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Advances in Brief

Overexpression of *cdc25A* and *cdc25B* Is Frequent in Primary Non-Small Cell Lung Cancer but Is Not Associated with Overexpression of c-*myc*[1]

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Abstract

Cyclin-dependent kinases can be activated by cdc25, which removes inhibitory phosphates from tyrosine and threonine residues. At least three *cdc25* genes (*cdc25A*, *cdc25B*, and *cdc25C*) have been identified in humans. Accumulating evidence indicates that *cdc25A* and *cdc25B* possess oncogenic properties. Recently, overexpression of *cdc25A* and of *cdc25B* was found in many breast and head and neck cancers. To determine potential roles of *cdc25s* in non-small cell lung cancer (NSCLC), we analyzed primary tumors and corresponding normal lung tissues from 40 patients with NSCLC for relative expression levels of these genes by multiplex reverse transcription PCR (RT-PCR). *cdc25A* was overexpressed in 60% (24 of 40) of the tumors and *cdc25B* in 45% (18 of 40) of the tumors, whereas *cdc25C* was not overexpressed in any of the tumors analyzed. Because c-*myc* can increase *cdc25A* and *cdc25B* expression, it may be a factor in *cdc25* overexpression. We found that c-*myc* was overexpressed in only 18% (7 of 40) of the tumors. We found no association between overexpression of c-*myc* and *cdc25A* or *cdc25B*. We also investigated whether the *cdc25B* gene was amplified in NSCLC and found this was true in 40% (8 of 20) of the tumors tested. However, this amplification was not correlated with gene expression status. Interestingly, among 24 tumors with *cdc25A* overexpression and 18 with *cdc25B* overexpression, 42% (10 of 24) and 44% (8 of 18) were poorly differentiated histological type. In contrast, well or moderately differentiated tumors had lower frequencies of *cdc25A* and *cdc25B* overexpression [19% (3 of 16) and 23% (5 of 22), respectively]. These data indicate that overexpression of *cdc25A* and *cdc25B* is frequent and that it may play an important role in NSCLC. However, it is unlikely that this overexpression is caused by c-*myc* stimulation or *cdc25B* gene amplification.

Introduction

Lung cancer is the leading cause of cancer-related death in the United States (1). It is believed that the development of lung cancer including NSCLC[3] is a multistep process involving the accumulation of genetic alterations that then lead to tumor initiation and progression. Cell cycle checkpoints are crucial in controlling cell proliferation and are frequently disrupted in tumors by the activation of oncogenes and the inactivation of tumor suppressor genes. Thus, identification and characterization of abnormalities in components of these checkpoints in NSCLC may extend our understanding of the tumorigenic process and reveal potential biomarkers for cancer detection, risk assessment, and treatment.

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; CDK, cyclin-dependent kinase; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cdc25s can dephosphorylate threonine 14, tyrosine 15, or both on CDKs and activate cyclin/CDK complexes to stimulate cell proliferation (2–3). In humans, at least three *cdc25* genes, *cdc25A*, *cdc25B* and *cdc25C*, have been identified (2–4). It has been suggested that *cdc25A* and *cdc25B* but not *cdc25C* possess oncogenic properties (5). Recent studies showed that the overexpression of *cdc25A* and *cdc25B* is frequent in primary breast tumors and head and neck tumors (5–6), suggesting the potential role of these genes in tumorigenesis. c-*myc*, a proto-oncogene belonging to a family of genes implicated in the control of normal cell proliferation and the induction of neoplasia as well as the induction of apoptosis (7–8), is amplified and overexpressed in some human cancers including NSCLC (9). Because c-*myc* may induce expression of *cdc25A* and *cdc25B* (10), it was speculated that overexpression of *cdc25A* and *cdc25B* in human cancers may be a consequence of c-*myc* overexpression (6).

To determine the potential role of *cdc25*s in NSCLC, we examined the relative expression levels of *cdc25A*, *cdc25B*, and *cdc25C* in 40 primary tumors and their corresponding normal lung tissue samples. The potential association between overexpreesion of *cdc25s* and c-*myc* was also examined in this study. Furthermore, association between *cdc25B* expression and *cdc25B* gene amplification was analyzed.

Materials and Methods

Patients. Forty patients with histologically confirmed NSCLC were included in this study. There were 20 patients with adenocarcinoma and 20 patients with squamous cell carcinoma. All of the patients were treated by curative surgical resection in the University of Texas M. D. Anderson Cancer Center. The study was reviewed and approved by the Institutional Review Board's Surveillance Committee. None of the patients had had chemotherapy before the surgery. General patient characteristics are shown in Table 1.

RNA Extraction and cDNA Synthesis. After surgical resection, samples of residual primary tumor and the distal normal lung tissue were obtained for each patient from the Department of Surgical Pathology. Tissues were transferred immediately to the research laboratory and stored at −80°C until the experiment.

Total RNA was isolated from tumors and their paired normal lung tissues using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's protocol after tissue homogenization. Five μg of total RNA from each sample was subjected to reverse transcription with random hexamer, dNTPs, and 200 units Superscript II Rnase H^- reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in a 20-μl reaction volume. The synthesized cDNA was either used immediately for PCR amplification or stored at −20°C for further analysis.

Multiplex PCR Analysis. The relative expression levels of *cdc25A*, *cdc25B*, *cdc25C* and c-*myc* were examined by using a modified multiplex PCR technique as described previously (11–12). Either β-actin or GAPDH was used as an internal control in each PCR study. To avoid amplification of possible contaminated genomic DNA, all primer sets were designed to flank at least one intron and tested to ensure amplification of only cDNAs. The primer sequences used in this study were as follows: (*a*) for β-actin, 5′-GTTGCTATCCAGGCTGTGC-3′ (sense) and 5′-GCATCCTGTCGGCAATGC-3′ (antisense); (*b*) for GAPDH, 5′-AACATCATCCCTGCCTCTAC-3′ (sense) and 5′-

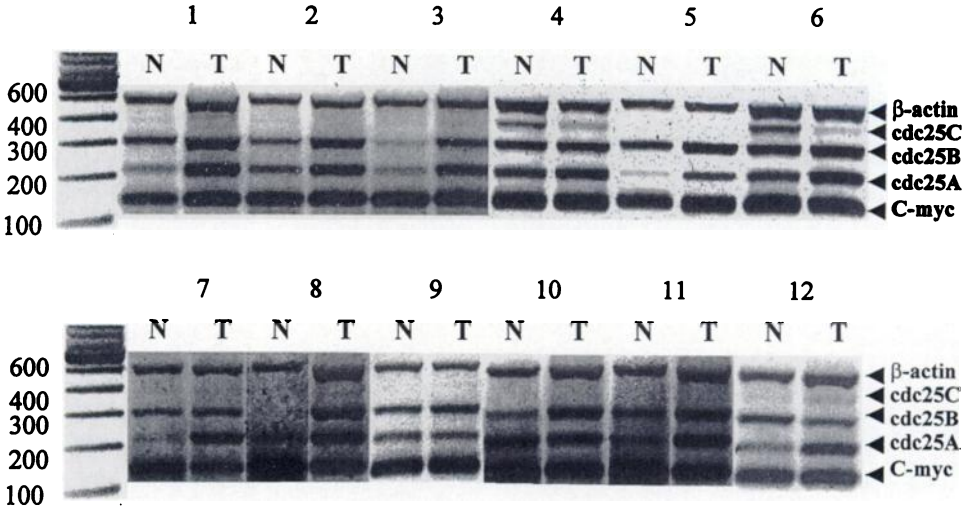

Fig. 1. Overexpression of *cdc25* and c-*myc* in NSCLC: photographic negative of an agarose electrophoresis gel showing expression of *cdc25A*, *cdc25B*, *cdc25C*, and c-*myc* by a multiplex RT-PCR. β-actin was used as an internal control. *T*, tumor; *N*, corresponding normal lung tissue. Overexpression of both *cdc25A* and *cdc25B* was shown in cases 1, 2, 3, and 8; *cdc25A* overexpression was shown in cases 6, 7, 11, and 12; *cdc25B* overexpression was shown in cases 9 and 10. Cases 1 and 5 showed c-*myc* overexpression; *cdc25s* and c-*myc* were not overexpressed in case 4.

Table 1 *Patient characteristics*

Case	Age (yr)/Sex	Cancer-type	Tumor grade	Disease stage	Smoking status	*cdc25A* overexpression	*cdc25B* overexpression
A1	74/F	AC[a]	MD	III	N	−	−
A2	53/F	AC	WD	I	N	−	−
A3	47/F	AC	PD	I	Y	+	+
A4	72/M	AC	PD	II	N	+	+
A5	54/M	AC	PD	II	Y	+	+
A6	56/F	AC	MD	II	Y	+	+
A7	69/M	AC	PD	I	Y	−	+
A8	46/M	AC	PD	II	Y	+	−
A9	70/M	AC	MD	I	Y	+	−
A10	43/F	AC	WD	III	Y	+	+
A11	67/F	AC	WD	I	Y	−	+
A12	64/M	AC	WD	I	N	−	−
A13	62/M	AC	WD	II	N	+	+
A14	63/M	AC	MD	I	Y	+	−
A15	59/F	AC	MD	III	N	+	−
A16	32/F	AC	MD	III	N	+	−
A17	67/M	AC	MD	II	N	−	+
A18	70/M	AC	WD	II	N	−	−
A19	82/F	AC	WD	I	N	−	−
A20	70/M	AC	PD	III	Y	−	+
S1	81/F	SCC	MD	I	Y	−	−
S2	59/M	SCC	MD	IIIA	N	−	+
S3	67/F	SCC	PD	II	Y	+	+
S4	61/F	SCC	MD	I	Y	−	−
S5	54/M	SCC	MD	I	Y	+	+
S6	73/M	SCC	MD	II	Y	−	−
S7	74/M	SCC	PD	IIIA	Y	+	−
S8	77/F	SCC	PD	IIIA	Y	+	+
S9	73/M	SCC	MD	I	Y	+	+
S10	74/F	SCC	PD	I	Y	+	+
S11	66/M	SCC	MD	I	Y	−	+
S12	78/M	SCC	MD	I	Y	+	−
S13	75/F	SCC	MD	I	N	+	−
S14	69/M	SCC	MD	II	Y	−	−
S15	72/M	SCC	PD	II	N	+	−
S16	73/M	SCC	MD	I	Y	+	−
S17	68/M	SCC	MD	I	Y	+	+
S18	66/F	SCC	PD	IIIA	N	+	−
S19	68/F	SCC	PD	I	Y	−	−
S20	51/M	SCC	MD	II	Y	+	−

[a] AC, adenocarcinoma; SCC, squamous cell carcinoma. WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

TCTCTCTCTTCCTCTTGTGC-3′ (antisense); (*c*) for *cdc25A*, 5′-ACCGTCACTATGGACCAGC-3′ (sense) and 5′-TTCAGAGCTGGACTACATCC-3′ (antisense); (*d*) for *cdc25B*, 5′-TCTCATCTGAGCGTGGGC-3′ (sense) and 5′-CTTCAGGCCTCGAAAGGC-3′ (antisense); (*e*) for *cdc25C*, 5′-CACTCCTACCGCGTCTTC-3′(sense) and 5′-CGTATCGCCCTCATCTGG-3′ (antisense); and (*f*) for c-*myc*, 5′-CTGGTGCTCCATGAGGAG-3′ (sense) and 5′-AGGTGATCCAGACTCTGAC-3′ (antisense). Each of the PCR reactions was performed in a 25-μl volume containing 0.5 μl of reverse transcription-reaction mixture, 3% dimethyl sulfoxide, 1.5 mM dNTP, 6.7 mM $MgCl_2$, 16.6 mM $(NH4)_2SO_4$, 67 mM Tris, 10 mM β-mercaptoethanol, 6.7 μM EDTA, 2.5 units of *Taq* Polymerase (Life Technologies), 0.4 μM each of the primers for *cdc25A*, 0.2 μM for *cdc25B*, 1.5 μM for *cdc25C*, 1.0 μM for c-*myc*, and 0.04 μM for β-actin or 1.5 μM of those for GAPDH. Thermal cycling was performed in a temperature cycler (Hybaid; Omnigene, Woodbridge, NJ) in

Table 2 *The relative expression levels of cdc25s and c-myc mRNA*

Case[a]	*cdc25A* (tumor:normal)	*cdc25B* (tumor:normal)	*cdc25C* (tumor:normal)	c-*myc* (tumor:normal)
A1	0.76 ± 0.47	0.66 ± 0.34	0	0.50 ± 0.34
A2	1.89 ± 0.44	0.92 ± 0.13	0	0.92 ± 0.01
A3	6.19 ± 0.06	3.20 ± 1.00	0	1.42 ± 0.28
A4	2.58 ± 0.36	5.50 ± 0.90	0	0.78 ± 0.36
A5	2.61 ± 0.54	2.23 ± 0.11	0.35 ± 0.06	0.99 ± 0.12
A6	2.72 ± 0.64	2.90 ± 0.24	0	0.75 ± 0.24
A7	1.17 ± 0.20	2.77 ± 0.07	0	0.65 ± 0.24
A8	3.00 ± 0.70	0.49 ± 0.06	0	0.85 ± 0.21
A9	3.73 ± 1.35	0.52 ± 0.07	0.60 ± 0.30	0.95 ± 0.17
A10	7.66 ± 1.43	8.54 ± 2.01	0	5.25 ± 0.75
A11	1.58 ± 0.11	5.85 ± 0.35	0	1.01 ± 0.32
A12	0.40 ± 0.15	0.86 ± 0.02	0	0.65 ± 0.10
A13	3.32 ± 0.93	2.29 ± 0.39	0.35 ± 0.23	2.06 ± 0.24
A14	3.30 ± 1.25	1.66 ± 0.18	0	1.51 ± 0.36
A15	3.62 ± 1.51	1.23 ± 0.30	0.71 ± 0.18	1.11 ± 0.06
A16	2.72 ± 0.32	1.90 ± 0.25	0	0.92 ± 0.04
A17	0.83 ± 0.08	3.23 ± 0.67	0	1.07 ± 0.44
A18	1.25 ± 0.20	1.02 ± 0.36	0	0.82 ± 0.04
A19	0.26 ± 0.17	0.42 ± 0.15	0	0.21 ± 0.06
A20	1.45 ± 0.23	3.31 ± 0.28	0	0.61 ± 0.02
S1	0.98 ± 0.37	1.13 ± 0.49	0	0.98 ± 0.37
S2	0.76 ± 0.35	3.41 ± 0.79	0	0.36 ± 0.05
S3	2.49 ± 0.29	2.60 ± 0.10	0	1.19 ± 0.01
S4	1.55 ± 0.44	1.13 ± 0.09	0	1.74 ± 0.15
S5	3.25 ± 0.71	3.02 ± 0.18	0.88 ± 0.38	2.60 ± 0.24
S6	0.99 ± 0.34	1.14 ± 0.04	0	1.43 ± 1.05
S7	2.82 ± 0.45	1.24 ± 0.07	0	0.89 ± 0.15
S8	2.81 ± 0.36	3.38 ± 0.32	0	1.16 ± 0.25
S9	4.81 ± 0.99	4.03 ± 0.94	0	3.14 ± 0.37
S10	4.36 ± 0.99	5.19 ± 0.32	0	3.40 ± 0.38
S11	1.92 ± 0.27	2.75 ± 0.26	0	1.98 ± 0.08
S12	2.86 ± 0.83	1.46 ± 0.15	0.15 ± 0.02	1.48 ± 0.51
S13	2.38 ± 0.14	0.95 ± 0.06	0	0.87 ± 0.17
S14	0.91 ± 0.45	1.01 ± 0.10	0	0.67 ± 0.39
S15	2.91 ± 0.74	1.84 ± 0.19	0	1.04 ± 0.20
S16	3.14 ± 0.56	1.42 ± 0.37	0	3.84 ± 0.60
S17	2.21 ± 0.02	2.93 ± 0.49	0	1.01 ± 0.26
S18	3.41 ± 0.46	1.19 ± 0.08	0	1.24 ± 0.58
S19	1.31 ± 0.41	0.98 ± 0.10	0.15 ± 0.06	1.58 ± 0.81
S20	3.81 ± 0.94	1.51 ± 0.20	0	3.45 ± 1.35
Total showing overexpression[b]	24 of 40 (60.0%)	18 of 40 (45%)	0 of 40 (0%)	7 of 40 (17.5%)

[a] A, adenocarcinoma; S, squamous cell carcinoma.

[b] Cases were considered to show overexpression when the ratio between expression in the tumor and in normal tissue was >2.

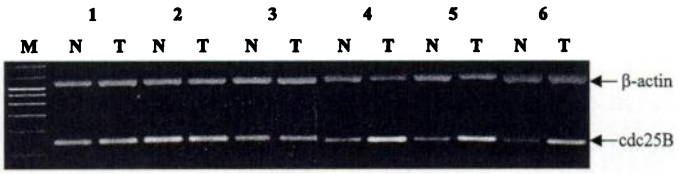

Fig. 2. Amplification of *cdc25B* gene. PCR was used to obtain a 144-bp *cdc25B* genomic DNA fragment in both NSCLCs (*T*) and corresponding normal lung tissues (*N*). The same amount of DNA template (as used for amplifying *cdc25B* gene) from each sample was also used to amplify a 637-bp genomic DNA fragment of β-actin as a control for DNA quantity. Cases 4, 5, and 6 showed amplification of *cdc25B* gene whereas cases 1, 2, and 3 showed an equal level of *cdc25B* amplification between normal and tumor tissues, which indicated that *cdc25B* was not amplified in these cases. *M*, molecular weight marker.

500-μl plastic tubes for one cycle of an initial denaturation at 95°C for 2 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 45 s, an extension at 70°C for 1 min, and final elongation step at 70°C for 5 min. All PCR experiments were performed in duplicate and included negative controls in which no cDNA template was added. The PCR products were then separated in a 2% agarose gel containing 0.05 μg/ml Ethidine Bromide.

Interpretation of Gene Expression. To quantify relative levels of gene expressions, the electrophoresed PCR products were visualized under UV light and photographed. The bands on the negatives of the photos were scanned by transmission densitometry (Model GS300 densitometer; Hoefer Scientific Instruments, San Francisco, CA). The areas of the wave peaks were calculated in arbitrary units. The variations in the amounts of RNAs and cDNAs at the start were normalized by comparing the band densities of the β-actin or GAPDH control between the tumor cDNA and the corresponding normal lung cDNA. Genes were considered to be overexpressed in tumors if the average ratio of expression level between tumor and normal lung tissues was more than 2.

DNA Extraction and Quantitative PCR Analysis. Genomic DNA was extracted from tumors and corresponding normal lung tissues using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's protocol. The primers for *cdc25B* used in this experiment were 5′-GCTATTCAAGAGGAAATGTC-3′ for sense and 5′-GCTCAGTGCTTTATTGAACC-3′ for antisense to amplify a 144-bp DNA fragment. β-actin was used as the internal control (an expected 637-bp PCR product). PCR reactions underwent one cycle of denaturation at 95°C for 3 min, 29 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 45 s, extension at 70°C for 1 min; and one cycle of extension at 70°C for 5 min. PCR experiments were repeated at least twice. *cdc25B* gene amplification was recorded when the density of *cdc25B* PCR fragment in tumors was at least two times more intense relative to the corresponding normal lung tissues.

Results and Discussion

We examined the relative expression levels of *cdc25A*, *cdc25B*, and *cdc25C* in 20 primary squamous cell carcinoma and 20 primary adenocarcinoma of the lung by a multiplex RT-PCR technique. Examples of gene overexpression patterns are shown in Fig. 1. We found that *cdc25A* and *cdc25B* were overexpressed in 60% (24 of 40) and 45% (18 of 40) of the tumors, respectively, but *cdc25C* was not overexpressed in any of these tumors (Table 2). Frequency of overexpression of these genes was not significantly different between the two cancer types. Taken together, 30 (75%) of 40 tumors overexpressed *cdc25A*, *cdc25B*, or both. There was no apparent association between overexpression of *cdc25A* and *cdc25B* (Table 1), suggesting that overexpression of these genes may be caused by distinct pathways and that it may play different roles in tumorigenesis. Two internal controls were used in this experiment to normalize the possible variations of gene expression among these housekeeping genes. It was noticed that rather wide variations of the gene expression existed in some cases when different internal control markers were used (Table 2). We, therefore, thought that multiple control markers might be required to accurately determine relative gene expression levels.

It was shown in previous studies (5, 6, 13) that *cdc25A* and *cdc25B* were overexpressed in primary breast cancers, head and neck cancers, and non-Hodgkin's lymphomas. In primary head and neck cancer, *cdc25A* and *cdc25B* were overexpressed in 80% and 50% of tumors, respectively (6). In human primary breast cancer, *cdc25B* was overexpressed in 32% of the tumors analyzed. There was strong association between *cdc25B* overexpression, microvessel density, and higher histological tumor grade (5). In our study, 42% (10 of 24) of the tumors with *cdc25A* overexpression and 44% (8 of 18) of the tumors with *cdc25B* overexpression were of poorly differentiated histological type; only 19% (3 of 16) and 23% (5 of 22) of the tumors without *cdc25A* or *cdc25B* overexpression were poorly differentiated. This observation is consistent with previous findings and suggests that *cdc25A* and *cdc25B* overexpression may be associated with tumors consisting of poorer differentiated cells.

How *cdc25A* and *cdc25B* are overexpressed during tumorigenesis is unclear. It has been indicated that *cdc25* is a positive regulator in cell cycle progression by dephosphorylating and activating CDKs (14). Recently, several studies demonstrated that *cdc25* is important in DNA-damage-induced cell cycle checkpoint control (15–18). In human cell lines, DNA damage can cause phosphorylation and activation of Chk1 kinase which in turn phosphorylates cdc25s (16). It has been shown that cdc25C is phosphorylated on Ser216, which allows binding of 14–3-3 protein and is then sequestered by 14–3-3, thus preventing activation of cdc2-cyclinB complex and mitotic entry (16–18). It was also suggested that *cdc25A* and *cdc25B* are required for G1-S phase checkpoint regulation through a similar mechanism (16).

Although evidence from previous studies supports the concept that overexpression of *cdc25A* and *cdc25B* may play an important role in certain human cancers, the mechanisms causing such overexpression are not known. c-*myc* is a proto-oncogene overexpressed in about 30% of NSCLCs (9). The product of c-*myc*, in partnership with max, forms a transcriptional factor that can promote either oncogenic transformation or apoptosis. A recent study showed that *cdc25s* including *cdc25A* and *cdc25B* are direct transcriptional targets of c-*myc* (10). It has been speculated that overexpression of *cdc25A* and *cdc25B* in head and neck cancer may be a consequence of c-*myc* overexpression (6). Indeed, in a recent study, overexpression of *cdc25B* was associated with overexpression of c-*myc* in non-Hodgkin's lymphomas (13). To address the possibility that the overexpression of *cdc25A* and *cdc25B* are the down stream events of c-*myc* overexpression, we examined the expression levels of c-*myc* and its association with *cdc25A* and *cdc25B* expression status in a multiplex RT-PCR assay. We found that 18% of tumors overexpressed c-*myc* (Table 2.). However, the overexpression of c-*myc* is probably not the cause of overexpression of *cdc25s* in NSCLC because 75% (18 of 24) of the tumors with *cdc25A* overexpression and 78% (14 of 18) of the tumors with *cdc25B* overexpression did not coincidentally overexpress c-*myc*. The data suggest that *cdc25A* and *cdc25B* may play an important role different from that of c-*myc* in NSCLC. However, we cannot rule out the possibility that the overexpression of *cdc25A* and *cdc25B* may be associated with expression status of max, since the transcriptional function of c-*myc* required the association of max.

It is also possible that overexpression of *cdc25s* is a consequence of the amplification of *cdc25* genes. Using comparative genomic hybridization, a recent study showed that about 45% of primary NSCLCs and NSCLC cell lines contain an amplified 20p13 region where *cdc25B* gene is located (19). To determine the frequency of *cdc25B* gene amplification and its potential role in gene overexpression, quantitative PCR studies were performed. Among 20 tumors (10 exhibited *cdc25B* overexpression and the other 10 did not) examined, the *cdc25B* gene was found amplified in 40% (8 of 20) of the tumors.

However, *cdc25B* overexpression was not associated with *cdc25B* gene amplification because tumors with and without *cdc25B* overexpression showed the same rate of gene amplification [40% (4 of 10) and 40% (4 of 10), respectively] (Fig. 2.). The data further suggest that one or more oncogenes may be found in the 20p13 amplicon and that these oncogenes are important in NSCLC.

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