4	IGCA	ND	MSI-H
49	IGCA	ND	MSI-H
55	IGCA	ND	MSI-H
87	IGCA	ND	MSI-H
191	IGCA	ND	MSI-H
195	IGCA	ND	MSI-H

DGCA, diffuse-type gastric cancer; IGCA, intestinal-type gastric cancer; LOH-N, LOH-non-detectable; LOH-L, LOH-low; LOH-H, LOH-high; MSS, microsatellite-stable; MSI-L, MSI-low; MSI-H, MSI-high; ND, not detected

[a] MSI-H and LOH are considered to be mutually exclusive [15]

Table 4. Numbers and proportions of MSI and LOH phenotypes in gastric cancers

		IGCA ($n = 25$)	DGCA ($n = 12$)	
LOH type	MSI type	n (%)	n (%)	P value[a]
ND	MSI-H	6 (24.0)	1 (8.3)	$P = 0.027$[b]
LOH-H	MSI-L	5 (20.0)	0	$P = 0.015$[c]
LOH-H	MSS	1 (4.0)	0	
LOH-L	MSI-L	1 (4.0)	0	
LOH-L	MSS	1 (4.0)	1 (8.3)	
LOH-N	MSI-L	4 (16.0)	1 (8.3)	$P = 0.023$[d]
LOH-N	MSS	7 (28.0)	9 (75.1)	$P = 0.035$[e]

ND, not detected

[a] Fisher's exact test

[b] Difference in prevalence of LOH-H and MSI-H in IGCA vs DGCA

[c] Difference in prevalence of MSI-L in LOH-H vs LOH-N tumors

[d] Difference in prevalence of MSI-L between non-MSI IGCA vs DGCA

[e] Difference in prevalence of LOH-N/MSS genotype in IGCA vs DGCA

Table 5. Clinicopathological features of the gastric cancers

Microsatellite phenotype		LOH-N (n = 21)	LOH (n = 9)	MSI-H (n = 7)	P value[a]
Sex (M/F)					
All		14/7	5/4	3/4	0.519
Site					
IGCA	Distal	10	5	5	0.303
	Proximal	1	3	1	
DGCA	Distal	5	0	1	0.368
	Proximal	5	1	0	
Tumor					
IGCA	T0–T2	2	2	0	0.544
	T3–T4	9	6	6	
DGCA	T0–T2	2	0	0	0.787
	T3–T4	8	1	1	
Nodes					
IGCA	N0–N1	9	6	6	0.435
	N2–N3	2	2	0	
DGCA	N0–N1	8	1	1	0.787
	N2–N3	2	0	0	
Stage					
IGCA	I–II	4	2	5	0.139
	III–IV	7	6	1	0.056[b]
DGCA	I–II	6	1	0	0.345
	III–IV	4	0	1	
Grade					
IGCA	G I	3	2	0	0.119
	G II–III	8	6	6	
Mean age[f]					
All		67.5	73.0	76.3	0.062[c]
IGCA		70.4	74.8	77.2	0.011[d]
DGCA		64.4	58.8[e]	70.6[e]	

[a] χ^2 test
[b] P value for stage of MSI-H vs LOH-N and LOH genotypes in IGCA (Fisher's exact test)
[c] P value for age at detection of MSI-H vs LOH-N tumors (t-test)
[d] P value for mean age at diagnosis; IGCA vs DGCA (t-test)
[e] only one patient
[f] At diagnosis

Table 4). However, the difference between the two cancer types in the prevalence of MSI-H or LOH-H alone did not reach statistical significance. MSI-L was found in 40% (12/30) of the non-MSI-H GCAs, and was associated significantly (P = 0.023) with IGCA (57.9%; 11/19) as compared with DGCA (9.1%; 1/11). Further, MSI-L was found in 83% (5/6) of LOH-H, 33% (1/3) of LOH-L, and in 24% (5/21) of LOH-N tumors. Among these non-MSI-H GCAs, a significant (P = 0.015) concordance between the rates of MSI-L and LOH was noted (Table 4). Collectively, 43% of the sporadic GCAs were found to be genetically stable (LOH-N/MSS), with a significant (P = 0.035) association with DGCA (75%), as compared with IGCA (28%).

No significant differences were found between the various clinicopathological parameters of GCA and the microsatellite phenotypes (Table 5). MSI-H tumors tended to represent a lower clinical stage than LOH-N/LOH tumors. On average, the DGCA patients were younger (64.6 years; P = 0.011) than the IGCA patients (74.8 years). Age at the time of detection of the MSI-H cancers tended to be higher (76.3 years; P = 0.062) than the age of LOH-N patients (67.5 years).

Discussion

Initially, we evaluated the validity of the tetranucleotide markers in LOH detection by comparing the rates of LOH at four chromosomal regions with the previously published data, as summarized in Table 2. The results show that very similar LOH rates (about 20%–40%) were observed for the tetranucleotide markers and for markers used in other studies. In addition, a perfect match in the MSI-H detection between our panel of 15 markers and BAT-26 was observed (data not shown). We thus conclude that the markers used here are valid for the determination of LOH and MSI in IGCA and DGCA.

The higher prevalence of MSI-H in IGCA than in DGCA found in our study is in line with previous results of 15%–23% and 2.5%–7.7% for MSI-H in IGCA

and DGCA, respectively [7,11,12,36–38]. Previously, IGCA has been reported to show more LOH than DGCA [12]. Accordingly, we found significantly more LOH at the marker loci and a higher prevalence of LOH-H in IGCA than in DGCA.

In the present study, MSI-L was found in 40% of the non-MSI-H GCAs and was associated more often with IGCA than with DGCA. As reported recently by Halford et al. [18], MSI-L is present in about 30% of colorectal, endometrial, and ovarian cancers, in contrast to 3% in breast cancer. Those authors concluded, based on its frequent presence, that MSI-L occurred as a real phenomenon in cancers that were associated with the HNPCC syndrome. However, GCA, which has also been linked to the HNPCC syndrome, was not analyzed in the study [18].

In total, 43% of the GCAs studied here were found to be genetically stable (LOH-N/MSS), with a significant association with DGCA. Previous studies have found the LOH-N type to be more prevalent (51%–60%) in DGCA than in IGCA (13%–50%) [12,26]. However, comparison of the results is not straightforward, because the LOH-N tumors in the aforementioned studies [12,26] were not stratified according to their MSI-L or MSS status. Recently, a microsatellite and chromosomally stable subgroup similar to LOH-N/MSS has been described in 17%–38% of colorectal cancers [29,30]. Accordingly, our data imply, as a novel finding for GCA, that the LOH-N/MSS phenotype represents a distinct subgroup which is more prevalent in DGCA than in IGCA. The carcinogenic mechanism behind this stable GCA phenotype is not understood at present, but it may involve epigenetic alterations of multiple genes, e.g., by hypermethylation of their promoter regions ([39], reviewed in [6]).

In the present study, the MSI-L phenotype was found to be significantly linked to LOH-H cancers (Table 4). In support, Goel et al. [30] found 69.7% (30/43) of MSI-L colorectal cancers to have at least one LOH event in the seven markers analyzed. Tang et al. [29] found chromosomal instability in 91.7% (11/12) of MSI-L colorectal cancers, as assessed either by flow cytometry or by LOH analysis. Accordingly, in our study on GCA, 54.5% (6/11) of the MSI-L tumors showed at least one LOH. Tang et al. [29] and Goel et al. [30] suggest that, in colorectal cancers, a subgroup exists where MSI and LOH pathways overlap. Taking our findings together with these previous findings [29,30], it is conceivable that, also in GCA, MSI-L represents an independent phenomenon which may be associated with the LOH pathway.

Variations in genetic stability may associate differently with clinicopathological parameters and survival. MSI-H has been associated with expanding growth, a high number of tumor-infiltrating lymphocytes, poor differentiation, lower clinical stage, intestinal type, lower likelihood of distant metastases, older age at detection, and better prognosis [9,11,40–43]. LOH has been shown to relate to cancer progression, where a transition from LOH-L to LOH-H is thought to reflect an increase in chromosomal instability during tumor advancement [7,11,12]. A poor outcome for GCA with LOH-H and LOH-N phenotypes has been reported [12]. In the present study, most likely due to the limited number of samples, the only clinicopathological differences were the younger age of DGCA patients and the higher age at the time of detection of MSI-H cancers. Also, MSI-H tumors tended to represent a lower clinical stage than LOH-N/LOH tumors. These results are in agreement with earlier observations [9,11,39–43].

In conclusion, the overall frequencies of MSI and LOH determined by the tetranucleotide markers were in line with previous results for GCA. Our data corroborate previous reports showing that, of the two major types of gastric cancer, IGCA comprises significantly more genomic instability, whereas in DGCA, a stable phenotype is predominant. Contrary to previous views that MSI-L would represent merely an inherent genomic instability of cancer, our data suggest that MSI-L is a true phenomenon, especially in the intestinal type of gastric cancer, and that MSI-L may be associated with the LOH pathway by a mechanism that is unknown at present.

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