GASTRIC CANCER

Is gastric cancer part of the tumour spectrum of hereditary non-polyposis colorectal cancer? A molecular genetic study

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Background: Gastric cancer is the second most common extracolonic malignancy in individuals with hereditary non-polyposis colorectal cancer (HNPCC)/Lynch syndrome. As gastric cancer is relatively common in the general population as well, it is not clear whether or not gastric cancer is a true HNPCC spectrum malignancy.
Aim: To determine whether or not gastric cancer is a true HNPCC spectrum malignancy.
Subjects and methods: The molecular and clinicopathological profiles of gastric cancers (n = 13) from HNPCC mutation carriers were evaluated and compared with the profiles of sporadic gastric cancers (n = 46) stratified by histology and microsatellite instability (MSI) status.
Results: This study on sporadic and HNPCC gastric cancers revealed several important universal associations. Loss of heterozygosity in the adenomatous polyposis coli (APC) region was associated with intestinal histology regardless of the MSI (p = 0.007). KRAS-mutations (p = 0.019) and frameshift mutations in repeat tracts of growth-regulatory genes ($p<0.001$) were associated with MSI tumours being absent in microsatellite stable (MSS) tumours. The average number of methylated tumour suppressor gene loci among the 24 genes studied (methylation index) was higher in MSI than in MSS tumours regardless of histology ($p<0.001$). Gastric cancers from HNPCC mutation carriers resembled sporadic intestinal MSI gastric cancers, except that MLH1 promoter methylation was absent ($p<0.001$) and the general methylation index was lower (p = 0.038), suggesting similar, but not identical, developmental pathways. All these lacked the mismatch repair protein corresponding to the germline mutation and displayed high MSI.
Conclusion: The present molecular evidence, combined with the previous demonstration of an increased incidence relative to the general population, justify considering gastric cancers as true HNPCC spectrum malignancies.

Hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome) is among the most prevalent hereditary cancers in humans and is associated with germline mutations in DNA mismatch repair (MMR) genes, usually MLH1 or MSH2, and microsatellite instability (MSI) in tumour tissue.[1] Besides cancer in the colon and rectum, individuals with HNPCC develop cancers in several other organs. Among these, cancer of the endometrium, small bowel, ureter and renal pelvis are considered to have sufficiently high relative risks in HNPCC compared with the average population, and hence warrant their inclusion in the Amsterdam criteria II, the clinical consensus criteria for the diagnosis of HNPCC.[2]

Despite the fact that gastric cancer is the second most common extracolonic malignancy in HNPCC,[3 4] and in some populations even the most common extracolonic cancer,[5 6] at present it is not included in the Amsterdam criteria II. This is because, despite the currently decreasing trends of this cancer in the Western world, it is relatively common in the general population making the specificity of the association of HNPCC difficult to prove. Although the risk of extracolonic cancers, including gastric cancer, could be higher in MSH2 than MLH1 mutation carriers,[7] the correlation between germline mutation and clinical phenotype is generally poor in the case of HNPCC. For a further assessment of the relationship between gastric cancer and HNPCC, comprehensive molecular characterisation of gastric carcinoma tumours from patients with HNPCC would be necessary; however, the existing literature is surprisingly scarce in this regard.

At the same time, the knowledge of the molecular pathogenesis of sporadic gastric cancers has increased enormously. Gastric carcinoma can histologically be classified into two variants, an intestinal type and a diffuse type,[8] and these two types seem to develop by distinct molecular pathways.[9–12] Intestinal gastric carcinoma is often preceded by chronic atrophic gastritis with intestinal metaplasia,[13] and is related to environmental exposures such as diet, smoking, alcohol and *Helicobacter pylori* infection.[11] High degree of MSI and, possibly, increased loss of heterozygosity (LOH) frequencies have been associated with intestinal-type differentiation.[14 15] Gastric cancers with MSI often display MLH1 promoter methylation and a consequent lack of MLH1 protein.[16–19] The above-described changes are rare in the diffuse type of gastric cancer, which is a poorly differentiated tumour that often starts from the gastric mucosa with normal appearance, and is seen only in the stomach. Diffuse carcinomas occur more often among younger patients. It could have a primary genetic aetiology, hereditary diffuse gastric cancer, owing to germline mutations in the E-cadherin gene.[10] Additionally, somatic E-cadherin mutations have been detected in most diffuse-type tumours but not in intestinal-type gastric cancers.[20 21]

We examined gastric carcinomas arising in families with HNPCC for predisposing mutations and tumour alterations to

Abbreviations: APC, adenomatous polyposis coli; BRAF, B-Raf mutations; CIMP, CpG island methylator phenotype; HNPCC, hereditary non-polyposis colorectal cancer; IHC, immunohistochemistry; KRAS, K-ras mutation; LOH, loss of heterozygosity; MLPA, multiplex ligation-dependent probe amplification; MMR, DNA mismatch repair; MSI, microsatellite instability; MS-MLPA, methylation-specific MLPA; MSS, microsatellite stable; SNuPE, single nucleotide primer extension

clarify whether gastric cancer is a true HNPCC spectrum malignancy. The tumour profiles of HNPCC gastric cancers were compared with those of sporadic gastric carcinomas, stratified by histology and MSI status. Our findings shed light on the molecular pathogenesis of gastric cancer in both HNPCC and sporadic settings.

MATERIALS AND METHODS

Patients and specimens

The study material consisted of 61 gastric carcinoma samples. Of these, 15 tumours were from patients belonging to families with verified HNPCC, with HNPCC according to the Amsterdam criteria[2 22] or the revised Bethesda guidelines.[23] All families were known to have segregate germline mutations in MMR genes. The other 46 tumours represented sporadic gastric cancers. To obtain subgroups with defined histology and MSI status, a larger cohort of sporadic late-onset gastric carcinomas was first subjected to MSI screening, which made it possible to construct three subgroups (not selected for any other characteristics): intestinal MSI (n = 10), intestinal microsatellite stable (MSS) (n = 20) and diffuse MSS (n = 16). Due to the infrequent occurrence of MSI among diffuse cancers, no "diffuse MSI" group could be assembled. The stage of tumours was determined according to the TNM classification.[24]

Formalin-fixed paraffin-wax-embedded specimens of tumours and matching normal tissues were collected from the pathology departments of different hospitals in Finland. Based on histological verification, areas with pure normal or high tumour percentages were selected and subsequently dissected out for DNA extraction. DNA was prepared from archival tissue samples according to the method of Isola *et al*.[25] In addition, 5 µm thick tissue sections were cut, mounted on glass slides (Dako, Glostrup, Denmark) and air-dried overnight at 37℃ for immunohistochemical (IHC) analysis. The relevant institutional review boards of the Helsinki University Central Hospital, Helsinki Finland and Jyväskylä, Finland, Central Hospital, Jyväskylä approved this study.

Histology and *H pylori* status

Gastric carcinomas were classified according to Laurén[8] into intestinal and diffuse types of tumours, and the differentiation grade was determined according to the World Health Organization classification.[26] Chronic gastritis, atrophy, intestinal metaplasia and *H pylori* infection were evaluated and graded according to the Sydney classification.[27] *H pylori* status was verified using hematoxylin and eosin (H&E) and Alcian-blue/periodic acid Schiff stains added with modified Giemsa.

IHC analysis

Formalin-fixed, paraffin-wax-embedded tissue sections were stained by IHC methods using the following primary antibodies of mouse: anti-MLH1 (clone G168-15, Pharmingen, San Diego, California, USA), anti-MSH2 (clone FE-11, Calbiochem, San Diego, California, USA /Oncogene Research, San Diego, California, USA), anti-MSH6 (clone 44, Transduction Laboratories, San Diego, California, USA), anti-β-catenin antibody (clone 14, BD Transduction Laboratories) and anti-p53 (clone DO7, DakoCytomation, Dako).[28] The DAKO EnVision+ System (DakoCytomation, Dako) was applied according to the manufacturer's instructions with antigen-retrieval step by microwave boiling for 15 min in citrate buffer pH 6.0. IHC results for the MMR proteins were interpreted as described previously.[29] β-Catenin expression was considered aberrant if there was nuclear staining in >10% (not observed in the matching normal tissue). In reporting p53 protein stabilisation a cut-off level of >10% positive tumour cells was used.

MSI analysis

MSI status was determined by using the Bethesda panel (BAT25, BAT26, D5S346, D2S123 and D17S250).[30] Tumours with two or more unstable markers were considered to have MSI-H, whereas those with no unstable markers were considered as MSS.

Loss of heterozygosity

Single nucleotide primer extension (SNuPE) based on a polymorphism in exon 8 was used to analyse the LOH at MLH1.[29] In the carriers of a genomic deletion of MLH1 exon 16, multiplex ligation-dependent probe amplification (MLPA) was also applied to detect locus-restricted LOH.[31] LOH at adenomatous polyposis coli (APC) was examined by SNuPE analysis using a polymorphism in exon 11 as well as flanking microsatellite markers, D5S1965 (200 kb upstream of APC) and D5S346 (<100 kb downstream of APC).[32] In both SNuPE and microsatellite marker LOH analyses, ratios of allelic peak areas in normal tissue to tumour were calculated, and values <0.6 or >1.67 (indicating that the transcript of one allele had decreased >40%) were considered to be strict LOH[33] and ratios between 0.61–0.75 and 1.66–1.33 were considered to be putative LOH.[14 15 34]

Mutation analysis

To examine whether patients with HNPCC gastric cancer carried the mutations previously identified in their families, the respective exons of MLH1 and MSH2 were screened by direct sequencing of genomic PCR products using primers from Chadwick *et al*.[35] For the genomic deletion affecting MLH1 exon 16, a direct assay described in Nyström-Lahti *et al*[36] was used.

KRAS, CTNNB1 and BRAF were screened for mutations by single-strand conformation polymorphism analysis of genomic DNA, followed by sequencing, to determine the exact nucleotide changes. KRAS exon 2 was studied with primers from Deng *et al*.[37] The primers for the CTNNB1 exon 3 were forward 5′-TGCTAATACTGTTTCGTATTTATAGC-3′ and reverse 5′-TGACTTTCAGTAAGGCAATGA-3′. BRAF exon 15 was studied using primers from Abdel-Rahman *et al*.[28]

Eight mononucleotide repeats in six frameshift-prone target genes were studied using primers shown in table 1.

Methylation-specific MLPA

The SALSA methylation-specific (MS)-MLPA ME001 (MRC Amsterdam, Holland) tumour suppressor probe mix detects aberrant methylation of DNA using probes containing a digestion site for the methylation-sensitive HhaI enzyme. All reactions were carried out and the results were analysed according to the manufacturer's instructions (http://www.mrc-holland.com). The 24 different tumour-suppressor genes whose methylation status could be monitored are TP73, CASP8, VHL, RARB, MLH1, RASSF1, FHIT, APC, ESR1, CDKN1B, CDKN2A/p14ARF, CDKN2B, DAPK1, PTEN, CD44, GSTP1, ATM, IGSF4, CHFR, BRCA1, BRCA2, CDH13, HIC1 and TIMP3. Normal DNA specimens derived from the lymphocytes of healthy controls and tumour cell lines (HCT116, HCT15, RKO, HEC59, LoVo, SW48), with verified methylation status, were included in every assay. For each MLPA reaction, 100–150 ng of DNA extracted from paraffin-wax-embedded tissue was used.

The MS-MLPA technique was validated on tumour cell lines by demonstrating high concordance relative to the results obtained by an independent method (methylation-specific PCR). On the basis of our titration experiments with cell lines known to have full methylation or complete lack of methylation of a given gene and, in the case of MLH1, correlating methylation level and protein expression, a dosage ratio of >0.15 (corresponding to 15% of methylated DNA) was

Table 1 Target genes with intragenic mononucleotide repeats screened for mutations

Gene	Description	Repeat (site)	Primers (5′–3′)	Reference
PTEN	Phosphatase and tensin homologue Mutated in multiple advanced cancers 1(MMAC1) (phosphatidylinositol 3-phosphatase)	A6 (exon 7)	GACGGGAAGACAAGTTCAT TTTGGATATTTCTCCCAATG	Kuismanen *et al*[62]
		A6 (exon 8)	CAGAGGAAACCTCAGAAAAA TTGGCTTTGTCTTTATTTGC	Kuismanen *et al*[62]
ACVR2	Activin A type II receptor (ACTRII) (serine-threonine kinase)	A8 (exon 3)	AAAAACACTTGTTGTAGGGTCAG CGCTGTGTGACTTCCATCTC	Jung *et al*[59]
		A8 (exon 10)	GTTGCCATTTGAGGAGGAAA CCTCTGAAAAGTGTTTTATTGGAA	Jung *et al*[59]
BLM	Bloom's syndrome protein (DNA helicase)	A9 (exon 7)	CACCAGGAAGAATCTTTTTGAA TGCTTGTGAGAACATTTCCTG	This study
MBD4	Methyl-CpG-binding endonuclease (MED1)	A10 (exon 3)	CTCAGTGTGACCAGTGAAGAAAA TCTGAGTCTTTGGCTGAACAAA	This study
TGFβ RII	Tumour growth factor-β type II receptor (serine-threonine kinase)	A10 (exon 3)	CTAGAGACAGTTTGCCATGACC TGTTGTCATTGCACTCATCAGA	This study
MRE11A	Meiotic recombination 11 homologue A, accessory splicing signal within intron 4	T11 (intron 4)	AGTCAGTTTGCCTATGATTGC TCTTGATAGTTCACCCATGGAA	This study

regarded to indicate promoter methylation. This threshold value also provided the best discrimination of tumour DNA relative to paired normal DNA where no methylation was generally expected.

Statistical analysis

Significance level for the differences between groups (p value) was determined using Fisher's or Student's *t* test as appropriate. All reported p values were two-tailed, and values <0.05 were considered significant.

RESULTS

Clinicopathological characteristics

All gastric cancer specimens (n = 15) from families with HNPCC, with known mutations in MMR genes, wherever adequate normal and tumour specimens were available, were collected and subjected to molecular analyses, and 13 tumours turned out to originate from germline mutation carriers (see below). The average age at diagnosis for HNPCC gastric cancer was 58 years. For comparison, a cohort of sporadic late-onset gastric cancers assigned to the following three subgroups was examined: intestinal MSI (n = 10; average age at diagnosis 76 years), intestinal MSS (n = 20; 73 years) and diffuse MSS (n = 16; 69 years). Table 2 gives the results from Sydney classification[27] and the distribution of grades and stages. HNPCC and the three sporadic subgroups did not reveal essential differences in the Sydney classification. *H pylori* infection was rare in all groups. Lymphocyte infiltration of MSI colorectal cancers[23] was not common, being present in only 3/10, 1/10, 2/20 and 2/16 among HNPCC, intestinal MSI, intestinal MSS and diffuse MSS groups, respectively (data not shown). Poor differentiation was relatively common in HNPCC (4/9) and was more frequent for MSI than for MSS cancers among sporadic intestinal cancers (5/10 vs 1/20, p = 0.009). Among the MSI cancers (HNPCC and sporadic), the tumour stages were relatively evenly distributed, whereas most sporadic intestinal MSS gastric cancers were diagnosed at stages I and II and most diffuse gastric cancers at stages III and IV.

MMR genes

Of the 15 examined patients with HNPCC, 13 were found to have the predisposing MMR gene germline mutation of their families, 11 in MLH1 and 2 in MSH2 (table 3). These cancers were mainly of intestinal histology (12/13, 92% and 1 diffuse). All 13 patients lacked the MMR protein corresponding to the germline mutation (case 122:1 additionally lacked MSH6, as expected for a MSH2 mutation carrier),[38] and displayed a high degree of MSI. Among the initial 15 patients with tumours, 2 did not to have the predisposing MMR gene germline mutation of their families, making a phenocopy frequency of 13%. These tumours were MSS and showed no evidence of abnormal MMR protein expression. The two phenocopies were omitted from further calculations.

Among the sporadic gastric cancers that were studied for comparison, all intestinal MSI gastric cancers showed the absence of MLH1 protein by IHC (table 4). The mechanism of MLH1 inactivation was different between HNPCC and sporadic gastric cancers. Among sporadic MSI cancers, it was probably due to MLH1 promoter methylation that was present in 7/10 (70%), and the degree of methylation (by MS-MLPA, see Materials and methods section) suggested the methylation of both alleles. HNPCC cancers showed no significant MLH1 promoter methylation (0/13; $p<0.001$ for differences relative to the sporadic MSI tumours). Instead, by SNuPE and MLPA, LOH at MLH1 seemed to be an important mechanism for somatic inactivation of the wild-type allele in HNPCC gastric cancers (4/8, 50%). Only two of the 10 sporadic intestinal MSI tumours were informative (constitutionally heterozygotic) for the intragenic MLH1 polymorphism used in SNuPE, and one showed putative LOH. This tumour was one of those three that did not show MLH1 promoter hypermethylation in this group.

Wnt pathway genes

In the HNPCC group, strict or putative LOH at APC occurred in 5 (42%) of the 12 informative tumours. In general, APC-LOH was typical of intestinal tumours, whether hereditary or sporadic and whether MSI or MSS (20/39, 51%), and was significantly less common in diffuse tumours (2/17, 12%; p = 0.001; tables 3 and 4). Nuclear localisation of β-catenin was seen in 2/10 (20%) of gastric cancers from inherited MMR gene mutation carriers and in sporadic cancers with comparable frequencies (table 4). Among the 16 tumours with nuclear localisation of β-catenin, APC-LOH co-occurred in 9 cases (56%). No mutations in the β-catenin gene, CTNNB1, were found in any group.

p53, KRAS/BRAF

The number of tumours with positive/stabilised p53 ranged from 20–30% in all four groups (table 4). Somatic mutations in

Table 2 Results from the Sydney classification in HNPCC and sporadic gastric cancers

	HNPCC	Sporadic		
Sydney classification	**Intestinal MSI n = 10***	**Intestinal MSI n = 10**	**Intestinal MSS n = 20**	**Diffuse MSS n = 16**
Chronic inflammation				
Normal/none	0	0	0	1
Mild	1	2	6	4
Moderate	2	4	10	7
Marked	7	4	4	4
Neutrophils				
Normal/none	3	6	6	11
Mild	7	3	11	2
Moderate	0	1	3	3
Marked	0	0	0	0
Atrophy				
Normal/none	3	5	4	10
Mild	2	2	10	2
Moderate	5	3	5	4
Marked	0	0	1	0
Metaplasia				
Normal/none	4	3	7	14
Mild	4	5	8	2
Moderate	2	2	2	0
Marked	0	0	3	0
H pylori				
Normal/none	8	5	9	12
Mild	2	3	5	4
Moderate	0	2	3	0
Marked	0	0	3	0
Grade	n = 10*	n = 10	n = 20	n = 16
Well differentiated (grade 1)	1	1	5	–
Moderately differentiated (grade 2)	4	4	14	–
Poorly differentiated (grade 3)	4	5	1	–
N/A	1	0	0	16
Stage	n = 13	n = 10	n = 20	n = 16
I	2	1	8	3
II	5	4	6	1
III	5	3	5	6
IV	1	2	1	6

HNPCC, hereditary non-polyposis colorectal cancer; MSI, microsatellite instability; MSS, microsatellite stable; N/A, not available/not applicable.
*Of the 13 MMR gene mutation-positive gastric cancers, 10 were available for evaluation.
Differentiation grade and tumour stage are also shown.

KRAS were only found in MSI tumours, 4/23 (17%), as opposed to none among 36 MSS tumours ($p = 0.019$). All KRAS mutations consisted of G12D. No somatic mutations in BRAF were found in any group.

Mononucleotide repeats within growth-regulatory genes

The tumours were screened for mutations in six growth-regulatory target genes known to be prone to inactivation by frameshift mutations as they contain mononucleotide repeats in their coding regions (PTEN, ACVR2, BLM, MBD4, TGFβRII) or in an adjacent intronic splice site (MRE11A). All genes have previously been implicated in the development of sporadic gastric or colorectal cancers and the protein products of at least three (BLM,[39] MBD4,[40] MRE11A[41]) interact with MLH1. As expected, frameshift mutations were limited to MSI gastric cancers only, whether hereditary or sporadic, and were absent in MSS tumours (68/135 vs 0/97, $p<0.001$). ACVR2, TGFβRII and MRE11A were highly mutable in MSI tumours, with mutation frequencies ranging between 62% and 100%; MBD4 was mutated in 18–40%, and BLM and PTEN in <10% (tables 3 and 4). The high rate of ACVR2 and TGFβRII frameshift mutations might be explained by the similarity between the activin and TGFβ signalling systems: as both systems use the same set of SMADs, they may share common regulatory mechanisms.[42]

Promoter methylation of tumour-suppressor genes

MS-MLPA method was used to examine the methylation status of the promoters for 24 tumour-suppressor genes, including MLH1 (see Materials and methods section). Table 4 gives the average numbers of methylated genes among these 24 for each group of tumours as well as the methylation frequencies for selected loci that showed frequent methylation in tumour DNA (but not in paired normal DNA). As described above, MLH1 methylation was absent in HNPCC gastric cancers in contrast to sporadic MSI gastric cancers ($p<0.001$). The average number of genes with promoter methylation among all 24 genes studied was significantly associated with MSI, being 4.9 for the MSI tumours (HNPCC and sporadic intestinal) compared with 2.0 for the MSS tumours (sporadic intestinal and diffuse; $p<0.001$). The present sporadic tumours, in particular, highlighted the fact that MSI, and not histology, determined the general methylation profile, as among intestinal tumours, the

Table 3 Histology and molecular features of gastric cancers from patients with hereditary non-polyposis colorectal cancer with germline mutations in DNA mismatch repair genes

ID # (criteria)	Histology	MMR gene germline mutation	MLH1 LOH	Immunohistochemistry			MSI status	APC LOH	β-catenin IHC/ mutation	p53 IHC	KRAS/BRAF mutation	Repeat containing genes					
				MLH1	MSH2	MSH6						PTEN	ACVR2	BLM	MBD4	TGFβRII	MRE11A
1:32 (A)	Intestinal	MLH1 exon 16 deletion[36]	N	–	+	+	MSI	N	Mem/N	Negative	N/N	N	U	N	U	U	U
1:80 (A)	Intestinal	MLH1 exon 16 deletion[36]	LOH	–	+	+	MSI	LOH?	Mem/N	Positive (60%)	N/N	N	U	N	N	N	U
3:39 (A)	Intestinal	MLH1 exon 16 deletion[36]	LOH	–	+	+	MSI	N	Nuc/N	Negative	N/N	N	U	N	U	U	U
4:4 (A)	Intestinal	MLH1 exon 3–5 deletion[73]	N	–	+	+	MSI	LOH	Nuc/ND	Negative	N/N	N	U	ND	ND	N	U
7:18 (A)	Intestinal	MLH1 exon 17, G→C at 1976, R659P[73]	LOH?	ND	ND	ND	MSI	N	ND/N	ND	G12D/N	N	U	N	N	U	U
73:1 (A)	Intestinal	MLH1 exon 3, C→T at 298, R100X[29]	LOH	ND	ND	ND	MSI	LOH	ND/ND	ND	N/N	N	U	N	ND	U	U
83:18 (A)	Intestinal	MLH1 exon 17, C→T at 1975, R659X[73]	N	–	+	+	MSI	N	Mem/N	Negative	N/N	N	U	N	N	U	U
83:45 (A)	Intestinal	MLH1 exon 17, C→T at 1975, R659X[73]	NI	–	+	+	MSI	N	Mem/N	Negative	N/N	N	U	N	N	N	U
90:60 (A)	Intestinal	MLH1 intron 5, G→A at 454–1[36]	NI	–	+	+	MSI	N	Mem/N	Negative	N/N	N	U	N	N	U	U
122:1 (B)	Intestinal	MSH2 exon 12, G→A at 1807, D603N[33]	NI	+	+	–	MSI	NI	Mem/ND	Positive (20%)	N/N	N	U	N	N	U	U
130:1 (A)	Intestinal	MSH2 exon 11, C→T at 1720, Q574X	NI	ND	ND	ND	MSI	LOH?	ND/N	ND	N/N	N	U	N	N	U	U
12:20 (A)	Intestinal/mucinous	MLH1 exon 16 deletion[36]	N	–	+	+	MSI	N	Mem/N	Negative	N/N	N	U	N	N	N	U
67:15 (A)	Diffuse	MLH1 exon 4, T>G at 320, I107R[73]	NI	–	+	+	MSI	LOH?	Mem/N	Negative	N/N	N	U	N	N	N	N

A, Amsterdam criteria I or II; APC, adenomatous polyposis coli; B, Bethesda criteria; IHC, immunohistochemistry; LOH, loss of heterozygosity; LOH?, putative LOH; MSI, microsatellite instability; MMR, DNA mismatch repair; MSS, microsatellite stable; Mem, membranous; N, normal; ND, not done; NI, not informed; Nuc, nuclear; U, unstable.

average number of methylated genes was significantly higher for MSI than MSS tumours (5.9 vs 2.3, $p<0.001$), whereas among MSS tumours, intestinal and diffuse tumours showed no difference (2.3 vs 1.6; table 4). Note that there was a significant difference within MSI tumours depending on whether the tumours represented the HNPCC or the sporadic intestinal MSI group (4.1 vs 5.9; $p = 0.038$). As the MLH1 methylation status was drastically different in these two groups (table 4), one could conclude that the general methylation profile did not entirely follow the methylation status of MLH1 even if the whole study series showed a strong correlation between MLH1 methylation and methylation of other tumour-suppressor-promoter loci ($p<0.001$).

DISCUSSION

Associations of molecular alterations with histology and MSI status

The present study of 61 gastric cancer samples revealed several important universal associations that increase the understanding of gastric tumourigenesis. First, LOH in the APC region was associated with intestinal histology regardless of MSI ($p = 0.007$). Other investigators have also noted an association between APC-LOH and intestinal histology,[43 44] although contradictory reports exist.[45] The Wnt pathway, in general, was found to be involved in nearly half (27/59, 46%) of the present gastric carcinomas, through either alterations in β-catenin expression (nuclear localisation) or APC-LOH. We found no mutations in CTNNB1, which is in agreement with many published studies (eg, Sasaki *et al*[46] and Kim *et al*[47]), whereas fairly high mutation frequencies (up to 38%) and variable associations with histology have also been reported.[48 49]

Second, KRAS mutations ($p = 0.019$) and frameshift mutations in repeat-containing tumour-suppressor genes ($p<0.001$) were limited to MSI tumours, being absent in MSS tumours, whether intestinal or diffuse. Other investigators have also described a preferential occurrence of KRAS mutations in MSI gastric cancers.[50 51] Our data on instability in repeat-containing target genes (association with MSI tumours, overall frequencies of frameshift mutations) are broadly compatible with the previous reports on sporadic gastric cancers.[52–56] The instability profile we report for gastric cancer shares features with gastrointestinal tumours in general, such as high frequency of TGFβRII, ACVR2 and MRE11A mutations.[57–60] Specifically, the instability profile for our HNPCC gastric cancers resembles that reported for colorectal or small bowel tumours from patients with HNPCC.[61–64]

Third, the average number of methylated tumour-suppressor-gene loci among the 24 loci that were studied was significantly higher in MSI versus MSS tumours, regardless of histology ($p<0.001$). Our results are broadly compatible with some previous reports, which found by studying a variable number of indicator genes (typically <10) that MSI gastric cancers are associated with MLH1 hypermethylation and CpG island methylator phenotype (CIMP).[47 65] Recent observations on colorectal cancers suggest the existence of a CIMP+, non-MSI group that shows different clinicopathological characteristics relative to the CIMP+, MSI group.[66] Although the average number of methylated loci in our sporadic MSS gastric cancers was low (2 out of 24 loci studied), a small subgroup (3/36 tumours) did exist that had 6–7 methylated loci per tumour, implying that a CIMP+, non-MSI phenotype also exists among gastric cancers. Interestingly, two of these tumours were diagnosed at stage I and one at stage II. MSS gastric cancers, in general, showed an inverse trend between the overall methylation index and tumour stage (with average methylation indices of 2.5, 3.1, 1.6 and 0.71 for stages I, II, III and IV, respectively). Among the MSI gastric cancers (HNPCC and

Table 4 Summary of molecular data on hereditary non-polyposis colorectal cancer (mismatch repair gene germline mutation positive) and sporadic gastric cancers

	HNPCC mut+		Sporadic	
	Intestinal*, MSI	Intestinal, MSI	Intestinal, MSS	Diffuse, MSS
Decreased MMR protein expression:	n (%)	n (%)	n (%)	n (%)
MLH1	9/10 (90)	10/10 (100)	ND	ND
MSH2	1/10 (10)	0/10 (0)	ND	ND
MSH6	1/10 (10)	0/10 (0)	ND	ND
Nuclear β-catenin	2/10 (20)	3/10 (30)	8/20 (35)	3/16 (19)
CTNNB1 exon 3 mutation	0/10 (0)	0/10 (0)	0/12 (0)	0/16 (0)
APC LOH (strict)	2/12 (17)	3/8 (38)	8/20 (40)	0/16 (0)
APC LOH (strict + putative)	5/12 (42)	4/8 (50)	12/20 (60)	1/16 (6)
p53 positive IHC	2/10 (20)	3/10 (30)	6/20 (30)	4/16 (25)
KRAS exon 2 mutation	1/13 (8)	3/10 (30)	0/20 (0)	0/16 (0)
BRAF exon 15 mutation	0/13 (0)	0/10 (0)	0/20 (0)	0/16 (0)
Proportion of tumours with unstable loci:				
PTEN (A6)	0/13 (0)	1/10 (10)	0/20 (0)	0/16 (0)
ACVR2 (A8)	13/13 (100)	10/10 (100)	ND	ND
BLM (A9)	0/12 (0)	1/10 (10)	ND	ND
MBD4 (A10)	2/11 (18)	4/10 (40)	ND	ND
TGFβRII (A10)	8/13 (62)	9/10 (90)	0/20 (0)	0/16 (0)
MRE11 (T11)	12/13 (92)	8/10 (80)	0/11 (0)	0/14 (0)
Average number of methylated genes†	4,1	5,9	2,3	1,6
MLH1 promoter methylation	0/13 (0)	7/10 (70)	0/20 (0)	0/16 (0)
TIMP3 promoter methylation	5/13 (38)	6/10 (60)	3/20 (15)	2/16 (13)
ESR1 promoter methylation	5/13 (38)	7/10 (70)	6/20 (30)	5/16 (31)
CHFR promoter methylation	7/13 (54)	9/10 (90)	5/20 (25)	2/16 (13)

HNPCC, hereditary non-polyposis colorectal cancer; MMR, DNA mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable; ND, not done.
*Twelve intestinal and one diffuse
† Of the 24 studied genes
Values are given as proportion of tumours with alterations (percentage) unless specified otherwise.

sporadic), the methylation index was similar for all stages (being 4.3, 4.9, 5.3 and 4.3 according to increasing stage). Besides the CIMP+ non-MSI group, the relatively high methylation index in the present HNPCC gastric cancers, which was associated with MSI but did not involve MLH1 promoter, may represent another variant of CIMP. *H pylori* infection has been suggested to induce methylation of CpG islands in gastric mucosa.[67] In our study, *H pylori* infection was rare in all groups and therefore it is unlikely to explain the observed methylation differences.

Differences and similarities between HNPCC and sporadic gastric cancers

The present gastric cancers from germline carriers of MMR gene mutations had mostly intestinal histology; most showed the lack of MLH1 protein and all displayed a high-degree of MSI. Thus, the closest sporadic counterpart was intestinal MSI gastric cancer, most of which were also MLH1-deficient as observed in this and in previous studies.[68] According to our findings, the tumourigenic events in HNPCC gastric cancers closely resembled those in sporadic intestinal MSI gastric cancers, except for the mechanism of MLH1 inactivation and overall epigenetic changes. Thus, although biallelic methylation of the MLH1 promoter underlies MSI and the absence of MLH1 protein in most sporadic cancers, no significant MLH1 promoter methylation was seen in HNPCC, and, instead, the wild-type allele was inactivated by LOH. Even, more importantly, the overall frequency of tumour-suppressor-gene loci with promoter methylation was significantly ($p = 0.038$) lower in HNPCC than in sporadic intestinal MSI gastric cancers, let alone sporadic MSS gastric cancers, which suggests that despite many similarities, the tumourigenic mechanisms in HNPCC and sporadic intestinal MSI gastric cancers are not identical. The methylation rates of certain loci are known to increase with age,[69] and as our patients with HNPCC gastric cancers were diagnosed at a younger age than patients with sporadic intestinal MSI gastric cancers (average 58 vs 76 years, respectively) one might argue that age played a role. This may partly be true as, for example, the oestrogen receptor α locus whose methylation has typically been connected with age was less often methylated in HNPCC than sporadic intestinal MSI tumours; by contrast, the possible association with age did not extend to sporadic MSS cancers (table 4).

Gastric cancers and HNPCC tumour spectrum

According to our study, HNPCC gastric cancer (MMR gene germline mutation positive) has the following characteristics: intestinal histology (92%), MSI-high (100%), absence of the MMR protein corresponding to the germline mutation (100%), frequent APC-LOH as typical of intestinal gastric cancer, patterns of KRAS mutation and mutations in repeat-containing target genes similar to sporadic MSI gastric cancers and a unique pattern of promoter methylation of tumour-suppressor genes. Additionally, our previous investigation shows rare changes in DNA copy number by comparative genomic hybridisation.[70]

At present, gastric cancer is not included in the Amsterdam II criteria[2] but is included in the revised Bethesda criteria.[23] Evidence to support the idea that a given type of tumour from a MMR gene germline mutation carrier is part of the HNPCC tumour spectrum would include the increased incidence in HNPCC compared with the average population and the demonstration of MMR deficiency as the driving force for tumourigenesis.[71] The relative risk of gastric cancer in HNPCC mutation carriers compared with the general population has been reported to be higher by 4–19-fold[3 4 72] in populations of the Western world and at least by twofold in endemic areas in Asia.[5] Although all tissues of a MMR gene germline mutation carrier are genetically predisposed to cancer as all cells carry the "first hit", tumour development additionally requires somatic inactivation of the remaining wild-type allele ("second hit") in a target tissue. If a second hit does not occur, there will be no loss of MMR protein, no MSI and no MMR deficiency-driven tumourigenesis. This was not the case with the present HNPCC gastric cancers, which showed MLH1-LOH as a second hit,

MMR protein loss, MSI-high and a number of other tumourigenic events typical of MMR deficiency. Together, these data justify considering gastric cancer as a true HNPCC spectrum malignancy.

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