

RAB32 hypermethylation and microsatellite instability in gastric and endometrial adenocarcinomas

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The recently described gene, *RAB32*, is a *ras* proto-oncogene family member that encodes an A-kinase-anchoring protein. *RAB32* has been found to be frequently hypermethylated in microsatellite instability-high (MSI-H) colon cancers. We sought to determine the prevalence of *RAB32* hypermethylation in gastric and endometrial adenocarcinomas, the 2 other major tumor types in which MSI-H is common. Moreover, we delineated the association of *RAB32* hypermethylation with microsatellite instability (MSI) and *hMLH1* hypermethylation. MSI status and hypermethylation of the *RAB32* and *hMLH1* genes were studied in paired primary normal and tumor tissues from 48 patients with gastric cancer. An additional 80 endometrial cancer patients were studied for *RAB32* methylation and MSI status. Thirteen (27%) of 48 gastric cancers demonstrated evidence of *RAB32* hypermethylation. MSI status was determined in 46 of the tumors, with 7 (100%) of 7 MSI-H tumors, 1 (33%) of 3 MSI-low (MSI-L) tumors and 4 (11%) of 36 microsatellite-stable (MSS) tumors found to harbor *RAB32* hypermethylation. *RAB32* methylation was significantly associated with intestinal type histology and concomitant *hMLH1* hypermethylation in gastric cancer. In contrast, *RAB32* methylation occurred in only 1 of 80 endometrial cancers, including 20 MSI-H, 8 MSI-L and 52 MSS tumors. Hypermethylation of *hMLH1* was noted in 16 (20%) of 80 endometrial tumors. We conclude that although *RAB32* methylation is rare in endometrial cancers, it is strongly associated with *hMLH1* hypermethylation and MSI in gastric adenocarcinomas. Given its similar involvement in colon cancer, *RAB32* inactivation may represent a component of the oncogenic pathway of microsatellite-unstable gastrointestinal adenocarcinomas.

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Hypermethylation of promoter region CpG islands is well recognized as an important mechanism of transcriptional repression and loss of gene function.¹ As a key example, hypermethylation of *hMLH1* is strongly associated with sporadic cases of microsatellite instability-high (MSI-H) cancers. There is growing evidence to suggest that concurrent hypermethylation of multiple additional genes may represent an important component in the development and progression of colorectal cancers.^{2–8} This propensity for methylation in such tumors has served as an interesting feature to exploit in the search for novel methylation targets.

We have recently applied a global expression profiling-based technique to identify potential novel methylation targets in MSI-H colorectal cancers. One of the genes discovered to have significant differential methylation in microsatellite-unstable cancers was *RAB32*, a mitochondrial *ras* family member that encodes an A-kinase anchoring protein. *RAB32* was found to be methylated exclusively in MSI-H colon cancers, with no evidence of methylation in either non-MSI-H tumors or normal adjacent mucosal tissue.⁹

RAB32 has the ability to anchor the cyclic AMP-dependent kinase, PKA, by binding to its regulatory subunit.¹⁰ *RAB32* inactivation may alter the phenotype of the cell through dysregulation of mitochondrial/AMP-PKA-mediated cellular functions such as apoptosis and energy production.^{11,12}

In addition to colorectal carcinoma, 2 other cancer types characterized by frequent microsatellite instability (MSI) are sporadic gastric and endometrial cancers.¹³ As with MSI-H colorectal cancers, concomitant methylation of multiple genes is associated with the neoplastic progression of sporadic MSI-H gastric cancers. Hypermethylated genes showing an association with MSI-H gastric cancers include *hMLH1*,^{14,15} *MGMT*,¹⁶ *E-cadherin*,^{16,17} *HPPI*,¹⁸ *p16*,¹⁷ *ID4*¹⁹ and *TGF- β receptor type I*.²⁰ In the case of endometrial cancer, the association between MSI and gene hypermethylation is less well defined. In the current study, we sought to determine the prevalence of *RAB32* hypermethylation in gastric and endometrial adenocarcinomas and to define any associations of this event with both MSI status and *hMLH1* hypermethylation.

Material and methods

Primary tumor samples

Forty-eight primary gastric adenocarcinomas and matched normal gastric mucosae were obtained and flash-frozen at surgical resection. Eighty primary endometrial cancers and matching normal endometrial tissues were similarly processed. Genomic normal and tumor DNAs were extracted using standard protocols.²¹ Clinicopathologic data were recorded for gastric cancer patients, but such data were unavailable for endometrial cancer patients.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed using Quantitect One-step RTPCR kit (Qiagen, CA). Briefly, the PCR mixture contained 1× RTPCR Master Mix, 500 nM each of forward and reverse primers, 200 nM of 6-FAM-labeled TaqMan probe, 0.2 μ l of Quantitect RT Mix and 5 ng of template total RNA in a total of 20 μ l. PCR reaction and real-time data collection were performed using an ABI7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The thermal cycling protocol consisted of reverse transcription at 50°C for 30 min, DNA polymerase activation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 60°C for 30 sec. Data for β -actin was used for normalization of the data. A colon cancer cell line expressing *RAB32*, SW620, was used as the quantification standard. The expression index was cal-

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culated according to the following formula for the relative expression of target mRNA:

$$\text{Expression index} = (\text{TarS}/\text{TarC})/(\text{rActS}/\text{rActC})$$

TarS and TarC represent levels of mRNA expression for the target gene in the sample and control specimens, respectively, while rActS and rActC correspond to the amplified ribosomal RNA levels in the sample and control specimens, respectively.

Real-Time quantitative methylation-specific PCR

Real-time quantitative methylation-specific PCR (MSP) using TaqMan technology was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Briefly, 2 μg of genomic DNA were treated with sodium bisulfite according to the standard protocol, and resuspended in 100 μl of water.²²

Subsequent real-time quantitative MSP using primers and probes specific to sequences corresponding to methylated DNA sequences was then performed as described previously.⁹ To normalize data, duplex PCR with β -actin primer and probe sequences containing no CpGs was performed. Primer and probe sequences for *RAB32*, *hMLH1* and β -actin were the same as those in our previous report.⁹ CpGenome Universal Methylated DNA (Intergen Purchase, NY) was used to generate a standard curve for each

reaction. Reaction mix without template DNA was used as a negative control.

An MSP value was calculated by dividing the ratio of GENE: β -actin for a sample by the GENE: β -actin ratio for Universal Methylated DNA. As per our previous publication, an MSP value of 0.2 was designated as the cutoff point for classifying a result as positive (>0.2) or negative (≤ 0.2) for methylation.⁹

Statistics

Comparisons between methylation prevalence and clinicopathologic variables were performed using Fisher's exact test.

MSI status

The MSI status of each tumor was determined using previously published methods with 5 microsatellite loci (*D2S123*, *D5S346*, *D17S250*, *BAT25* and *BAT26*).²³ Tumors were categorized as MSI-H (2 loci unstable), MSI-low (MSI-L; 1 locus unstable) or MS-stable (MSS; no loci unstable). MSI status for 2 gastric cancer specimens could not be obtained due to insufficient genomic DNA quantity. One of these 2 specimens demonstrated positive *RAB32* methylation. For endometrial cancers, matched white blood cell DNA samples were used as controls.

TABLE I – GASTRIC CANCER PATIENT DEMOGRAPHICS

Parameter	<i>RAB32</i> METH + (n = 13)	%	<i>RAB32</i> METH – (n = 35)	%	Total	p-value*
Age						
≤ 60	3	23.1	10	28.6	13	0.51
> 60	10	76.9	25	71.4	35	
Gender						
M	6	46.1	18	51.4	24	0.50
F	7	53.9	17	48.6	24	
Site						
Upper third	1	7.7	12	34.3	13	0.06
Middle or distal third	12	92.3	23	65.7	35	
Lauren						
Intestinal or mixed	11	84.6	17	48.6	28	0.02
Diffuse	2	15.4	18	51.4	20	
Differentiation						
Poor	9	69.2	23	65.7	32	0.55
Well or moderate	4	30.8	12	34.3	16	
LVI						
Yes	6	46.2	17	48.6	23	0.57
No	7	53.8	18	51.4	25	
Perineural						
Yes	7	53.9	19	54.3	26	0.62
No	6	46.1	16	45.7	22	
T stage						
T1	0	0.00	2	5.7	2	0.074**
T2	10	76.9	15	42.8	25	
T3	3	23.1	17	48.6	20	
T4	0	0.00	1	2.9	1	
N stage						
Negative	4	33.3	9	25.7	13	0.50
Positive	8	66.6	26	74.3	34	
M stage						
M0	13	100.00	30	85.7	43	0.19
M1	0	0.00	5	14.3	5	
TNM stage						
IA	0	0.00	2	5.7	2	0.168 ¹
IB	3	23.1	3	8.6	6	
II	5	38.4	9	25.7	14	
IIIA	4	30.8	10	28.6	14	
IIIB	0	0.00	3	8.6	3	
IV	1	7.7	8	22.8	9	
<i>hMLH1</i> methylation						
Positive	9	69.2	1	2.9	10	<0.001
Negative	4	30.8	34	97.1	38	

*By Fisher's exact test. **T1/T2 vs. T3/T4. ¹Stage I/II vs. III/IV.

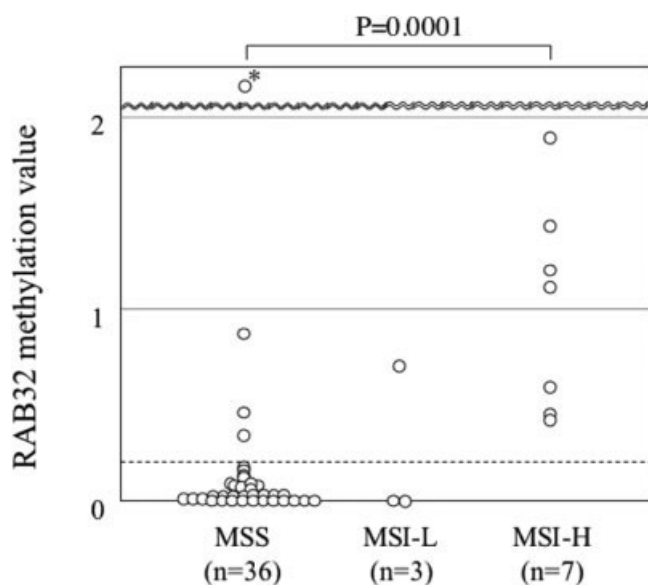


FIGURE 1 – Scatter plot representing *RAB32* methylation in primary gastric cancer specimens. The dashed line represents the cut-off value for *RAB32* methylation (0.2). The data point denoted by an asterisk represents an MSS specimen with a high *RAB32* methylation value (2.7).

Results

Gastric cancer patient demographics and tumor characteristics

The median age of patients was 67 years (range, 27–94), with the population consisting of 24 males and 24 females. The distributions of patient demographics and characteristics are included in Table I.

MSI status

Sufficient DNA for MSI analysis was available for 46 of the 48 gastric cancer patients. Seven (15%) tumors were classified as MSI-H, 3 (7%) as MSI-L and 36 (78%) as MSS. All 80 endometrial cancer specimens were evaluable for MSI. Twenty (25%) tumors were categorized as MSI-H, 8 (10%) as MSI-L and 52 (65%) as MSS.

Analysis of *RAB32* methylation

Gastric cancer. Using real-time quantitative MSP, we demonstrated that *RAB32* gene methylation was present in 13 (27%) of 48 of gastric cancers. Seven (100%) of 7 MSI-H tumors, 1 (33%) of 3 MSI-L tumors and 4 (11%) of 36 MSS tumors demonstrated *RAB32* methylation (Figs. 1 and 2). There was no methylation noted in normal adjacent mucosa (data not shown). The distribution of clinicopathologic variables between *RAB32* methylation-positive and -negative tumors is outlined in Table I. By univariate analysis, *RAB32* methylation was found to have a statistically significant association with Lauren intestinal/mixed type *vs.* diffuse type cancers ($p = 0.02$) and *hMLH1* methylation ($p < 0.001$). There was a nonstatistically significant trend towards *RAB32* methylation association with nonproximal location ($p = 0.06$).

Endometrial cancer. Only 1 of 80 endometrial cancer specimens was noted to harbor *RAB32* promoter hypermethylation (Fig. 2). This case represented an MSS tumor. *RAB32* methylation was not detected in any normal endometrial specimens.

Correlation of *RAB32* methylation and expression in gastric tissues

A representative analysis of 3 sets of gastric cancers and adjacent normal mucosal tissue was conducted to assess the correla-

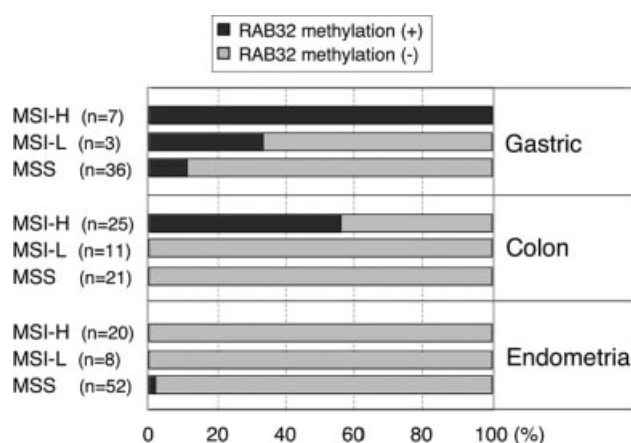


FIGURE 2 – *RAB32* methylation status in gastric, colon and endometrial cancers. This bar graph displays the ratio of specimens with (dark gray) or without (light gray) *RAB32* methylation in gastric, colon and endometrial cancers stratified by MSI status. *RAB32* was highly methylated in gastric and colon MSI-H cancers (100% and 56%, respectively) while *RAB32* was methylated in none of the endometrial MSI-H cancers. These colon cancer data are based upon our previous analysis.⁹

tion between the level of *RAB32* methylation and concomitant mRNA expression. We have confirmed that promoter methylation of *RAB32* was associated with diminished mRNA expression. Concordantly, lack of methylation was associated with strong expression of *RAB32* (Fig. 3).

Analysis of *hMLH1* methylation

Gastric cancer. Ten (21%) of 48 gastric tumors were found to have *hMLH1* hypermethylation. There was no evidence of *hMLH1* methylation in any normal adjacent gastric mucosae. Six (85.7%) of 7 MSI-H cancers demonstrated *hMLH1* methylation as compared to 4 (10.3%) of 39 non-MSI-H tumors ($p < 0.001$).

Endometrial cancer. We detected *hMLH1* hypermethylation in 16 (20%) of 80 endometrial cancers. Normal endometrial specimens did not harbor *hMLH1* methylation. Twelve (60%) of 20 MSI-H endometrial tumors demonstrated *hMLH1* hypermethylation as opposed to only 4 (6.6%) of 60 non-MSI-H cancers ($p < 0.00001$).

Correlation between *RAB32* and *hMLH1* methylation in gastric cancers

There was a strong, statistically significant correlation (Table I) between concomitant methylation of the *RAB32* and *hMLH1* genes in our gastric cancer population. Nine of 10 tumors with *hMLH1* methylation demonstrated concurrent methylation at the *RAB32* locus ($p < 0.001$). Conversely, 9 of 13 tumors with *RAB32* methylation also showed *hMLH1* methylation ($p < 0.001$).

Discussion

RAB32 methylation in gastric cancer

We have demonstrated that hypermethylation of the A-kinase anchoring protein-encoding gene, *RAB32*, occurred in ~25% of the gastric cancers analyzed. There appeared to be a strong correlation between concurrent methylation at the *hMLH1* and *RAB32* loci. Moreover, *RAB32* methylation was present in all of 7 MSI-H specimens from our patient population.

These findings are in concordance with our previous results examining *RAB32* methylation in colorectal cancers. We have recently demonstrated that in colorectal cancers, *RAB32* methylation occurred exclusively in the setting of MSI-H tumors and more specifically, *hMLH1* inactivation by methylation.⁹ The association

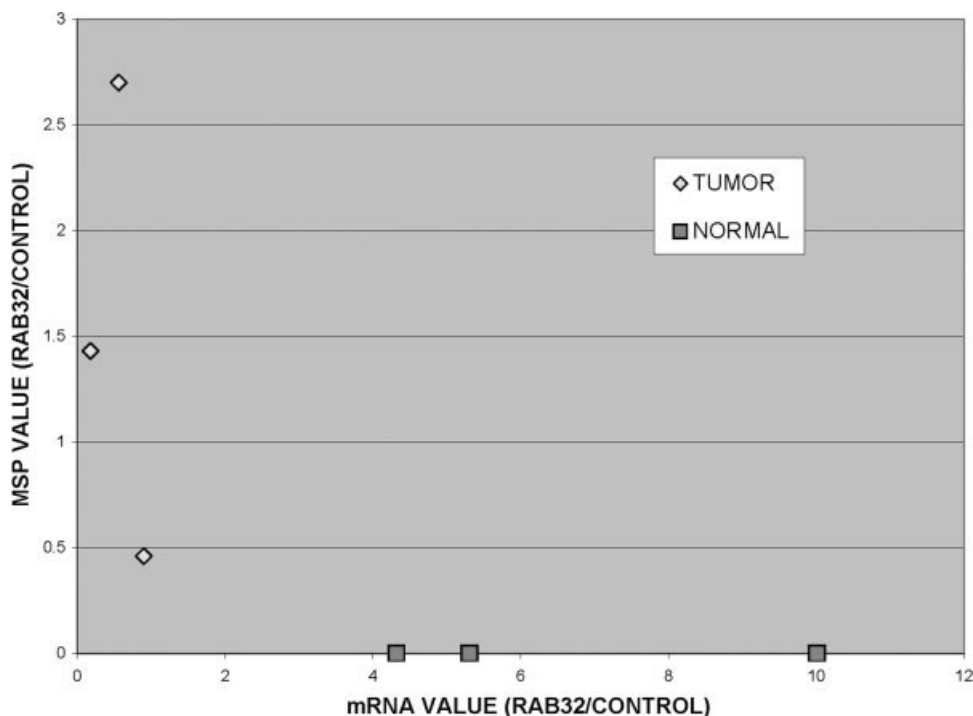


FIGURE 3 – Correlation of RAB32 methylation and expression. Analysis of representative samples of gastric tumor and normal tissues illustrate that high levels of RAB32 methylation was associated with a suppression of mRNA expression as quantitated by real-time RT-PCR. Strong expression of RAB32 was accompanied by an absence of methylation.

of *hMLH1* methylation and the similar epigenetic activation of other genes would add further evidence to support a methylator phenotype for this particular subset of microsatellite-unstable tumors.

Interestingly, a recent study has reported that another AKAP known as *AKAP12* or Gravin is frequently inactivated by promoter methylation in gastric carcinoma. Gravin organizes the PKA/PKC complex and is involved in the regulation of the β -2 adrenergic receptor complex. In structure–function analyses, the restoration of Gravin in nonexpressing cells resulted in growth suppression and apoptosis.²⁴ The MSI status of the tumors in that study was not evaluated.

RAB32 methylation in endometrial cancer

In contrast to colorectal cancer, in our analysis of 80 endometrial cancers, only 1 microsatellite-stable tumor demonstrated evidence of RAB32 methylation (Fig. 2). Our finding of a 25% incidence of MSI-H endometrial cancers is consistent with previously reported estimates which range from 17–25%.²⁵ Of the 20 MSI-H endometrial cancers in our series, 12 (60%) were found to harbor *hMLH1* hypermethylation.

Although endometrial cancers are characterized by a relatively high rate of MSI, there is significant evidence to suggest that the subsequent underlying molecular determinants may be quite different from those of their microsatellite-unstable gastrointestinal counterparts. There is growing evidence to suggest that gene promoter methylation may represent an important phenomenon in endometrial cancer as is the case in gastrointestinal malignancies.^{26–28} To date, however, only a handful of these events, such as methylation of *hMLH1*,²⁹ *PTEN*,³⁰ and *APC*,³¹ appear to be associated with MSI. Furthermore, in gastrointestinal cancers affected by MSI, it is believed that an important mechanism of neoplastic progression is frameshift mutation of coding region microsatellite repeats.²³ Gurin *et al.* analyzed microsatellite-unstable endometrial cancers for somatic mutations in the microsatellite coding regions of genes (*TGFBIIR*, *IGFIIR*, *BAX*, *E2F4*, *MSH3*, *MSH6*, *BRCA1* and *BRCA2*) known to be frequently affected in microsatellite-unstable gastrointestinal cancers. They concluded that somatic mutations in these selected genes were very rare.²⁵ Given that the molecular

pathway underlying MSI-H endometrial cancer appears discrete from that of gastric and colorectal cancers, it is not surprising that our current results are also discordant.

Clinicopathologic differences among subtypes of gastric cancer

With colorectal cancers, the morphologic and histopathologic differences between MSI-H and MSS tumors have been well defined. Even within microsatellite-unstable tumors there appear to be differences between those associated with hereditary non-polyposis colon cancer (HNPCC) and those with sporadic MSI-H. Lymphocytic infiltration, tumor budding and co-existing adenomas are frequently noted in HNPCC. Mucin secretion, poor differentiation, tumor heterogeneity, glandular serration and co-existing serrated polyps are characteristic of sporadic MSI-H colorectal cancers.^{32,33} Distinct clinical and histopathologic features of MSI-H gastric as compared to microsatellite-stable cancers are not as well established. However, there have been studies suggesting that microsatellite-unstable gastric cancers are associated with older age of presentation, distal tumor location, early disease staging and better overall prognosis.³⁴ Kim *et al.* demonstrated that MSI-positive gastric cancer cases were significantly correlated with intestinal type, late onset and early stage disease.³⁵ In this current study, we have shown that the presence of RAB32 methylation correlates in statistically significant fashion with mixed or intestinal type cancer and *hMLH1* methylation. There was a nonstatistically significant trend towards RAB32 methylation association with nonproximal location. Although we were unable to complete MSI analysis of the entire population (one of the specimens that could not be analyzed for MSI status was RAB32 methylation-positive), these trends are likely an indirect manifestation of underlying MSI. We did not show any age-associated relationship with RAB32 in our current study. Similarly, we did not demonstrate any significant association with nodal status or tumor stage.

The biologic role of RAB32

The GTP-binding Rab family of proteins are thought to play important roles in vesicle and granule targeting.³⁶ The novel member, RAB32, was initially cloned and isolated from human platelets and demonstrated low steady state GTPase activity.³⁷ Subse-

quently, RAB32 was demonstrated to function as an A-kinase anchoring protein and interact with the type II regulatory subunit of cAMP-dependent protein kinase (PKA). In this fashion, it is felt that RAB32 contributes to the tethering of PKA specifically to the surface of mitochondria. Transfection of a GTP-binding-deficient mutant of RAB32 was shown to result not only in morphologic alteration of mitochondrial structure, but also in the aberrant accumulation of mitochondria.¹⁰ The physiologic consequences of RAB32 disruption on mitochondria remain to be elucidated. However, mitochondrial alterations are thought to result in changes in cellular energy capacities, oxidative stress, reactive oxygen species (ROS)-mediated DNA damages and cellular apoptosis.³⁸ Alterations in such parameters are thought to be potentially important contributors to the development of a neoplastic phenotype.

In the setting of MSI-H colorectal cancers, *K-ras* mutations are rare. However, activating mutations of *BRAF* are strongly associated with these tumors and may functionally compensate for the lack of *K-ras* mutation.³⁹ Conversely, in gastric cancers, *BRAF* mutations are exceedingly rare and it appears that *K-ras* mutation is strongly associated with microsatellite-unstable lesions.⁴⁰ Notably, PKA, a postulated RAB32-interactor, is thought to be a modulating component of the *RAS/RAF/ERK/MAP* kinase cascade and may therefore represent a potential unifying link between RAB32 and microsatellite-unstable colorectal and gastric cancers.

Methylator phenotype

Our study demonstrates an association of hypermethylation of RAB32, another gene that is strongly associated with microsatellite-unstable gastric cancer. This finding is in agreement with similar previous studies showing hypermethylation of several genes such as *hMLH1*,^{14,15} *MGMT*,¹⁶ *E-cadherin*,^{16,17} *HPP1*,¹⁸ *p16*,¹⁷ *ID4*¹⁹ and *TGF- β receptor type I*²⁰ occurring preferentially in the setting of MSI-H cancers. Thus, there is growing evidence to support the existence of an aberrant methylator phenotype in the neo-

plastic progression of microsatellite-unstable gastric oncogenesis. This finding may be similar to the description of a CpG island methylator phenotype (CIMP) that has been reported in colorectal cancers.^{41,42}

Conclusion

We conclude that RAB32 promoter methylation is very rare in both sporadic and microsatellite-unstable endometrial cancers, further emphasizing its difference from molecular pathways underlying MSI-H tumorigenesis in gastrointestinal cancers. RAB32 is more frequently a target of methylation-mediated epigenetic inactivation in gastric adenocarcinoma. Similar to the situation in colorectal cancers, there is a strong association of this event with concurrent *hMLH1* methylation and underlying MSI. Inactivation of RAB32 may potentially contribute to gastrointestinal tumorigenesis by alterations in mitochondrial physiology as well as by interactions with the RAS signaling pathway. Additional investigations are warranted to further elucidate the biologic function of RAB32. Our study also supports the theory that microsatellite-unstable gastric cancers may potentially be characterized by specific clinicopathologic features such as intestinal type and distal location. Further studies of MSI-H gastric cancers may yield evidence of a pattern of methylation events that would support a distinct molecular pathway in this subset of tumors.

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