Short Communication

Frequent Hypermethylation of the *hMLH1* Gene Promoter in Differentiated-Type Tumors of the Stomach with the Gastric Foveolar Phenotype

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Hypermethylation of the *bMLH1* mismatch repair gene promoter has been revealed to lead to microsatellite instability (MSI). Previously, we demonstrated a high prevalence of MSI in differentiated-type gastric tumors showing distinct features of gastric foveolar epithelium (foveolar type). To clarify the significance of *bMLH1* promoter hypermethylation in the development of this tumor type, we studied promoter methylation status and expression of *hMLH1* in foveolar-type tumors and their surrounding non-neoplastic mucosae, as well as in tumors with other cellular phenotypes. The results were compared to MSI status. After phenotypical analyses using mucin histochemistry and immunohistochemistry, 41 differentiated-type tumors with distinct cellular phenotypes were classified into three categories: foveolar type, intestinal type (tumors with the distinct cellular phenotype of the intestine), and combined type (tumors with both foveolar and intestinal phenotypes). Methylation-specific polymerase chain reaction (MSP) was performed to determine the methylation status of *bMLH1* promoter. *hMLH1* protein expression was immunohistochemically examined. MSI was detected in 57% of the foveolar type, 8% of the intestinal type, and 67% of the combined-type tumors. Hypermethylation of *bMLH1* promoter was found in 74% of the foveolar type, 33% of the intestinal type, and 83% of the combined-type tumors. Of 18 MSI-positive tumors, all but one were hypermethylated. Methylation status of *bMLH1* promoter correlated well with protein expression in foveolar-type tumors. Moreover, hypermethylation was also detected frequently (71%) in the non-neoplastic surrounding mucosa of the hypermethylated tumors. Hypermethylation of *bMLH1* promoter is an initial, vital event in the development

of foveolar-type tumors of the stomach. (*Am J Pathol* 2000, 157:717-722)

Gastric carcinomas are histopathologically divided into two types, differentiated and undifferentiated type¹ or intestinal and diffuse type,² based on their tendency to form glands. Differentiated-type tumors have long been believed to develop from intestinal metaplasia and have a predominantly intestinal cellular phenotype.¹⁻³ However, differentiated-type tumors with the phenotype of gastric foveolar epithelium (foveolar-type tumor), which are considered to derive from gastric proper epithelium, have been revealed with the development of phenotypical analyses using mucin histochemical and immunohistochemical procedures.⁴⁻¹³ The frequency of this type tumor was reported to be 18 to 41% in the intramucosal differentiated-type tumors of the stomach.^{6,8,9,11} In one of our recent studies,¹³ we made it clear that genetic backgrounds of differentiated-type tumors were quite different among cellular phenotypes. We demonstrated frequent microsatellite instability (MSI) and infrequent *p53* mutations in foveolar-type tumors and, conversely, infrequent MSI and considerable *p53* mutations in tumors with a distinct intestinal cellular phenotype (the intestinal-type tumor). MSI is believed to be caused by a deficient DNA mismatch repair. Recent studies revealed that epigenetic methylation-associated inactivation of the *hMLH1* mismatch repair gene is a potent trigger of MSI, especially of high-rate MSI, in gastrointestinal carcinomas.¹⁴⁻¹⁷ Foveolar-type tumors contain several histopathological problems as follows: 1) They are prone to lose their glandular structure and progress to undifferentiated-type tumors,^{5,11} and thus should be regarded as precursors of

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the undifferentiated-type tumors;¹¹ and 2) histopathological diagnosis is difficult; ie, it is often difficult to distinguish such lesions from regenerative or inflammatory changes in the foveolar epithelium, rather than the neoplastic lesion.^{5,11}

To clarify the significance of *hMLH1* promoter hypermethylation in the development of foveolar-type tumors, we studied promoter methylation status and expression of *hMLH1*, and the results were compared to MSI status. In addition, we pursued the possibility that these molecular biological and immunohistochemical methods would be helpful in the diagnosis of this perplexing but obvious tumor.

Materials and Methods

Phenotypical Analysis and Classification

Representative sections from paraffin blocks were examined for phenotypical analysis. As markers for gastric foveolar phenotype, galactose oxidase-Schiff (GOS) mucin stain,^{8,18} and immunostain of human gastric mucin (45 M1, Novocastra, Newcastle, UK)¹⁹ were used. To detect intestinal phenotypical expressions, immunostains of MUC2 (Ccp58, Novocastra), a marker for intestinal goblet-cell mucin,²⁰ and CD10 (56C6, Novocastra), which detect the brush border of absorptive cells, a marker for small intestine (complete-type intestinal metaplasia),¹⁰ were performed. Using these procedures, 41 lesions from 39 patients, which showed marked phenotypical expressions, were selected. To investigate the relationship between the genetic analyses and the phenotypic expressions of tumors, tumors without overt phenotypical expressions were excluded from this study. Tumors were classified as follows: (i) 23 foveolar-type tumors that had frequent gastric foveolar-type mucin (>50% of neoplastic cells are positive for the GOS stain or human gastric mucin), but infrequent intestinal markers (<5% of MUC2- and CD10-positive cells) (Figure 1, A and B); (ii) 12 intestinal-type tumors that had predominantly either MUC2- or CD10-positive cells or both (>30% of neoplastic cells); and (iii) 6 combined-type tumors that showed both foveolar and intestinal phenotypes (>30% of neoplastic cells express both foveolar and intestinal type markers). We followed the Padova international classification²¹ that was recently proposed to estimate the intramucosal lesion of the stomach, about which there is a difference of opinion between Western and Japanese pathologists.²² Clinicopathological findings are summarized in Table 1.

DNA Extraction

DNA was extracted from tumors and normal mucosa surrounding tumors as described by Goelz et al²³ from ten 10- μ m-thick formalin-fixed, paraffin-embedded serial sections.

Methylation-Specific Polymerase Chain Reaction (MSP)

DNA methylation patterns in the *hMLH1* promoter were determined by MSP, as described previously.^{14,15,17} MSP distinguishes unmethylated from methylated alleles of a given gene based on sequence changes that are produced following bisulfite treatment of DNA, which converts unmethylated cytosines to uracils, while leaving methylated cytosines unaffected. Subsequent polymerase chain reaction (PCR) using primers specific to sequences that correspond to either methylated or unmethylated DNA was performed. Primer sequences of each methylated and unmethylated sequence of the *hMLH1* promoter were also described in the protocols.¹⁴ Briefly, 2 μ g of genomic DNA were denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were then purified using a Wizard DNA purification resin (Promega, Madison, WI), treated with NaOH, precipitated with ethanol, and resuspended in 30 μ l of water. Modified DNA was amplified in a total volume of 20 μ l 1 $\times$ GeneAmp PCR Gold Buffer (PE Applied Biosystems, Foster City, CA) containing 1.0 mmol/L MgCl₂, 1 μ mol/L of each primer, 0.2 mmol/L dNTPs, and 1 unit of *Taq* polymerase (Ampli Taq Gold DNA Polymerase, PE Applied Biosystems). After activation of the *Taq* polymerase at 95° for 10 minutes, the PCR was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles consisting of denaturation at 95° for 15 seconds, annealing at 60° for 15 seconds, and extension at 72° for 30 seconds, followed by a final 7-minute extension at 72° for all primer sets. The PCR products were then loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

MSI

We screened MSI using the mononucleotide repeats BAT26 and BAT25 according to the published protocols.^{13,24} They are sensitive and recommended to detect MSI-positive, especially high-rate MSI tumors.^{25–28} The bands of different molecular weights in tumor DNA, not observed in normal DNA, were designated as MSI-positive.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections using the standard labeled streptavidin-biotin system (Nichirei, Tokyo, Japan). Mouse monoclonal antibody to the *hMLH1* gene product, G168–728 (PharMingen, San Diego, CA), was used at 1:50 dilution after antigen retrieval by microwave. Expression was evaluated by comparison to normal tissue in the same section using three categories: markedly decreased (decreasing in more than half of the tumor tissue), lightly decreased (decreasing in less than half of the tumor tissue), and not decreased.

Figure 1. **A:** Typical foveolar-type tumor (case 11). Glands were lined by cells with clear to slightly basophilic cytoplasm and oval-to-round, basally oriented nuclei, mimicking well the foveolar epithelium (H&E; original magnification, $\times 200$). **B:** Diffuse apical staining for GOS, case 11. (GOS; original magnification, $\times 200$). **C:** Case 7, foveolar-type tumor with MSI and hypermethylated *bMLH1* (H&E; original magnification, $\times 100$). **D:** Case 7, hMLH1 protein expression is markedly diminished in the tumor compared to the adjacent normal mucosa (streptavidin-biotin peroxidase stain with methyl green counterstain; original magnification, $\times 100$).

Sequencing Analysis

The methylated and unmethylated DNA-derived PCR products from several tumors were directly sequenced using a terminator cycle sequencing kit (dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kits; PE Applied Biosystems) and a DNA sequencer (310 Genetic Analyzer; PE Applied Biosystems).

Statistical Analysis

Statistical comparisons were performed using Fisher's exact test with a criterion of $P < 0.05$.

Results

The results are summarized in Table 1 and Figure 2. MSI was detected in 57% (13/23) of the foveolar type, 8% (1/12) of the intestinal type, and 67% (4/6) of the combined-type tumors. In foveolar-type tumors, the frequency of MSI was significantly higher in early lesions, mucosa (m) and submucosa (sm) (71%; 12/17) than advanced lesions, subserosa (ss) and exposed beyond the subserosa (se) (17%; 1/6). The *hMLH1* promoter hypermethylation was detected more extensively than MSI among all phenotypes (Figures 2 and 3A). The frequencies were 74% (17/23) of the foveolar type, 33% (4/12) of

Table 1. Characteristics of Gastric Epithelial Lesions and the Results of Genetic Analyses

	Case No.	Sex ^{a)}	Age	Depth of invasion ^{b)}	Size of the tumor (mm)	Histology ^{c)}	<i>hMLH1</i> protein reduction ^{d)}	MSI ^{e)}	Methylation in tumor ^{f)}	Methylation in normal ^{g)}
foveolar type	1	M	64	m	4	NIN-L				
	2	M	83	m	8	NIN-L				
	3	M	72	m	10	NIN-L				
	4	M	80	m	13	NIN-L				
	5	F	84	m	14	NIN-L				
	6	F	66	m	15	NIN-L				
	7	F	75	m	22	IA				
	8	M	64	m	25	NIN-H				
	9	F	71	m	26	NIN-L				
	10	F	82	m	35	IA				
	11	M	67	m	56	NIN-L				
	12	M	70	m	58	NIN-L				
	13	F	76	sm	15	IA				
	14	M	51	sm	20	IA				
	15	F	67	sm	34	IA				
	16	F	84	sm	37	IA				
	17	M	84	sm	77	IA				
	18	F	63	ss	42	IA				
	19	M	54	se	28	IA				
	20	M	75	se	65	IA				
	21	M	73	ss	65	IA				
	22	M	72	se	70	IA				
	23	F	60	ss	83	IA				
combined type	24	M	71	m	17	NIN-L				
	25	F	69	m	19	IA				
	26	M	78	m	30	NIN-L				
	27	F	66	sm	11	IA				
	28	M	68	sm	18	IA				
	29	F	68	sm	24	IA				
intestinal type	30	M	62	sm	15	IA				
	31	M	59	sm	15	IA				
	32	M	74	sm	18	IA				
	33	M	70	sm	25	IA				
	34	M	70	sm	27	IA				
	35	M	79	sm	12	IA				
	36	M	62	sm	19	IA				
	37	F	72	sm	27	IA				
	38	M	75	sm	33	IA				
	39	M	82	sm	37	IA				
	40	M	63	sm	53	IA				
	41	M	65	se	55	IA				

- a) M, male; F, female
b) m, mucosa; sm, submucosa; ss, subserosa; se, exposed beyond the subserosa
c) NIN-L, noninvasive neoplasia, low grade; NIN-H, noninvasive neoplasia, high grade; IA, invasive adenocarcinoma
d) ■, markedly decreased; ▨, lightly decreased; □, not decreased; blank, not done
e)-g) ■, present; □, absent; blank, not done.

the intestinal type, and 83% (5/6) of the combined-type tumors. The frequency of both MSI and hypermethylation of *hMLH1* was significantly higher in the foveolar type than in the intestinal-type tumors ($P = 0.006$ for MSI, and $P = 0.02$ for hypermethylation). Taking the foveolar- and the combined-type tumors together, the differences were more obvious ($P = 0.003$ for MSI, $P = 0.01$ for hypermethylation). In total, the *hMLH1* promoter hypermethylation occurred in 94% (17/18) of the MSI+ cases.

Direct sequencing of the methylated DNA PCR product confirmed the retention of cytosines at all CpGs within the PCR product, whereas cytosines were all converted to thymines in the unmethylated DNA PCR product (Figure 3B).

All foveolar-type tumors, in which frequent *hMLH1* hypermethylation was detected, were immunohistochemi-

Figure 2. Comparison of frequency of MSI and promoter hypermethylation of *hMLH1* among three phenotypic types.

cally tested for *hMLH1* protein expression (Figure 1, C and D). In early lesions, the cases with both MSI and the hypermethylation of *hMLH1* showed decreased expression of *hMLH1* in tumor nuclei without exception, but this was not the case in advanced lesions.

The surrounding mucosa within 2 cm of 17 foveolar-type hypermethylated tumors was also tested for *hMLH1* promoter hypermethylation. Methylated *hMLH1* alleles were

Figure 3. A: MSP in foveolar-type tumors. In cases 16 and 18, a hypermethylated *hMLH1* band is observed. B: Sequencing histograms of the PCR products of methylated and unmethylated *hMLH1*. Cytosines at all CpGs within the methylated DNA PCR product are retained, whereas all cytosines were converted to thymines in the unmethylated DNA PCR product. The number indicates the case number; U, unmethylated *hMLH1*; M, hypermethylated *hMLH1*.

detected in 71% (12/17) of the non-neoplastic mucosa surrounding the tumors (Table 1); however, no decreases in *hMLH1* protein expression and MSI were observed.

Discussion

We have demonstrated considerably high prevalences of MSI and *hMLH1* promoter hypermethylation in both the foveolar- and combined-type tumors, but not in the intestinal-type tumors. These results indicate that MSI and *hMLH1* promoter hypermethylation affect the tumors showing gastric foveolar phenotypical expression, irrespective of intestinal phenotypical expression. Actually, we could not make morphological distinctions between the combined- and foveolar-type tumors, and the intestinal phenotypical expressions in the combined-type tumors were all revealed after mucin histochemical and immunohistochemical examinations.

Previous studies have reported infrequent MSI in early, but frequent MSI in advanced, differentiated-type carcinomas of the stomach (early, 9–19% *versus* advanced, 27–38%).^{24,29,30} These results suggest that the MSI phenotype does not play an important role in the early phase of differentiated-type tumors of the stomach. The MSI phenotype would be accumulated secondarily during tumor progression. The high incidence of MSI in early foveolar-type tumors (71%) contrasts sharply with these results. In the foveolar-type tumor, the MSI phenotype would be deeply involved from the early stage of tumorigenesis. In early foveolar-type tumors, hypermethylation associated with *hMLH1* protein reduction caused MSI without exception. Hypermethylation alone, not associated with *hMLH1* protein reduction, did not cause MSI at all. These results are consistent with those of previous studies.^{14,15,17} They demonstrated that enough (biallelic or complete) hypermethylation of *hMLH1* promoter caused protein reduction and sequentially induced MSI.^{14,31} However, this sequence did not apply to half of the advanced foveolar-type tumors (case numbers 20–22). We may have to discriminate between the advanced and early foveolar-type tumors from the standpoint of genetic alterations; however, further studies will be necessary to answer this question.

In the colon and rectum, considerable hypermethylation of *hMLH1* promoter in non-neoplastic mucosa adjacent to the tumors, especially in MSI-negative tumors, was reported.³² No such methylation in adjacent mucosa was reported in carcinomas of the stomach.³³ Frequent *hMLH1* hypermethylation in non-neoplastic mucosa adjacent to foveolar-type tumors strongly suggests that hypermethylation of *hMLH1* is an initial vital event in the early tumorigenesis of foveolar-type tumors.

The difficulty in histopathological diagnosis of foveolar-type tumors, especially of early lesions, has been pointed out.^{5,11} This type of tumor often shows a low-grade cytologic atypia even in cases with overt invasion, but intramucosal tumorous elements are usually well preserved. Indeed, 75% (9/12) of intramucosal foveolar-type tumors were classified as non-invasive neoplasia, low-grade. Therefore, a histopathological diagnosis is diffi-

cult, especially based on superficial biopsy samples alone. Detection of *hMLH1* promoter hypermethylation and reduced *hMLH1* protein expression may be useful as potential diagnostic markers because more than half of the early foveolar-type lesions showed these features, even though the expression was well preserved in surrounding non-neoplastic mucosa. Moreover, the assessment of the status of *hMLH1* promoter hypermethylation in non-neoplastic gastric mucosa may be useful in early detection of foveolar-type tumors.

References

1. Nakamura K, Sugano H, Takagi K: Carcinoma of the stomach in incipient phase: its histogenesis and histological appearances. *GANN* 1968, 59:251–258
2. Lauren P: The two histological main types of gastric carcinoma; diffuse and so-called intestinal type carcinoma. *Acta Pathol Microbiol Scand* 1965, 64:31–49
3. Jass JR, Filipe MI: The mucin profiles of normal gastric mucosa, intestinal metaplasia and its variants and gastric carcinoma. *Histochem J* 1981, 13:931–939
4. Hattori T: Morphological range of hyperplastic polyps and carcinomas arising in hyperplastic polyps of the stomach. *J Clin Pathol* 1985, 38:622–630
5. Ishiguro S: Histological significance of foveolar type tubular adenocarcinoma of the stomach-its histogenesis and relationship to undifferentiated carcinoma. *Med J Osaka Univ* 1987, 39:507–514 (in Japanese with an English abstract)
6. Sasaki I, Yao T, Nawata H, Tsuneyoshi M: Minute gastric carcinoma of differentiated type with special reference to the significance of intestinal metaplasia, proliferative zone, and p53 protein during tumor development. *Cancer* 1999, 85:1719–1729
7. Kushima R, Hattori T: Histogenesis and characteristics of gastric-type adenocarcinomas in the stomach. *J Cancer Res Clin Oncol* 1993, 120:103–111
8. Tatematsu M, Ichinose M, Miki K, Hasegawa R, Kato T, Ito N: Gastric and intestinal phenotypic expression of human stomach cancers as revealed by pepsinogen immunohistochemistry and mucin histochemistry. *Acta Pathol Jpn* 1990, 40:494–504
9. Egashira Y, Shimoda T, Ikegami M: Mucin histochemical analysis of minute gastric differentiated adenocarcinoma. *Pathol Int* 1999, 49:55–61
10. Endoh Y, Tamura G, Motoyama T, Ajioka Y, Watanabe H: Well-differentiated adenocarcinoma mimicking complete-type intestinal metaplasia in the stomach. *Hum Pathol* 1999, 30:826–832
11. Endoh Y, Tamura G, Watanabe H, Ajioka Y, Motoyama T: The common 18-base pair deletion at codons 418–423 of the E-cadherin gene in differentiated-type adenocarcinomas and intramucosal precancerous lesions of the stomach with the features of gastric foveolar epithelium. *J Pathol* 1999, 189:201–206
12. Fiocca R, Villani L, Tenti P, Cornaggia M, Finzi G, Riva C, Capella C, Bara J, Samloff IM, Solcia E: The foveolar cell component of gastric cancer. *Hum Pathol* 1990, 21:260–270
13. Endoh Y, Sakata K, Tamura G, Ohmura K, Ajioka Y, Watanabe H, Motoyama T: Cellular phenotypes of differentiated-type adenocarcinomas and precancerous lesions of the stomach are dependent on the genetic pathways. *J Pathol* 2000, 191:257–263
14. Fleisher AS, Esteller M, Wang S, Tamura G, Suzuki H, Yin J, Zou TT, Abraham JM, Kong D, Smolinski KN, Shi YQ, Rhyu MG, Powell SM, James SP, Wilson KT, Herman JG, Meltzer SJ: Hypermethylation of the *hMLH1* gene promoter in human gastric cancers with microsatellite instability. *Cancer Res* 1999, 59:1090–1095
15. Kang GH, Shim YH, Ro JY: Correlation of methylation of the *hMLH1* promoter with lack of expression of *hMLH1* in sporadic gastric carcinomas with replication error. *Lab Invest* 1999, 79:903–909
16. 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
17. Leung SY, Yuen ST, Chung LP, Chu KM, Chan AS, Ho JC: *hMLH1*

- promoter methylation and lack of hMLH1 expression in sporadic gastric carcinomas with high-frequency microsatellite instability. *Cancer Res* 1999, 59:159–164
18. Katsuyama T, Ono K, Nagata T: Application of galactose oxidase mucosubstance histochemistry: galactose oxidase-Schiff reaction. *J Histochem Cytochem* 1982, 96:555
19. Kushima R, Muller W, Stolte M, Borchard F: Differential p53 protein expression in stomach adenomas of gastric and intestinal phenotypes: possible sequences of p53 alteration in stomach carcinogenesis. *Virchows Arch* 1996, 428:223–227
20. Tytgat KM, Buller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J: Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. *Gastroenterology* 1994, 107:1352–1363
21. Rugge M, Correa P, Dixon MF, Hattori T, Leandro G, Lewin K, Riddell RH, Sipponen P, Watanabe H: Gastric dysplasia: the Padova international classification. *Am J Surg Pathol* 2000, 24:167–176
22. Schlemper RJ, Itabashi M, Kato Y, Lewin KJ, Riddell RH, Shimoda T, Sipponen P, Stolte M, Watanabe H, Takahashi H, Fujita R: Differences in diagnostic criteria for gastric carcinoma between Japanese and western pathologists. *Lancet* 1997, 349:1725–1729
23. Goelz SE, Hamilton SR, Vogelstein B: Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochem Biophys Res Commun* 1985, 130:118–126
24. Tamura G, Sakata K, Nishizuka S, Maesawa C, Suzuki Y, Terashima M, Eda Y, Satodate R: Allelotype of adenoma and differentiated adenocarcinoma of the stomach. *J Pathol* 1996, 180:371–377
25. Zhou XP, Hoang JM, Li YJ, Seruca R, Carneiro F, Sobrinho-Simoes M, Lothe RA, Gleeson CM, Russell SE, Muzeau F, Flejou JF, Hoang-Xuan K, Lidereau R, Thomas G, Hamelin R: Determination of the replication error phenotype in human tumors without the requirement for matching normal DNA by analysis of mononucleotide repeat microsatellites. *Genes Chromosomes Cancer* 1998, 21:101–107
26. Zhou XP, Hoang JM, Cottu P, Thomas G, Hamelin R: Allelic profiles of mononucleotide repeat microsatellites in control individuals and in colorectal tumors with and without replication errors. *Oncogene* 1997, 15:1713–1718
27. Cravo M, Lage P, Albuquerque C, Chaves P, Claro I, Gomes T, Gaspar C, Fidalgo P, Soares J, Nobre-Leitao C: BAT-26 identifies sporadic colorectal cancers with mutator phenotype: a correlative study with clinico-pathological features and mutations in mismatch repair genes. *J Pathol* 1999, 188:252–257
28. Halling KC, Harper J, Moskaluk CA, Thibodeau SN, Petroni GR, Yustein AS, Tosi P, Minacci C, Roviello F, Piva P, Hamilton SR, Jackson CE, Powell SM: Origin of microsatellite instability in gastric cancer. *Am J Pathol* 1999, 155:205–211
29. Buonsanti G, Calistri D, Padovan L, Luinetti O, Fiocca R, Solcia E, Ranzani GN: Microsatellite instability in intestinal- and diffuse-type gastric carcinoma. *J Pathol* 1997, 182:167–173
30. Luinetti O, Fiocca R, Villani L, Alberizzi P, Ranzani GN, Solcia E: Genetic pattern, histological structure, and cellular phenotype in early and advanced gastric cancers: evidence for structure-related genetic subsets and for loss of glandular structure during progression of some tumors. *Hum Pathol* 1998, 29:702–709
31. Wheeler JM, Beck NE, Kim HC, Tomlinson IP, Mortensen NJ, Bodmer WF: Mechanisms of inactivation of mismatch repair genes in human colorectal cancer cell lines: the predominant role of hMLH1. *Proc Natl Acad Sci USA* 1999, 96:10296–10301
32. Kuismanen SA, Holmberg MT, Salovaara R, Schweizer P, Aaltonen LA, de La Chapelle A, Nystrom-Lahti M, Peltomaki P: Epigenetic phenotypes distinguish microsatellite-stable and -unstable colorectal cancers. *Proc Natl Acad Sci USA* 1999, 96:12661–12666
33. Toyota M, Ahuja N, Suzuki H, Itoh F, Ohe-Toyota M, Imai K, Baylin SB, Issa JP: Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Res* 1999, 59:5438–5442