E-cadherin and DAP kinase in pancreatic adenocarcinoma and corresponding lymph node metastases

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Abstract. E-cadherin and DAP kinase have been implicated as 'invasion suppressor' genes in human cancer. The aim of this study was to analyze the methylation status of E-cadherin and DAP kinase and the expression of the protein in the metastatic lesions and to compare it with the expression in the primary tumor. Methylation-specific PCR of the DAP kinase and E-cadherin promoter was performed in 28 primary adenocarcinomas of the pancreas and in 13 corresponding regional lymph node metastases. The presence of E-cadherin and DAP kinase protein was assessed by immunohistochemistry. Metastatic lymph nodes showed a significant different expression profile from the primary tumor. E-cadherin methylation was observed in 8/28 (29%) and loss of protein expression was observed in 16/28 (57%) of pancreatic carcinomas. E-cadherin methylation was observed in 7/13 (54%) and loss of protein expression was observed in 11/13 (85%) lymph node metastases (p=0.047). DAP kinase methylation occurred in 11/28 (39%) pancreatic carcinomas and loss of protein expression was observed in 13/28 (46%). DAP kinase was methylated in 6/13 (46%) lymph node metastases and loss of protein expression was observed in 10/13 (77%) (p=0.039). Comparing primary tumor and corresponding lymph node metastases in 13 cases, the status of E-cadherin methylation was discordant in 2 cases. The protein expression pattern of E-cadherin and DAP kinase was discordant in 4 and 3 cases respectively. Unmethylated tumor samples did not express E-cadherin in 12 and DAP kinase protein in 6 cases. Our results demonstrate that reduction of E-cadherin and DAP kinase expression is more frequent in lymph node metastases than in the primary tumor and methylation of the promoter region contributes to this reduction; however, an alternative mechanism of inactivation seems to exist.

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Introduction

Ductal adenocarcinoma of the pancreas is one of the leading causes of cancer-related deaths in Western countries, with a median survival of 4 to 6 months after diagnosis (1). One of the principal reasons for the poor survival rate is a high potential of pancreatic cancer cells to metastasize. Regional lymph node metastases are present at the time of diagnosis in more than 50% of patients with pancreatic cancer (2,3). However, the molecular mechanisms involved in the metastatic invasion of lymph nodes in pancreas carcinogenesis are poorly understood at present. Multiple genetic alterations have been described including mutational activation of K-ras oncogenes and inactivation of tumor suppressor genes, Dpc4, p53, and p16 (4,5). Also, hypermethylation of gene promoters is a possible mechanism for gene inactivation. More than 50% of all human genes contain GC-rich DNA sequences within the promoter and first exon region (6). Aberrant methylation of the normally unmethylated CpG islands has been associated with transcriptional inactivation of cancer-related genes, including hMLH, p16, DAP kinase and E-cadherin (7-9).

E-cadherin is a transmembrane glycoprotein that mediates homotypic calcium-dependent cell-cell adhesion in epithelial tissue. It plays an important role in modulating the metastatic ability of various human tumors, such as gastric, cervical and nasopharyngeal cancer (10-12). In pancreatic cancer, down-regulation of E-cadherin expression has been shown to be associated with advanced tumor stage and poor survival (9,13,14).

Another gene reported to be regulated by methylation is DAP kinase, calcium-regulated serine/threonine kinase (15). The down-regulation of DAP kinase expression through hypermethylation of the promoter region has been demonstrated in cells derived from human breast, urinary bladder and renal cell carcinoma and in clinical specimens from lung and B-cell malignancies (16). The loss of DAP kinase expression was associated with metastatic potential in lung cancer (17).

Distant spread is the major cause of eventual treatment failure in patients suffering from pancreas carcinoma. To date, however, E-cadherin expression has only been investigated in primary tumors. The aim of this study was to evaluate the methylation status and protein expression of E-cadherin and DAP kinase in metastatic lesions and to compare it with the expression in the primary tumor.

Materials and methods

We used paraffin-embedded tissue from 28 patients with ductal adenocarcinoma of the pancreas (17 men, 11 women; median age 63 years, range 46-76). In addition, we obtained cooresponding regional lymph node metastases with a high content of tumor cells (>30%) from 13 pN1 patients (patient: 2, 3, 5, 10, 11, 14, 15, 18, 19, 23, 24, 25, 28) (Table II). All patients were operated on with curative intent (R0 resection).

All of the patients underwent pancreatic surgery during the period of 1996 to 2001 in the Surgery Department (University of Leipzig). Staging and grading were performed according to the TNM classification of the International Union Against Cancer (UICC) and WHO criteria (18,19).

The clinicopathological characteristics of the 28 patients are summarized in Table I. In all cases, the specimens were re-diagnosed and re-classified according to the new UICC classification system (18).

MSP[3] (methylation-specific PCR). Genomic DNA of each tumor was extracted from formalin-fixed, paraffin-embedded tissue blocks. Two 10-μm-thick sections were cut from each block and placed into a microcentrifuge tube. Genomic DNA was isolated from paraffin-embedded tissue by digestion with 100 μg/ml proteinase K followed by standard phenol-chloroform extraction and ethanol precipitation. Since it has been shown that formalin fixation and long-term storage may contribute to false-negative PCR as a result of DNA degradation, all unmodified DNA samples were examined for GAPDH-PCR (not shown).

DNA methylation patterns in the CpG islands of DAP kinase and E-cadherin genes were determined by chemical treatment with sodium bisulfite and PCR analysis. Treatment of DNA with sodium bisulfite converts unmethylated cytosines to uracil, producing sequence changes between methylated and unmethylated DNA. PCR primers that distinguish between these methylated and unmethylated DNA sequences were then used.

The bisulfite treatment was performed as follows: DNA (2 μg) was denatured by adding NaOH to a final concentration of 0.3 M and incubating for 15 min at 37˚C. 12 μl of a 100 mM hydroquinone (Sigma, St. Louis, MO) and 208 μl of 3.6 M bisulfite (Sigma) at pH 5.0 (both freshly prepared) were added and samples were incubated for 16 h at 55˚C. Modified DNA was purified using Wizard DNA purification resign (Promega Corp.) according to the manufacturer's instructions. Modification was completed by NaOH treatment (final concentration, 300 mM) for 15 min at 37˚C. The DNA was ethanol-precipitated, dried, resuspended in water, and stored at -20˚C.

For each PCR reaction, 100 ng of bisulfite-modified DNA were amplified using the following primers. The primer sequences for unmethylated E-cadherin PCR were 5'-TAATT TTAGGTTAGAGGGTTATTGT-3' (sense) and 5'-CACAAC CAATCAACAACACA-3' (antisense), which amplify a 97-bp product; and for methylated E-cadherin PCR were 5'-TTAG GTTAGAGGGTTATCGCG-3' (sense) and 5'-TAACTAAA AATTCACCTACCGAC-3' (antisense), which amplify a 116-bp product (7). The primer sequences for unmethylated DAP kinase PCR were 5'-GGAGGATAGTTGGATTGAGTT-AATGTT-3' (sense) and 5'-CAAATCCCTCCCAAACACC AA-3' (antisense), which amplify a 106-bp product; and for methylated DAP kinase PCR were 5'-GGATAGTCGGATC GAGTTAACGTC-3' (sense) and 5'-CCCTCCCAAACGCC GA-3' (antisense), which amplify a 98-bp product (7).

Table I. Clinicopathological characteristics of 28 patients with pancreatic adenocarcinoma.

	No. of patients (%)
TNM stage[a]	
I A	3 (10.7)
I B	1 (3.6)
II A	6 (21.4)
II B	4 (14.3)
III	8 (28.6)
IV	6 (21.4)
pT categories[a]	
pT1	1 (3.6)
pT2	7 (25)
pT3	18 (64.3)
pT4	2 (7.1)
Grading[b]	
G1	1 (3.6)
G2	21 (75)
G3	6 (21.4)
Location	
Head	25 (89.3)
Body	3 (10.7)
Tail	0

[a]According to UICC (2002). [b]According to WHO (2000).

PCR conditions consisted of denaturation at 94˚C for 5 min followed by 40 cycles of denaturation at 94˚C for 45 sec, primer annealing at temperatures from 58˚C (methylated and unmethylated E-cadherin) and 60˚C (methylated and unmethylated DAP kinase) for 45 sec, and primer extension at 72˚C for 45 sec. Each PCR reaction (20 μl) was loaded onto a 2% agarose gel and visualized under UV illumination. If a visible PCR product for both the unmethylated and methylated primers or methylated primer alone could be seen, the gene promoter was considered to be methylated.

DNA from the peripheral blood of healthy individuals that were positive for the E-cadherin and DAP kinase-unmethylated PCR reaction was used as an unmethylated positive control. The same DNA samples treated with SssI methyltransferase (Biolabs), which generates a completely methylated DNA at all CpG sites, were used as a methylation-positive control. Water blanks were also performed in all PCRs. Methylation negative samples were additionally verified by light cycler in real-time PCR in duplicate.

Table II. Methylation and expression analysis of DAP kinase and E-cadherin in primary pancreas carcinoma (tumor) and lymph nodes (L. node).

Patient no.	Grading	UICC	MSP E-cad.	Imm.	MSP DAPK	Imm.
1 Tumor	3	I A	u	-	m	-
2 Tumor	2	IV	u	+	m	-
3 Tumor	1	IV	u	+	u	++
4 Tumor	2	II A	u	-	m	-
5 Tumor	2	III	u	++	u	+
6 Tumor	2	II A	m	-	u	+
7 Tumor	3	III	u	-	u	+
8 Tumor	2	II A	m	-	u	+
9 Tumor	3	II B	u	-	u	+
10 Tumor	2	III	m	-	m	-
11 Tumor	2	III	u	+	m	-
12 Tumor	2	II A	u	-	u	++
13 Tumor	2	IV	u	+	u	+
14 Tumor	2	III	m	-	u	+
15 Tumor	2	III	u	+	m	-
16 Tumor	2	I A	m	-	u	-
17 Tumor	2	IV	u	+	u	++
18 Tumor	2	III	m	-	u	-
19 Tumor	3	II B	u	-	u	-
20 Tumor	2	I B	u	+	m	-
21 Tumor	2	II A	u	+	m	-
22 Tumor	3	II A	u	+	u	++
23 Tumor	2	IV	m	-	u	+
24 Tumor	2	III	u	-	m	-
25 Tumor	2	II B	m	-	m	-
26 Tumor	3	IV	u	+	u	+
27 Tumor	2	I A	u	-	m	-
28 Tumor	2	II B	u	++	u	+
2 L. node			m	-	m	-
3 L. node			u	+	u	-
5 L. node			m	-	u	+
10 L. node			m	-	m	-
11 L. node			u	-	m	-
14 L. node			m	-	u	-
15 L. node			u	-	m	-
18 L. node			m	-	u	-
19 L. node			u	-	u	+
23 L. node			m	-	u	-
24 L. node			u	-	m	-
25 L. node			m	-	m	-
28 L. node			u	+	u	+

[a]Promoter methylation status of E-cadherin and DAP kinase genes were evaluated by MSP analysis (U, unmethylated; M, methylated). [b]The results in the immunohistochemistry were placed into three categories: absent (-), positive (+) and strong positive (++) depending on the percentage and intensity of immunoreactive tumor cells.

Table III. E-cadherin methylation and loss of protein expression within primary tumors and lymph node metastases of pancreatic adenocarcinoma.

	Primary tumor (%)	LNM (%)
MSP		
Methylated	8/28 (29)	7/13 (54)
Unmethylated	20/28 (71)	6/13 (46)
Imm.		
Absent	16/28 (57)	11/13 (85)
Positive	12/28 (43)	2/13 (15)

MSP, methylation-specific PCR; Imm., immunohistochemistry; LNM, lymph node metastasis.

Immunohistochemistry. As previously described (21), the 5 μm-thick paraffin sections deparaffinated in xylene and rehydrated in graded alcohols were heated in a microwave oven 3 times for 5 min at 850 W in 10 mM citrate buffer, pH 6.0. The slides were dipped into 98% methanol with 1% H_2O_2 for 20 min to avoid a non-specific reaction due to endogenous peroxidase. After washing with PBS and pretreatment with 2% skim milk, slides were incubated at 37˚C for 1 h with the primary antibodies (E-cadherin 1:50, DAP kinase 1:80). A rabbit polyclonal antibody raised against DAP kinase (Santa Cruz) and mouse monoclonal antibody against E-cadherin (Novocastra) were used.

The sections were run through the Biotin Streptavidin-Amplified (B-SA) detection system (BioGenex). The immunoreaction was visualized by developing sections with AEC chromogene. Negative controls in which non-immune sera was substituted for the primary antibody were also included.

Only membranous staining of E-cadherin antibody was assessed for the scoring, since E-cadherin is an adhesion molecule located at the membrane. DAP kinase immunohistochemistry exhibited only cytoplasmic staining.

Qualitative and quantitative evaluation of the percentage of immunostained tumor cells and the intensity of staining were combined for scoring of E-cadherin and DAP kinase immunohistochemistry.

Quantitative evaluation: 1) 0-20% of the tumor cells are positive, 2) 20-70% of tumor cells are positive, 3) over 70% of tumor cells are positive. Qualitative evaluation: (A) intense red staining, (B) light red staining, (C) no staining. The degree was scored as absent (1-B, 1-C), positive (2-A, 2-B) and strong- positive (3-A, 3-B).

Statistical analysis. For statistical analysis, the frequency of decreased expression and promoter methylation of E-cadherin and DAP kinase in node-negative and node-positive types of tumors, and in lymph node metastases was determined. Associations between variables were determined by χ^2 analysis using a Statistical Package for the Social Sciences (SPSS/PC 9.0, Chicago). A $p<0.05$ was defined as being statistically significant.

Results

E-cadherin. The results of E-cadherin MSP analysis are summarized in Table II. There was no evidence of homozygous deletion in the examined region, since all of the samples were amplified by PCR, either for methylated DNA or for unmethylated DNA. We detected a methylation of the E-cadherin promoter region in 8 of 28 (29%) primary tumors and in 7 of 13 (54%) lymph node metastases (Table III and Fig. 1a). Promoter methylation in primary tumors did not appear to be related to tumor stage, grade or lymph node status.

All tissue specimens were examined immunohistochemically to determine the relationship between E-cadherin promoter methylation and protein expression (Fig. 2a-c). Data obtained from E-cadherin immunohistochemistry are presented in Tables II and III.

The loss of E-cadherin protein expression was detected in 57% (16/28) of primary tumors and in 85% (11/13) of lymph node metastases (Table IV). The loss of E-cadherin was more frequent in lymph node metastases (85%) compared to primary tumors (57%) ($p=0.047$).

None of the cases with promoter methylation showed E-cadherin staining in the immunohistochemistry. Of the samples with a loss of protein, 56% (15 of 27) showed methylation of the E-cadherin gene promoter. However, in

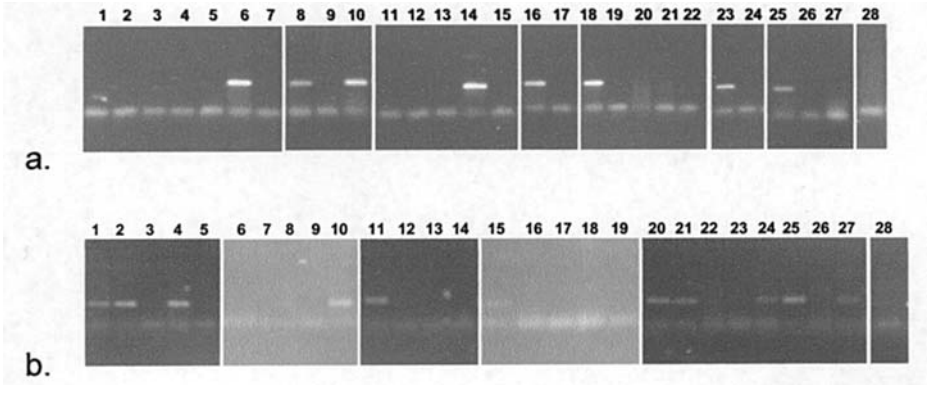

Figure 1. MSP analysis for methylated E-cadherin (a) and DAP kinase (b) gene promoters in primary and metastatic pancreatic carcinoma.

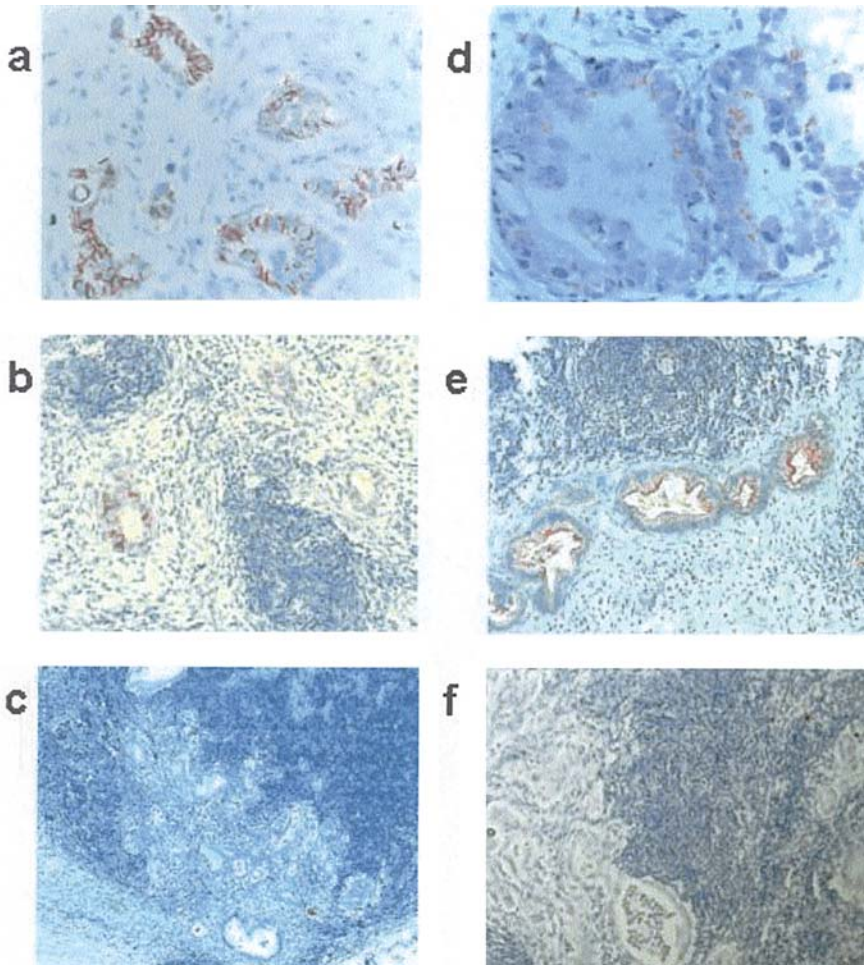

Figure 2. Expression pattern of E-cadherin and DAP kinase in primary and metastatic pancreatic carcinoma. E-cadherin expression in a primary tumor (a) and in lymph node metastasis (b), and loss of E-cadherin expression in lymph node metastasis (c) (a, original magnification x40; b and c, original magnification x20). DAP kinase expression in a primary tumor (d) and in lymph node metastasis (e), and loss of DAP kinase expression in lymph node metastasis (f) (d, original magnification x40; e and f, original magnification x20).

Table IV. DAP kinase methylation and loss of protein expression within primary tumors and lymph node metastases of pancreatic adenocarcinoma.

	Primary tumor (%)	LNM (%)
MSP		
Methylated	11/28 (39)	6/13 (46)
Unmethylated	17/28 (61)	7/13 (54)
Imm.		
Absent	13/28 (46)	10/13 (77)
Positive	15/28 (54)	3/13 (23)

MSP, methylation-specific PCR; Imm., immunohistochemistry; LNM, lymph node metastasis.

12 samples (8 primary tumors, 4 lymph node metastases) the loss of E-cadherin expression was not associated with promoter methylation, indicating that there are alternative mechanisms of inactivation.

Among matched samples of primary tumors and lymph node metastases from 13 patients, concordant methylation of the E-cadherin promoter was observed in 5 cases whereas, in two cases, methylation of the E-cadherin promoter was found only in lymph node metastases and not in primary tumors (Table V).

In immunohistochemistry, the loss of E-cadherin expression was concordant in 7 cases. In 4 cases, only lymph node metastases revealed loss of E-cadherin expression whereas the corresponding primary tumors were positive for E-cadherin staining.

DAP kinase. Methylation of the DAP kinase gene promoter was detected in 39% (11/28) of primary tumors and in 46% (6/13) of lymph node metastases. In immunohistochemistry, the loss of DAP kinase protein expression was detected in 46% (13/28) of primary tumors and in 77% (10/13) of lymph node metastases (Table IV).

No correlation between DAP kinase protein expression and the clinicopathological characteristics of pancreatic cancer was observed. Moreover, lymph node metastases showed a higher frequency of DAP kinase protein loss compared to primary tumors (p=0.039).

Table V. Promoter methylation and loss of protein expression of E-cadherin and DAP kinase in paired samples of primary tumors and corresponding lymph node metastases.

Gene	Primary tumor, n=13	LNM, n=13
E-cadherin		
Promoter methylation	5 (39)	7 (54)
Loss of protein	7 (54)	11 (85)
DAP kinase		
Promoter methylation	6 (46)	6 (46)
Loss of protein	7 (54)	10 (77)

All 17 cases (11 primary tumors, 6 lymph node metastases) with promoter methylation showed loss of DAP kinase expression. Of specimens with a loss of DAP kinase expression, 74% (17/23) revealed promoter methylation. However, 6 unmethylated samples (2 primary tumors, 4 lymph node metastases) did not express DAP kinase, indicating that there are, at present, other unknown mechanisms of inactivation.

Amongst the 13 matched samples of primary tumors and corresponding lymph node metastases, each of the 6 patients who had methylated DAP kinase promoter in the lymph node metastasis had identical alterations in the primary tumor DNA (Table V). In immunohistochemistry, loss of DAP kinase expression was also detected in 7 paired lymph node metastases and primary tumors (Table V, Fig. 2d-f). In 3 cases, the loss of DAP kinase expression was obtained only in lymph node metastases and not in corresponding primary tumors.

Discussion

In this study, we provided evidence that the loss of E-cadherin expression and DAP kinase expression is significantly lower in metastatic lesions than in the primary tumor. To our knowledge, this has not previously been reported, but our findings are in accordance with other study groups. In our study, a down-regulation of E-cadherin expression was found only in lymph node metastases and not in corresponding primary tumor tissue in 4 cases. Two of these cases were associated with methylation of the E-cadherin promoter. This may reflect the discordant methylation pattern in different maturation stages of the same tumor cell lineage, as was recently demonstrated in paired pancreatic cancer cell lines and corresponding primary tumors (22). In this study, we found a significantly higher frequency of DAP kinase expression loss in lymph node metastases than in primary tumors of the pancreas. Among the matched samples of primary tumors and lymph node metastases, the loss of DAP kinase expression was subsequently obtained only in lymph node metastases and not in corresponding primary tumors in 3 cases. The results of this study support the proposition that DAP kinase inactivation is associated with the metastatic behavior of tumor cells. This data is consistent with *in vivo* studies demonstrating a suppressive role of DAP kinase expression for formation of metastases by increasing the occurrence of apoptosis (23). The loss of DAP kinase expression has been demonstrated to contribute to the formation of lung metastases and to be a positive selective advantage for metastasizing tumor cells (16). In squamous cell cancer, DAP kinase promoter methylation was significantly correlated with lymph node metastases (24).

Another possible explanation could be the accumulating evidence, from research at the cellular, chromosomal, and molecular genetic levels, that epithelial cancer is remarkably heterogenous. It is possible that the metastatic cells are subclones of the primary tumor which have lost E-cadherin and DAP kinase expression before they metastasize. As we know, down-regulation or complete shutdown of E-cadherin-mediated cell adhesion correlates with the loss of epithelial morphology and the acquisition of metastatic potential by the carcinoma cells in different tumors (25-28). In pancreatic cancer cell lines, the loss of E-cadherin/beta-catenin adhesion complex was shown to contribute to tumor progression (29).

In our series, 56% of samples with a loss of E-cadherin showed methylation of the E-cadherin gene promoter. This data confirmed the hypothesis that hypermethylation of CpG islands in the promoter region is one mechanism for loss of E-cadherin gene function, since mutations of the E-cadherin gene were rarely detected in human cancers (30). However, in twelve samples (8 primary tumors, 4 lymph node metastases) the loss of E-cadherin expression was not associated with promoter methylation, indicating a presence of alternative mechanisms of inactivation. The fact that 74% of cases with loss of DAP kinase expression showed methylation at the gene promoter supports the notion that promoter methylation might be an important mode of inactivation. Six unmethylated samples (2 primary tumors, 4 lymph node metastases) did not express DAP kinase, indicating also an alternative mechanism of inactivation. In accordance with other studies, our study suggests that promotor hypermethylation is the main mechanism involved in promotor silencing of E-cadherin and DAP kinase, although not the only one. Loss of heterozygosity and/or point mutation can also contribute to the down-regulation of these two tumor suppressor genes (30).

In conclusion, the present study provides the first evidence that loss of E-cadherin and DAP kinase is significantly more frequent in metastatic lesions than in the primary tumor in pancreatic cancer. Promotor hypermethylation is one important event in silencing tumor suppressor genes but future studies, probably investigating the status of these two genes at different stages (e.g. premalignant versus malignant lesions), might help to understand the onset and underlying cause of inactivation.

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