Oncogene (2009) 28, 899–909

www.nature.com/onc

ORIGINAL ARTICLE

Normal colorectal mucosa exhibits sex- and segment-specific susceptibility to DNA methylation at the *hMLH1* and *MGMT* promoters

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Silencing of gene expression by aberrant cytosine methylation is a prominent feature of human tumors, including colorectal cancers. Epigenetic changes of this type play undisputed roles in cell transformation when they involve genes that safeguard genome stability, and they can also be detected in precancerous lesions and seemingly normal peritumoral tissues. We explored physiological conditions associated with aberrant promoter methylation involving two DNA-repair genes in normal colorectal mucosa. Samples of cecal, transverse colon, sigmoid and rectal mucosa collected from 100 healthy individuals undergoing screening colonoscopy were analysed for *hMLH1* and *MGMT* promoter methylation with a quantitative PCR assay. Positivity in at least one colon segment was common in both sexes, with methylation involving 0.1–18.8% of the alleles (median = 0.49%). Samples from males showed no consistent patterns for either promoter, but there were striking age- and colon segment-specific differences in the female subgroup. Here, the prevalence of *hMLH1* and *MGMT* methylation increased significantly with age, particularly in the right colon, where there was also an age-related increase in the percentage of alleles showing *hMLH1* methylation. Concomitant methylation of both promoters was also significantly more common in the right colon of women. These findings paralleled immunohistochemical patterns of hMLH1 and MGMT protein loss in an independent series of 231 colorectal cancers and were consistent with current epigenetic profiles of colorectal cancer subsets. They suggest the intriguing possibility that the epigenetic signatures of cancers may have early-stage, normal-tissue counterparts that reflect potentially important aspects of the initial carcinogenetic process.
Oncogene (2009) **28,** 899–909; doi:10.1038/onc.2008.444; published online 8 December 2008

Keywords: *hMLH1*; *MGMT*; promoter methylation; colorectal mucosa; colorectal cancer

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Received 2 September 2008; revised 20 October 2008; accepted 6 November 2008; published online 8 December 2008

Introduction

Aberrant methylation of CpG islands in gene promoters is a frequent occurrence in cancer, and it can affect critical steps in the process of carcinogenesis (Momparler, 2003). The molecular mechanisms underlying these epigenetic changes are largely unknown. Cell-lineage-specific CpG methylation patterns are established mainly during embryogenesis, and their maintenance in the adult organism is critical for tissue homeostasis (Reik, 2007). Consequently, CpG methylation levels in somatic cells are meant to remain stable, and the changes that occur must be considered accidental. Some gene loci appear to undergo methylation as a function of age; in others methylation occurs more selectively in neoplastic tissues (Toyota *et al*., 1999). It is unclear whether these age- and cancer-related phenomena are reflections of distinct pathways of *de novo* methylation or simply successive stages of a 'methylator condition' that confers a general predisposition to cancer. The latter view is supported by reports of acquired hypermethylation at multiple genetic loci (Toyota and Issa, 1999; Goel *et al*., 2007) in a fairly large portion of colorectal cancers, that is, those with a CpG island methylator phenotype (CIMP).

Colon cancer-associated DNA methylation changes at certain loci may not be direct causes of tumorigenesis, but the impact of others is almost certain. These include alterations affecting genes that are critical to the maintenance of DNA stability, such as *hMLH1* and *MGMT* (Giovannucci and Ogino, 2005). Approximately 15% of all colorectal cancers show high-level microsatellite instability (MSI) (Aaltonen *et al*., 1993; Ionov *et al*., 1993; Thibodeau *et al*., 1993) reflecting dysfunction of the postreplicative DNA mismatch repair system, and in most of these tumors (roughly 10% of all colon cancers), the instability stems from CpG methylation-mediated silencing of the mismatch repair gene *hMLH1* (Kane *et al*., 1997). Indeed, *hMLH1* promoter methylation is a defining feature of CIMP-positive colorectal cancers, which are preferentially located in the right colon and usually affect older women (Malkhosyan *et al*., 2000; Percesepe *et al*., 2001; Slattery *et al*., 2001). An even larger percentage of colorectal cancers (20–40%) are characterized by promoter methylation-mediated silencing of *MGMT* (Esteller *et al*., 2000; Whitehall *et al*.,

2001). This gene encodes *O*-6-methylguanine DNA methyltransferase, which removes the mutagenic methyl group from *O*-6-alkylated guanines. It is therefore the cell's major defense against G→A mutations that may arise during DNA replication across these lesions (Gerson, 2004).

Given their indisputable relevance to colorectal carcinogenesis, aberrant promoter methylation involving the *hMLH1* and *MGMT* genes might be expected to occur at an early stage in this process (Greenspan *et al*., 2007; Menigatti *et al*., 2007), before the gut mucosa exhibits gross changes. Systematic analysis of normal-appearing colorectal mucosa for cancer-relevant DNA methylation changes at these two gene promoters could thus provide insight into the pathological significance of similar changes found in colorectal cancers. It could also shed light on the biological processes involved in the maintenance and alteration of epigenetic states in normal gut mucosa. Both issues are fundamental for better understanding of the early events that occur during colorectal carcinogenesis, and they have already been explored in different ways by other groups (see Discussion). A major novelty of our study is its focus on the normal colorectal mucosa of healthy participants with no evidence of intestinal disease. Our study is also the first attempt to determine how epigenetic changes in *hMLH1* and *MGMT* are influenced by age, sex, and colon segment, factors that seem to be related—in one way or another—to the deficient expression of these genes in colorectal cancers.

Results

hMLH1 and MGMT promoter methylation in the normal colorectal mucosa

Samples of normal-appearing colorectal mucosa were collected from the cecum (C), transverse colon (TC), sigmoid colon (SC) and rectum (R) of 100 individuals (47 females, 53 males, aged 50–80 years) with no evidence of intestinal disease (details on participant enrollment are provided in Materials and methods). DNA extracted from each sample was analysed with a quantitative methylation-specific PCR (qMSP) protocol. As summarized in Supplementary Figures 1–4, we measured methylation levels (that is, the percentage of alleles showing methylation) at the *MGMT* promoter and in the distal region of the *hMLH1* promoter. Methylation of the latter region has been shown in the normal mucosa of patients with colorectal cancer (Kuismanen *et al*., 1999; Menigatti *et al*., 2001; Miyakura *et al*., 2001; Nakagawa *et al*., 2001; Kawakami *et al*., 2006). It does not *per se* affect *hMLH1* transcription, but it has been shown to be the starting point for progressive methylation (Deng *et al*., 1999; Menigatti *et al*., 2001; Miyakura *et al*., 2001; Nakagawa *et al*., 2001) that spreads toward the transcription start site and eventually does silence expression of this mismatch repair gene (Deng *et al*., 1999; Miyakura *et al*., 2001; Nakagawa *et al*., 2001; Truninger *et al*., 2005; Capel *et al*., 2007). Involvement of the distal promoter thus reflects the earliest stage of this process, and as such, it is more likely to be observed in the normal mucosa of participants who have no apparent colorectal disease. In some experiments, however, we also evaluated the proximal region of the *hMLH1* promoter (Supplementary Figure 4) for evidence of more advanced-stage methylation.

Each DNA sample was tested in triplicate (total number of reactions per target gene: 1200). If all three assays showed methylation involving ⩾0.1% of the alleles (a cutoff reflecting the lowest threshold for reproducible measurement), the specimen was considered promoter methylation-positive. Individuals with promoter methylation positivity in at least one of the four colon segments analysed were classified as promoter methylation-positive patients.

The results are summarized in Table 1. Distal *hMLH1* promoter methylation-positive participants were significantly more common than those positive for *MGMT* promoter methylation (70 vs 44% of all participants). For both genes, the percentages of women classified as methylation-positive (72% for *hMLH1,* 47% for

Table 1 *hMLH1* and *MGMT* methylation in the normal colorectal mucosa of healthy individuals

Promoter methylation levels[a]	*hMLH1*				*MGMT*			
	Women, n = *47*		*Men,* n = *53*		*Women,* n = *47*		*Men,* n = *53*	
	<60 years (n = 20)	*⩾60 years (n = 27)*	*<60 years (n = 27)*	*⩾60 years (n = 26)*	*<60 years (n = 20)*	*⩾60 years (n = 27)*	*<60 years (n = 27)*	*⩾60 years (n = 26)*
NEG: <0.1%	10 (50)	3 (11)	14 (52)	3 (12)	15 (75)	10 (37)	16 (59)	15 (58)
POS: ⩾0.1%	10 (50)	24 (89)	13 (48)	23 (88)	5 (25)	17 (63)	11 (41)	11 (42)
Positivity range	0.1–6.4	0.1–11.4	0.1–1.5	0.1–6.2	0.1–3.6	0.1–18.8	0.1–8.0	0.1–5.0
0.1–0.49%	4 (20)	6 (22)	6 (22)	6 (23)	4 (20)	3 (11)	6 (22)	7 (27)
0.5–1.99%	3 (15)	11 (41)	7 (26)	13 (50)	0	4 (15)	2 (7.4)	1 (3.8)
2.0–4.99%	2 (10)	2 (7.4)	0	3 (12)	1 (5)	5 (19)	1 (3.7)	2 (7.7)
⩾5%	1 (5)	5 (19)	0	1 (3.8)	0	5 (19)	2 (7.4)	1 (3.8)

[a]Percentage of alleles showing promoter methylation. Values shown reflect the maximum level observed in the colon of each participant. NEG, participants with promoter methylation levels <0.1% in all four colon segments; POS, participants with promoter methylation level ⩾0.1% in at least one colon segment. Sex- and age-specific (under 60 vs 60 years or over) frequencies are expressed in absolute numbers with percentages in parentheses.

MGMT) were similar to those observed in men (68% for *hMLH1*, 42% for *MGMT*). Methylation levels varied considerably (ranges: 0.1–11.4% for *hMLH1*, 0.1–18.8% for *MGMT*), but median levels for both genes were quite low (0.506 and 0.458%, respectively). (The impact of such changes on protein expression are most likely to be limited to single cells or crypts and, as noted by other investigators (Shen *et al*., 2005), such alterations are very difficult to document with immunohistochemistry.) As shown in Supplementary Figure 5, detection of promoter methylation in one colon segment significantly increased the likelihood that the same promoter would also be methylated in one or two other segments.

Concomitant methylation of both promoters in the same biopsy was a frequent finding, especially in older participants, but it was statistically significant (cecum: odds ratio (OR) = 8.2; $P = 0.003$; transverse colon; OR = 3.7; $P = 0.042$) only in the right colon of women (Figure 1). The likelihood of this finding declined in the sigmoid colon and disappeared completely in the rectum. In men, concomitant *hMLH1* and *MGMT* promoter methylation occurred in the rectum, but its statistical significance was marginal (OR = 4.4; $P = 0.042$). These findings show that the relation between methylation of these two gene promoters may be age-, sex- and colon segment-dependent.

Promoter methylation in the normal-appearing colorectal mucosa of participants with incident polyps

Colorectal polyps are found in roughly one-third of all asymptomatic adults undergoing colonoscopy in developed countries. Nonpolypoid lesions are less common: they have been detected in roughly 5% of patients undergoing screening with high-resolution colonoscopy and mucosal staining (Soetikno *et al*., 2008). Lesions of the latter type were not found in any of the participants we examined with standard colonoscopy, but 1–3 small (generally $\leqslant 10$ mm) polyps were found in 31% (Supplementary Table).

Analysis of normal mucosal data for the subgroups with and without polyps showed no significant differences in terms of the prevalence of promoter methylation-positive patients (71 vs 70% for *hMLH1*, 42 vs 45% for *MGMT*) or methylation levels (Table 1 and data not shown). This finding tends to exclude a direct relationship between the methylation status of the normal mucosa and the occurrence of benign colorectal polyps. To further explore this issue, however, we evaluated promoter methylation in the polyps themselves. As only formalin-fixed, paraffin-embedded tissues were available for this analysis, methylation in these samples was assessed semiquantitatively by combined bisulfite restriction analysis (COBRA) (see Materials and methods and Figure 2a), which is highly specific but less sensitive than qMSP.

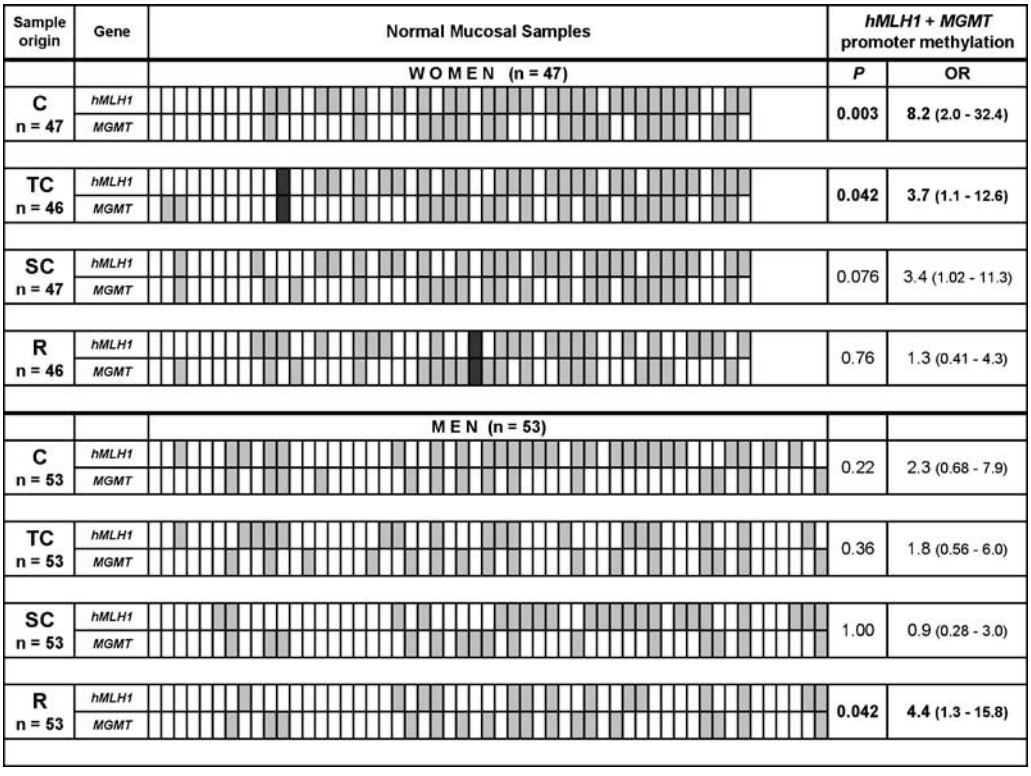

Figure 1 Methylation of the *hMLH1* and *MGMT* promoters in 400 specimens of normal mucosa. Promoter methylation was defined as methylation involving $\geqslant 0.1\%$ of all alleles. Data are shown separately for women and men. The rectangles shown in the central lanes represent normal mucosal specimens from the cecum (C), transverse colon (TC), sigmoid colon (SC) and rectum (R). Specimens are stratified according to participant age (increasing from left to right) and color-coded (gray: specimens showing promoter methylation; white: specimens without promoter methylation; black: tissue specimens not available for testing). Concomitant methylation of the *hMLH1* and *MGMT* promoters was significantly associated with the right colon in women (C, TC) and with the rectum (R) in men. Contingency tables were evaluated with a two-tailed Fisher exact test; *P*-values of 0.05 were considered significant. Odds ratios (OR) are presented with 95% confidence intervals.

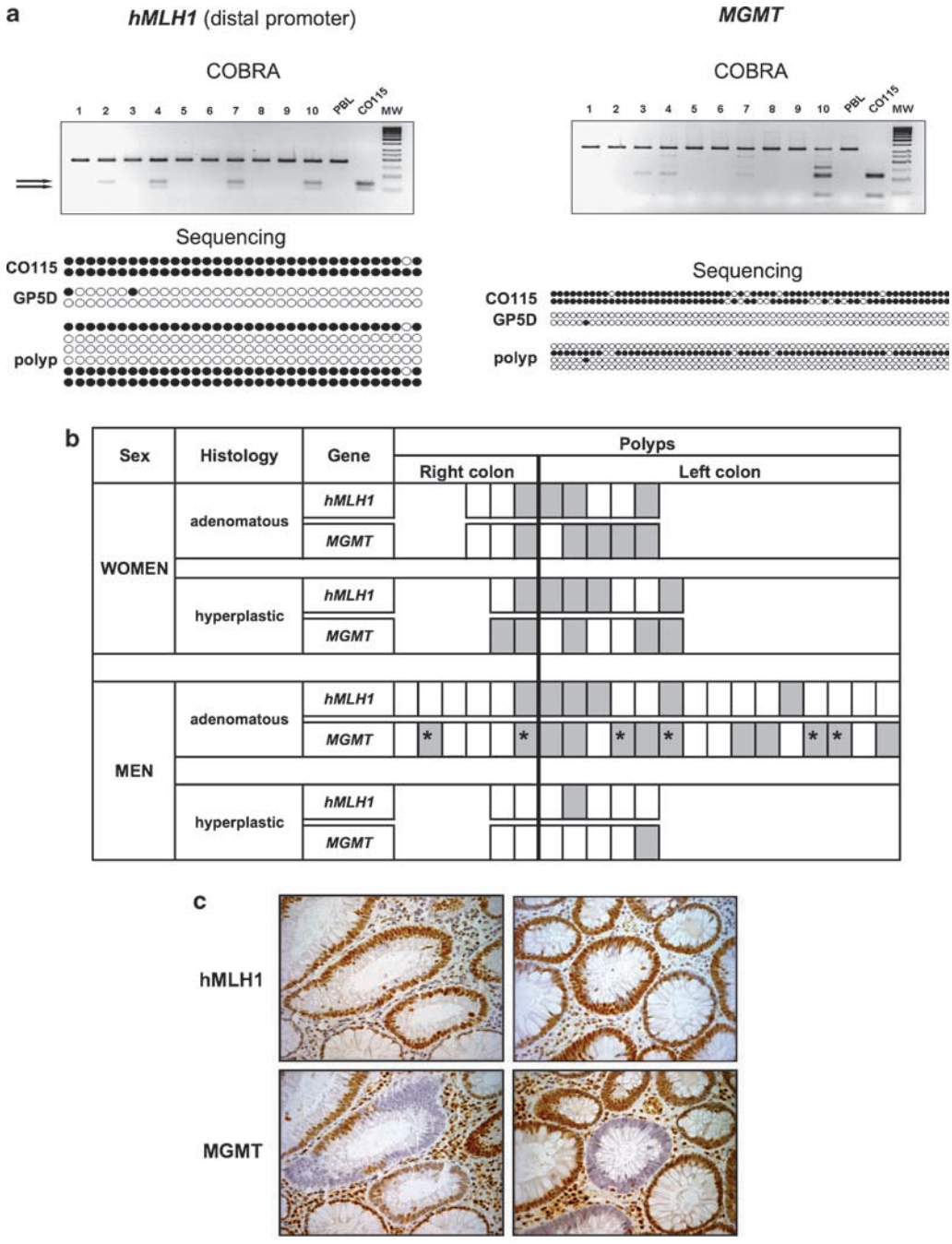

Figure 2 *hMLH1* and *MGMT* promoter methylation and expression in polyps. (**a**) Upper panel: combined bisulfite restriction analysis (COBRA) of *hMLH1* and *MGMT* promoter methylation in colorectal polyps. Data are shown for 10 (1–10) of the 44 polyps analysed. (As shown in Supplementary Table, 2 of the 46 polyps removed were not available for this analysis). PBL, bisulfite-treated DNA from peripheral blood leukocytes used as a negative control for methylation; CO115, positive-control DNA from the colon cell line CO115. Arrows indicate *BstUI*-digested DNA fragments representing methylated alleles; slower migrating fragments correspond to undigested, unmethylated DNA. Lower panel: COBRA results for randomly chosen samples were verified by bisulfite sequencing of the *hMLH1* and *MGMT* promoters in polyps and in the colon cancer cell lines GP5D and CO115. Each row shows the methylation status of a cloned target sequence, with circles representing unmethylated (open) and methylated (filled) CpG dinucleotides. (**b**) *hMLH1* and *MGMT* promoter methylation status of the 44 polyps analysed. Polyps are sorted according to sex, histotype (adenomatous or hyperplastic) and location in the colorectum (right colon, cecum or transverse colon; left colon, sigmoid colon or rectum) and are color-coded (gray: specimens showing promoter methylation; white: specimens without promoter methylation). Asterisks indicate polyps with patchy expression of the MGMT protein documented by immunohistochemistry (example in panel c, below). (**c**) Immunostaining of two adenomatous polyps with antibodies directed against hMLH1 (top panels) and MGMT (bottom panels). Both polyps exhibited normal expression of hMLH1 and patchy loss of MGMT expression.

MGMT promoter methylation was observed in 25 (57%) of the 44 polyps tested, whereas only 16 out of the 44 (36%) exhibited distal *hMLH1* promoter methylation ($P = 0.08$) (Figure 2b), and none had involvement of the proximal region of this promoter (data not shown). We then compared the promoter methylation status of each polyp with that of the normal mucosa from the segment of the colon where the polyp had been found (although the statistical power of this analysis was limited by the small number of observations). There was no significant correlation between the *hMLH1* promoter methylation statuses of the two tissue sets ($P = 0.21$), but *MGMT* methylation in polyps was positively correlated with that of the same-segment normal mucosa ($P = 0.021$, OR = 6.7, 95% confidence interval 1.4–31.0), particularly in men ($P = 0.013$, OR = 22, 95% confidence interval 1.8–241.0). Immunohistochemical assessment of hMLH1 and MGMT protein expression showed hMLH1 in all 44 polyps, whereas MGMT protein expression was abnormal (undetectable in 1, patchy in 5) in 6 (31%) of the 19 adenomatous polyps with methylated *MGMT* (Figures 2b and c).

Promoter methylation in the normal-appearing colorectal mucosa of polyp-free participants

To eliminate the possible effects of the presence of benign lesions on the methylation status of the normal mucosa, we next examined the promoter methylation statuses and levels of the normal mucosa (276 samples) from the 69 polyp-free participants.

Promoter. methylation positivity. The prevalence of promoter methylation in this subgroup showed age-related differences that were gene-, sex- and segment-specific (Figure 3). In women, distal *hMLH1* promoter and *MGMT* promoter methylation rates were significantly increased after the age of 60 years in all segments but the rectum (Figures 3a and b). In men (Figures 3c and d), the only significant difference between the over- and under-60 years age groups was an increased frequency in the older participants of distal *hMLH1* promoter methylation in the sigmoid mucosa. Details on age-related trends for methylation of the two gene promoters are shown in Supplementary Figure 6.

Promoter methylation levels. The impact of age on the levels of methylation observed in the polyp-free subgroup also presented gene-, sex- and segment-related differences. In women, distal *hMLH1* promoter methylation levels in the cecum and sigmoid colon increased steadily after the age of 60 years (Figure 4a, Table 1). In men, these levels remained low in all segments, although there was a trend towards age-dependent increase in the rectum (Figure 4c). As for *MGMT*, no significant

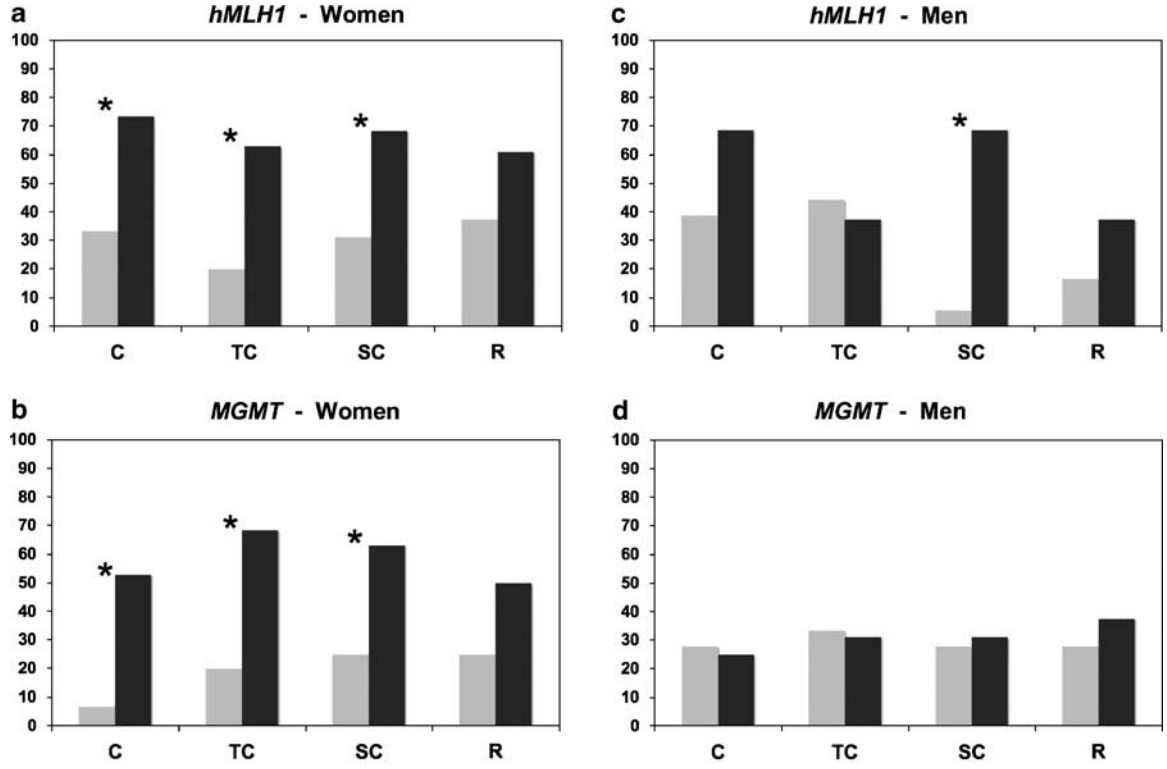

Figure 3 Age- and sex-related variability in the prevalence of *hMLH1* and *MGMT* promoter methylation in the normal colorectal mucosa of polyp-free participants. Panels **a** and **b** show the percentages of polyp-free women ($n = 35$) with methylation (involving $\geqslant 0.1\%$ of the alleles) of the distal *hMLH1* promoter and the *MGMT* promoter, respectively. Corresponding data for the polyp-free men ($n = 34$) are shown in panels **c** and **d**. Data are presented for each colon segment: cecum (C), transverse colon (TC), sigmoid colon (SC) and rectum (R). Percentages are shown for patients under 60 years of age (gray bars) and those 60 years or over (black bars). Asterisks indicate significant differences between the two age groups ($P \leqslant 0.05$) (contingency tables, two-tailed Fisher's exact tests).

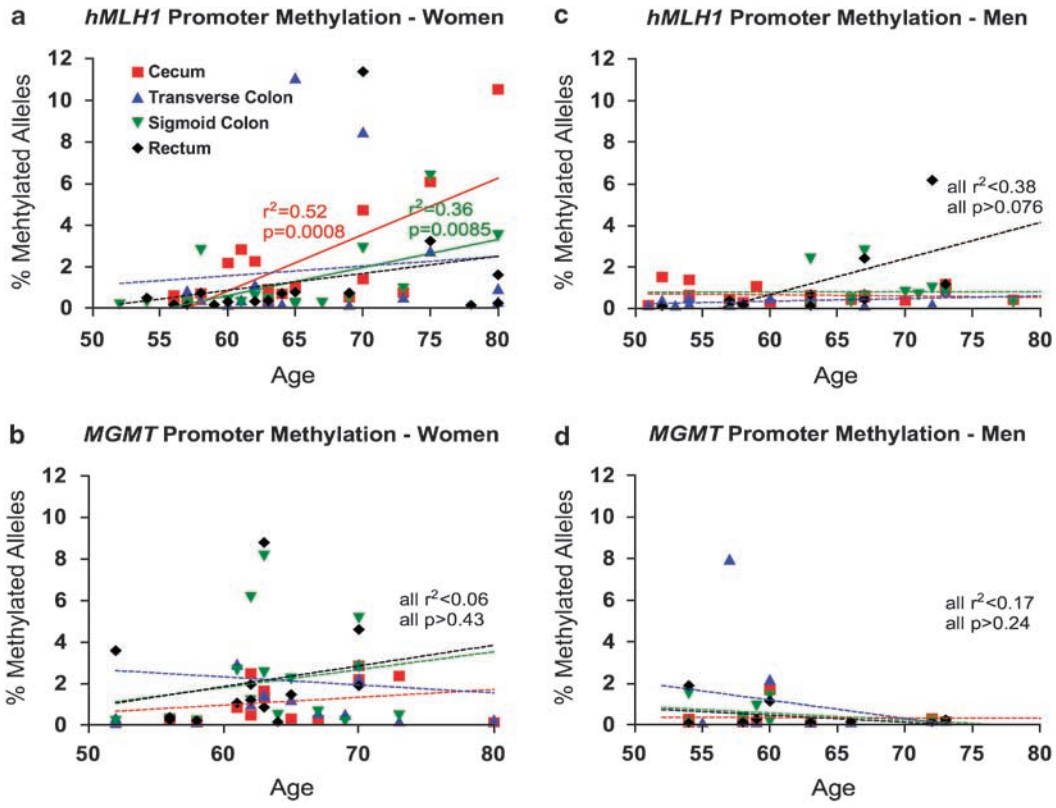

Figure 4 Age-, sex- and colon segment-related variability in *hMLH1* and *MGMT* promoter methylation levels in the normal colorectal mucosa of polyp-free participants. The analysis included all biopsies from polyp-free participants with methylation positivity in at least one colon segment. Percentages of methylated alleles (as determined by quantitative methylation-specific PCR) found in different segments of the colon (color-coded symbols) are plotted against participant age. Data are shown separately for women (**a** and **b**) and men (**c** and **d**). Lines represent linear regressions with r^2 and *P*-values for significant correlations in panel **a** (solid lines). In panels **b**–**d**, where correlations were nonsignificant—dashed lines—the highest r^2 and lowest *P*-values are reported. In women, the age-dependent increase in *hMLH1* methylation levels in the normal mucosa was particularly significant in the cecum.

age-dependent changes in promoter methylation levels were found in any areas of the colon in either sex (Figures 4b and d).

The fact that distal *hMLH1* promoter methylation levels were significantly increased in older women suggested that more advanced cytosine methylation—reflected by the spread of involvement to the proximal region of this promoter—might conceivably be present in some of the individuals in this subset (Deng *et al*., 1999; Miyakura *et al*., 2001; Nakagawa *et al*., 2001). Assessment of the normal mucosal specimens from the 27 women over 60 years disclosed low levels (<1% of the alleles) of proximal *hMLH1* promoter methylation in samples (two from the cecum, one from the rectum) collected from 3 of the 24 *hMLH1* methylation-positive participants in this subgroup (see Table 1).

hMLH1 *and* MGMT *expression in a series of consecutively collected colorectal cancers*

Direct assessment of the carcinogenic potential of the methylation patterns described above would require long-term follow-up (2–3 decades) of a much larger cohort of healthy volunteers than the one examined here. In the absence of such data, we analysed this issue indirectly by investigating the frequency of transcriptional silencing of *hMLH1* and *MGMT* in an independent series of archival colorectal cancer tissues from the Canton of Lucerne, where our screening population resided.

As detailed in Figure 5, immunohistochemical analysis showed loss of MGMT in 48 of these 231 cancers (20.8%), loss of hMLH1 in 20 (8.7%) and loss of both proteins in 11 cancers (4.7%). Nonexpression of MGMT was slightly more frequent in cancers from women 25/91 (27.5%) vs 23/140 (16.4%) in men; OR = 1.92, $P = 0.048$), but it was not related to the site of the tumor in either sex. Twenty (8.7%) of the 231 cancers failed to express hMLH1. This finding was much more common in the subgroup of cancers from women (17/91 (18.7%) vs 3/140 (2.1%) cancers in men; OR = 10.5; $P < 0.001$), where it occurred predominantly in right-colon tumors (15/42 (35.7%) vs 2/49 (4.1%) left-colon cancers; OR = 13; $P < 0.001$). Eleven of these 20 cancers (4.7% of the total series) also exhibited concomitant loss of MGMT. Ten of the 11 cancers came from women (OR = 5.6, $P = 0.005$), and 8 of the 10 occurred in the right colon. All 20 cancers that failed to express hMLH1 were MSI-positive. Random testing of 30 of the hMLH1-proficient cancers showed no other

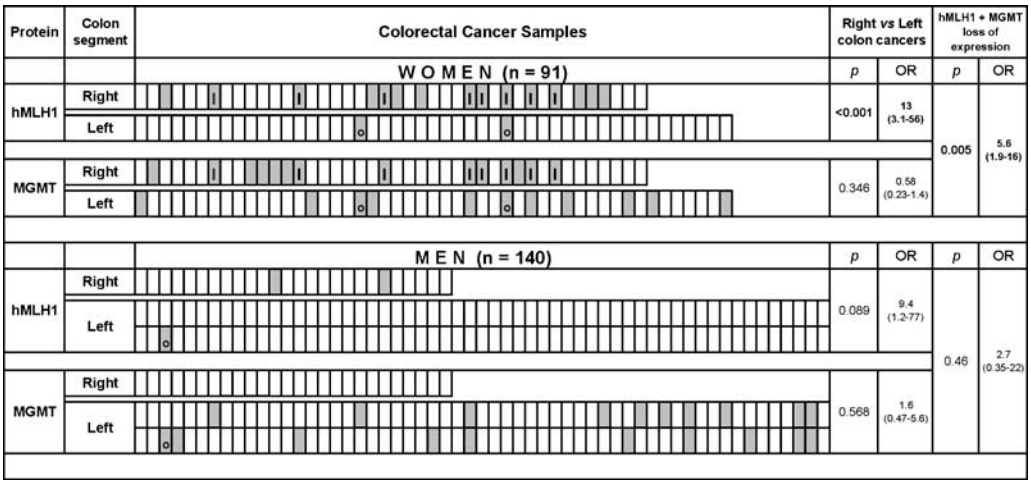

Figure 5 Loss of hMLH1 and MGMT expression in an unselected series of 231 colorectal cancers. Data are shown separately for cancers from women ($n=91$) and men ($n=140$) and for cancers from the right colon (cecum or transverse colon) and left colon (sigmoid or rectum). Each row of rectangles represents a subset of cancers. Specimens are stratified by participant age (increasing from left to right) and color-coded to indicate hMLH1 or MGMT expression status (gray: cancers with undetectable expression; white: specimens expressing the protein. The latter category includes four cancers with patchy loss of MGMT expression in a limited area of the tumor). Concomitant loss of hMLH1 and MGMT protein expression occurred in eight right-colon cancers (gray rectangles with a vertical bar) and three left-colon cancers (gray rectangles with a circle). Statistical analysis (contingency tables, Fisher exact test) showed a highly significant correlation between loss of hMLH1 expression and right-colon cancers in women ($P=0.001$) but not men. Concomitant loss of expression of both proteins was also significantly more common in cancers from women ($P=0.005$).

cases of MSI (Truninger *et al*., 2005; and data not shown).

Two patterns emerged from this analysis that showed intriguing parallels with those of promoter methylation observed in the normal mucosa of the individuals without colon cancer: (1) methylation of the *hMLH1* promoter in the normal mucosa of healthy individuals (Figures 3 and 4) and loss of hMLH1 in colon cancers both showed significant associations with the right colon in women (but not men); and (2) the same sex- and segment-specific correlations emerged for concomitant promoter methylation of *hMLH1* and *MGMT* in normal mucosa (Figure 1) and concomitant losses of the proteins they encode in colon cancers.

Discussion

Epigenetic changes involving multiple gene promoters are common in virtually all types of human neoplastic cells (Esteller, 2008). Because they frequently play important roles in the transformation process, many studies have focused on their identification in precancerous or even apparently normal tissues. Studies of normal gut mucosa collected near, or in some cases immediately adjacent to, colorectal tumors (benign or malignant) have showed methylation involving the promoters of various genes (Issa *et al*., 1994, 2001; Ahuja *et al*., 1998; Xiong *et al*., 2001; Suzuki *et al*., 2004; Kawakami *et al*., 2006; Minoo *et al*., 2006; Ye *et al*., 2006; Shen *et al*., 2007) including *hMLH1* (Miyakura *et al*., 2001; Nakagawa *et al*., 2001; Xiong *et al*., 2001; Chan *et al*., 2002; Kawakami *et al*., 2006; Minoo *et al*., 2006; Ye *et al*., 2006; Al-Ghnaniem *et al*., 2007) and *MGMT* (Shen *et al*., 2005, 2007; Minoo *et al*., 2006; Ye *et al*., 2006). These changes have never been associated with significant loss of hMLH1 or MGMT protein expression in the nontumoral tissues, even in those cases in which complete silencing of the methylated gene has been immunohistochemically documented in the tumors themselves. However, their relevance to the tumorigenic process is supported by an increasing body of evidence, including the demonstration by Shen *et al*. (2005) of significant correlation between *MGMT* promoter methylation in colorectal cancers and corresponding samples of normal-appearing mucosa (including that >10 cm from the tumor) and the significantly higher levels of *hMLH1* promoter methylation documented by Kawakami *et al*. (2006) in the normal mucosa of guts harboring CIMP+ cancers (compared with those with CIMP-negative tumors).

Field defects of this type were not analysed in the present study, as normal mucosal tissues were not available for the archival cancer specimens we analysed. The main focus of our study was in fact the earlier-stage epigenetic alterations in *hMLH1* and *MGMT*, which might be found before the onset of clinically evident colorectal tumorigenesis. Few attempts have been made to characterize epigenetic changes in these genes in tumor-free human colons, and in all of these studies, the analysis was limited to one segment (generally the rectum) (Shen *et al*., 2005; Ye *et al*., 2006; Al-Ghnaniem *et al*., 2007). In contrast, our study was pancolonic in scope and involved systematic, segment-by-segment, quantitative analysis of *hMLH1* and *MGMT* promoter methylation.

At the whole-colon level, this approach showed a high prevalence of low-level methylation involving both

promoters (*hMLH1* 70%, *MGMT* 44%). On subgroup analysis, no consistent patterns could be discerned in the prevalence or levels of methylation in samples from men, but striking age- and colon segment-specific variations were found in the female subgroup. Here, increasing age was associated with significantly higher frequencies of methylation at each promoter, markedly increased levels of *hMLH1* promoter methylation and a higher percentage of samples showing concomitant methylation of both promoters. And remarkably, all these differences were much more evident in the right colon.

Similar patterns emerged when we analysed hMLH1 and MGMT expression in an independent series of colorectal cancers from the same area in which our screening population resided. Indeed, the right-colon cancer in women was significantly associated with hMLH1 silencing and with concordant silencing of hMLH1 and MGMT. These findings are fully consistent with the epidemiological features of sporadic hMLH1- and MGMT-deficient colorectal cancers: the latter tumors have never shown any clear associations with age, sex or colon segment (Esteller *et al*., 2000; Whitehall *et al*., 2001; Kim *et al*., 2003), but cancers characterized by hMLH1-deficiency show a clear predilection for the right colon segments of women over 60 years (Malkhosyan *et al*., 2000; Lindblom, 2001; Slattery *et al*., 2001), and those showing MSI (a hallmark of mismatch repair deficiency) are also found mainly in the right colon.

Although the 100 individuals we studied were all apparently healthy, colonoscopy showed small, benign adenomatous or hyperplastic polyps in 31 individuals. The similarity between the *hMLH1* and *MGMT* promoter methylation findings for the normal mucosa of these 31 individuals and those of the polyp-free subgroup suggests that detectable methylation in one or both of these promoters in normal tissue does not *per se* increase the risk for developing benign colorectal polyps. However, it could conceivably increase the likelihood of *hMLH1* or *MGMT* methylation in polyps that do arise.

Our attempt to investigate this possibility was hampered by the size of the subgroup with polyps, but the results it yielded certainly merit further investigation. For instance, *MGMT* promoter methylation in normal mucosa seems to be positively correlated with methylation of this promoter in polyp tissues, especially among men. Furthermore, in six polyps, *MGMT* methylation had already silenced the expression of this gene in several mildly dysplastic crypts (Figures 2b and c). In contrast, *hMLH1* promoter methylation in normal and polyp tissues seemed to be unrelated. The markedly lower frequency of methylation found in polyps (36 vs 70% in normal mucosa) might be partly related to the sensitivity of the method used to assess methylation in these tissues (COBRA). However, the *hMLH1* and *MGMT* methylation profiles of the polyps we examined are fully consistent with our current knowledge of the different pathways of colorectal carcinogenesis. MGMT-deficient colorectal cancers seem to arise in polypoid lesions with conventional adenomatous histology, which were found in a substantial proportion of our screening colonoscopies, but numerous lines of evidence indicate that hMLH1-deficient colon cancers originate in the right colon from nonpolypoid lesions, which are frequently characterized by a serrated pattern on histology (reviewed by Jass *et al*., 2002). These lesions, which are much less common than colorectal polyps, were not represented at all in the group of benign lesions we analysed. Under these circumstances, the relatively low frequency of polyps with *hMLH1* methylation is not surprising. In light of these considerations, our data suggest that methylation of the distal region of the *hMLH1* promoter may be common in the normal mucosa. In contrast, progression of this phenomenon (heralded by involvement of proximal regions of the promoter near the transcription start site and ultimately, biallelic involvement) is a less frequent occurrence, which is more likely to be observed in the right colon of a woman. (We probably detected an early step in this process in the three normal mucosal biopsies from older women that showed low-level methylation of both the distal and proximal regions of the *hMLH1* promoter.) Here, within the context of a nonpolypoid lesion, progressive methylation might be sustained or even accelerated by the presence of a phenotype such as CIMP (Hiraoka *et al*., 2006), and this effect would also increase the likelihood of biallelic methylation leading to complete silencing of *hMLH1* expression.

The existence of a CIMP colorectal cancer phenotype was originally suggested by the observations of simultaneous methylation of multiple gene promoters in a subset of colon cancers (Toyota *et al*., 1999; Issa, 2004; Ogino *et al*., 2006; Weisenberger *et al*., 2006). Although *hMLH1* promoter methylation is consistently identified as a CIMP component, the data on *MGMT*'s role in this phenotype are far less conclusive. In colorectal cancer, the relation between promoter methylation involving these two genes emerged from one study as mutual exclusiveness (Fox *et al*., 2006); other investigators found that the two events were completely independent of one another (Ogino *et al*., 2007), and a third group reported that they were positively associated (Anacleto *et al*., 2005). Our data support the latter relation but only in the right colon of women: in other settings, promoter methylation of *hMLH1* appears to be totally unrelated to that of *MGMT*. This conclusion seems to apply not only to the normal colorectal mucosa but also to the colorectal cancers we investigated with immunohistochemistry. The contradictory reports cited above might thus be related to the features of the cancers and patients represented in the different series. In that case, we would be faced with the possibility of multiple CIMP variants, each showing sex- and colon segment-specific features.

In conclusion, early epigenetic changes involving genes whose silencing plays crucial roles in carcinogenesis can be readily detected in normal tissues at risk for transformation. Similar correspondence between normal and neoplastic tissues might exist in all organs that are prone to cancer, and its presence might even be a

reflection of different transformation pathways in the same tissue. How is this phenomenon related to ageing, sex and anatomic location? Why is the progression toward gene silencing accelerated in some individuals? Answers to these questions will expand our understanding of the changes we are witnessing in cancer epidemiology as a result of increasing population ageing. Studies similar to ours could pave the way for the identification of early diagnostic biomarkers whose use could improve the efficacy of current tumor surveillance strategies.

Materials and methods

Patients and tissues

The study protocol was approved by the central medical ethics committee of the canton of Lucerne, Switzerland. Participants were randomly recruited during a colonoscopy screening program that was open to all residents of the canton aged 50–80 years. Candidates were evaluated with a precolonoscopy questionnaire designed to exclude patients with lower gastrointestinal tract disease (reflected by history, symptoms and/or previous colonoscopy within the 5 years preceding enrolment). All enrolled participants provided written informed consent. Each underwent standard pancolonoscopy with biopsy of normal-appearing mucosa from the cecum, transverse colon, sigmoid colon and rectum. These four samples, plus any benign polyps removed during endoscopy, were analysed, as specified in Results. We also evaluated formalin-fixed, paraffin-embedded specimens of 231 consecutively diagnosed colorectal cancers from the Pathology Archives of the Cantonal Hospital of Lucerne. For a portion of these tumors, hMLH1 immunohistochemistry data and MSI statuses were obtained in a previous study (Truninger *et al*., 2005).

DNA extraction, bisulfite conversion, quantitative methylation-specific PCR, combined bisulfite restriction analysis, bisulfite sequencing and MSI analysis

Genomic DNA was extracted from the freshly collected samples of normal mucosa and formalin-fixed, paraffin-embedded polyp tissues with the QIAamp DNA Mini Kit (Qiagen, Basel, Switzerland) and subjected to sodium bisulfite conversion, as previously described (Menigatti *et al*.,. 2001) with minor modifications (see Supplementary Methods). Promoter methylation of *hMLH1* and *MGMT* was analysed in bisulfite-modified DNA with a qMSP assay developed and validated in our laboratory (Supplementary Figures 1 and 2). COBRA was used to assess promoter methylation in bisulfite-modified DNA from paraffin-embedded polyp tissues. In randomly selected DNA samples from normal mucosa and polyps, bisulfite sequencing of the h*MLH1* and *MGMT* promoters was also performed (Supplementary Figure 3). The locations of CpG sites in the *hMLH1* and *MGMT* gene promoters and the regions explored with qMSP, COBRA and bisulfite sequencing assays are shown in Supplementary Figure 4. Further details on all three assays are provided in Supplementary Methods. MSI analysis (based on the analysis of the mononucleotide repeat *BAT26*) was performed as previously described (Truninger *et al*., 2005).

Immunohistochemistry

As previously described (Truninger *et al*., 2005), sections of each formalin-fixed, paraffin-embedded polyp and cancer were immunostained with primary monoclonal antibodies (1.2 μg/ml) against hMLH1 (Ab 13271A, PharMingen, Erembodegern, Belgium) and MGMT (Ab MS-470, NeoMarkers, Runcorn, Cheshire, UK). Lack of protein expression in tumor cells was clearly reflected by the absence of nuclear staining in these cells (compared with positive staining observed in proliferating cells of the normal crypts and stroma).

Statistical analysis

Contingency tables were constructed with the following categorical variables: patient age and sex; origin of specimen (colon segment); methylation status (methylated vs unmethylated, as defined in the Results); and methylation level (percentage of methylated alleles). Two-tailed *P*-values for observed vs expected rates were then calculated with the Fisher exact or χ^2-test, as appropriate. Null hypotheses were rejected at $P \leqslant 0.05$. Age and percentage of methylated alleles were also treated as continuous variables for regression and correlation analyses. Analyses were performed using the R2.6.1 and Prism 5.0 statistical software packages for Macintosh computers.

Abbreviations

CIMP, CpG island methylator phenotype; COBRA, combined bisulfite restriction analysis; MSI, microsatellite instability; qMSP, quantitative methylation-specific PCR.

Acknowledgements

We thank Josef Jiricny at the Institute of Molecular Cancer Research of the University of Zurich and our colleagues at the Department of Biomedicine of the University of Basel for helpful discussions and support throughout the project. We are also grateful to Patric Urfer and Christophe Kunz for critical reading of the manuscript, Ritva Haider for technical assistance, and Marian E Kent for editorial assistance. The study was supported by the Swiss National Science Foundation (to GM), the Krebsliga Zentralschweiz (to GM), the Krebsliga Beider Basel (to PS), Julius-Müller Stiftung, Zürich (to KT) and the Hanne-Liebermann Stiftung, Zürich (to KT).

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)