Expression profile of the tumor suppressor genes DLC-1 and DLC-2 in solid tumors

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Abstract. Several years after the isolation of deleted in liver cancer 1 (DLC-1), a gene that encodes a Rho GTPase activating protein, the closely related DLC-2 gene was identified. DLC-1 and DLC-2 are ~50% identical and share the same SAM-RhoGAP-START domain organization. Since DLC-1 and -2 are located at chromosome regions that are commonly deleted in cancer cells and have been found to function as tumor suppressor genes, we sought to compare their expression profiles in several common types of cancer and to determine whether *dlc1* and *dlc2* proteins cooperate in tumor development. Using cancer-profiling arrays, we detected for the first time down-regulation of DLC-1 expression in renal, uterine and rectal cancers and down-regulation of DLC-2 expression in lung, ovarian, renal, breast, uterine, gastric, colon and rectal tumors. Since DLC-1 also functions as a metastasis suppressor gene in breast cancer, DLC-1 and DLC-2 expression were examined in a series of primary ductal carcinomas derived from patients with regional lymph node metastases. Using quantitative RT-PCR we detected a significantly lower expression of DLC-1 and DLC-2 in high percentage of tumors, suggesting that deficiency of either DLC gene facilitates dissemination of breast carcinoma cells to secondary sites. We examined DLC-2 expression in DLC-1-negative cell lines derived from human breast, non-small cell lung, and hepatocellular carcinomas, that could be rendered less or non-tumorigenic by ectopic expression of DLC-1. DLC-2 transcripts were detected in all cell lines, indicating that none of the cells were deficient in both members of the DLC family. This comparative expression analysis of DLC-1 and -2 identifies down-regulation of the two emerging bona fide tumor suppressor genes in additional types of solid tumors. The large spectrum of cancers with dysregulated DLC genes underlines the involvement of this family of genes in cancer development.

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Introduction

DLC-1 (deleted in liver cancer 1), a gene that encodes a Rho GTPase-activating protein was cloned as a genomic DNA segment under-represented in a human primary hepatocellular carcinoma (HCC) (1). DLC-1 is located on chromosome 8p21-22, a region of recurrent loss of DNA-copy-number and loss of heterozygosity (LOH) in a variety of human cancers (1). DLC-1 is frequently inactivated or down-regulated in a variety of solid tumors and hematological malignancies and functions as a tumor suppressor gene in breast, lung, ovarian and liver cancers and as a metastasis suppressor gene in breast cancer (2-9). The regulation of Rho GTPase proteins is critical to the neoplastic process and metastasis (10-14). An altered balance between active GTP-bound and inactive GDP-state determines the activity of Rho proteins. DLC-1-mediated negative regulatory effect of cell growth and tumorigenicity is primarily due to RhoGAP ability to inactivate Rho proteins. DLC-1 is a GTP-ase activating protein specific for RhoA and Cdc42, that are dramatically overexpressed in various cancers (8,15). Frequent structural and functional alterations of the DLC-1 gene and its anti-oncogenic activity suggest potential clinical applications in prevention and early detection of cancer. A high-throughput single nucleotide polymorphism genotyping and gene expression profiling identified DLC-1 as a candidate breast cancer susceptibility gene and the high incidence of DLC-1 methylation in prostate carcinomas may serve as a useful biomarker and in combination with other methylated genes for early detection of prostate cancer (16-18). Cells derived from different types of cancer such as breast, lung, liver or ovarian cancers are highly sensitive to reactivation of DLC-1 function. Thus, the potential for an effective therapy based on DLC-1 transfer to tumor cells appears high.

Two additional members of the DLC family have recently been identified: DLC-2 (STARD13) and DLC-3 (KIAA0189, also known as STARD8) that are ~50% identical to DLC-1 and share the same SAM-RhoGAP-START domain organization (19-22). DLC-2 is located on chromosome 13q12 and is emerging as a tumor suppressor gene due to its inhibitory effect on Ras-induced transformation of rodent cells and suppression of the growth of human breast and liver tumor cells (20,21,23). Like DLC-1, DLC-2 GTP-ase activating

protein is specific for RhoA and Cdc42 (23). Given the homology and the suppressive activity on proliferation of tumor cells of DLC-1 and DLC-2, the present study was, therefore undertaken to compare the expression of both genes in several types of cancer.

Materials and methods

Cell culture and primary samples. The cell lines from our laboratory repository or purchased from the American Type Culture Collection (Manassas, VA) were cultured in DMEM/F12 media with 10% fetal bovine serum and antibiotics (Biosource, CA) (1). RNA from primary breast tumors and their normal counterparts were obtained from Oncomatrix (San Marcos, CA) while normal liver tissue was from our laboratory repository.

DLC-1 and -2 expression profiling. A cancer profiling array I, containing normalized cDNA from 241 tumors, metastases and corresponding normal tissues from individual patients, was purchased from BD Biosciences (Palo Alto, CA). The DLC-1 probe was amplified from the HLE liver cancer cell line using primers: forward 5' CACAGGACAACCGTTG CCTCGA 3' and reverse 5' CTCTTCAGGGTGTTGAGAT GGA 3'. DLC-2 probe was amplified from HLE liver cancer cell line using primers forward: 5' AGCCCCTGCCTCA AAGTATT 3' and reverse: 5' ATGGGCGTCATCTGATT CTC 3' (14). PCR of both genes was carried out as follows: (95˚C, 1 min; 55˚C, 1 min; 72˚C, 1 min) 35 cycles, 72˚C, 7 min. Purified PCR products were labeled with α-^{32}P-dATP using a Prime-It™ II random primer labeling kit (Stratagene, CA). Labeled probe was purified by column chromatography and 15-20x10^6 cpm of the probe was hybridized overnight with the cancer profiling array I according to the manufacturer's protocol. Autoradiographs were scanned and analyzed by Gene Snap™ densitometry software. A ubiquitin cDNA probe was prepared using the same protocol from the manufacturer's ubiquitin cDNA and it was used as a hybridization control and for data normalization. DLC-1 and -2 expressions were first normalized to the expression of ubiquitin control. The difference in the gene expression between normal and tumor tissue was revealed for each sample as a ratio of normal/tumor values of normalized expression. Significant difference was considered when the ratio of normal/tumor was >1.25 and <0.75.

Real-time RT-PCR of DLC-1 and DLC-2. RNA (5 μg) from 24 breast tumors originating from patients with regional lymph node metastases and matching normal breast tissue was transcribed into cDNA using a cDNA archive kit (Applied Biosystems, NJ). Real-time RT-PCR of DLC-1 used the manufacturer's Taq Man® probe DLC1-1_P and primers DLC1-1499R, DLC1-1416F (Applied Biosystems). Real-time RT-PCR of DLC-2 (STARD13) was performed using SYBR Green I PCR master mix (Superarray, MD) and commercial primers designed for specific real-time RT-PCR of STARD13 (Superarray). The specific peak of DLC-2 was checked by melting-curve analysis. Real-time RT-PCR of GAPDH was carried out according to the manufacturer's protocol using commercial Taq Man probe and primers (TaqMan GAPDH

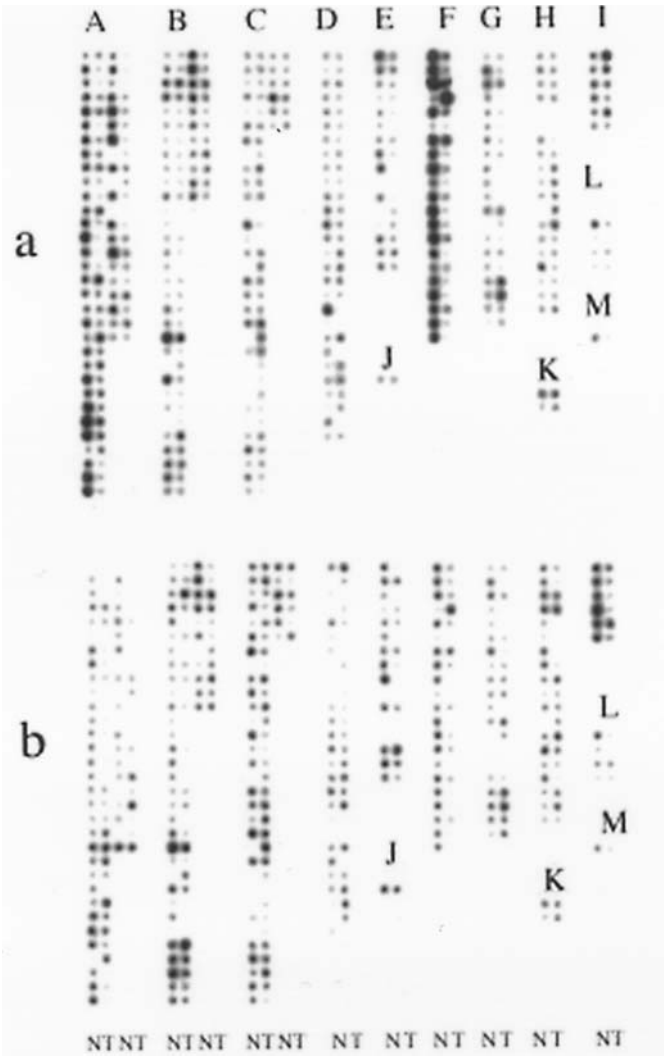

Figure 1. DLC-1 (a) and DLC-2 (b) expression in tumor and normal tissues detected by hybridization of DLC-1 and DLC-2 probes with the cancer profiling array I, containing normalized cDNA from tumors and corresponding normal tissues from individual patients. A, breast; B, uterus; C, colon; D, stomach; E, ovary; F, lung; G, kidney; H, rectum; I, thyroid; J, cervix; K, small intestine; L, prostate; M, pancreas; N, normal tissue; T, tumor counterparts.

Control Reagents, Applied Biosystems). The same reaction efficiency of all PCR systems was revealed from standard curves. All real-time PCR reactions were carried out using an ABI PRISM 7900. DLC-1 and -2 expression was first normalized to GAPDH control gene. The difference in the gene expression between normal and tumor tissue was revealed by the $2^{-\Delta\Delta ct}$ method for each sample. Significant difference was considered when $2^{-\Delta\Delta ct}$ was $\geq$2 and $\leq$0.5.

RT-PCR of DLC-2. Total RNA from all cancer cell lines was isolated by RNeasy™ mini kit (Qiagen, CA) and 5 μg of RNA was transcribed using the cDNA archive kit (Applied Biosystems). RT-PCR of DLC-2 used primers and conditions as described above with the exception of 30 cycles instead of 35. GAPDH was used as an internal control gene. PCR of GAPDH was carried out as follows: (95˚C, 1 min; 61˚C, 1 min; 72˚C, 1 min) 33 cycles, 72˚C, 7 min using primers: reverse 5' AGGGGAGATTCAGTGTGGTG 3' and forward 5' CGA CCACTTTGTCAAGCTCA 3'.

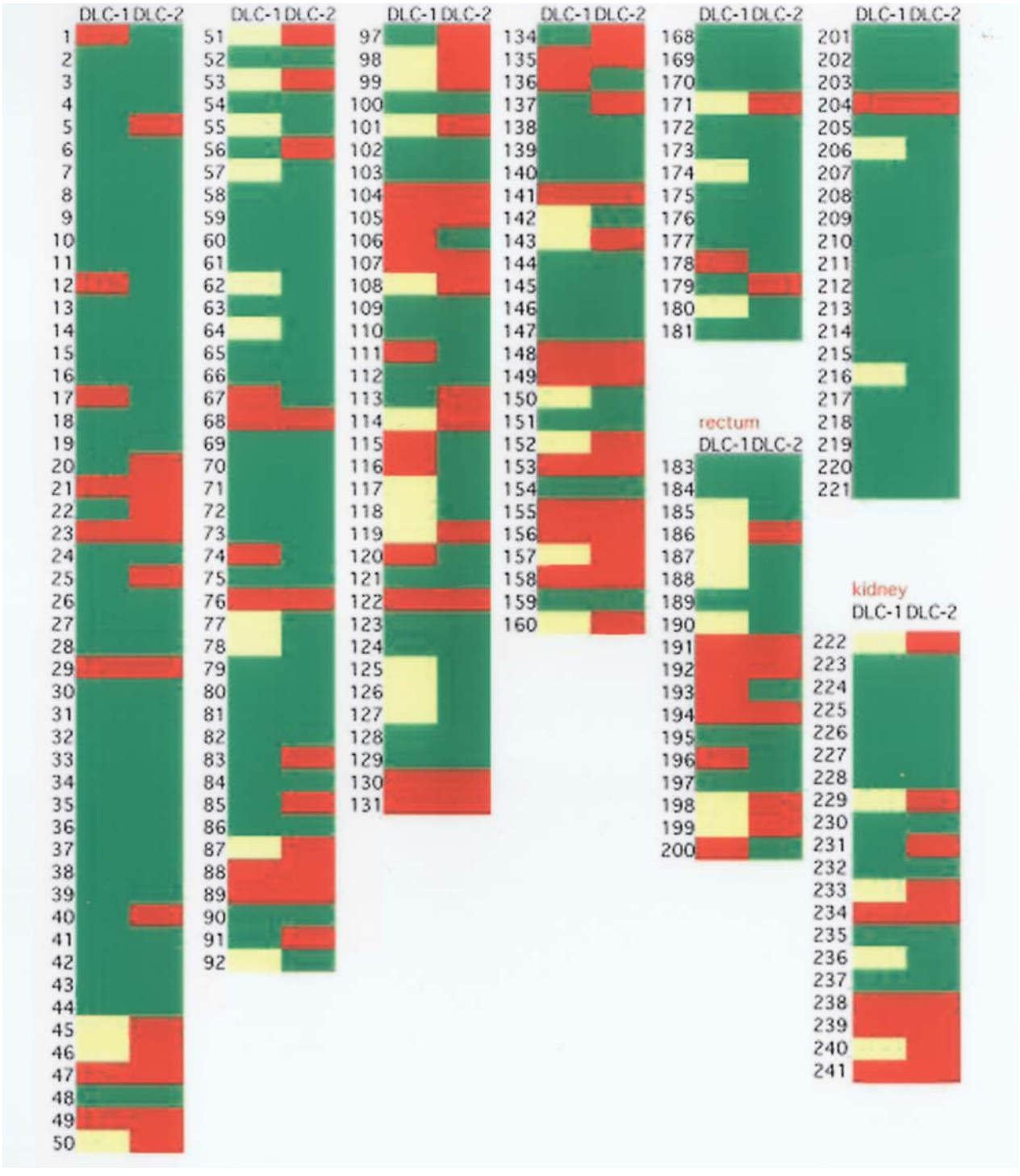

Figure 2. Summary of DLC-1 and DLC-2 expression in different tissues obtained after data normalization to ubiquitin control and analysis by Gene Snap™ densitometry software. Samples are divided in groups according to tissue of origin. Up-regulation (red), down-regulation (green) and no change (yellow) of DLC-1 and DLC-2 expression is shown for each sample. Numbers refer to appropriate sample identification on the cancer profiling array I. The difference between normal and tumor tissue was revealed for each sample as a ratio of normal/tumor values of ubiquitin-normalized expressions.

Results

Expression of DLC-1 and DLC-2 in tumor and normal tissues. To assess the involvement of DLC-1 and DLC-2 in various types of cancer we compared their expression by using a cancer profiling array I containing 241 tumor and normal tissue samples derived from patients with breast, uterine, colon, gastric, ovarian, lung, renal and rectal cancer. Array expression analysis showed that both genes are recurrently down-regulated in several forms of solid tumors. We detected a reduced expression of DLC-1 and DLC-2 in a significant number of tumor samples derived from lung, ovarian, renal, breast, uterine, gastric, colon and rectal cancers as compared to the matched normal tissue (Fig. 1, Table I). The percentages of tumor samples exhibiting down-regulation of both genes ranged from <50% in colon and gastric tumors to 60-80% in uterine, breast, renal and ovarian tumors to as high as 90% in lung tumors (Table I). In rectal tumors DLC-2 was more

Table I. DLC-1 and DLC-2 expression in various types of solid tumors.

Tissue (total number of samples)	DLC-1 (%)[a]		DLC-2 (%)[a]		Loss of DNA copy-number (%)[b]	
	Up	Down	Up	Down	8p	13q
Lung (21)	5	90	9	91	40-69	70-100
Ovary (14)	7	79	14	86	40-69	40-69
Kidney (20)	5	75	20	65	<40	<40
Breast (50)	14	76	20	72	<40	40-69
Uterus (42)	12	64	24	64	-	40-69
Colon (35)	37	43	37	46	70-100	-
Stomach (27)	41	41	52	37	-	-
Rectum (18)	39	28	22	61	-	-

[a]Frequency of tumors exhibiting an increase or decrease in DLC-1 and DLC-2 expression relative to appropriate normal tissue. [b]Majority of tumors with a reduced expression of DLC-1 and DLC-2 also exhibit with high frequency loss of 8p and 13q as depicted by comparative genomic hybridization (reviewed in ref. 28).

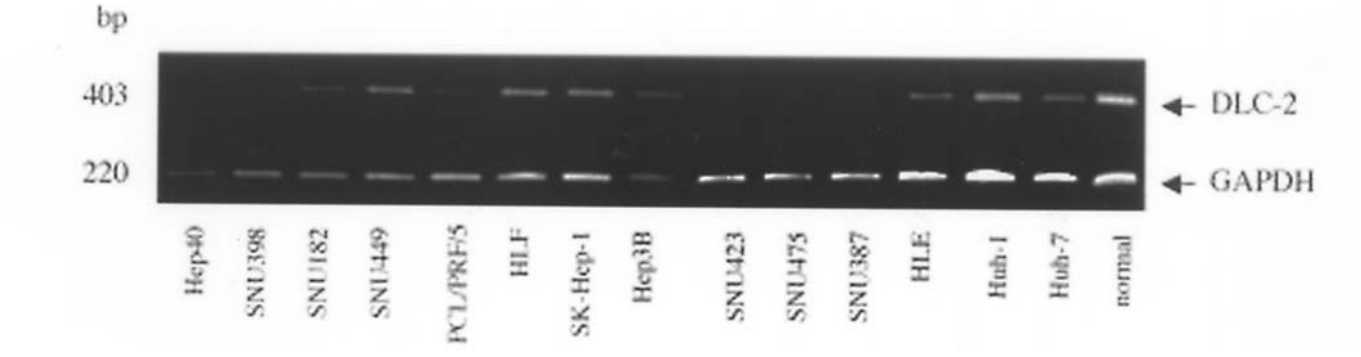

Figure 3. Expression of DLC-2 in human hepatocellular carcinoma cell lines and a normal liver tissue sample. RT-PCR of DLC-2 and GAPDH genes was performed as described in Material and methods. The expression of DLC-1 gene in human hepatocellular carcinoma cell lines for comparison of both genes was reported earlier (18). GAPDH was used as an internal control of amplification.

frequently down-regulated than DLC-1 (Table I). A schematic representation of these data in Fig. 2 and the percentages shown in Table I illustrate that both DLC genes are consistently down-regulated at approximately the same frequency in a variety of solid tumors.

Expression of DLC-2 in liver cancer cell lines. Since samples derived from HCC were not included in the cancer profiling array I and given the fact that both DLC-1 and 2 genes are located at regions prone to deletion and LOH in HCC, we examined the expression of DLC-2 by RT-PCR in 14 HHC cell lines previously characterized for DLC-1 expression (24). While DLC-2 was expressed in normal liver tissue, five HCC cell lines, Hep40, SNU398, SNU-423, SNU-475 and SNU-387, lacked expression of DLC-2 mRNA expression (Fig. 3). A comparison of both DLC genes expression determined in the same cell lines demonstrated that DLC-2 is more frequently down-regulated than DLC-1 in HCC cell lines (24).

Expression of DLC-1 and DLC-2 in metastatic breast tumors. Previous investigations with breast cancer have shown that DLC-1 is frequently down-regulated or silenced in cell lines derived from aggressive metastatic tumors (4,9). Restoration of DLC-1 expression in cell lines derived from metastatic breast adenocarcinomas with low levels of endogenous gene expression caused significant growth inhibition and prevented the development of tumors and reduced the ability of metastatic cells to form pulmonary metastases in athymic mice (4,9). The expression of DLC-1 and DLC-2 was examined by real-time RT-PCR in 12 breast ductal carcinomas from patients with regional lymph node metastases and their corresponding normal breast tissue. A significant level of DLC-1 and DLC-2 down-regulation was detected in 91% and 73% of tumors, respectively, that disseminated to 1 or to as many as 8 lymph nodes (Fig. 4).

Expression of DLC-2 in cancer cell lines lacking expression of DLC-1. To see whether DLC-2 interferes with the ectopic expression of DLC-1, the expression of DLC-2 was examined in two breast (MDA-MB-361, MDA-MB-468), two HCC (Focus, 7703) and two non-small cell lung carcinoma (H23, H358) cell lines lacking DLC-1 mRNA expression. Restoration of DLC-1 expression in these lines by transfection of DLC-1 cDNA suppressed *in vitro* cell proliferation and tumorigenicity *in vivo* (4-6). DLC-2 mRNA was expressed at different levels in all six cell lines (Fig. 5).

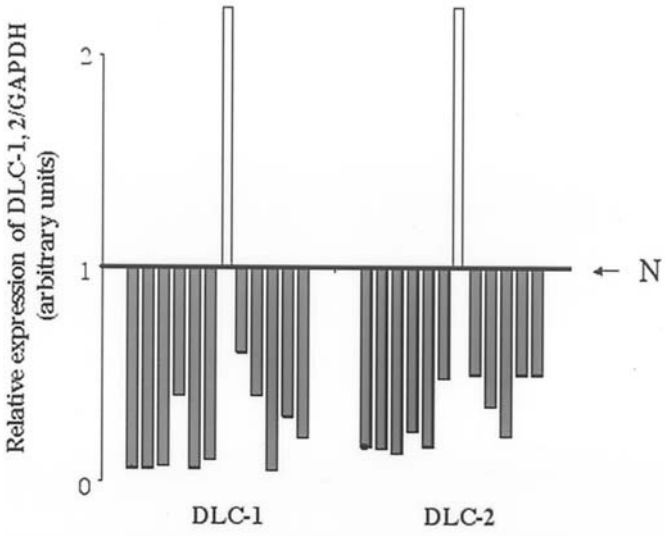

Figure 4. Expression of DLC-1 and DLC-2 in 12 breast ductal carcinomas from patients with regional lymph node metastases and their corresponding normal tissue. Real-time RT-PCR of DLC-1, DLC-2 and control GAPDH genes was described in Material and methods. DLC-1 and DLC-2 expression was normalized to the expression of control GAPDH and the difference between normal and tumor tissue was revealed by the $2^{-\Delta\Delta ct}$ method. Significant difference was considered when $2^{-\Delta\Delta ct}$ was ≥2 and ≤0.5. Levels of gene expression in normal tissue (N) are set at 1 and indicated by an arrow. Both values, which are over the normal level, belong to the same patient.

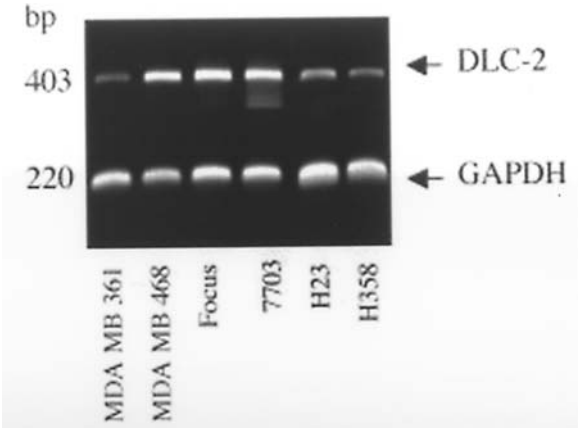

Figure 5. DLC-2 expression in breast carcinoma (MDA-MB-361, MDA-MB-468), hepatocellular carcinoma (Focus, 7703) and non-small cell lung carcinoma (H23, H358) cell lines lacking DLC-1. RT-PCR of DLC-2 was performed for comparison of DLC-2 expression with that of DLC-1 gene described earlier (4-6) in the same cell lines. GAPDH was used as an internal control of amplification.

Discussion

This comparative expression analysis provides a wider view of the involvement of DLC-1 and DLC-2 in human cancer. Cancer profiling array and RT-PCR analyses of tumor cell lines revealed a high frequency of down-regulation of both DLC genes in a variety of solid tumors. Down-regulation or inactivation are hallmarks of tumor suppressor genes and current results underline the notion that both DLC-1 and DLC-2 are emerging as bona fide tumor suppressor genes. Strikingly, both genes are down-regulated in lung, ovarian, renal, breast, uterine, gastric, liver, prostate, colon and rectal tumors as well as in HCC cell lines. Among these cancers, alterations of DLC-1 in renal, uterine and rectal tumors were identified for the first time. Prior to this study alterations of DLC-2 were reported only in liver and breast cancer (20,21). Therefore, both DLC genes may be implicated in considerably more forms of cancer than previously known.

The high incidence of various tumors eliciting down-regulation of DLC-1 and DLC-2 can be attributed to both genetic and epigenetic mechanisms. An interesting observation is that the majority of tumors with a reduced expression of DLC-1 and DLC-2 also exhibit a high frequency of loss of 8p and 13q as depicted by comparative genomic hybridization and recently compiled in a review article (25) (Table I). This is particularly obvious for lung, ovary, kidney and breast cancer. In addition, the loci of both DLC-1 and DLC-2 are located in regions of LOH in breast, lung and HCC (26). Tumor suppressor genes that are down-regulated or silenced by promoter hypermethylation are often located in genomic regions of deletion (27). Undoubtedly, promoter hypermethylation or histone deacetylation are major mechanisms responsible for down-regulation or silencing of DLC-1 in a variety of solid tumors and hematological malignancies (28-34). Most likely, epigenetic modifications account for dysregulation of DLC-2 as well.

DLC-1 expression was previously examined in several different HCC cell lines (1,2,8,24,29). In our series of cell lines, DLC-2 was more frequently down-regulated than DLC-1. However, larger numbers of primary tumors should be examined to validate the extent of DLC-1 and -2 dysregulation in HCC.

Based on previous investigations demonstrating that DLC-1 functions as a metastasis-suppressor gene in breast cancer (9), we examined DLC-1 and DLC-2 expression in breast tumors from patients with axillary lymph node metastases. Down-regulation of both DLC genes in a high number of cases underscores the role of RhoGAP deficiency in the acquisition of invasiveness and tumor cell dispersion at secondary sites. The spread of primary tumor cells to secondary sites is a coordinated and complex process involving interactions between the tumor cells and tissues of the host, changes in actin cytoskeleton organization, alterations of cell adhesion to extracellular matrix proteins, or disruption of cell-to-cell junctions (35-37). Overexpression of either DLC-1 or DLC-2 can disrupt cytoskeleton organization and reduce cell motility (25,29). In addition, rat DLC-1/p122 RhoGAP is localized to focal adhesions, structures that link the actin stress fibers and extracellular matrix receptors (38). DLC-1/p122 RhoGAP was also localized to caveolin-1-enriched plasma membrane domains, suggesting that DLC-1/p122 may play an important role in caveolin distribution through reorganization of the actin cytoskeleton (39).

Our previous investigations with several solid tumor cell lines, consistently demonstrated that ectopic expression of DLC-1 in DLC-1-deficient cell lines, reduced or abolished their tumorigenicity in nude mice. All these lines express DLC-2 at different levels, raising the possibility that DLC proteins cooperate in suppression of cancer cell proliferation. Due to their oncosuppressive function, DLC genes have attracted considerable interest for potential clinical applications in the

prevention, early detection and therapy of cancer and current observations implicate alterations of both DLC-1 and -2 in additional forms of cancer.

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