Aberrant Methylation and Deacetylation of *Deleted in Liver Cancer-1* Gene in Prostate Cancer: Potential Clinical Applications

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Abstract **Purpose:** The *deleted in liver cancer-1* (*DLC-1*) gene that encodes a Rho GTPase-activating protein with tumor suppressor function is located on chromosome 8p21-22, a region frequently deleted in prostate carcinomas. This study was designed to determine whether *DLC-1* is deregulated in prostate carcinomas and to assess the contribution of *DLC-1* alterations to prostate carcinogenesis.

Experimental Design: Primary prostate carcinomas, prostate carcinoma cell lines, benign prostatic hyperplasias, and normal prostatic tissues were examined for detection of functional and structural alterations of the *DLC-1* gene by real-time PCR, methylation-specific PCR, and Southern and Western blots.

Results: Down-regulation or loss of *DCL-1* mRNA expression was detected in 10 of 27 (37%) prostate carcinomas, 3 of 5 (60%) prostate carcinoma cell lines, and 5 of 21 (24%) benign prostatic hyperplasias. *DLC-1* promoter methylation was identified in 13 of 27 (48%) prostate carcinomas and 2 matching normal tissues and in 15 of 21 (71%) benign prostatic hyperplasias but was absent in 10 normal prostatic tissues from noncancerous individuals. Genomic deletions were found in only 3 prostate carcinomas and 1 benign prostatic hyperplasia. DLC-1 protein was not detected in 8 of 27 (30%) prostate carcinomas and 11 of 21 (52%) benign prostatic hyperplasias. Methylation of *DLC-1* correlated with age in prostate carcinoma patients ($P = 0.006$) and with prostate-specific antigen blood levels in benign prostatic hyperplasia patients ($P = 0.029$). Treatment of the three prostate carcinoma cell lines (PC-3, LNCaP, and 22Rv1) expressing a low level of *DLC-1* transcripts with inhibitors of DNA methyltransferase or histone deacetylase increased *DLC-1* expression.

Conclusions: These results show that the transcriptional silencing of DLC-1 by two epigenetic mechanisms is common and may be involved in the pathogenesis of prostate carcinomas and benign prostatic hyperplasias and could have potential clinical application in the early detection and gene therapy of prostate cancer.

Prostate cancer remains a major cause of cancer-related deaths worldwide, with an estimated 232,000 new cases in United States and 30,000 deaths in 2005 (1). Molecular and genetic mechanisms implicated in the development of this disease have only recently been characterized (2). Prostate carcinomas, like several other types of solid tumors and hematologic malignancies, exhibit recurrent loss of DNA copy number and loss of heterozygosity on the short arm of chromosome 8, particularly at region 8p21-22 (2-7).

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Region 8p21-22 contains the gene *deleted in liver cancer-1* (*DLC-1*), a regulator of the Rho family of small GTPases that is highly homologous to the rat p122RhoGAP (8, 9). The regulation of Rho GTPase proteins may be critical to the neoplastic process (10-13). An altered balance between active GTP-bound and inactive GDP-bound forms determines the activity of Rho proteins. *DLC-1*-mediated negative regulatory effect on cell growth and tumorigenicity could be due to the ability of RhoGAP to inactivate Rho proteins. *DLC-1* RhoGAP has been found to enhance the *in vitro* GTPase activity of RhoA and Cdc42, which are overexpressed in cancer cells (13-15).

DLC-1 has been considered a candidate tumor suppressor gene, because it was initially cloned as a genomic DNA segment underrepresented in a primary human hepatocellular carcinoma and was found to be frequently deleted, down-regulated, or inactivated in several forms of cancer (8). Tumor suppressor genes that are deregulated or silenced by promoter hypermethylation are often located in genomic regions that are frequently deleted in neoplasias (16). This explains why down-regulation or inactivation of the *DLC-1* gene is commonly mediated at the genomic level by heterozygous or homozygous deletion and at the transcriptional level by aberrant promoter methylation in breast, liver, colon, lung, stomach, and brain tumors (8, 15, 17-22). Remarkably, promoter methylation of

DLC-1 is also common in hematologic malignancies, as methylation of *DLC-1* has been found in >70% of various types of childhood and adult leukemias (23, 24). It has been proposed that *DLC-1* methylation status in non-Hodgkin's lymphomas could be used as a diagnostic marker (23).

Evidence that DLC-1 can function as a tumor suppressor has come from experiments in which *DLC-1* cDNA was transfected into human tumor cells that do not express the endogenous gene. Overexpression of *DLC-1* induced apoptosis and inhibited cell proliferation, colony formation, and cell migration *in vitro*. It also reduced or prevented the development of tumors after inoculation of breast, liver, lung, and ovarian carcinoma cells in athymic nude mice (17, 25–29). Recently, *DLC-1* was identified as a potential breast cancer susceptibility gene using high-throughput single nucleotide polymorphism genotyping and as a breast metastasis suppressor gene (30–32). In addition, *DLC-1* is essential for embryonic development, as mice homozygous mutant embryos do not survive beyond midterm gestation (33).

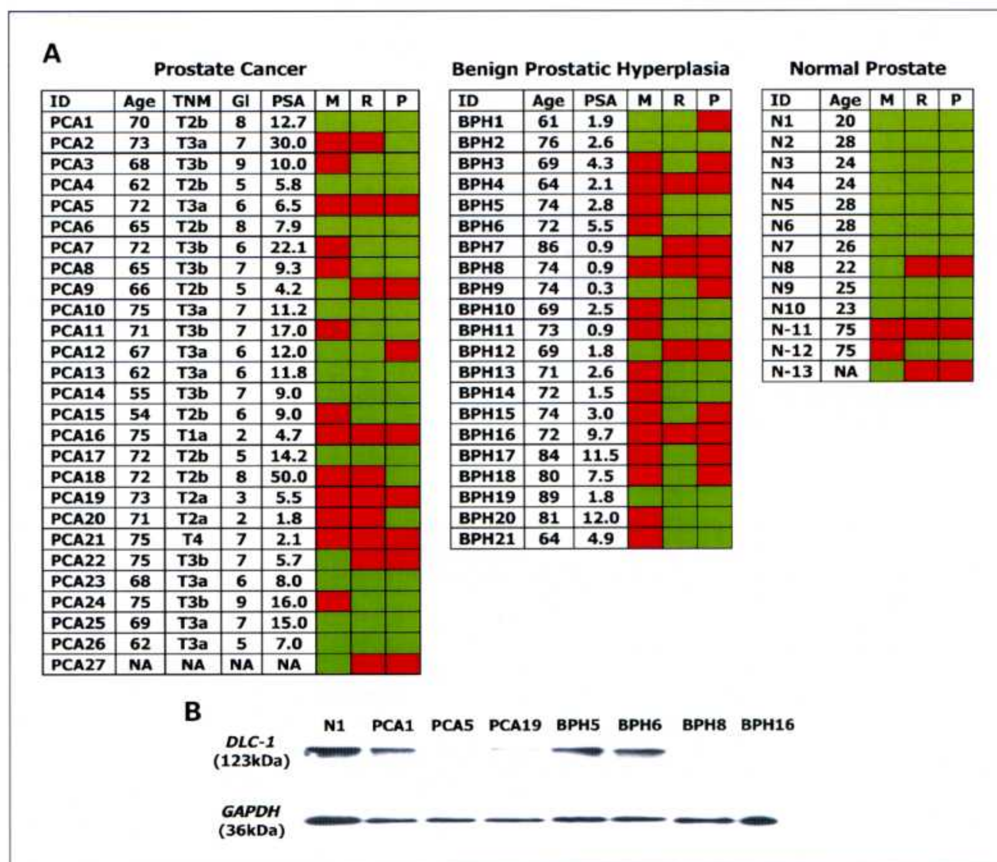
Given the high frequency of loss of heterozygosity and loss of DNA copy number on 8p21-22 in human prostate carcinomas, we sought to find out whether alterations of the *DLC-1* gene are recurrent and to determine their nature and relevance to this neoplasia. In this study, we identified recurrent functional alterations, implicating *DLC-1* gene in prostate carcinogenesis.

Materials and Methods

Patients. Tissue specimens from 27 patients with prostate carcinoma that underwent radical prostatectomy and from 21 patients with benign prostatic hyperplasia that underwent suprapubic or transurethral resection were collected within a 3-year period (from October 2000 to September 2003) at the Department of Urology, University Hospital of Heraklion (Crete, Greece). In 3 prostate carcinoma cases, normal adjacent prostatic tissue was also available. Median age at diagnosis was 70.5 years (range, 54-75) for prostate carcinoma subjects and 73 years (range, 61-89) for benign prostatic hyperplasia patients. Prostate carcinoma stage (tumor-node-metastasis) and grade (Gleason score) were determined by histologic examination using H&E-stained slides. Because the prostate gland is not normal in old individuals, we used specimens from 10 young subjects (age 20-28 years) as a source of normal prostatic tissue. Specimens were collected postmortem, within 8 hours of the time of death, at the same hospital. All normal, benign, and malignant samples were immediately frozen and stored at −80°C until used. The clinical and histopathologic characteristics of all samples are listed in Fig. 1A. The ethics committees of the University of Crete and the University Hospital of Heraklion approved this study, and written informed consent was obtained from all patients or their relatives.

Cell culture. The human prostate carcinoma cell lines PC-3, LNCaP, DU145, and 22Rv1 (American Type Culture Collection, Manassas, VA) and SP3031 (kindly provided by Dr. Sen Pathak, M. D. Anderson Cancer Center) were maintained in RPMI 1640 (BioSource, Camarillo, CA) supplemented with 10% fetal bovine serum. The immortalized normal prostate epithelial cell lines RWPE-1 and PWR-1E (American Type Culture Collection) were cultured in keratinocyte serum-free medium supplemented with 5 ng/mL human recombinant epidermal growth factor and 0.05 mg/mL bovine pituitary extract (Invitrogen, Carlsbad, CA).

Quantitative and semiquantitative reverse transcription-PCR. *DLC-1* mRNA expression was evaluated by quantitative real-time reverse transcription-PCR. Total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.



A

Prostate Cancer

ID	Age	TNM	Gl	PSA	M	R	P
PCA1	70	T2b	8	12.7			
PCA2	73	T3a	7	30.0			
PCA3	68	T3b	9	10.0			
PCA4	62	T2b	5	5.8			
PCA5	72	T3a	6	6.5			
PCA6	65	T2b	8	7.9			
PCA7	72	T3b	6	22.1			
PCA8	65	T3b	7	9.3			
PCA9	66	T2b	5	4.2			
PCA10	75	T3a	7	11.2			
PCA11	71	T3b	7	17.0			
PCA12	67	T3a	6	12.0			
PCA13	62	T3a	6	11.8			
PCA14	55	T3b	7	9.0			
PCA15	54	T2b	6	9.0			
PCA16	75	T1a	2	4.7			
PCA17	72	T2b	5	14.2			
PCA18	72	T2b	8	50.0			
PCA19	73	T2a	3	5.5			
PCA20	71	T2a	2	1.8			
PCA21	75	T4	7	2.1			
PCA22	75	T3b	7	5.7			
PCA23	68	T3a	6	8.0			
PCA24	75	T3b	9	16.0			
PCA25	69	T3a	7	15.0			
PCA26	62	T3a	5	7.0			
PCA27	NA	NA	NA	NA			

Benign Prostatic Hyperplasia

ID	Age	PSA	M	R	P
BPH1	61	1.9			
BPH2	76	2.6			
BPH3	69	4.3			
BPH4	64	2.1			
BPH5	74	2.8			
BPH6	72	5.5			
BPH7	86	0.9			
BPH8	74	0.9			
BPH9	74	0.3			
BPH10	69	2.5			
BPH11	73	0.9			
BPH12	69	1.8			
BPH13	71	2.6			
BPH14	72	1.5			
BPH15	74	3.0			
BPH16	72	9.7			
BPH17	84	11.5			
BPH18	80	7.5			
BPH19	89	1.8			
BPH20	81	12.0			
BPH21	64	4.9			

Normal Prostate

ID	Age	M	R	P
N1	20			
N2	28			
N3	24			
N4	24			
N5	28			
N6	28			
N7	26			
N8	22			
N9	25			
N10	23			
N-11	75			
N-12	75			
N-13	NA			

Fig. 1. Methylation and expression analysis of *DLC-1* gene in prostate cancer. *A*, summary of clinical and pathologic characteristics and molecular analysis of malignant, benign, and normal prostatic tissues. *TNM*, tumor-node-metastasis; *GI*, Gleason score; *M*, methylation status; *R*, mRNA levels; *P*, protein levels; *NA*, not available; *N-11*, adjacent normal tissue of PCA21; *N-12*, adjacent normal tissue of PCA24; *N-13*, adjacent normal tissue of PCA27; *N*, normal prostate; *PCA*, prostate cancer; *BPH*, benign prostatic hyperplasia. *Red*, methylation in *column M* and reduced expression in *columns R* and *P*; *green*, lack of methylation in *column* M and normal expression in *columns R* and *P*. *B*, representative examples of DLC-1 protein expression after Western blot analysis in prostate carcinomas, benign prostatic hyperplasias, and a normal prostatic tissue (N_1). *GAPDH* was used as an internal control.

Table 1. Primer sequences used for reverse transcription-PCR, MSP, bisulfite DNA sequencing, and chromatin immunoprecipitation

Primers	Sequence (5′-3′)	PCR product size (bp)
DLC-1F	CACAGGACAACCGTTGCCTCAG	465
DLC-1R	CTCTTCAGGGTGTTGAGATGGA	
GAPDH-F	GAAGGTGAAGGTCGGAGTCA	226
GAPDH-R	GAAGATGGTGATGGGATTTC	
MSP-F	TTTAAAGATCGAAACGAGGGAGCG	172
MSP-R	CCCAACGAAAAAACCCGACTAACG	
USP-F	TTTTTAAAGATTGAAATGAGGGAGTG	178
USP-R	AAACCCAACAAAAAAACCCAACTAACA	
Bis-DLC-F	GTTTTTAGTTAGGATATGGT	292
Bis-DLC-R	ACTTCTTTCTACACATCAAACAC	
ChIP-F	AGAGGAGAGGCGGGGCCT	124
ChIP-R	CTTAGCGACGGGCTGTTCTCC	

Reverse transcription reactions were carried out on 1 μg total RNA with the SuperScript II First-Strand Synthesis System using the oligo(dT) primer (Invitrogen). For real-time PCR, sequence-specific PCR primers and TaqMan probes for *DLC-1* (Assay ID: Hs00183436) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Applied Biosystems, Foster City, CA) were used. *DLC-1* was amplified on the same plate with the reference, GAPDH, using the TaqMan Universal PCR Master Mix. All reactions were carried out in the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative fold difference of *DLC-1* mRNA expression in all prostate tissue samples compared with the average ratio of normal samples. Two-fold increased or decreased expression was considered significant.

Semiquantitative reverse transcription-PCR for the detection of *DLC-1* expression in prostate cell lines was carried out using HotStarTaq DNA polymerase (Qiagen). Samples were defined as *DLC-1* positive if a PCR product was detectable after 35 cycles of amplification. The primers used (*DLC-1*F and *DLC-1*R; *GAPDH*-F and *GAPDH*-R) are listed in Table 1.

Southern blot. For each tissue sample and cell line, 10 μg DNA was digested to completion with *Eco*RI and resolved on 0.8% agarose gels. *DLC-1* fragments (465 bp), amplified with primers DLC-1F and DLC-1R, were labeled with [α-^{32}P]dCTP using the Prime-It II Random Primer Labeling kit (Stratagene, La Jolla, CA), hybridized overnight, and exposed for 72 hours on a Storm PhosphorImager (Amersham Biosciences, Piscataway, NJ). ImageQuant software (Amersham Biosciences) was used for quantifying the signal intensity of the fragments. The results were normalized by *GAPDH* before data analysis for all prostate tissue samples.

Methylation-specific PCR. Genomic DNA extracted from each sample was modified by sodium bisulfite using EZ DNA Methylation kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. For the detection of *DLC-1* gene aberrant methylation, the modified DNA was amplified using primers specific for the methylated sequence (MSP-F and MSP-R; Table 1). For quality control of the bisulfite modification process, the modified DNA was also amplified using primers specific for the unmethylated sequence of this gene (USP-F and USP-R; Table 1). PCR was done in a 50 μL reaction volume using HotStarTaq DNA polymerase. CpGenome Universal Methylated DNA (Chemicon, Temecula, CA) was used as a positive control for the methylated reactions. Products were run on 2% agarose gels and visualized under UV illumination.

Sodium bisulfite DNA sequencing. The bisulfite-modified DNA was also amplified by PCR using the primers Bis-DLC-F and Bis-DLC-R (Table 1). PCR products were then subcloned into the pCR2.1-TOPO vector using a TA Cloning kit (Invitrogen). To determine the methylation status of the 5′ CpG island of the DLC-1 gene, four clones from each plate were sequenced using an ABI PRISM Dye Deoxyterminator Cycle Sequencing kit and analyzed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Trichostatin A and 5-aza-2′-deoxycytidine treatment. Cells from DLC-1-negative prostate carcinoma cell lines, plated at a density of 2×10^6 per 100-mm dish, were treated with 1 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC; a DNA methyltransferase inhibitor; Sigma-Aldrich, St. Louis, MO) for 72 hours, 500 nmol/L trichostatin A [a histone deacetylase (HDAC) inhibitor] for 12 hours (Sigma), or a combination of 5-aza-dC (for 72 hours at 1 μmol/L) and trichostatin A (added only during the last 12 hours at 500 nmol/L). All experiments were conducted in triplicates.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY) was used according to the method recommended by the manufacturer. Briefly, cells were plated at a density of 10^6 to 10^7 per 100-mm dish and cultured for 24hours followed by 12 hours of culture with 500 nmol/L trichostatin A. Subsequently, chromatin was solubilized and subjected to sonication to obtain DNA fragments with an average size of 200 to 1,000 bp. Chromatin immunoprecipitation was carried out by incubation with 4 μg anti-acetyl histone H3 antibody (Upstate Biotechnology) and a no-antibody immunoprecipitation as control. Ten percent of precleared lysate was saved for each sample to determine the input chromatin amount. Immunoprecipitated DNA was used as a template for PCR of the *DLC-1* promoter. The primers used (ChIP-F and ChIP-R) are listed in Table 1.

Western blot. Tissues and cells were lysed in 100 μL CelLytic MT Cell Lysis Reagent (Sigma) containing a protease inhibitor cocktail (Sigma). Total protein concentration was determined by the BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of total protein were resolved by SDS-PAGE and subjected to Western blot analysis. Human monoclonal anti-*DLC-1* (1:100; clone 3; BD Biosciences PharMingen, Mountain View, CA) and anti-GAPDH (1:5,000; clone 6C5; Chemicon) were used as primary antibodies. A bovine anti-mouse IgG horseradish peroxidase conjugate (1:4,000; Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibody. Proteins were transferred from the gels to nitrocellulose membranes (Invitrogen), which were developed using the SuperSignal West Pico Chemiluminescent kit (Pierce). Films were scanned and protein band signal intensity was measured with Adobe Photoshop 7.0 (Adobe, San Jose, CA). After normalization with *GAPDH*, the relative fold difference of DLC-1 protein expression in all prostate tissue samples compared with the average expression of normal samples was calculated. Two-fold increased or decreased expression was considered significant.

Statistical analysis. The association of *DLC-1* methylation and mRNA and protein expression with patients' clinical and histopathologic variables was analyzed with the χ^2 test (for categorical data), using Fisher's exact test when indicated by the analysis, or with the

Mann-Whitney *U* test (for continuous variables). For the trichostatin A and 5-aza-dC experiments, mean and SE were calculated. Student's *t* test was used to compare these values with the ones obtained from the corresponding control experiments. All statistical analyses were two sided and done with SPSS 11.5 (SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

To determine whether *DLC-1* is deregulated in prostate cancer and benign prostatic hyperplasia, we examined functional and structural alterations of the gene. The results of *DLC-1* methylation and mRNA and protein expression in prostate carcinomas, adjacent normal tissue from prostate carcinoma patients, benign prostatic hyperplasias, and normal prostatic tissues from noncancerous individuals are schematically represented in Fig. 1*A*. Whereas *DLC-1* mRNA was abundant in 9 of 10 (90%) normal prostatic tissues, down-regulation or loss of *DCL-1* expression was found by quantitative real-time reverse transcription-PCR in 10 of 27 (37%) prostate carcinomas and 5 of 21 (24%) benign prostatic hyperplasias. Loss of DLC-1 protein expression was detected by Western blot in 8 of 27 (30%) prostate carcinomas and in 11 of 21 (52%) benign prostatic hyperplasias. Representative examples are shown in Fig. 1*B*. DLC-1 mRNA and protein were not expressed in 2 of 3 adjacent tissues from patients with prostate carcinoma and in 1 normal prostatic tissue (Fig. 1*A*).

To see whether the loss of *DLC-1* mRNA expression was associated with genomic deletions, we subjected all normal, benign, and malignant prostate samples along with the prostate carcinoma cell lines to Southern blot. The analysis of genomic DNA from normal tissue samples revealed a fragment of ~4.5 kb. Although no homozygous deletions were detected in either primary prostate carcinomas, benign prostatic hyperplasias, or prostate carcinoma cell lines, in 3 prostate carcinoma cases (PCA19, PCA21, and PCA22) and 1 benign prostatic hyperplasia sample (BPH12), a 50% decrease in the signal intensity of the *DLC-1* band was observed, suggesting that genomic deletion is unlikely to be responsible for the loss of DLC-1 expression in benign or malignant prostatic disease (Fig. 2).

To determine whether the loss of *DLC-1* expression was mediated by promoter hypermethylation, the methylation status of the 5′ CpG island of DLC-1 gene was determined by methylation-specific PCR (MSP). DLC-1 promoter contains CpG islands of 700 bp, with a GC content of 72.5% and a CpG observed/expected ratio of 0.88, thus satisfying the criteria for a CpG island (Fig. 3*A*). MSP assay was employed to assess the methylation status of several CpG dinucleotides within the 5′ CpG island. A few examples are illustrated in Fig. 3*B*. All tissue samples exhibited the bands that correspond to either unmethylated (178 bp) or methylated (172 bp) CpG island. The unmethylated band, which was observed in all prostate carcinomas and benign prostatic hyperplasias, is probably due to inherent contamination with normal cells or partial methylation. MSP analysis revealed methylation in 13 of 27 (48%) prostate carcinomas and 15 of 21 (71%) benign prostatic hyperplasias (Fig. 1*A*). Both methylated and unmethylated bands were detected by MSP in LNCaP cells. The methylation level was significantly higher in prostate carcinomas and benign prostatic hyperplasias compared with normal samples (Fisher's exact test, $P = 0.006$ and $P < 0.001$, respectively). In prostate carcinomas, the frequency of *DLC-1* methylation was higher in patients ages >70 years compared with younger patients (χ^2 test, $P = 0.006$), whereas DLC-1 mRNA expression was lower (Fisher's exact test, $P = 0.011$; Table 2). There was no statistically significant correlation between *DLC-1* hypermethylation and mRNA or protein expression and prostate-specific antigen (PSA) levels, tumor-node-metastasis staging, or Gleason score in prostate carcinoma samples (Table 2). However, there was a significant association between *DLC-1* methylation status and PSA blood levels in benign prostatic hyperplasia specimens (Mann-Whitney *U* test, $P = 0.029$; Table 3).

To gain more information on the methylation status, particularly for the upstream region of the basic promoter and around the translation start sites, a 292-bp fragment of the *DLC-1* promoter region (Bis-DLC), containing 35 CpG dinucleotides (Fig. 3*A*), was sequenced, after sodium bisulfite modification, in a normal prostatic tissue (N_1), a prostate carcinoma cell line (LNCaP), and in the 13 prostate carcinoma samples that were found to be methylated by MSP (Fig. 1*A*; ref. 22). Consistent with the MSP data, we found that the CpG island was unmethylated in the normal prostate sample but exhibited frequent, localized methylation in the LNCaP cell line and in 13 prostate carcinomas. Representative examples, displaying extensive hypermethylation at the CpG dinucleotides in a prostate carcinoma sample (PCA3) and lack of methylation in the normal prostate sample N_1, are shown in Fig. 3*C*. Bisulfite DNA sequencing revealed a variable pattern of methylated cytosine residues in the priming sites and elsewhere, especially at 2, 7, 9, 13, 15, and 17 CpG loci (Fig. 3*D*), which may have affected the efficiency of MSP and could be responsible for the lack of correlation between methylation and *DLC-1* mRNA in certain prostate carcinoma samples.

An alternative epigenetic mechanism for gene silencing or down-regulation is histone deacetylation. In the prostate carcinoma cell lines PC-3, LNCaP, and 22Rv1, *DLC-1* mRNA and protein (data not shown) were either undetectable or reduced in abundance (Fig. 4*A*). To ascertain whether HDAC activity, which is essential for maintaining histone deacetylation levels, is involved in the repression of *DLC-1* expression, the three *DLC-1*-negative cell lines (PC-3, LNCaP, and 22Rv1) were treated with trichostatin A, a HDAC inhibitor. A 9.6-fold (Student's *t* test, $P = 0.004$), 29.8-fold ($P < 0.001$), and 41.1-fold ($P < 0.001$) increase in *DLC-1* transcripts was detected in PC-3, LNCaP, and 22Rv1 trichostatin A–treated cells, respectively (Fig. 4*B*). Combined 5-aza-dC (a DNA methyltransferase

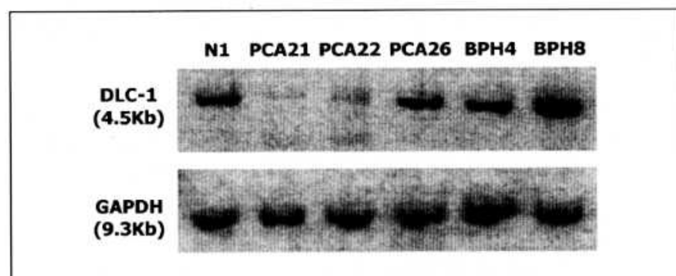


Fig. 2. Southern blot analysis of DLC-1 gene. Genomic DNA was digested with restriction enzyme *Eco* RI for the detection of DLC-1 or with *Bam* HI for the detection of GAPDH. Hybridization was done using a DLC-1 cDNA specific probe, which corresponds to 4.5 kb, and a control probe (GAPDH) for DNA loading (which corresponds to 9.3 kb).

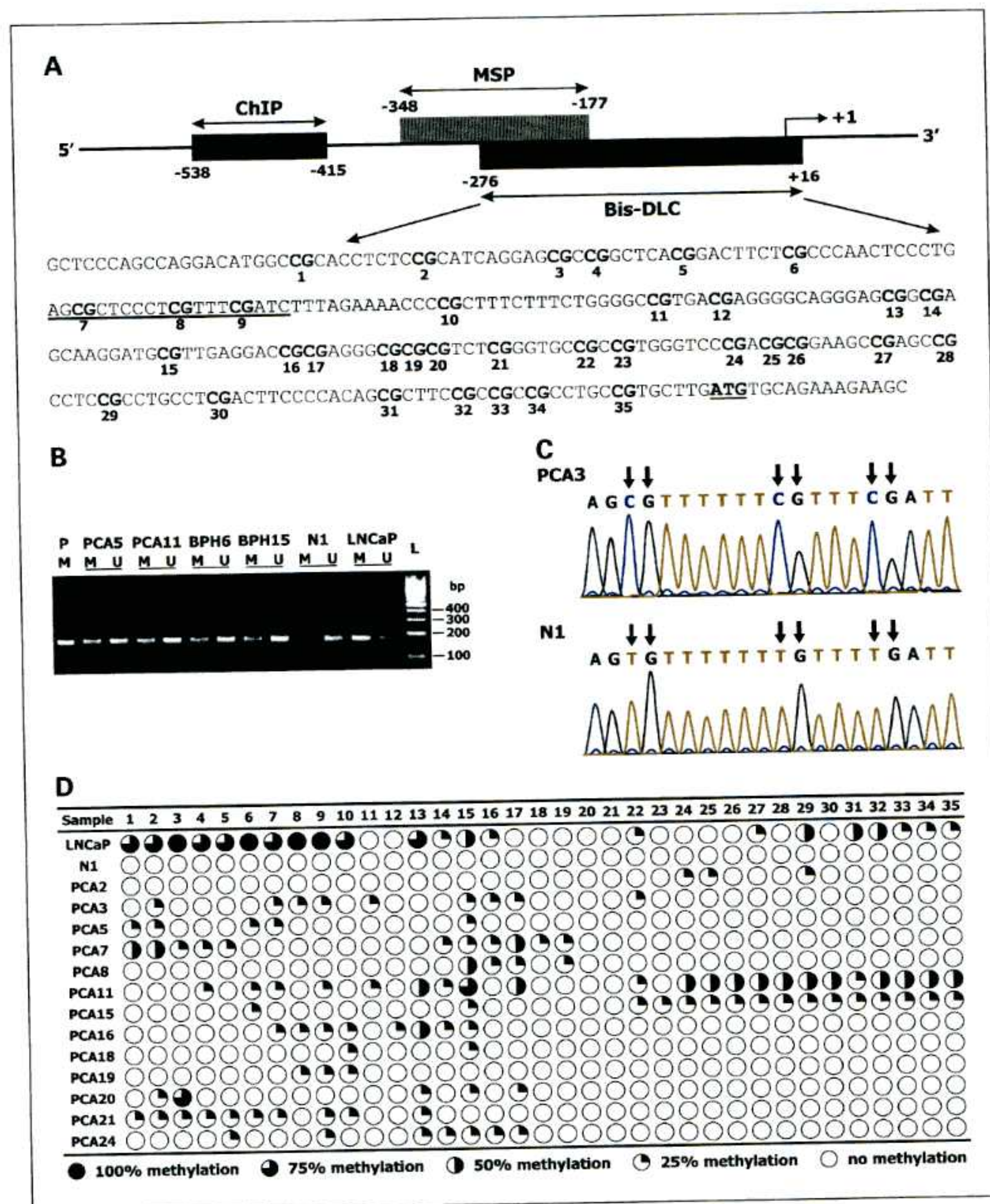


Fig. 3. Methylation status of the DLC-1 5′ CpG island region in prostate cancer. *A*, schematic depiction of *DLC-1* promoter-associated CpG island, which spans the region from −538 to +16 with respect to the ATG start site (+1; *bold and underlined*). Regions of chromatin immunoprecipitation (*ChIP*) analysis, MSP, and bisulfite genomic sequencing (*Bis-DLC*). *Bis-DLC* has a size of 292 bp and contains 35 CpG dinucleotides (the nucleotide sequence used as an example of the bisulfite DNA sequencing in *C* is *underlined*). *B*, representative examples of MSP of the *DLC-1 5′* CpG island in prostate carcinomas, benign prostatic hyperplasias, a normal prostatic tissue (N_1), and a commercial positive control sample (*P*). *M*, methylated; *U*, unmethylated. Both methylated and unmethylated bands were detected by MSP in LNCaP cells (*L*; 100-bp DNA ladder). *C*, examples of a highly methylated *DLC-1 5′* CpG island in sample PCA3 (*top*) and an unmethylated CpG island in sample N_1 (*bottom*) as determined by bisulfite sequencing analysis. *Arrows*, positions of CpG dinucleotides. *D*, methylation pattern of the Bis-DLC region of the *DLC-1 5′* CpG island in normal sample N_1, LNCaP cell line, and the 13 prostate carcinoma tumor samples that were found to be methylated by MSP. Each circle in the figure represents a single CpG site. For each DNA sample, the percentage of methylation at a single CpG site was calculated from the sequencing results of four independent clones.

inhibitor) and trichostatin A treatment of LNCaP cells, which have a partially methylated allele, exerted a synergistic effect on the level of *DLC-1* reexpression. The effect of each drug alone was a 5.4-fold increase ($P = 0.025$) for 5-aza-dC and a 29.8-fold increase ($P < 0.001$) for trichostatin A. Combining 5-aza-dC and trichostatin A resulted in a 122.8-fold increase ($P = 0.003$) of *DLC-1* transcripts (Fig. 4*C*).

Chromatin immunoprecipitation assay, using anti-acetylated histone H3 antibody, was used to provide direct evidence that trichostatin A mediates *DLC-1* mRNA reexpression by modulating acetylation status of the promoter region. From 22Rv1 cells, which were highly responsive to trichostatin A treatment, formaldehyde cross-linked protein chromatin complexes were immunoprecipitated and genomic DNA was analyzed by PCR using primers that recognize the *DLC-1* promoter region. A significant amount of acetylated histone H3 associated with the *DLC-1* promoter was detected only in trichostatin A–treated cells (Fig. 4*D*).

Discussion

Our results show that the transcriptional silencing of *DLC-1* by two epigenetic mechanisms is common and may be involved in the pathogenesis of prostate cancer and benign prostatic hyperplasia. Although region 8p21-22, which harbors DLC-1, does not correspond with a fragile site, its propensity for deletion is similar to that of the most unstable and vulnerable fragile sites (34, 35). However, inactivation or down-regulation of *DLC-1* mRNA and protein expression in prostate carcinomas, prostate carcinoma cell lines, and benign prostatic hyperplasias was primarily caused by promoter hypermethylation and histone deacetylation and only a small fraction of cases exhibited heterozygous genomic deletions. Previously, it has been shown that the frequency of 8p22 deletion is higher in cancer patients with tumor-node-metastasis stage T_3 or higher than T_2 (6). On the contrary, the *DLC-1* locus was not deleted in this series of prostate carcinomas. Promoter

Table 2. Correlation between *DLC-1* methylation and mRNA and protein expression with clinical and histologic variables in prostate carcinoma patients

	Methylation		P*	mRNA expression		P†	Protein expression		P†
	Present	Absent		Normal	Reduced		Normal	Reduced	
Age (y)									
≤70	3	10	**0.006**	12	1	**0.011**	11	2	0.378
>70	10	3		5	8		8	5	
PSA (ng/mL)									
<10	7	7	1.000	7	7	0.110	8	6	0.081
≥10	6	6		10	2		11	1	
Stage (tumor-node-metastasis)									
T_1-T_2	5	5	1.000	5	5	0.234	7	3	1.000
T_3-T_4	8	8		12	4		12	4	
Gleason score									
2-6	6	7	0.695	8	5	1.000	8	5	0.378
7-10	7	6		9	4		11	2	

NOTE: Bolded entries indicate significance.
*χ^2 test.
†Fisher's exact test.

methylation seems to be associated with human cancer at least as frequently as disruption of tumor suppressor genes by mutation or deletion, and many genes modified by promoter methylation have classic tumor suppressor functions (16, 36). Frequent structural and functional alterations of the *DLC-1* gene and its antioncogenic activity in several common cancers indicate that this gene is emerging as a bona fide tumor suppressor gene. Recently, by microarray analysis of 5-aza-dC-treated prostate carcinoma cell lines, *DLC-1* was identified among 50 candidate genes for epigenetic silencing and with possible involvement in tumor suppression in prostate cancer (37).

Histone deacetylation is also an important component of *DLC-1* silencing as shown in our experiments with the cell lines derived from prostate carcinomas. In the three *DLC-1*-negative cell lines, trichostatin A restored *DLC-1* expression to various degrees. In LNCaP cells, in which *DLC-1* promoter is partially methylated, the combined 5-aza-dC and trichostatin A treatment had a synergistic effect on *DLC-1* expression. This cell response is consistent with the evidence generated in several studies, showing that gene silencing associated with methylation and histone deacetylation can be converted to changes adequate for gene activation (reviewed in ref. 36).

Our results show that *DLC-1* gene promoter is methylated in prostate carcinomas, adjacent normal tissue from patients with prostate carcinoma, and benign prostatic hyperplasias but not in normal prostatic tissue samples from noncancerous individuals. The incidence of prostate cancer increases considerably with age and our statistical analysis revealed an association of *DLC-1* aberrant methylation with aging (38). This correlation remains to be confirmed with increasing sample size. The significance of *DLC-1* methylation in adjacent normal tissue of two prostate carcinoma patients is unclear. One can speculate that it may reflect a wider spread of malignancy than was originally determined by the histologic examination.

Table 3. Correlation between *DLC-1* methylation and mRNA and protein expression with clinical variables in benign prostatic hyperplasia patients

	Age (mean ± SE, y)	P*	PSA (mean ± SE, ng/mL)	P*
Methylation				
Present (n = 15)	72.9 ± 1.4	0.411	4.78 ± 0.96	**0.029**
Absent (n = 6)	75.8 ± 4.3		1.56 ± 0.34	
mRNA expression				
Normal (n = 16)	73.9 ± 1.8	0.678	4.10 ± 0.87	0.283
Reduced (n = 5)	73.0 ± 3.7		3.07 ± 1.67	
Protein expression				
Normal (n = 10)	74.1 ± 2.2	0.887	3.72 ± 1.02	0.833
Reduced (n = 11)	73.4 ± 2.3		3.98 ± 1.16	

NOTE: Bolded entry indicates significance.
*Mann-Whitney U test.

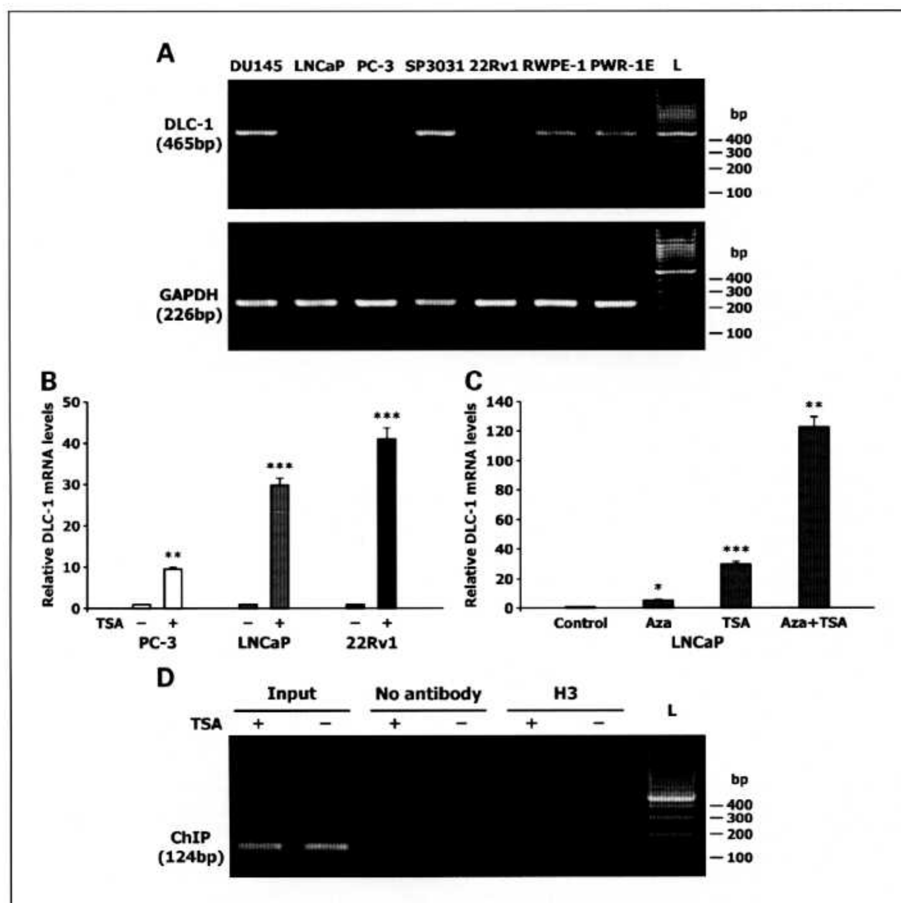


Fig. 4. Restoration of *DLC-1* expression *in vitro* by DNA methyltransferase and HDAC inhibitors. *A*, reverse transcription-PCR analysis of *DLC-1* expression in prostate carcinoma cell lines. Among 5 prostate carcinoma lines (DU145, LNCaP, PC-3, SP3031, and 22Rv1) and 2 normal prostate epithelial lines (RWPE-1 and PWR-1E), *DLC-1* is expressed at a negligible level in three prostate carcinoma cell lines. *GAPDH* was used as an internal control (*L*; 100-bp DNA ladder). *B*, expression of *DLC-1* mRNA in PC-3, LNCaP, and 22Rv1 cells after 12-hour exposure to trichostatin A. The level of expression is represented as fold increase over the control untreated cells. *Bars*, SE of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, relative to control. *C*, increased expression of *DLC-1* mRNA in LNCaP cells after treatment with 5-aza-dC (1 μmol/L) for 72 hours or trichostatin A (*TSA*; 500 nmol/l) for 12 hours or by combined treatment with both agents. The level of expression is represented as fold increase over the control untreated cells. *Bars*, SE of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, relative to control. *D*, chromatin immunoprecipitation analysis of histone H3 acetylation on the *DLC-1* promoter. 22Rv1 cells were treated with 500 nmol/L trichostatin A for 12 hours and the cell lysate was immunoprecipitated with an antibody to acetylated histone H3. DNA fragments from chromatin immunoprecipitation assays were amplified using promoter primer sets. Trichostatin A increased acetylation of histone H3 on the *DLC-1* promoter. Input for each reaction was used for internal control of samples loading. An aliquot precipitated without antibody was employed as negative control (*L*; 100-bp DNA ladder).

A high incidence of *DLC-1* methylation was also detected in benign prostatic hyperplasias. In contrast to high-grade prostatic intraepithelial neoplasia and proliferative inflammatory atrophy, benign prostatic hyperplasias are not considered premalignant or predisposing lesions to the development of prostate carcinomas (39). Benign prostatic hyperplasias develop in the transitional zone of the prostate, whereas prostate carcinomas usually develop in the peripheral zone of the gland. However, ~25% of prostate carcinomas are detected in the transitional zone; thus, one cannot exclude the possibility that the two diseases are linked (40). Benign prostatic hyperplasias are diagnosed in men over their fifties (38). The average age of our benign prostatic hyperplasia patients was 73 years, and there was a statistically significant correlation between *DLC-1* methylation and PSA blood levels. It is possible that down-regulation or silencing of *DLC-1*, which encodes a protein with tumor suppressor function, or of other putative tumor suppressor genes commonly methylated in benign prostatic tissue are age-dependent early events that promote initiation and progression of cellular hyperplasia (38, 41).

Methylation changes in normal tissues seem to indicate a risk for developing cancer rather than the presence of cancer in an individual (16). The absence of *DLC-1* methylation in normal prostatic tissues from our noncancerous subjects is consistent with a mutation study showing that *DLC-1* is not linked, as was previously suspected, to a prostate cancer susceptibility gene (42, 43). Because all normal prostate samples (N_1-N_{10}; Fig. 1*A*) derived from young individuals, future studies are warranted to examine the methylation of DLC-1 in nonneoplastic tissues from older subjects.

Our understanding of the mechanisms responsible for gene silencing in neoplasias has clinical relevance for the risk assessment, early diagnosis, prognostic monitoring, treatment, and prevention of cancer (36). A recent comprehensive study highlighted the limitations of the PSA test for prostate cancer screening and underlined the need for the development of biomarkers for early detection and prognosis of the disease (44). Aberrant methylation is one of the earliest alterations in the development of cancer, including prostate carcinomas (38). Methylation of *DLC-1*, as a part of a panel of biomarkers, could be useful for the detection and risk assessment of prostate cancer and of its treatment (38, 45).

Because inhibitors of DNA methyltransferase and HDAC can induce the restoration of *DLC-1* expression, the DLC-1 protein may also represent a potential target for novel therapies. Zebularine, a new and highly effective DNA demethylating agent, and several HDAC inhibitors are attractive therapeutic approaches (46, 47). Lastly, if *DLC-1* has tumor suppressive activity in prostate cancer, its silencing by promoter methylation may increase the risk of metastasis. Therefore, DLC-1 up-regulation may be a good candidate for gene therapy of prostate cancer.

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