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Molecular progression of promoter methylation in intraductal papillary mucinous neoplasms (IPMN) of the pancreas

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To understand the role of gene promoter methylation in neoplastic evolution and progression, the methylation changes associated with 15 candidate tumor suppressor genes were studied throughout stages of tumor progression involving intraductal papillary mucinous neoplasms (IPMN) of the pancreas. Genomic DNA from 28 pancreatic IPMN tissue samples, categorized histologically as non-invasive intraductal IPMN ($n = 3$), IPMN with carcinoma *in situ* ($n = 7$), IPMN with microinvasion <1 mm ($n = 4$), and infiltrative IPMN with associated adenocarcinoma ($n = 14$), was modified by bisulfite treatment and analyzed with methylation-specific PCR (MSP). Promoter methylation of at least one tumor suppressor gene was present in 26/28 (92%) of the IPMNs. The cell cycle control genes, p16 and p73, were methylated frequently (>50%) in both non-invasive and invasive tumors. APC methylation was discovered in <10% of the non-invasive IPMNs versus 45% of the IPMNs associated with infiltrative adenocarcinoma, $P = 0.040$. Mismatch repair genes, hMLH1 and MGMT, were frequently methylated in the invasive IPMNs compared with the non-invasive tumors (38 versus 10% and 45 versus 20%, respectively) as was E-cadherin (38 versus 10%), $P = 0.11$. Multiple gene methylation at greater than three loci was present in 55% of the invasive tumors compared with 20% of the non-invasive tumors, $P = 0.075$. Lymph node status did not predict multi-gene methylation among tumors associated with invasive cancer. Compared with non-invasive IPMNs of the pancreas, IPMNs associated with adenocarcinoma demonstrate higher rates of aberrant tumor suppressor gene methylation. The sequential acquisition of hypermethylation at multiple gene promoter sites may explain tumor progression in IPMNs and other malignancies. Detection of methylation within selected genes may afford an accurate diagnostic molecular marker and predictor of neoplastic behavior.

Introduction

Since its first description by Ohhashi *et al.* in 1982, intraductal papillary mucinous neoplasm (IPMN) of the pancreas has become an increasingly recognized cystic tumor with unique histopathologic findings including massive dilatation of the pancreatic duct, mucin hypersecretion and papillary epithelial projections into the pancreatic duct tributaries (1–3). While 25% of IPMNs are associated with an infiltrating adenocarcinoma capable of regional and systemic metastasis, the biology of these neoplasms affords patients a relatively good prognosis after surgical resection compared with their counterpart ductal adenocarcinomas (1,2,4,5). In a recent retrospective study by Sohn *et al.* (6) at The Johns Hopkins Hospital, the mean 5-year survival for patients ($n = 60$) undergoing complete resection for IPMN was 57% compared with 19% for patients with ductal adenocarcinoma. Interestingly, there was no significant difference in overall 3- and 4-year survival between patients undergoing resection for non-invasive IPMN versus those with IPMNs associated with an infiltrating adenocarcinoma (6,7). Although the cause of death for the patients in each IPMN group was uncertain in many cases, tumor recurrence was indeed found in some patients with margin-negative resections for non-invasive IPMNs.

The favorable clinical behavior of IPMNs attracts interest into the potential genetic and epigenetic factors that foster this tumor's evolution and progression from pancreatic ductal epithelium. Unfortunately, only limited data exist to characterize the genetic alterations that occur during IPMN tumorigenesis. Loss of heterozygosity, detected through microsatellite analysis of IPMNs, has been discovered at several chromosomal locations (i.e. 6q, 8p, 9p, 17p, 18q) including the 19p locus housing the STK11/LKB1 gene (1,8). Additionally, selected expression of DPC4 (SMAD4), a nuclear transcription factor that is lost in >50% of pancreatic ductal adenocarcinomas, is preserved in monoclonal immunohistochemical stains of IPMNs (9–11). Scientific appreciation for the epigenetic as well as the genetic changes associated with solid tumor formation and progression continues to grow. Of the several epigenetic mechanisms that play a role in tumorigenesis, DNA methylation of tumor suppressor gene promoter sites has gained attention in recent years. Silencing of tumor suppressor gene expression by promoter hypermethylation at CpG-rich islands is common among several human malignancies (12–17). The hypermethylation of promoter regions for p16, p15, E-cadherin, VHL and hMLH1 correlates directly with the loss of transcription of these tumor suppressor genes in a variety of tumors (12,13,15,18). In studying methylation patterns among pancreatic ductal adenocarcinomas using methylation-specific PCR (MSP), Ueki *et al.* (16) discovered aberrant methylation involving at least one gene locus in 60% of all cancers. Methylation has been found for several tumor suppressor genes (i.e. p16, RAR-b, TIMP-3, E-cadherin, DAPK-1, hMLH1, cyclin G, ppENK) involving ductal adenocarcinoma of the pancreas (16,17).

Characterization of the methylation patterns of candidate genes, established to play a role in human tumorigenesis, may afford significant insight into the peculiar biology associated with pancreatic IPMNs. The tumor-specific methylation profile for each IPMN may be predictive of tumor recurrence, micro-

Abbreviations: IPMN, intraductal papillary mucinous neoplasm; MSP, methylation-specific PCR; PanIN, pancreatic intraductal neoplasia.

invasion, and risk of tumor formation in the remnant pancreas following margin-negative surgical resection. With these goals in mind and using a modified MSP technique, we examined IPMNs from 28 patients for abnormal promoter hypermethylation associated with p16, p14, p15, p73, APC, hMLH1, E-cadherin, TIMP-3, BRCA-1, ER, DAPK-1, GST-p, RAR-b, MGMT and SOCS-1.

Materials and methods

Human tissue samples

Tumor samples were obtained from 28 resected IPMN specimens that presented to the Department of Pathology at The Johns Hopkins Hospital between 1998 and 2001. Permission for cataloging and processing all samples for this study was obtained in accordance with the guidelines set forth by the institution's review board and joint committee for clinical research. The IPMN specimens were paraffin-embedded and sectioned sequentially at a thickness of 10 mm each. Histopathological characterization of the IPMN samples, collected from 28 individual patients, was determined by unanimous consensus among three separate gastrointestinal pathologists examining the tissue sections. Subpopulations of IPMNs were categorized histologically as non-invasive intraductal IPMN ($n = 3$), IPMN with carcinoma *in situ* ($n = 7$), IPMN with $\leqslant$1 mm microinvasion ($n = 4$) and infiltrative IPMN with associated adenocarcinoma ($n = 14$). Samples from two cases of resected chronic pancreatitis were also included. Sections from the tumor-free, histologically normal tissue margins adjacent to the pancreatic IPMNs for each patient were designated as normal pancreatic tissue, despite the fact that these tissues were derived from cancer patients. Table I indicates the tumor characteristics and associated patient demographics for each of the samples included in this study. There were no significant differences in race or gender between the tumor subpopulations. Overall, 60% of the tumors were derived from male patients, and 80% from Caucasian patients.

Table I. Tumor characteristics

Histology	Total samples	Average patient age (range)	Average tumor size (range)
Non-neoplastic			
Chronic pancreatitis (CP)	2	74 (71–6)	N/A
Non-invasive			
Intraductal IPMN (IPMN)	3	77 (74–79)	4.2 cm (2.5–5.0)
IPMN w/carcinoma *in situ* (Cis)	7	70 (56–78)	4.4 cm (2.5–6.0)
Invasive			
IPMN w/microinvasion (MI)	4	63 (54–72)	8.0 cm (7.0–9.0)
IPMN w/adenocarcinoma (Ca), LN–	5	70 (60–77)	2.7 cm (1.5–3.5)
IPMN w/adenocarcinoma (Ca), LN+	9	66 (55–77)	4.1 cm (1.5–8.0)

LN–, resected lymph nodes for tumor; LN+, positive for tumor.

DNA preparation

Two sequential 10 micron sections from each tumor and normal pancreas were de-paraffinized with xylene and digested overnight at 50°C with proteinase K buffered in 1% SDS (pH = 8). DNA was isolated by phenol–chloroform extraction and ethanol precipitation (19). Approximately 5 µg of DNA was partially purified from the two 10 micron tissue sections.

MSP

The methylation status of the promoter regions for 15 tumor suppressor genes was determined by the method of MSP further modified as a nested two-step approach in order to increase the sensitivity of detecting allelic hypermethylation at targeted sequences and to facilitate the examination of multiple gene loci (20,21). Initially, 1 µg of tissue DNA was bisulfite treated according to previously described protocols to render unmethylated cytosines to uracil (20). The bisulfite-treated DNA was column-purified over Wizard clean-up resin (Promega, Madison, WI) and ethanol precipitated. Step one of the nested MSP was carried out with primer sets (sense and anti-sense) for five individual genes in each reaction. Step one primers flanked the CpG-rich promoter regions of the respective targeted genes. Hence, these primers did not discriminate between methylated and unmethylated nucleotides following bisulfite treatment. PCR products of step one were diluted 1:1000 and subjected to the second step of MSP that incorporated one set of primers for each gene [labeled as unmethylated (U) or methylated (M)] that were designed to recognize bisulfite-induced modifications of unmethylated cytosines. Table II lists the primer sequences and PCR product sizes for each gene during this nested-MSP approach. Both steps of the nested MSP utilized a 25 µl reaction volume, 0.5 µl of Jump Start Red *Taq* DNA polymerase (Sigma, St Louis, MO), and 1 ml of DNA template. The PCR conditions for step one of the nested MSP were as follows: 95°C hot start ×5 min, then 40 repetitive cycles of denaturation (95°C ×30 s), annealing (56°C ×30 s), extension (72°C ×30 s) followed by a final 5 min extension at 72°C. Step two of the nested MSP was performed in a similar fashion with the exception of a few slight modifications including: addition of the Jump Start Red *Taq* DNA polymerase after the initial 5 min hot start, adjustments to the annealing temperature (range 58–61°C) for each gene to allow for optimal template discrimination, and PCR cycle number from 25 to 30. DNA isolated from normal peripheral lymphocytes from healthy individuals served as a negative methylation control. Human placental DNA was treated *in vitro* with SssI methyltransferase (NEB, Beverly, MA) to create completely methylated DNA at all CpG-rich regions. *In vitro* methylated DNA (IVD) served as the positive methylation control. MSP products were analyzed on 6% PGE.

Statistical analysis

The Fisher exact probability test was used to analyze the correlation between the methylation status of tumor suppressor genes and the histopathological features of the IPMNs (invasive versus non-invasive). A two-tailed *P*-value of <0.05 was considered statistically significant.

Results

Methylation among human tissues

Applying our nested-MSP technique to 15 candidate tumor suppressor genes (Figure 1), we found aberrant methylation involving at least one gene promoter site in 26/28 (92%) of the neoplastic cases. While one of these tumors without promoter region methylation at the tested loci was a non-invasive intraductal IPMN, the other was an infiltrating IPMN associated with lymph node metastasis. Two specimens of chronic pancreatitis demonstrated no promoter site methylation except at the p16 locus. This finding of p16 methylation in pancreatic tissue, verified histologically as chronic pancreatitis, has been observed previously by Gerdes *et al.* (22) who found inactivating hypermethylation of the p16 promoter in 20% of

Table II. Primer Sets for Nested MSP

GENE	5′ PRIMER	3′ PRIMER	SIZE (bp)
	External (stage 1)	**External (stage 1)**	
p14	TAGTTTGTAGTTAAGGGGGTAGGAG	CRCTACCCACTCCCCCRTAAACC	207
p15	GAYGTYGGTTTTTTGGTTTAGTTGA	AACRCAACCRAACTCAAAACC	139
p16	AGAAAGAGGAGGGGTTGGTTGG	ACRCCCRCACCTCCTCTACC	193
P73	GGGTYGGGTAGTTYGTTTTGTTTT	CRACCCTAAACCTCCTACCTACAACC	158
APC	TGGGYGGGGTTTTGTGTTTTATT	TACRCCCACACCCAACCAATC	136
hMLH1	GGAGTGAAGGAGGTTAYGGGTAAGT	AAAAACRATAAAACCCTATACCTAATCTATC	182
E-Cad	GTGTTTTYGGGGTTTATTTGGTTGT	TACRACTCCAAAAACCCATAACTAACC	186
TIMP3	GYGGTATTATTTTTTATAAGGATTTGA	ACCRAATAATATAACRCTAAACCCC	185
BRCA-1	GAGAGGTTGTTGTTTAGYGGTAGTTTT	TCTAAAAAACCCCCACAACCTATCC	143
ER	GGYGAGGTGTATTTGGATAGTAGTAAGTT	CRAACTCRAAAACACRCTATTAAATAAAA	204
DAPK-1	YGGAGGATAGTYGGATYGAGTTAA	ACRAAAACACAACTAAAAAATAAATAAAAAAC	140
GST-p	GGGATTTTAGGGYGTTTTTTTG	ACCTCCRAACCTTATAAAAATAATCCC	159
RAR-b	TATGYGAGTTGTTTGAGGATTGGGA	AATAATCATTTACCATTTTCCAAACTTA	204
MGMT	GYGTTTYGGATATGTTGGGATAGTT	AAACTCCRCACTCTTCCRAAAAC	135
SOCS-1	**GYGTAAGATGGTTTYGGGATTTA**	**RAAATTAAAAAAAATACRAACCAAATTCTC**	**204**
	Internal Methylated (stage 2)	**Internal Methylated (stage 2)**	
p14	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	122
p15	GGTTTTTTATTTTGTTAGAGCGAGGC	TAACCGCAAAATACGAACGCG	68
p16	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150
P73	GGACGTAGCGAAATCGGGGTTC	ACCCCGAACATCGACGTCCG	60
APC	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	98
hMLH1	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	115
E-Cad	TGTAGTTACGTATTTATTTTTAGTGGCGTC	CGAATACGATCGAATCGAACCG	112
TIMP3	CGTTTCGTTATTTTTTGTTTTCGGTTTC	CCGAAAACCCCGCCTCG	116
BRCA-1	TCGTGGTAACGGAAAAGCGC	AAATCTCAACGAACTCACGCCG	75
ER	ACGAGTTTAACGTCGCGGTC	ACCCCCCAAACCGTTAAAAC	110
DAPK-1	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98
GST-p	TTCGGGGTGTAGCGGTCGTC	GCCCCAATACTAAATCACGACG	91
RAR-b	TGTCGAGAACGCGAGCGATTC	CGACCAATCCAACCGAAACGA	149
MGMT	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	81
SOCS-1	**TTCGCGTGTATTTTTAGGTCGGTC**	**CGACACAACTCCTACAACGACCG**	**160**
	Internal Unmethylated (stage 2)	**Internal Unmethylated (stage 2)**	
p14	TTTGGTGTTAAAGGGTGGTGTAGT	CACAAAAACCCTCACTCACAACAA	132
p15	GGTTGGTTTTTTATTTTGTTAGAGTGAGGT	AACCACTCTAACCACAAAATACAAACACA	80
p16	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151
P73	AGGGGATGTAGTGAAATTGGGGTTT	ATCACAACCCCAAACATCAACATCCA	69
APC	GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	108
hMLH1	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124
E-Cad	TGGTTGTAGTTATGTATTTATTTTTAGTGGTGTT	ACACCAAATACAATCAAATCAAACCAAA	120
TIMP3	TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	CCCCCAAAAACCCCACCTCA	122
BRCA-1	TTGGTTTTTGTGGTAATGGAAAAGTGT	CAAAAAATCTCAACAAACTCACACCA	86
ER	TGTTGTTTATGAGTTTAATGTTGTGGTT	AAAAAAACCCCCCAAACCATT	124
DAPK-1	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	106
GST-p	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAACA	97
RAR-b	TTGGGATGTTGAGAATGTGAGTGATTT	CTTACTCAACCAATCCAACCAAAACAA	161
MGMT	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	93
SOCS-1	**TTATGAGTATTTGTGTATTTTTAGGTTGGTT**	**CACTAACAACACAACTCCTACAACAACCA**	**175**

cases of chronic pancreatitis with concomitant pancreatic intraductal neoplasia (PanIN). While no surgical specimens of completely normal pancreas were available for this investigation, we performed MSP on the macrodissected, histologically normal neck margins of the surgical specimens harboring IPMN lesions. No promoter methylation was detected for any of the candidate tumor suppressor genes among the disease-free margins, even those adjacent to IPMNs demonstrating positive promoter methylation at several gene loci (Figure 2). In addition, MSP was carried out on several non-neoplastic human tissues, including stomach, esophagus, colon and peripheral leukocytes. Consistent with previous work, hypermethylation was not found among tissue specimens devoid of tumor or chronic inflammation. Because of the limited number of tumors within each IPMN subpopulation, differences in methylation patterns according to age, race, gender and environmental exposures were not analyzed.

Methylation frequency among individual tumor suppressor genes

The methylation profile for each of the specimens included in this study is presented in Figure 3. The most frequently methylated promoter regions were associated with genes implicated in cell cycle control, namely p16, APC and p73. Whereas 10% of non-invasive IPMNs (including intraductal IPMNs and IPMNs with carcinoma *in situ*) harbored APC hypermethylation, nearly 50% of the infiltrative IPMNs tested positive, $P = 0.040$. Approximately 50–60% of both the

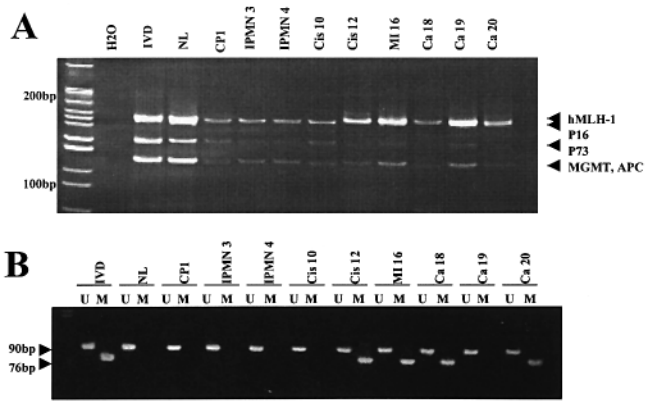

Fig. 1. Multiplex products following step one of the nested MSP approach (**A**). The multiplex products were obtained using primer sets flanking the CpG-rich promoter regions of five tumor suppressor genes (*P16*, *P73*, *APC*, *MGMT*, *hMLH-1*). Product sizes are consistent with anticipated values. Sterile water (H20) was used as the system control. *In vitro* methylated DNA (IVD) and normal human lymphocytes (NL) served as the positive and negative methylation controls, respectively. (**B**) The amplified products after step two of the nested MSP for MGMT. Lanes marked (U) contain products derived from unmethylated DNA templates; whereas, products amplified from methylated templates are found in lanes (M). Tissue samples according to histopathologic diagnosis: chronic pancreatitis (CP), intraductal IPMN (IPMN), IPMN with carcinoma *in situ* (Cis), IPMN with microinvasion <1 mm (MI) and IPMN associated with adenocarcinoma (Ca).

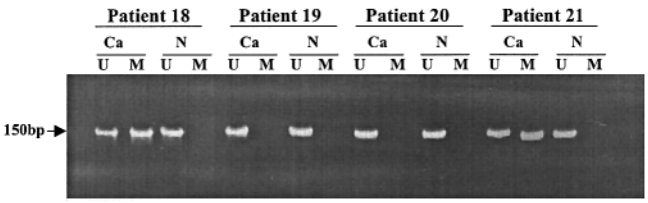

Fig. 2. The absence of methylation of p16 in histologically normal tissue (N) from the cancer-free neck margin of pancreaticoduodenectomy specimens containing an isolated, invasive IPMN (Ca). The findings of unmethylated (U) and methylated (M) alleles for p16 are shown for the separated normal and neoplastic tissues from four representative patients. An MSP product of the appropriate molecular weight (151 bp for U, 150 bp for M) indicates the presence of unmethylated and/or methylated p16 alleles in each sample.

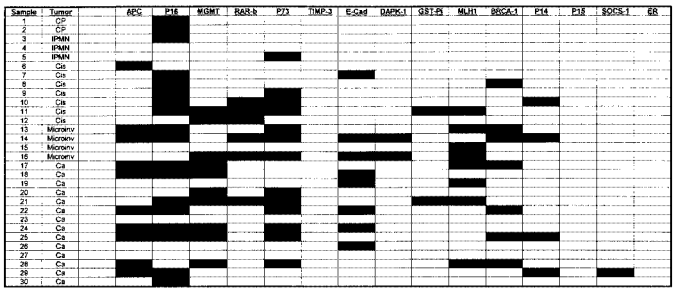

Fig. 3. Methylation profile of 15 tumor suppressor gene promoter regions involving tissue specimens of chronic pancreatitis (CP), intraductal IPMN (IPMN), IPMN with carcinoma *in situ* (Cis), IPMN with microinvasion <1 mm (MI) and IPMN associated with adenocarcinoma (Ca). Black grid squares, methylated sites. Open grid squares, unmethylated sites.

invasive and non-invasive IPMNs showed p16 methylation. The mismatch repair genes, hMLH1 and MGMT, were frequently methylated in the invasive IPMNs compared with the non-invasive tumors (38 versus 10% and 45 versus 20%, respectively), $P < 0.20$. Interestingly, a clear difference in E-cadherin methylation frequency was found between invasive

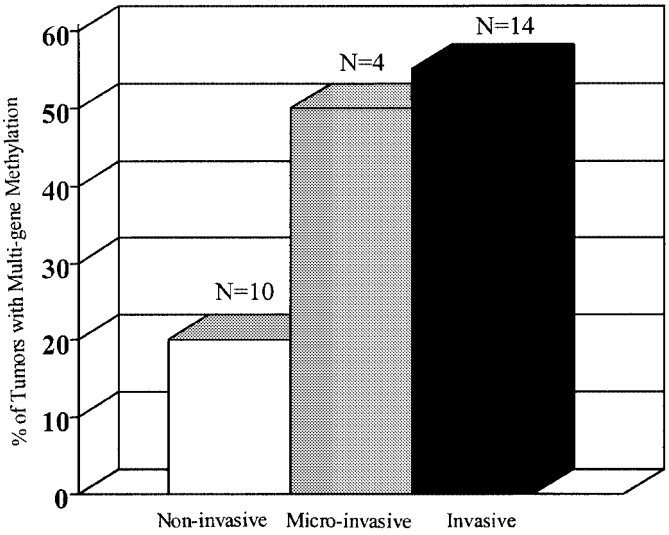

Fig. 4. The frequency of multiple gene (≥3) methylation among IPMN tumors. Non-invasive tumors include intraductal IPMN and IPMN with carcinoma *in situ.* These tumors were compared with IPMN with microinvasion <1 mm (micro-invasive) and IPMN associated with adenocarcinoma (invasive).

IPMNs (38%) and non-invasive IPMNs (10%), $P = 0.11$. Also noteworthy, E-cadherin was methylated in 50% of tumors determined histologically as IPMN with microinvasion <1 mm ($n = 4$).

Methylation differences among classes of tumor suppressor genes

Of the 15 gene loci examined in this study, interesting patterns of aberrant methylation were detected in promoter regions of genes with well-characterized roles in tumor suppression. Of the genes related to tissue infiltration and locoregional metastasis, E-cadherin methylation was found frequently in the invasive IPMNs; whereas, TIMP-3 methylation was not discovered in any of the tumors. Of the genes responsible for cell cycle control (p15, p16, p73, APC), hypermethylation of each gene locus was found in approximately half of all the IPMNs except for p15, which demonstrated no methylation at the promoter region examined. Aside from GST-p, the DNA repair genes (MGMT and hMLH1) were methylated in over 30% of all the IPMNs. As stated above, invasive IPMNs accounted for the majority of methylation among the repair genes. Methylation of any of the three DNA repair genes was detected in 45% of all the IPMNs. hMLH1 methylation, uncommon among pancreatic cancer xenografts in a study by Ueki *et al.* (16), was found in 29% of the IPMNs (mostly involving the invasive group of tumors). Interestingly, microsatellite instability has been discovered previously in IPMNs (1).

Multi-gene methylation

In comparing the frequency of methylation at multiple tumor suppressor gene loci, hypermethylation involving three or more promoter regions was present in only 20% of the non-invasive IPMNs. In comparison, 55% of the IPMN-associated adenocarcinomas were methylated at three or more gene promoters (Figure 4), $P = 0.075$. All of the non-invasive IPMNs found to have multi-gene (≥3) hypermethylation were associated with carcinoma *in situ*, as none of the intraductal IPMNs demonstrated methylation at more than one of the selected genes in this study. Despite the small number of IPMNs with microinvasion included in this study, these tumors

showed multi-gene hypermethylation more frequently than IPMNs with carcinoma *in situ.* No differences in multi-gene methylation were apparent among subpopulations of infiltrative IPMNs classified according to lymph node status.

Discussion

The orchestration of genetic and epigenetic events that underlies tumor evolution and progression in human tissues continues to be defined by the loss of tumor suppressor gene function (12). One of these epigenetic events, aberrant hypermethylation of gene promoter regions and subsequent loss of gene expression, is encountered in several human neoplasms (23,24). In this study, we characterized the methylation status of 15 candidate tumor suppressor genes within primary IPMNs of the pancreas in order to unveil a potential molecular marker that could be predictive of the biologic behavior of these peculiar tumors.

The frequency of methylation among the infiltrative IPMNs included in this investigation is consistent with that discovered in previous analyses of different gene loci in ductal adenocarcinomas of the pancreas (17). After examining nearly 50 primary adenocarcinomas, Ueki *et al.* (17) found hypermethylation of at least one CpG-rich island in 94% of the tumor specimens. In the methylation profile of seven candidate gene regions (cyclin G, ppENK, ZBP, MICP25, MICP27, MICP36, MICP38), multi-gene hypermethylation ($\geq$3 gene sites) was present in 34% of the primary cancers. In comparison to 35 pancreatic cancer xenografts, the invasive IPMNs in our study demonstrated a much higher rate of methylation for several gene loci, namely for p16 (55 versus 14%), E-cadherin (38 versus 3%), MGMT (45 versus 0%) and hMLH1 (38 versus 6%) (16). These striking differences may reflect a combination of sample size, sample population and our modification of the MSP technique to increase sensitivity; however, it could be hypothesized that epigenetic changes play a larger role than genetic mutations in the development and progression of IPMNs compared with adenocarcinomas of the pancreas. The unique behavior of IPMNs may in fact be determined by the methylation status of one or multiple tumor suppressor gene sites.

The well-defined cytologic and histologic features of IPMNs afford an opportunity to correlate methylation changes with distinct stages of tumor progression. Whereas multi-gene methylation was not found in the purely intraductal IPMNs, it was discovered frequently among IPMNs with carcinoma *in situ* (20%) and among infiltrative IPMNs associated with adenocarcinoma (55%). Interestingly, 50% of the IPMNs containing only microinvasive disease ($<$1 mm) were found to have multi-gene methylation. Although these data support a hypothetical model of 'multi-hit' methylation as an explanation for tumor progression in IPMNs, our findings only demonstrate a strong association of a molecular marker with a pathologically defined homogeneous tumor.

Previous genetic studies of IPMNs have demonstrated that patterns of allelic loss are consistent with clonal progression in these tumors (1,25,26). Fujii *et al.* (1) illustrated a stepwise increase in the loss of heterozygosity among the invasive components of IPMNs compared to the non-invasive components of the same tumors. Likewise, Zgraggen *et al.* (26) discovered a higher frequency of K-ras mutations with each stage of IPMN tumor progression. In this study, we demonstrated that the prevalence of epigenetic changes, namely methylation of CpG islands of selected tumor suppressor genes, increases with advanced stages of IPMN progression.

In characterizing the methylation status of multiple tumor suppressor genes in progressive stages of IPMNs, we sought to determine some of the epigenetic changes contributing to the molecular events responsible for the clinical and biological behavior of this uncommon, albeit interesting, pathological entity. Furthermore, methylation at specific genomic sites may predispose patients to tumor recurrence after complete surgical extirpation and may be predictive of regional and/or systemic metastasis. The methylation profile of the surgical resection margin in the absence of histologic disease may be used as a biologic marker for detecting a remnant pancreas at risk for tumor recurrence or for harboring multi-focal disease throughout the gland.

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