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Epigenetic alterations in intraductal papillary mucinous neoplasms of the pancreas

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Abstract
Intraductal papillary mucinous neoplasm (IPMN), an increasingly recognized cystic neoplasm of the pancreas with a broad spectrum of malignant potential, has been considered a precursor to infiltrating ductal adenocarcinoma. Because of its unique clinical, radiological, pathological, and molecular features, IPMN has attracted considerable interest among clinicians and researchers. Although some genetic alterations have been described in IPMNs, the molecular features that characterize the evolution and progression of these neoplasms are largely unknown. Recent studies have shown that aberrant methylation of the promoter cytosine-phospho-guanine (CpG) island is a common mechanism associated with the silencing of tumor-suppressor and cancer-related genes in IPMNs. Importantly, the prevalence of such methylation increases along with the grade of neoplasia, suggesting that these epigenetic events may contribute to the progression of IPMNs. Further studies of epigenetic alterations in IPMN will shed light on the molecular pathogenesis of this unique neoplasm and lead to the identification of epigenetic markers that can be applied in the clinical setting.

Key words Epigenetics · Hypermethylation · IPMN · Precursor · Pancreatic cancer

Introduction

Intraductal papillary mucinous neoplasm (IPMN), originally known from its peculiar endoscopic finding of mucin extrusion through an enlarged orifice of the ampulla of Vater,[1,2] is a mucin-producing cystic neoplasm of the pancreas with unique clinicopathological features.[3–17] IPMN is, by definition, a grossly visible noninvasive neoplasm that arises in the main pancreatic duct or its major branches.[15,18] This distinguishes IPMNs from pancreatic intraepithelial neoplasias (PanINs), which are smaller (<5 mm) and usually involve small branch ducts.[18] In addition, IPMNs lack the ovarian stroma characteristic of mucinous cystic neoplasms. The papillary epithelial component of IPMNs, the degree of mucin production, and the cystic dilatation are variable. Although IPMNs are, by definition, noninvasive neoplasms, they are often diagnosed in the setting of an associated invasive adenocarcinoma.[11–13,19–21] The majority (around 70%) of IPMNs arise in the head of the pancreas, although they also arise in the body or tail of the pancreas and, in some cases, they diffusely involve the entire gland.[22] IPMNs are being identified with increased frequency and now account for around 20% of all pancreatic resections at many academic centers.[22] It is also notable that some IPMNs arise in association with inherited syndromes such as Peutz-Jeghers syndrome[23] and familial adenomatous polyposis (FAP).[24]

Clinically, most IPMNs are less aggressive and survival after surgical resection is better than that for conventional ductal adenocarcinoma.[10–14,20,21] However, a subset of patients with IPMNs experience recurrence or develop disseminated pancreatic adenocarcinoma after surgical resection and die of their disease, especially when their IPMNs have an associated infiltrating carcinoma.[21,25,26] Importantly, an associated invasive carcinoma is sometimes found in a distant location of the pancreatic gland where no IPMN is identifiable, suggesting that IPMNs serve not only as a precursor to invasive carcinoma but also as a predictor of an independent invasive carcinoma.[27] Recent screening studies performed on asymptomatic individuals at high risk for developing pancreatic neoplasia suggest that many individuals with an inherited susceptibility to develop pancreatic ductal adenocarcinoma may initially develop IPMNs prior to developing invasive pancreatic adenocarcinoma.[28,29] These screening studies have identified six patients with IPMN (including one patient who also

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had a microinvasive adenocarcinoma) and all six patients underwent curative resection. These observations together highlight the importance of IPMN as a detectable precursor to invasive pancreatic adenocarcinoma.

In contrast to our increasing knowledge about the clinical and pathological manifestations of IPMNs, the molecular background underlying these neoplasms remains poorly understood. Several studies have identified genetic alterations, such as those seen in invasive pancreatic ductal adenocarcinoma, in IPMN; however, the prevalence of such genetic events is generally lower than that in conventional ductal adenocarcinoma.[9] The infrequent genetic alterations in IPMNs may partly reflect the fact that IPMNs are noninvasive neoplasms, but available evidence suggests that IPMNs have a lower prevalence of known genetic alterations than another form of noninvasive ductal lesions, PanINs.[30] These findings raise the possibility that alternative mechanisms of tumor-suppressor gene inactivation, such as promoter cytosine-phospho-guanine (CpG) island hypermethuylation,[31] play an important role in the development of IPMNs. Although a number of studies have revealed frequent alterations in DNA methylation (aberrant hypermethylation and hypomethylation) in infiltrating pancreatic ductal adenocarcinoma,[32–41] the demonstration of frequent epigenetic alterations in IPMNs has only been described recently.[42,43] In this article, we will briefly review recent advances in our understanding of the molecular events that occur in IPMNs, with special attention paid to epigenetic alterations.

Genetic alterations in IPMNs

Several investigators have studied a series of IPMNs for genetic alterations that have been previously identified in invasive pancreatic adenocarcinoma. Z'graggen et al.[44] found activating point mutations in the *K-ras* oncogene in at least one of the microdissected lesions from 13 (81%) of 16 IPMNs, with a stepwise increase in the frequencies from papillary hyperplasia (adenoma), to low-grade dysplasia (borderline), and to carcinoma in situ and invasive carcinoma. Fujii et al.[45] analyzed 13 IPMNs for polymerase chain reaction (PCR) amplification of multiple microsatellite markers and found frequent loss of heterozygosity (LOH) at several chromosomal loci, including 6q (54%), 8p (31%), 9p (62%), 17p (38%), and 18q (38%), suggesting that inactivation of the *p16* gene (at chromosome 9p), the *p53* gene (at 17p), and the *DPC4/SMAD4* gene (at 18q) may occur in these neoplasms. However, it appears that biallelic genetic inactivation of these tumor-suppressor genes occurs less frequently in IPMNs than it does in ductal adenocarcinomas. For example, mutations in the *p53* tumor-suppressor gene were detected in only 8% of IPMNs[46] (compared to around 75% in invasive pancreatic adenocarcinoma).[47] Loss of DPC4/SMAD4 protein expression, a surrogate marker for *SMAD4* genetic inactivation, which has been observed in more than 50% of invasive adenocarcinomas[48] and 30% of PanIN-3 lesions,[49] is rarely seen in noninvasive IPMNs and remains an infrequent event (around 15%) even among IPMNs with invasive adenocarcinoma.[50–52] In support of this finding, Inoue et al.[53] reported no mutation of the *DPC4/SMAD4* gene in 18 IPMNs. It has also been shown that LOH at 19p13.3, the *STK11/LKB1 Peutz-Jeghers* gene locus, is common (>30%) in IPMNs.[23] A recent immunohistochemical study has also demonstrated that inactivation of STK11/LKB1 is likely to be more common in IPMNs than in pancreatic ductal adenocarcinoma.[54]

Global analysis of gene expression revealed the overexpression of a number of genes (including *lipocalin 2*, *galectin 3*, *claudin 4*, *cathepsin E*, and trefoil factor family [*TFF1*, *TFF2*, and *TFF3*]) in IPMNs.[54] A comparable gene expression analysis of IPMNs with and without an associated invasive carcinoma identified a subset of genes (such as *claudin 4*, *CXCR4*, *S100A4*, and *mesothelin*) associated with the invasive phenotype of these neoplasms.[56] That study also identified a number of genes differentially expressed in IPMNs that have not been implicated in invasive ductal adenocarcinomas,[56] suggesting a specific gene expression signature that characterizes IPMNs. These findings may lead to a hypothesis that the molecular targets in IPMNs differ from those in infiltrating ductal adenocarcinoma. Alternatively, it is possible that other molecular mechanisms, such as epigenetic alterations, could play a role in the pathogenesis of IPMNs; this possibility has been recently explored by us and other investigators.

Aberrant DNA methylation in IPMNs

Using methylation-specific PCR (MSP), we analyzed a total of 51 IPMNs with different histological grades for the methylation status of seven CpG islands (including *p16* and preproenkephalin [*ppENK*], which encodes for a native opioid peptide with tumor-suppressor properties) previously identified as aberrantly methylated in pancreatic adenocarcinoma.[42] We found that aberrant hypermethylation of at least one of these CpG islands was detected in a majority (more than 80%) of the IPMNs. In most of the CpG islands analyzed (including *p16*), the methylation frequencies in IPMNs were similar to or slightly lower than those in invasive pancreatic adenocarcinomas.[33,34] Importantly, hypermethylation of *ppENK* and *p16* was detected at a significantly higher frequency in high-grade (in situ carcinoma) IPMNs than

in low-grade (adenoma/borderline) IPMNs, and the overall number of methylated loci was significantly higher in high-grade IPMNs than in low-grade IPMNs. We also demonstrated that aberrant methylation was indeed associated with loss of expression in IPMNs; for example, loss of nuclear staining of p16 protein was detected in 5 of 6 (83%) IPMNs with methylated *p16* but in only 8 of 33 (24%) IPMNs with unmethylated *p16* ($P = 0.01$). These findings are the first to demonstrate that aberrant methylation of promoter CpG islands is a common event in IPMNs, and suggest that the methylation-associated silencing of *p16*, *ppENK*, and other genes may contribute to the malignant transformation of IPMNs.

Another group examined the methylation status of a panel of 15 genes (including *p14*, *p15*, *p16*, *p17*, *APC*, *hMLH1*, *E-cadherin*, and others) in 28 IPMNs (10 noninvasive and 18 invasive IPMNs).[43] Using a modified (nested, two-step) MSP, the authors of that study demonstrated a high prevalence of aberrant methylation in their series of IPMNs (methylation of at least one of the markers analyzed in 92% of IPMNs) with some of the genetic loci (such as *APC*) being more frequently methylated in invasive IPMNs than in noninvasive IPMNs. In agreement with our study, they also showed that the prevalence of methylation of multiple genes (3 or more) was increased from 20% in noninvasive IPMNs to 55% in invasive IPMNs. However, more methylation was detected at several genetic loci (such as *p16*, *E-cadherin*, *MGMT*, and *hMLH1*) in their series than in our series of IPMNs and invasive pancreatic adenocarcinoma, probably due to differences in the number of samples analyzed, primer location, and MSP assays.

Other genes have also been identified as aberrantly methylated in IPMNs, at varying frequencies; these genes include *cyclin D2* (50%),[57] *SOCS-1* (6%),[58] and *TFPI-2* (60%).[40] Importantly, *TFPI-2* (tissue factor pathway inhibitor 2), encoding a broadspectrum serine proteinase inhibitor, was aberrantly methylated at a significantly higher frequency in high-grade (carcinoma in situ) IPMNs than in low-grade (adenoma/borderline) IPMNs (85% vs 17%; $P = 0.0002$).[40] Restored expression of *TFPI-2* by stable gene transduction in pancreatic cancer cells lacking *TFPI-2* expression resulted in marked suppression of their proliferation, migration, and invasiveness. We have also demonstrated that, in a subset of pancreatic cancers, LOH at the *TFPI-2* gene locus (7q22 region) is associated with complete methylation (but not mutation) of the remaining allele, raising the possibility of two-hit inactivation through LOH and methylation. These results suggest that epigenetic alterations could play a major role in the neoplastic development of IPMNs.

Recently, microarray-based expression profiling has been used in identifying genes affected by aberrant DNA methylation in cancer.[36,59,60] We used oligonucleotide microarrays (Affymetrix; Santa Clara, CA, USA) to identify genes that were specifically downregulated in IPMNs compared to normal pancreatic ductal epithelium.[61] Using this approach, we identified a large panel of underexpressed genes in IPMNs (http://pathology2.jhu.edu/pancreas/IPMNdown300/index.htm), some of which may be associated with epigenetic mechanisms. One of the genes identified was the cyclin-dependent kinase inhibitor *CDKN1C/p57KIP2*, which was demonstrated to be underexpressed at both the transcriptional and the protein levels in a significant proportion of IPMNs. We further investigated the mechanisms for the *CDKN1C* downregulation in IPMNs and in pancreatic cancer cell lines, and our results revealed the potential mechanism to be a combination of DNA methylation, histone deacetylation, and loss of the maternal allele at 11p15.5 expressing *CDKN1C*. This study suggests that gene expression profiling using microarrays may help to identify potential targets affected by epigenetic alterations in IPMNs.

Clinical implications of aberrant methylation in IPMNs

Because IPMN has a diverse spectrum of biological and clinical behaviors, accurate diagnosis and preoperative assessment of the neoplastic grade of IPMNs is critical to determine the optimal management for these patients. It is still difficult, however, with currently available imaging techniques, conventional cytology in pancreatic juice, and serum tumor markers (such as carbohydrate antigen [CA]19-9) to adequately differentiate between benign and malignant IPMNs or to detect an associated invasive carcinoma preoperatively.[61–64] Only a few molecular markers (such as *K-ras* gene mutations and telomerase activity) have been evaluated in an attempt to improve the diagnosis of IPMNs.[66,67]

As has been proposed for other cancers,[68,69] the detection of aberrantly methylated genes in secondary sources (such as pancreatic juice obtained endoscopically) may be useful in refining the preoperative diagnosis or the postoperative follow-up of patients with IPMNs. For example, genes frequently methylated in IPMNs but not methylated in either normal pancreatic ductal epithelium or in non-neoplastic cystic lesions (pseudocysts) could potentially be used to diagnose IPMNs or to monitor patients for recurrent disease following surgery, while genes preferentially methylated in IPMNs with an associated invasive carcinoma (invasive IPMNs) could be used preoperatively to predict the presence of such an invasive focus in IPMNs. Indeed, we have been able to detect methylated DNA in the pancreatic juice samples of patients with high-grade

IPMNs; in our preliminary MSP analyses of pancreatic juice samples collected during surgery, methylated *ppENK* was detected in four (44%) of nine juice pancreatic samples from patients with high-grade (carcinoma in situ) IPMNs (two of the four IPMNs had an associated invasive carcinoma), but in none of two pancreatic juice samples from patients with low-grade (borderline) IPMNs.[70] Furthermore, a recent study analyzing multiple methylation markers in a large series of pancreatic juice samples, using quantitative MSP (QMSP), revealed that the amount of methylation tended to increase with the grade of IPMN; more methylated genes were detected in pancreatic juice samples from high-grade IPMNs than in the samples from low-grade IPMNs.[71] These findings suggest that the quantification of methylated DNA in pancreatic juice could be useful as an aid to differentiate invasive IPMNs from noninvasive IPMNs preoperatively. Further studies are needed to determine how epigenetic markers identified in IPMNs can be best applied in the clinical setting.

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