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Short Communication

Loss of Dpc4 Expression in Colonic Adenocarcinomas Correlates with the Presence of Metastatic Disease

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DPC4* is a candidate tumor suppressor gene on chromosome 18q21, a region that shows high frequencies of allelic losses in pancreatic and colorectal adenocarcinomas. Biallelic inactivation of *DPC4* has been reported in half of pancreatic cancers, but are relatively infrequent in other tumor types. The role of *DPC4* inactivation in colorectal neoplasms has not been fully characterized. An immunohistochemical assay for Dpc4 protein expression has been recently developed and shown to be a sensitive and specific surrogate for alterations in the *DPC4* gene. In this study we examined the expression of Dpc4 protein in formalin-fixed archival tissue from 83 colorectal lesions, including 19 adenomas and 64 sporadic adenocarcinomas (11 stage I, 13 stage II, 17 stage III, and 23 stage IV cancers). None of the adenomas or stage I adenocarcinomas showed loss of Dpc4 expression, whereas one of 13 (8%) stage II, one of 17 (6%) stage III, and five of 23 (22%) of stage IV cancers showed loss of Dpc4 expression. There was a borderline significant difference in loss of Dpc4 reactivity in colorectal tumors with distant metastasis at presentation (22%) *versus* primary tumors without distant metastasis (5%) (Fisher's exact test, $P = 0.05$; $\chi^2 = 0.04$). Poorly differentiated histology or status of pericolonic lymph nodes did not affect Dpc4 expression. Alterations in *DPC4* are involved in the progression of a subset of colorectal carcinomas, especially those that present with advanced disease. In the sequential pathogenesis of colorectal tumors, inactivation of *DPC4* is likely to be a late event. *(Am J Pathol 2000, 157:1105–1111)

The DPC4 gene (for deleted in pancreatic carcinoma, locus 4) has been identified as a candidate tumor suppressor gene in pancreatic adenocarcinomas.[1,2] The *DPC4* gene is located on chromosome 18q21.1, a region that also contains the deleted in colon carcinoma (*DCC*) gene,[3] and is characterized by high frequency of allelic losses in pancreatic and colorectal carcinomas.[4–6] Inactivation of *DPC4* can occur by one of two identified mechanisms: a) intragenic mutation of one allele coupled with loss of the other allele, or b) deletion of both alleles (homozygous deletions). Both mutations and homozygous deletions of the *DPC4* gene have been observed in a high proportion of pancreatic carcinomas.[7] Germ-line mutations of *DPC4* were recently described in familial juvenile polyposis,[8–10] a condition that predisposes to colorectal cancer, and Dpc4 knockout mice have been reported to develop gastrointestinal polyps resembling juvenile polyps.[11] Moreover, compound mutant mice with both Dpc4 and Apc mutations have demonstrated a significant contribution of loss of Dpc4 function to progression of colorectal cancers.[12] In contrast, the role of *DPC4* in human colorectal cancers remains less well defined. Although as many as 60% of colorectal cancers show loss of heterozygosity (LOH) at the 18q21.2 locus,[6] only ~14% show mutations or homozygous deletions of *DPC4* (Table 1). It is possible that additional epigenetic mechanisms such as promoter hypermethylation[13] may contribute to inactivating the second *DPC4* allele in a subset of cases, but the frequency of this pathway has not been reported so far.

Recently, an immunohistochemical assay has been developed for the determination of Dpc4 expression in archival paraffin-embedded tissues.[14,15] The advantages of an immunohistochemical assay are: 1) down-regulation of protein expression can be directly measured irrespective of mechanism(s) of inactivation; 2) it can be applied to a large number of archived tissue samples in a routine manner; and 3) the specific cell types expressing the protein of interest can be assessed by morphol-

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Table 1. Review of Literature on *DPC4* Alterations in Colorectal Neoplasms

Author (reference)	System	Total tumors examined (*n*)	*DPC4* alteration (%)
Thiagalingam et al[6]	Xenografts	18	6 (33%)
MacGrogan et al[29]	Cell lines and primary tumors	17	4 (23%)
Takagi et al[30]	Primary tumors	31	5 (16%)
Akiyama et al[31]	Primary tumors	30	4 (13%)
Koyama et al[32]	Primary tumors	64	7 (11%)
Tarafa et al[33]	Xenografts	13	4 (31%)
Lei et al[34]	Primary tumors	10	0 (0%)
Hoque et al[35]	Primary tumors	6	1 (16%)
Miyaki et al[36]	Primary tumors	176	21 (12%)
Total		365	52 (14.2%)

ogy. In pancreatic adenocarcinomas, immunohistochemical labeling for Dpc4 has been found to be an extremely sensitive (91%) and specific (94%) surrogate for *DPC4* genetic alterations.[14] We investigated the expression of Dpc4 protein in 83 colorectal lesions, both adenomas and carcinomas, and correlated loss of Dpc4 expression with the stage of the lesion at presentation. This is the first study to systematically examine Dpc4 expression in a series of colorectal neoplasms that have been stratified by stage.

Materials and Methods

Eighty-three cases of colorectal lesions were retrieved from the surgical pathology archives of Parkland Memorial and Zale Lipshy Hospitals, affiliated with the University of Texas Southwestern Medical Center, Dallas. Clinicopathological correlates such as age, sex, site and size of lesions, pathological stage at presentation, histological grade, and presence of lymph node and distant metastases were retrieved from the corresponding surgical pathology reports. The pathological staging designated in the individual cases were jointly verified by two of the authors (AM and GL). The carcinomas were staged using criteria defined by the American Joint Committee on Cancer for staging of colorectal cancers.[16] Multiple hematoxylin and eosin-stained slides from each case were screened by light microscopy for selection of sections with both neoplastic and nonneoplastic colonic tissue. Unstained 5-μm sections were cut from the formalin-fixed paraffin-embedded block selected in each case, and deparaffinized by routine techniques. The slides were treated with sodium citrate buffer (Ventana BioTek Solutions, Tucson, AZ) and steamed at 80°C for 20 minutes. After cooling for 5 minutes, the slides were labeled with monoclonal antibody to Dpc4 (1:100 dilution, clone B8; Santa Cruz Biotechnology, Santa Cruz, CA) using the Bio TekMate 1000 automated immunostainer (Ventana). The detection step was performed by streptavidin-biotin labeling followed by counterstaining with hematoxylin. Positive and negative controls were analyzed in each run, with formalin-fixed normal pancreatic tissue being used as positive control. Normal pancreatic ducts show strongly positive Dpc4 reactivity, whereas moderate expression is present in the acini, islets of Langerhans, stromal fibroblasts, and lymphocytes.[14]

The immunohistochemical stains were independently evaluated by two of the authors (AM and GL). When present, Dpc4 is most often seen in the cytoplasmic compartment of cells, with focal nuclear staining only. A two-tier scoring system (positive and negative) was used for analysis, with negative labeling being defined as absence of Dpc4 expression in both the cytoplasmic and nuclear compartments. Positive labeling was defined as either diffuse expression of Dpc4 in the cytoplasm of neoplastic cells, with concomitant focal expression in nuclei or the presence of two distinct populations of cells, those that labeled with the antibody and those that did not. In other words, no distinction was made between diffuse- and focal-positive categories. This was primarily because in our study, the proportion of cases with focal-positive staining were too few to be analyzed separately, a finding also seen in a previous study on pancreatic adenocarcinomas.[14] Nonneoplastic colonic epithelium, stromal fibroblasts, and lymphoid aggregates, all of which expressed Dpc4 with a moderate to strong intensity, served as internal positive controls. The 83 colorectal lesions (including 11 cases in which metastatic lesions were examined simultaneously) were then stratified by stage at presentation and compared with regards to the presence or absence of Dpc4 expression.

Statistical analysis was performed using the Fisher's exact probability test and chi-square analysis using the SAS statistical software (Cary, NC), to determine whether loss of Dpc4 reactivity in colorectal tumors with distant metastases *versus* localized primary tumors was significantly different.

Results

Clinicopathological Parameters

The clinical and pathological data in the 83 patients whose colorectal lesions were analyzed in this study are summarized in Table 2. The patients were stratified into five subgroups for analysis: 19 patients were diagnosed with benign colorectal adenomas, whereas 64 patients had

Table 2. Clinical and Pathological Features in 83 Patients with Colorectal Lesions

	n	Sex ratio	Age range (mean)	Location (L:R:T)	Size range, cm (mean)	Histology	LN status	Distant metastasis
Overall	83	1:1.5	41–88 (65.1)	4.9:3.3:1	1–13 (4.2)	64 carcinomas 55G2: 9G3	6n0: 20N1: 14N2 (stage III–IV only)	23 metastases (stage IV only)
Adenomas	19		50–79 (62.5)	3.7:1:1.7	1–3 (1.3)	14T: 5TV	All N0	NA
Stage I	11		44–88 (67.8)	5:5:1	1–9 (3.6)	10G2: 1G3	All N0	NA
Stage II	13		46–85 (66.6)	1:1:0	3–12 (5.4)	12G2: 1G3	All N0	NA
Stage III	17		57–84 (68.8)	2.5:4.5:1	1–8 (4.5)	12G2: 5G3	12N1: 5N2	NA
Stage IV	23		41–85 (62.6)	16:6:1	3–13 (6.0)	21G2: 2G3	6N0: 8N1: 9N2	22 Liver; 1 uterus and ovaries

G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; N1, 1 to 3 pericolonic lymph nodes involved; N2, ≥4 pericolonic lymph nodes involved; L:R:T, left:right:transverse colon.

colorectal cancers (stage I , 11 patients; stage II, 13 patients; stage III, 17 patients; and stage IV, 23 patients). There were 33 males and 50 females (M:F ratio, 1:1.5), with an age range of 41 to 88 years (mean, 65.1 years). The mean ages for the five patient subgroups are summarized in Table 1. To the best of our knowledge, none of the 83 patients was diagnosed with familial adenomatous polyposis. There were 44 left-sided, 30 right-sided, and nine transverse colonic lesions (overall ratio, 4.9:3.3:1). The adenomas ranged in size from 1 to 3 cm (mean, 1.3 cm), whereas the carcinomas ranged in size from 1 to 13 cm (mean, 5.0 cm), with an overall mean of 4.2 cm. There were 14 tubular adenomas and five tubulovillous adenomas, two of which had high-grade dysplasia.The majority (55 of 64) of the carcinomas were moderately differentiated adenocarcinomas (G2), whereas nine were poorly differentiated (G3). Of the 40 stage III to IV cancers, six did not have lymph node metastasis in the pericolonic lymph nodes examined (N0), whereas 20 and 14 tumors, respectively, had less than four (N1) and greater than or equal to four (N2) involved lymph nodes in the resected specimen. There were 23 patients with distant metastases (stage

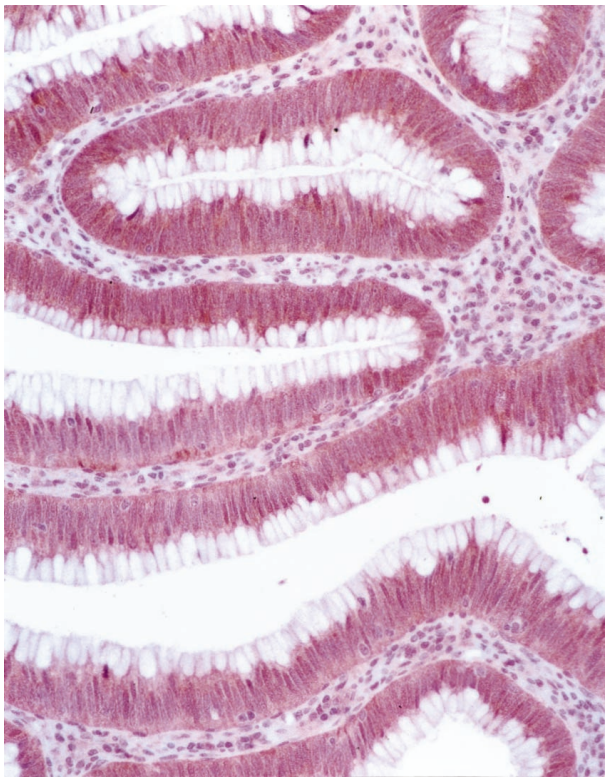

Figure 1. Expression of Dpc4 in tubular adenoma, hematoxylin counterstain. Original magnification, ×40.

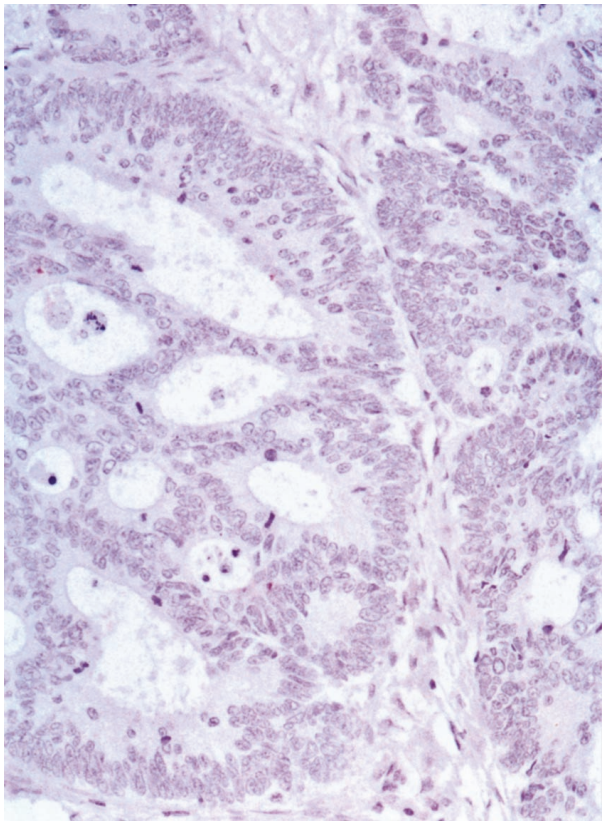

Figure 2. Loss of expression of Dpc4 in primary adenocarcinoma of the colon, hematoxylin counterstain. Original magnification, ×40.

Table 3. Immunohistochemical Expression of Dpc4 in 83 Colorectal Lesions

	Dpc4-positive	Dpc4-negative
Overall	76/83 (92%)	7/83 (8%)
Adenomas	19/19 (100%)	0
Stage I	11/11 (100%)	0
Stage II	12/13 (92%)	1/13 (8%)
Stage III	16/17 (94%)	1/17 (6%)
Stage IV	18/23 (78%)	5/23 (22%)

IV), 22 of which occurred in the liver. All patients with stage IV cancers had documentation of metastasis by pathological examination at the time of primary resection.

Immunohistochemical Analysis and Statistical Correlation

The results of immunohistochemical analysis are summarized in Table 3. Nonneoplastic colonic mucosa in all patients examined showed strong to moderate immunoreactivity for Dpc4.3 Similarly, 19 of 19 (100%) adenomas and 11 of 11 (100%) stage I lesions had positive Dpc4 expression in the neoplastic cells (Figure 1). In contrast, one of 13 (8%) stage II, one of 17 (6%) stage III, and five of 23 (22%) stage IV lesions showed loss of Dpc4 immunoreactivity in the tumor cells (Figures 2 and 3). Overall, three lesions had focal Dpc4 reactivity: two stage IV carcinomas had ~5% weakly positive cells whereas 95% of the tumor cells were nonreactive—these two cases were classified as negative for statistical analysis. In one stage III carcinoma, there were nearly equal proportions of totally negative and strongly positive islands of tumor cells—this case was classified as positive for statistical analysis. In 11 stage IV cancers, the concomitant metastatic tumors in the liver were also analyzed for Dpc4 expression. Ten of 10 (100%) lesions showed persistence of Dpc4 expression in both the primary and metastatic tumors, whereas one lesion showed loss of expression at both sites (Figure 4). Moderate Dpc4 expression was seen in the surrounding hepatocytes in all metastatic sections.

Table 4 summarizes the clinicopathological data in the seven cases with loss of Dpc4 expression. The presence of poorly differentiated histology did not affect Dpc4 expression, but this could be because of an overrepresentation of moderately differentiated adenocarcinomas in our series (Table 2). Similarly, no trend was seen between loss of Dpc4 expression and the presence or absence of nodal metastasis in the pericolonic lymph nodes, with both N0 and N1/N2 lesions showing loss of Dpc4 expression. There was a borderline statistically significant difference in loss of Dpc4 expression in colorectal carcinomas that presented with distant metastasis (5 of 23 or 22%) *versus* colorectal cancers without distant metastasis (2 of 41 or 5%) (Fisher's exact test, $P = 0.05$, chi-square = 0.04). There was a statistically significant difference in loss of Dpc4 expression in colorectal carcinomas that presented with distant metastasis *versus* all localized colorectal neoplasms, including adenomas

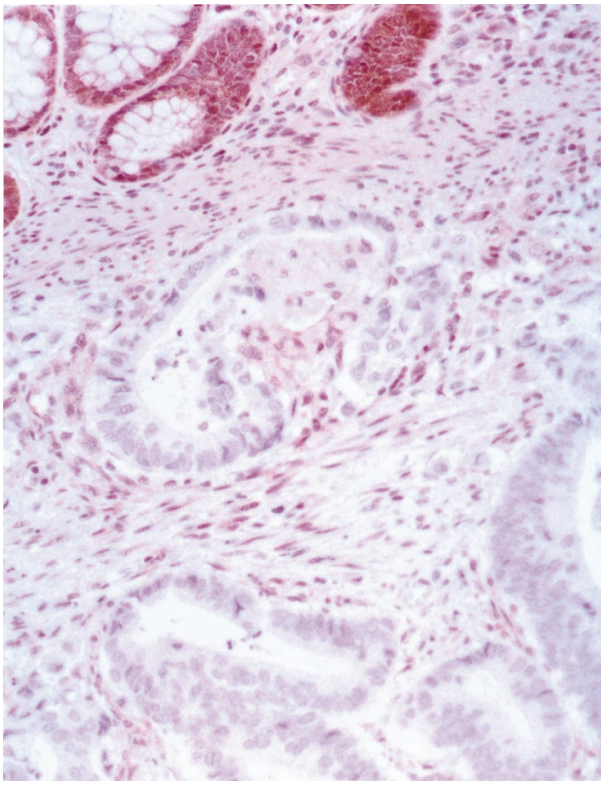

Figure 3. Loss of expression of Dpc4 in primary adenocarcinoma of the colon, with retention of strong immunoreactivity in the adjacent normal colonic mucosa, hematoxylin counterstain. Original magnification, ×40.

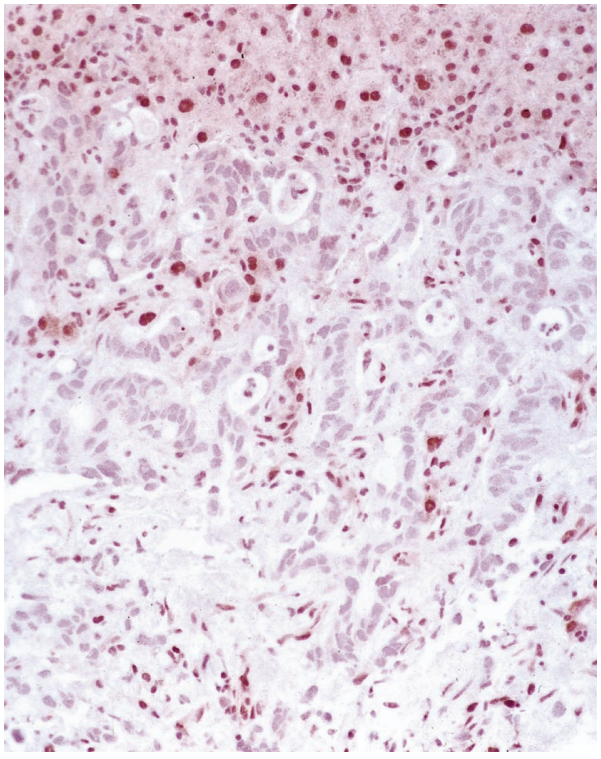

Figure 4. Loss of expression of Dpc4 in metastatic colon cancer, hematoxylin counterstain. Original magnification, ×40. Adjacent hepatocytes show Dpc4 immunoreactivity.

Table 4. Clinicopathological Characteristics of Seven Colorectal Tumors with Loss of Dpc4 Expression

	Stage	Age/Sex	Site	Size	Histology	LN status	Metastasis
Case 1	IV	59/F	Left	5	G2	N1	Liver
Case 2	IV	43/F	Right	10	G2	N0	Uterus/ovaries
Case 3	IV	56/M	Left	3	G2	N2	Liver
Case 4	IV	62/M	Left	5	G2	N1	Liver
Case 5	IV	64/M	Left	6	G2	N0	Liver
Case 6	III	75/F	Transverse	4	G2	N1	NA
Case 7	II	85/M	Left	4	G2	NA	NA

G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; N1, 1 to 3 pericolonic lymph nodes involved; N2, ≥4 pericolonic lymph nodes involved; L:R:T, left:right:transverse colon; NA, not applicable.

(2 of 60 or 3%) (Fisher's exact test, $P = 0.01$, chi-square = 0.007).

Discussion

Colorectal cancer accounted for an estimated 129,400 new cases in 1999, including 94,700 of colon cancer and 34,700 of rectal cancer (http://www.cancer.org/). Colorectal cancer is the third most common cancer in men and women. An estimated 56,600 deaths from colorectal cancers occurred in 1999, accounting for ~10% of cancer deaths (http://www.cancer.org/). Most colorectal carcinomas develop by a pathway of sequential progression from adenomas.[17] Considerable data has accumulated throughout the past decade on the underlying genetic changes that accumulate during the multistep pathogenesis of colorectal neoplasms, primarily based on studies in the two major forms of hereditary colon cancers: familial adenomatous polyposis and hereditary nonpolyposis colorectal cancers. It is now known that mutations of the *Ki-ras* oncogene,[18] *APC* (adenomatous polyposis coli gene),[19,20] *p53*,[5,21] and *DCC* (located at 18q21)[3,22] are important genetic events in the stepwise progression of colorectal cancers. The 18q21 region is thought to be particularly important locus because as many as 60% of colorectal cancers show LOH at this locus.[6] Despite the high proportion of allelic losses however, there have been few reports of inactivating mutations of *DCC* in these tumors. In 1996, a second candidate tumor suppressor gene, *DPC4*, was cloned in the vicinity of *DCC* raising the possibility that it may be the target of allelic losses at 18q21.[1] The product of *DPC4* belongs to the evolutionary conserved family of SMAD proteins which are involved in transforming growth factor-β signal transudation pathways.[23,24] Unlike pancreatic adenocarcinomas, where 50% of tumors show complete loss of *DPC4* function (20% mutations and 30% homozygous deletions),[4,7] biallelic inactivation of *DPC4* seems to be infrequent in other tumor types.[2,25–28]

In the context of colorectal cancers, Thiagalingam et al[6] reported mutations of *DPC4* in four of 18 xenografted tumors, and homozygous deletions in two additional tumors, that also spanned the *DCC* gene. Since that time, several investigators have examined the role of DPC4 in colorectal cancers at the genomic and transcriptional levels (Table 1). MacGrogan et al[29] described altered *DPC4* sequences in three of 12 colon cancer cell lines and one of five primary tumors, whereas Takagi et al[30] found mutations of *DPC4* in five of 31 (16%) primary tumors. Akiyama et al[31] described *DPC4* alterations in four of 30 (13%) of early superficial colorectal cancers; interestingly none of their cases harbored either *DCC* or smad2 alterations, despite a 65% frequency of LOH at 18q21. Similarly, Koyama et al,[32] who found no more than seven of 64 (11%) of tumors with *DPC4* mutations despite a 78% LOH at 18q21 in their stage II and III colorectal cancers. Tarafa et al[33] found loss of *DPC4* expression by reverse transcriptase-polymerase chain reaction in four of 13 (31%) colorectal xenografts; two tumors contained homozygous deletions and two had coding region mutations. In contrast, Lei et al[34] found no mutations in the coding region of *DPC4* in 10 cases of colorectal cancers arising in the background of ulcerative colitis, whereas Hoque et al[35] reported biallelic inactivation of *DPC4* in one of six colitis-associated neoplasia. A systematic analysis of *DPC4* alterations in progressive stages of colorectal tumors was performed by Miyaki et al[36] in 176 colorectal carcinomas, including 36 metatstatectomy specimens. The authors found *DPC4* mutations in six of 17 (35%) primary carcinomas with distant metastases, compared with 0%, 10%, and 7% *DPC4* mutations in adenomas, intramucosal carcinomas, and primary carcinomas without distant metastases, respectively. *DPC4* mutations were also present in 11 of 36 (31%) distant metastases, including four cases where both the primary and metastasis harbored identical mutations.[36] This was one of the strongest evidences to date that *DPC4* is a true target of inactivation in colorectal cancers, and loss of *DPC4* function correlated with advanced stages of malignancy, such as distant metastasis. Combining the data from literature on *DPC4* inactivation in colorectal neoplasms (including cell lines, xenografts, and primary tumors of all stages) yielded a total of 52 of 365 (14.2%) tumors that have shown genetic alterations of *DPC4* at the DNA and/or RNA level (Table 1).

We analyzed the expression of Dpc4 protein in a series of 83 colorectal neoplasms (19 adenomas, 11 stage I, 13 stage II, 17 stage III, and 23 stage IV) using a recently developed immunohistochemical assay applicable to paraffin-embedded material. The Dpc4 immunohistochemical assay has been shown to have a high degree of sensitivity and specificity for alterations in *DPC4* gene.[14] We found loss of Dpc4 expression in 8% of colorectal neoplasms, all of which were limited to cancers that were stage II or higher. Although this is lower than the proportion of tumors calculated on the basis of literature review

(Table 1, 14.2%), our series also includes adenomas and low-stage carcinomas, whereas many of the previous studies were performed on xenografts, cell lines, or advanced stage tumors. Both xenografts and cell lines have a potential for selective propagation of aggressive clones or acquisition of additional genetic changes during culture, including those of *DPC4*.[37–39] When stratified by stage, the highest percentage of loss of Dpc4 reactivity was found in stage IV cancers with distant metastasis (22%), compared with 0% in stage I, 8% in stage II, and 6% in stage III cancers (Fisher's exact test, $P = 0.05$, chi-square = 0.04).

The figures in our study correlated fairly well with what has been reported by Miyaki et al[36] in the stage-wise progression of colorectal cancers, although they had a slightly higher frequency of *DPC4* mutations in stage IV cancers. Taken in conjunction, these findings suggest that inactivation of *DPC4* 1) is a late event in colorectal carcinogenesis, and 2) might be permissive for acquisition of a metastatic phenotype in these tumors. Interestingly, we failed to find loss of Dpc4 expression in distant metastatic deposits from 10 lesions in which the primary tumor also expressed Dpc4. In one case, both the primary and metastasis showed loss of Dpc4 expression. Miyaki et al[36] also did not find significant differences in the rate of *DPC4* mutations in stage IV cancers *versus* distant metastases (36% *versus* 31%). This suggests that *DPC4* inactivation may be permissive for acquisition of a metastatic phenotype, but additional genetic changes at *DPC4* rarely occur during dissemination. In the current study, tumors with a poorly differentiated histology or presence of pericolonic nodal metastasis did not show any trend toward loss of Dpc4 expression.

In conclusion, increasing evidence from the literature suggests an important role for *DPC4* inactivation in a subset of colorectal cancers, based on genetic analyses. This is the first study to perform a systematic immunohistochemical analysis of Dpc4 expression in a series of sporadic colorectal tumors stratified by stage, and show that the loss of Dpc4 expression is a late event in colorectal tumors, that correlates with the presence of metastatic disease at presentation. The overall low frequency of Dpc4 inactivation in this study contrasts with the high frequency of LOH at 18q21, suggesting the existence of another putative tumor suppressor gene at this locus in addition to *DCC* and *DPC4*.

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