PERGAMON

European Journal of Cancer 39 (2003) 1222–1227

European Journal of Cancer

www.ejconline.com

Concurrent hypermethylation of gene promoters is associated with a MSI-H phenotype and diploidy in gastric carcinomas

B. Carvalho[a], M. Pinto[a], L. Cirnes[a], C. Oliveira[a], J.C. Machado[a,b], G. Suriano[a], R. Hamelin[c], F. Carneiro[a,b], R. Seruca[a,b,*]

[a]*IPATIMUP-Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Porto, Portugal*
[b]*Faculdade de Medicina do Porto and H. S. João, Porto, Portugal*
[c]*INSERM U434-CEPH, Paris, France*

Received 27 July 2002; received in revised form 11 December 2002; accepted 21 February 2003

Abstract

Changes in the pattern of DNA methylation are among the most common alterations observed in human cancers, such as gastric carcinomas. We analysed in a series of 51 sporadic gastric carcinomas the methylation status of the promoter regions of the *hMLH1*, *CDH1, MGMT* and *COX2* genes. We aimed to determine the frequency of CpG island hypermethylation and to find out whether the occurrence of concurrent hypermethylation is related to the clinicopathological features of the gastric carcinomas. Using methylation-sensitive restriction analysis/polymerase chain reaction (PCR) and methylation-specific PCR (MSP) strategies, we searched for the presence of hypermethylation on the promoter region of the 4 selected genes. All showed hypermethylation of their promoter regions with frequencies of 37, 51, 61 and 29% for *hMLH1*, *CDH1*, *MGMT* and *COX2*, respectively. Concurrent hypermethylation was more frequently observed in MSI-H ($P=0.0005$) and diploid ($P=0.029$) tumours. Hypermethylation of *hMLH1* was associated with MSI-H tumours ($P=0.0001$), whereas hypermethylation of *MGMT* was associated with MSI-H ($p=0.021$) and diploid tumours ($p=0.012$). Our results indicate that concurrent hypermethylation is a common event in gastric cancer, suggesting that global methylation changes play an important role in the development of sporadic gastric carcinoma. Moreover, inactivation of different gene promoters by hypermethylation is significantly associated with microsatellite instability (MSI-H) and diploidy: *hMLH1* determines MSI-H and *MGMT* the diploid status of gastric carcinomas.

Keywords: Methylation; Promoter; MSI; Ploidy; Gastric cancer; Concurrent hypermethylation; hMLH1; Cadherin; MGMT; COX2

1. Introduction

In neoplasia, genome-wide epigenetic disturbances result in altered DNA methylation patterns. Genome-wide hypomethylation and selective hypermethylation of DNA sequences is a common event in several cancers and became recognised as a hallmark of human cancers [1].

Methylation of regulatory regions of genes acts as an important alternative to genetic alteration for gene inactivation. Methylation of cytosines within CpG islands is also observed in physiological conditions as a common epigenetic event, such as chromosome X inactivation and ageing [2,3]. CpG island hypermethylation of normally unmethylated promoter regions correlates with loss of transcription [4].

Hypermethylation of several gene promoters has been described in sporadic gastric carcinomas, namely in genes of the repair pathway such as *hMLH1* and *MGMT*, cell cycle regulators such as the cyclin-dependent kinase inhibitor—*p16*, the mediator of epithelial cell growth *COX2*, and cell adhesion molecules such as *CDH1* [1,5,6]. Moreover, it was recently shown that hypermethylation of gene promoters increases along the pathway that evolves from chronic gastritis, intestinal metaplasia, and adenomas to carcinomas of the stomach [7,8]. Although epigenetic changes have been accepted as an important mechanism underlying gastric carcinoma progression, there are no data related to concurrent hypermethylation

* Corresponding author. Tel.: +351-225570700; fax: +351-225570799.
E-mail address: rseruca@ipatimup.pt (R. Seruca).

0959-8049/03/$ - see front matter
doi:10.1016/S0959-8049(03)00177-1

and the clinicopathological parameters of gastric tumours.

In this study, we searched for the promoter methylation status of the cancer-related genes *hMLH1*, *CDH1*, *MGMT* and *COX2*, in a series of 51 sporadic gastric carcinomas with the following aims: (1) To determine the frequency of CpG island hypermethylation and (2) to find out whether the occurrence of concurrent hypermethylation is related to clinicopathological features of gastric carcinomas.

2. Materials and methods

2.1. Samples

Haematoxylin-eosin stained sections were used to classify 51 gastric carcinomas according to Lauren's [9] and Carneiro's [10] classification. Invasion of the gastric wall, as well as the presence of lymph node metastases, was recorded in every case using the unified 1987 TNM system for pathological staging. Orcein stained sections were used for the detection of vascular invasion. Ploidy of the cases was determined by flow cytometry according to the method described by David and collaborators [11]. The cases were previously analysed for microsatellite instability (MSI) phenotype [12]. Tumours were classified as having a MSI-H phenotype whenever they presented a high frequency of microsatellite instability ($\geqslant 40\%$) at dinucleotide repeats and BAT26 markers. Cases were considered MSS/MSI-L whenever they presented instability at a low rate at dinucleotide repeats ($<40\%$), or did not show instability at any locus analysed. We considered MSS and MSI-L cases together because there is no evidence for a separate category of MSI-L nor a trimodal distribution of MSI (MSI-H, MSI-L, MSS). Moreover, MSI-low and MSS tumours do not differ in their clinicopathological features [13–18].

2.2. Methylation-specific PCR (MSP)

DNA methylation patterns in the CpG islands of *hMLH1* was analysed using *Hpa*II-sensitive methylation restriction analysis, followed by polymerase chain reaction (PCR) [17]. The promoter methylation analysis of the *CDH1*, *MGMT* and *COX2* genes was performed by chemical modification of unmethylated cytosines to uracil and subsequent PCR using primers specific for either methylated or the modified unmethylated DNA. One μg of genomic DNA was treated with bisulphite. DNA samples were then purified using the Wizard DNA purification kit (Promega), denatured with NaOH, and eluted in water. MSP was performed to examine the methylation status at the promoter regions. The primer sequences of each gene, for both methylated and unmethylated reactions were previously described [19–21]. Amplifications were performed in a 30 μl reaction mixture for 35 cycles with denaturation at 94×C for 30 s, annealing at 57 °C for 30 s for *CDH1* and *MGMT* and at 61 °C for *COX2*, and extension at 72 °C for 30 s. Initial and final extension steps were at 94 and 72 °C, respectively, for 5 min. Amplified products were separated by electrophoresis in a 2.5% agarose gel. DNA from blood samples was used as negative controls.

2.3. Statistical analysis

For the statistical analysis between concurrent methylation and the clinicopathological features of the cases, the number of genes affected by hypermethylation was the sole criterion used. Five different categories were defined according to the number of genes affected by hypermethylation and cases were ranked as follows: 0: with no methylation, 1: with methylation in one gene promoter, 2: with methylation in 2 gene promoters, 3: with methylation in 3 gene promoters, and 4: with hypermethylation in the promoter region of all genes.

The statistical analysis of concurrent hypermethylation and the clinicopathological features of the cases were evaluated with an analysis of variance, and Mann–Whitney and Kruskal–Wallis tests. Associations between the MSI and the ploidy status of the tumours and the methylation status of each gene promoter (*hMLH1*, *CDH1*, *MGMT* and *COX2*) were assessed by the χ^2 test. A P value of <0.05 was considered statistically significant.

3. Results

We searched for the presence of hypermethylation in the promoter region of 4 genes (*hMLH1*, *CDH1*, *MGMT*, *COX2*) in 51 sporadic gastric carcinomas. We observed hypermethylation of the promoter regions in 19 (37%), 26 (51%), 31 (61%) and 15 (29%) for the *hMLH1*, *CDH1*, *MGMT* and *COX2* genes, respectively. Except for the *hMLH1* gene promoter, all the cases under analysis showed hypermethylated sequences together with nonmethylated sequences (Fig. 1). Due to the technique used in the detection of hypermethylation in *hMLH1* promoter, in the hypermethylated cases, only methylated sequences could be detected. In the blood samples, used as negative controls, no hypermethylated sequences were found in any of the gene promoters.

The methylation status of the 4 gene promoters in each case is depicted in Fig. 2. Forty seven out of 51 (92%) carcinomas presented methylation in one or more loci under analysis. Only 4 (8%) carcinomas did not show hypermethylation in any of the studied regions. The profile of methylation shows that there are no preferential combinations of methylated loci

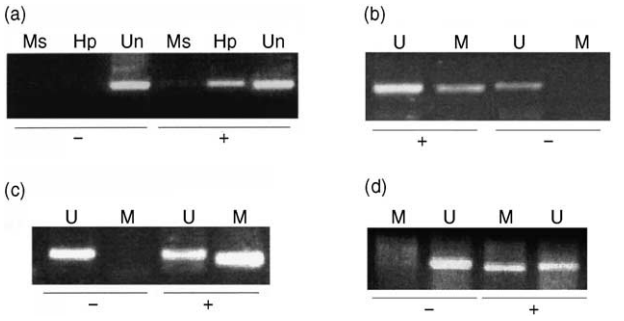

Fig. 1. Examples of cases with (+) and without (–) promoter region hypermethylation for the genes *hMLH1* (a), *CDH1* (b), *MGMT* (c) and *COX2* (d). Ms, methylation-specific PCR (MSPI) digestion; Hp, *HpaII* digestion; Un, undigested; U, unmethylated; M, methylated.

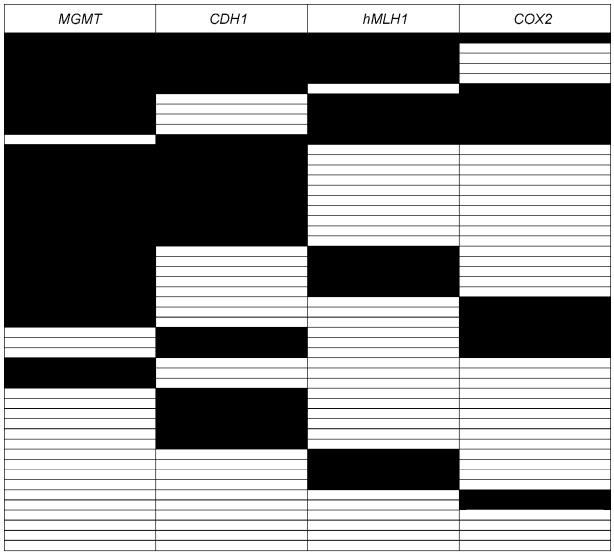

Fig. 2. Summary of methylation of *hMLH1*, *CDH1*, *MGMT* and *COX2* genes in the panel of 51 gastric carcinomas analysed. Black boxes, methylation; White boxes, nonmethylation.

throughout the tumours (Fig. 2). To determine the relationship between concurrent hypermethylation and the clinicopathological features of the patients and the tumours, we ranked the 51 gastric carcinomas in 5 groups with an increasing number of methylated loci (from without any affected locus to four methylated loci). The mean age of the patients within the 5 groups of carcinomas was not significantly different ($P=0.287$). There was no association between concurrent hypermethylation and the gender of the patients (Table 1). No significant association was found between concurrent hypermethylation and the histological classification of the carcinomas, wall invasion, presence of vascular invasion, lymph node metastases and TNM staging of the tumours (Table 1). The analysis between concurrent hypermethylation and the features of the tumours yielded significant associations with the MSI status ($P=0.0005$) and the ploidy of the tumours ($P=0.029$) (Table 1). Tumours with hypermethylation

Table 1
Relationship between concurrent hypermethylation and the clinicopathological characteristics of the gastric carcinoma cases[a]

Clinopathological features	Concurrent hypermethylation				
	Count	Range	Mean rank	Median	*P* value
Gender					
Male	26	0–3	25.4	2.0	0.78
Female	25	0–4	26.6	2.0	
Laurén's classification					
Intestinal	25	0–4	24.3	2.0	0.27
Diffuse	7	0–2	20.4	2.0	
Atypical	19	1–3	30.2	2.0	
Carneiro's classification					
Glandular	27	0–4	24.3	2.0	0.75
Isolated cells	5	1–2	25.5	2.0	
Solid	9	1–3	30.3	2.0	
Mixed	9	0–3	24.4	2.0	
Wall invasion					
T1 + T1sm	8	0–3	23.7	1.5	0.63
⩾T2	43	0–4	26.4	2.0	
Vascular invasion					
Absent	20	0–4	28.9	2.0	0.33
Present	30	0–3	23.9	2.0	
Lymph node metastases					
Absent	22	0–3	28.3	2.0	0.22
Present	28	0–4	23.3	2.0	
TNM staging					
I	19	0–3	29.4	2.0	0.27
II	14	0–4	24.9	2.0	
III	17	0–3	21.6	2.0	
MSI Status					
MSS/MSI-L	26	0–3	18.3	1.0	0.0005
MSI-H	23	1–4	32.6	2.0	
Ploidy					
Diploid	16	0–4	20.4	2.5	0.029
Aneuploid	16	0–3	12.6	1.0	

MSI, microsatellite instability; MSI-L, MSI-low; MSI-H, MSI-high; MSS, microsatellite stable.
[a] Some data is missing in some categories.

in a higher number of gene promoters were more frequently MSI-H and diploid (Fig. 3). To clarify if these associations were determined by the hypermethylation of a specific gene promoter, we looked for the relationship between both the MSI status and ploidy, and the methylation status of each gene promoter region (Table 2). We found that hypermethylation of the *hMLH1* promoter region was significantly associated with MSI-H ($P=0.0001$). Hypermethylation of the *MGMT* promoter region was significantly associated with MSI-H ($P=0.021$) and diploidy ($P=0.012$) (Table 2). Analysis of the relationship between concurrent hypermethylation and MSI status or ploidy of the tumours showed that the significant association between concurrent hypermethylation and MSI-H was lost when *hMLH1* was excluded ($P=0.09$), as well as the association with diploidy, when *MGMT* was excluded ($P=0.132$).

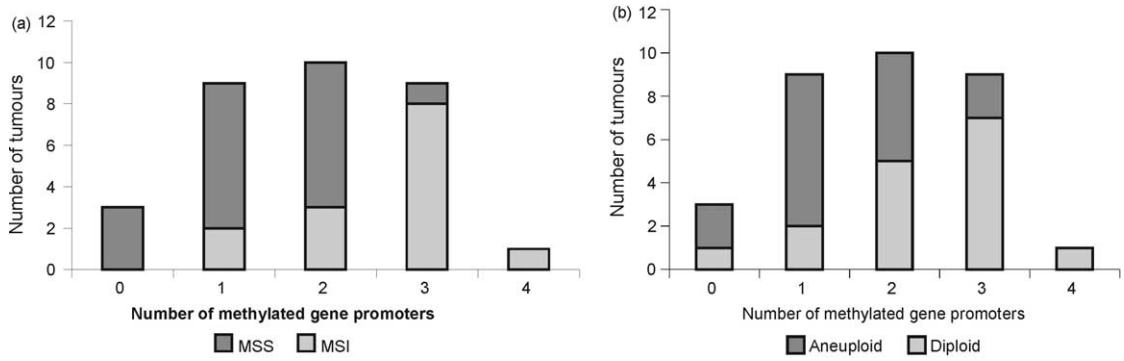

Fig. 3. Distribution of concurrent hypermethylation in (a) microsatellite stable (MSS) and microsatellite instability-high (MSI-H) gastric carcinomas, and in (b) aneuploid and diploid gastric carcinomas.

Table 2
Relationship between hypermethylation of *hMLH1*, *CDH1*, *MGMT* and *COX2* gene promoters and the MSI status and ploidy in gastric carcinoma cases

	No. total	*hMLH1* MET+ (%)	*P* value	*CDH1* MET+ (%)	*P* value	*MGMT* MET+ (%)	*P* value	*COX2* MET+ (%)	*P* value
MSI status	49		0.0001		NS		0.021		NS
MSS/MSI-L	26	2 (8)		16 (62)		23 (46)		4 (15)	
MSI-H	23	17 (74)		9 (39)		18 (78)		9 (39)	
Ploidy	32				NS		0.012		NS
Diploid	16	9 (56)	NS	8 (50)		13 (81)		7 (44)	
Aneuploid	16	5 (31)		9 (56)		6 (38)		3 (19)	

MET+, Positive for methylation. NS, non significant.

4. Discussion

In this study, we analysed the methylation status of the promoter region of four tumour-related genes—*hMLH1*, *CDH1*, *MGMT* and *COX2*—that were previously described to be silenced by promoter hypermethylation in gastric cancer. Hypermethylation was detected in all the genes, with increasing percentages from 29 and 37% for *COX2* and *hMLH1*, to 51% and 61% for *CDH1* and *MGMT* gene promoters, respectively. The lower frequency of promoter hypermethylation detected in *COX2* and *hMLH1* in comparison to *CDH1* and *MGMT* is likely to be related to the fact that in primary gastric cancer *COX2* and *hMLH1* hypermethylation is associated only to a subset of tumours. The high frequency of hypermethylation in *CDH1* and *MGMT* genes suggests that the inactivation of these tumour-related genes may play a pivotal role in gastric tumorigenesis.

Cyclooxygenase-2 (*COX2*) is upregulated in approximately 80% of gastric carcinomas [22], although a subset of cases does not express the gene [6]. In primary gastric carcinomas, aberrant methylation of the 5′ region of *COX2* was described in 12% of the cases [6]. Moreover, hypermethylation was linked with the loss of expression of *COX2* mRNA [23]. The percentage of cases with *COX2* hypermethylation found in our series (29%) seems to follow a similar trend, showing that only a subset gastric carcinomas evolved independently of *COX2* expression.

Regarding *hMLH1*, it is known that hypermethylation of its promoter region leads to diminished protein expression and is significantly associated with genome-wide instability of simple repeat sequences, referred to as microsatellite instability phenotype (MSI-H) [8,17,24,25]. The frequency of hypermethylation detected in all cases under study is 37% (19/51), which is in accordance with the results described by Leung and collaborators [26] in gastric carcinomas. Considering the MSI-H cases only, 17 of the 23 cases (74%) proved to be hypermethylated in the promoter region of *hMLH1*. Not surprisingly, significant associations were found between *hMLH1* promoter hypermethylation and MSI-H carcinomas ($P = 0.0001$), which is in agreement with what is known for MSI-H sporadic carcinomas in different MSI tumour types [27–29].

The percentage of *CDH1* promoter hypermethylation (51%) detected in this study is in accordance with previous results in sporadic gastric carcinomas [30]. *CDH1* is regarded as a key tumour suppressor gene in gastric cancer development of the diffuse type, in hereditary as well as sporadic forms [31–33]. Moreover,

hypermethylation of *CDH1* promoter was found to occur frequently in all histological types of gastric cancer [5,30,34], which is in agreement with what we also found in this study (data not shown).

MGMT is a repair protein responsible for the removal of O6-alkyl adducts produced by several carcinogens, including N-nitrosomethylurea. In primary gastric carcinomas as well as in many other types of malignancies, epigenetic silencing of the *MGMT* gene by promoter hypermethylation has been demonstrated [1,21,35]. Interestingly, we found for the first time in the setting of gastric neoplasms, that *MGMT* promoter hypermethylation was significantly associated with the MSI-H phenotype ($P = 0.021$), and with the diploid status of the tumours ($P = 0.012$). Our results contrast with those reported of Whitehall and collaborators [36], who suggested that *MGMT* promoter hypermethylation is the putative underlying epigenetic mechanism of MSI-L colon carcinomas. In our study, even when we separated the MSI-L cases (<40% of loci affected by dinucleotide instability and BAT26-negative) ($n = 7$) from the MSS cases (no loci affected by instability) ($n = 19$), we found only 2 MSI-L cases (29%) with *MGMT* hypermethylation compared with the 78% found in the MSI-H cases (data not shown). One hypothesis to explain the association between hypermethylation of *MGMT* and diploidy of the tumours stems from the function of the protein: when *MGMT* is silenced, it will not remove O6-alkyl adducts from the guanine nucleotides, leading the DNA polymerase β to misread guanines as adenines. This will promote G to A transitions [37], increasing the mutation rate. However, this explanation may be a bit simplistic as there is some evidence that silencing of *MGMT* will lead to some chromosomal instability [37].

In this study, we found no preferential combination of hypermethylated *loci*. Through the analysis of different tumour tissues, Esteller and collaborators [1] showed that for each human cancer there exists a unique profile of promoter hypermethylation, in which some gene changes are shared whereas others are cancer-type specific. In our panel of tumours, we found hypermethylation of all the gene promoters studied, and no particular profile was noticed. This is likely to depend on the specific promoter regions under analysis. We focused our study on candidate gene promoters that were previously shown to undergo epigenetic inactivation by hypermethylation in gastric cancers [1,5,6,23].

We observed a continuous distribution of the CpG island methylation in all of the tumours: the number of cases with methylated loci increased from no methylation to 2 methylated loci, and decreased from 2 methylated loci up to all four loci methylated. This is in contrast to the observations in colon cancer by Toyota and collaborators who found a bimodal distribution in all of the tumours [38]. The continuous distribution of hypermethylation of different promoter regions was also observed by Hawkins and collaborators [39] in a series of 426 colon carcinomas. A continuous distribution is in keeping with a generalised deregulation of CpG island methylation of promoters in cancer cells, in accordance to a model proposed by Nguyen and collaborators [4], in which a random and global defect in methylation leads to multiple abnormally methylated CpG islands.

We observed that concurrent CpG island methylation was significantly associated with MSI-H and diploid tumours. We have also demonstrated that the association of the 3 phenotypes is in keeping with a hypermethylation/MSI pathway leading to genetic instability, which in turn results in diploid or near-diploid tumours. To our knowledge, this is the first report describing the aforementioned association in gastric carcinomas. The most impressive associations are with regard to the tandem methylation of the *hMLH1* promoter and MSI-H phenotype, and the tandem methylation of *MGMT* promoter and the diploid status of the carcinomas.

In summary, in this study we verified that hypermethylation of several promoter regions occurs frequently in gastric cancer, reinforcing the idea that hypermethylation plays an important role in gastric carcinogenesis. Significant associations were found between concurrent hypermethylation, MSI status and ploidy of tumours. Inactivation of different gene promoters by hypermethylation is likely to lead to specific characteristics of the tumours: *hMLH1* determines MSI-H and *MGMT* the diploid status of gastric carcinomas.

Acknowledgements

We thank Professor M. Sobrinho-Simões for critically reviewing the manuscript. This study was funded by The Cancer Research Campaign [CRC], by Fundação para a Ciência e a Tecnologia, Portugal (Project SAU/14292/1998) and by Programa Operacional Ciência, Tecnologia e Inovação (POCTI), do QCA III. This study was funded by Fundação para a Ciência e a Tecnologia, Portugal (POCTI/35374/CBO/2000).

References

1. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001, **61**, 3225–3229.
2. Latham KE. X chromosome imprinting and inactivation in the early mammalian embryo. *Trends Genet* 1996, **12**, 134–138.
3. Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat Genet* 1994, **7**, 536–540.
4. Nguyen C, Liang G, Nguyen TT, *et al*. Susceptibility of nonpromoter CpG islands to de novo methylation in normal and neoplastic cells. *J Natl Cancer Inst* 2001, **93**, 1465–1472.

5. Machado JC, Oliveira C, Carvalho R, *et al*. E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma. *Oncogene* 2001, **20**, 1525–1528.
6. Kikuchi T, Itoh F, Toyota M, *et al*. Aberrant methylation and histone deacetylation of cyclooxygenase 2 in gastric cancer. *Int J Cancer* 2002, **97**, 272–277.
7. Kang GH, Shim YH, Jung HY, Kim WH, Ro JY, Rhyu MG. CpG island methylation in premalignant stages of gastric carcinoma. *Cancer Res* 2001, **61**, 2847–2851.
8. Fleisher AS, Esteller M, Tamura G, *et al*. Hypermethylation of the hMLH1 gene promoter is associated with microsatellite instability in early human gastric neoplasia. *Oncogene* 2001, **20**, 329–335.
9. Laurén P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965, **64**, 31–49.
10. Carneiro F, Seixas M, Sobrinho-Simões M. New elements for an updated classification of the carcinomas of the stomach. *Pathol Res Pract* 1995, **191**, 571–584.
11. David L, Seruca R, Nesland JM, *et al*. c-erbB-2 expression in primary gastric carcinomas and their metastases. *Mod Pathol* 1992, **5**, 384–390.
12. Oliveira C, Seruca R, Seixas M, *et al*. The clinicopathologic features of gastric carcinomas with microsatellite instability may be mediated by mutations of different "target genes": a study of the TGFβ RII, IGFII R, and BAX genes. *Am J Pathol* 1998, **153**, 1211–1219.
13. Gonzalez-Garcia I, Moreno V, Navarro M, *et al*. Standardized approach for microsatellite instability detection in colorectal carcinomas. *J Natl Cancer Inst* 2000, **92**, 544–549.
14. Jass JR, Do KA, Simms LA, *et al*. Morphology of sporadic colorectal cancer with DNA replication errors. *Gut* 1998, **42**, 673–679.
15. Thibodeau SN, French AJ, Cunningham JM, *et al*. Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. *Cancer Res* 1998, **58**, 1713–1718.
16. Yamamoto H, Perez-Piteira J, Yoshida T, *et al*. Gastric cancers of the microsatellite mutator phenotype display characteristic genetic and clinical features. *Gastroenterology* 1999, **116**, 1348–1357.
17. Pinto M, Oliveira C, Machado JC, *et al*. MSI-L gastric carcinomas share the hMLH1 methylation status of MSI-H carcinomas but not their clinicopathologic profile. *Lab Invest* 2000, **80**, 1915–1923.
18. Gryfe R, Kim H, Hsieh ET, *et al*. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* 2000, **342**, 69–77.
19. Graff JR, Herman JG, Myohanen S, Baylin SB, Vertino PM. Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation. *J Biol Chem* 1997, **272**, 22322–22329.
20. Akhtar M, Cheng Y, Magno RM, *et al*. Promoter methylation regulates Helicobacter pylori-stimulated cyclooxygenase-2 expression in gastric epithelial cells. *Cancer Res* 2001, **61**, 2399–2403.
21. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res* 1999, **59**, 793–797.
22. Uefuji K, Ichikura T, Mochizuki H, Shinomiya N. Expression of cyclooxygenase-2 protein in gastric adenocarcinoma. *J Surg Oncol* 1998, **69**, 168–172.
23. Song SH, Jong HS, Choi HH, *et al*. Transcriptional silencing of cyclooxygenase-2 by hyper-methylation of the 5′ CpG island in human gastric carcinoma cells. *Cancer Res* 2001, **61**, 4628–4635.
24. Toyota M, Ahuja N, Suzuki H, *et al*. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Res* 1999, **59**, 5438–5442.
25. Nakagawa H, Nuovo GJ, Zervos EE, *et al*. Age-related hypermethylation of the 5′ region of HMLH1 in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. *Cancer Res* 2001, **61**, 6991–6995.
26. Leung WK, Yu J, Ng EK, *et al*. Concurrent hypermethylation of multiple tumor-related genes in gastric carcinoma and adjacent normal tissues. *Cancer* 2001, **91**, 2294–2301.
27. Herman JG, Umar A, Polyak K, *et al*. Incidence and functional consequences of HMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA* 1998, **95**, 6870–6875.
28. Esteller M, Levine R, Baylin SB, Ellenson LH, Herman JG. HMLH1 promoter hypermethylation is associated with the microsatellite instability phenotype in sporadic endometrial carcinomas. *Oncogene* 1998, **17**, 2413–2417.
29. Fleisher AS, Esteller M, Wang S, *et al*. Hypermethylation of the HMLH1 gene promoter in human gastric cancers with microsatellite instability. *Cancer Res* 1999, **59**, 1090–1095.
30. Tamura G, Yin J, Wang S, *et al*. E-Cadherin gene promoter hypermethylation in primary human gastric carcinomas. *J Natl Cancer Inst* 2000, **92**, 569–573.
31. Guilford P, Hopkins J, Harraway J, *et al*. E-cadherin germline mutations in familial gastric cancer. *Nature* 1998, **392**, 402–405.
32. Machado JC, Soares P, Carneiro F, *et al*. E-cadherin gene mutations provide a genetic basis for the phenotypic divergence of mixed gastric carcinomas. *Lab Invest* 1999, **79**, 459–465.
33. Oliveira C, Bordin MC, Grehan N, *et al*. Screening E-cadherin in gastric cancer families reveals germline mutations only in hereditary diffuse gastric cancer kindred. *Hum Mutat* 2002, **19**, 510–517.
34. Lee TL, Leung WK, Chan MW, *et al*. Detection of gene promoter hypermethylation in the tumor and serum of patients with gastric carcinoma. *Clin Cancer Res* 2002, **8**, 1761–1766.
35. Park TJ, Han SU, Cho YK, Paik WK, Kim YB, Lim IK. Methylation of *O*(6)-methylguanine-DNA methyltransferase gene is associated significantly with K-ras mutation, lymph node invasion, tumor staging, and disease free survival in patients with gastric carcinoma. *Cancer* 2001, **92**, 2760–2768.
36. Whitehall VL, Walsh MD, Young J, Leggett BA, Jass JR. Methylation of *O*-6-methylguanine DNA methyltransferase characterizes a subset of colorectal cancer with low-level DNA microsatellite instability. *Cancer Res* 2001, **61**, 827–830.
37. Mitra G, Pauly GT, Kumar R, *et al*. Molecular analysis of O6-substituted guanine-induced mutagenesis of ras oncogenes. *Proc Natl Acad Sci USA* 1989, **86**, 8650–8654.
38. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999, **96**, 8681–8686.
39. Hawkins N, Norrie M, Cheong K, *et al*. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. *Gastroenterology* 2002, **122**, 1376–1387.