Down Regulation of High in Normal-1 (*HIN-1*) is a Frequent Event in Stage I Non-Small Cell Lung Cancer and Correlates with Poor Clinical Outcome

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ABSTRACT

Purpose: The aim of this study was to evaluate the prevalence and the clinical significance of HIN-1 mRNA expression in early stage non-small cell lung carcinomas (NSCLCs).

Experimental Design: A series of 91 NSCLC patients with stage I neoplastic disease was studied. HIN-1 expression was investigated by quantitative real-time reverse transcription-PCR on tumor specimens and matching normal lung tissues. Variables were analyzed by χ^2 test and Fisher's exact tests. Survival was evaluated with the method of Kaplan-Meier. Multivariate analysis was performed with Cox's proportional hazards model.

Results: Seventy one (78%) tumors showed a reduction of *HIN-1* mRNA compared with the normal counterpart. The range of reduction varied greatly, from −2-fold to −3350-fold. Setting a cutoff at −46-fold (median value of *HIN-1* mRNA reduction), 46 cases (51%) had a markedly reduced expression, and 45 cases (49%) showed a normal or slightly reduced expression. A statistically significant association between low *HIN-1* mRNA levels and T status was observed ($P = 0.036$). Univariate survival curves, estimated using the method of Kaplan-Meier, defined a significant association between *HIN-1* expression and both overall survival ($P = 0.0095$) and disease-free survival ($P = 0.0122$). A multivariate analysis, performed by Cox's proportional hazards regression model, confirmed that a low *HIN-1* expression was the only significant factor to predict poor prognosis.

Conclusions: Our data indicate that *HIN-1* expression, measured by real-time reverse transcription-PCR, is a possible prognostic factor in patients with stage I NSCLC. Additional studies are required to further validate this potential prognostic marker.

INTRODUCTION

Lung cancer is currently the leading cause of cancer death in both men and women in developed countries (1). Although new surgical procedures and techniques have been developed, the status of the treatment of this neoplasm and the median survival time of patients is not encouraging. The expected 5-year survival rate for all patients in whom non-small cell lung carcinoma (NSCLC) is diagnosed is 15% compared with 61% for colon cancer, 86% for breast cancer, and 96% for prostate cancer (2). The prognosis of patients is dependent on the stage of disease, and the expectations of surgical treatment are better for stage I NSCLC patients. However, 30–40% of stage I patients have tumor recurrence within 5 years and die despite complete surgical resection (3). The identification, in this latter group, of molecular markers with which to define a subset of individuals as candidates for new therapies could lead to an improvement in overall survival.

Molecular biological studies have revealed that lung cancer develops through sequential steps, with the accumulation of multiple genetic and epigenetic alterations affecting both tumor suppressor genes and dominant oncogenes (4). A recent study conducted by serial analysis of gene expression identified a putative tumor suppressor gene, *HIN-1*, which codes for a secretoglobin protein that negatively regulates cell growth that may act in a concentration-dependent autocrine manner (5). A high expression of *HIN-1* has been shown in organs that are composed of branching ductal epithelia, such as lung, breast, prostate, and salivary gland (5, 6). Abundant transcripts of *HIN-1* gene have been identified in normal mammary epithelial cells, although *HIN-1* expression was found to be significantly down-regulated or not detectable in 88% of human breast carcinomas and in several mammary cancer cell lines. Moreover, it has been observed that reintroduction of *HIN-1* into breast cancer cells inhibits their growth (5). These results suggest that *HIN-1* is a candidate tumor suppressor gene that appears inactivated in breast tumorigenesis.

In adult and developing mouse tissues, the highest *HIN-1* expression has been detected in the lung; this expression is restricted to terminally differentiated airway epithelial cells *in vivo* and *in vitro* (5, 6). Northern blot analysis of *HIN-1* expression in human tissues confirmed that normal lung exhibits the highest expression. In addition, using a dot blot expression array, a significant reduction of *HIN-1* mRNA was observed in

the vast majority of the 40 primary lung cancers investigated (5, 6). The *HIN-1* gene has been mapped to chromosome 5q33, a region potentially implicated in human carcinogenesis. Allelic deletion on chromosome 5q has been shown in different human tumors, including those of the lung (7–11). However, the loss of *HIN-1* expression is unlikely to be caused by genetic alterations (loss of heterozygosity or mutations). By a methylation-specific PCR assay, it has been shown in both breast and lung carcinomas that epigenetic events (promoter or gene methylation) are the main mechanisms of HIN-1 inactivation (5).

In this study, we report an analysis of *HIN-1* mRNA expression in a large series of stage I NSCLC patients by quantitative real-time reverse transcription (RT)-PCR. Our results indicate that *HIN-1* is down-regulated in primary tumors of most stage I NSCLC patients and that a reduced expression of this gene is an independent predictor of poor prognosis.

MATERIALS AND METHODS

Patients and Tissues. The tumors for this study were obtained from a consecutive series of 102 stage I (T_{1-2}, N_0, M_0) non-small cell lung cancer patients surgically treated at the Department of Surgery, University of Pisa (Pisa, Italy) between 1993 and 1994. The study was conducted on 91 of these patients for whom tissues and complete follow-up data were available. Informed consent was obtained from all patients under study. In each case, tumor and macroscopically normal lung tissue samples (taken as far as possible from the neoplastic area) were snap-frozen in liquid nitrogen within 10 min of excision and stored at −80°C. Pieces of tumor and normal tissues, immediately adjacent to the corresponding frozen samples, were fixed and processed for light microscopy. In all tumor specimens, the amount of tumor cells equaled or exceeded 80% of the overall sample, confirmed by histopathological examination. Similarly, all of the macroscopically normal samples were judged to be histologically normal.

The study population consisted of 85 men (93%) and 6 women (7%), with a mean age of 63.1 years (range 43–74 years). Patients underwent lobectomy (84% of cases) or pneumonectomy (16% of cases) with hylar and mediastinal lymph node sampling. Patient stage at the time of diagnosis was determined according to the guidelines of the American Joint Committee on Cancer (12). On the basis of sizes, 29 (32%) tumors were classified as T_1 and 62 (68%) tumors as T_2. Histological type and tumor cell differentiation were determined according to the WHO criteria (13). The most common histological type was squamous cell carcinoma (50 cases, 55%), followed by adenocarcinoma (37 cases, 41%) and large cell carcinoma (4 cases, 4%). Twenty-four (26%) tumors were well-differentiated (G_1), 36 (40%) moderately differentiated (G_2), and 31 (34%) poorly differentiated (G3). Smoking history was available for 78 patients. Forty-five (58%) were smokers, 31 (40%) were former smokers (stopped smoking at least 1 year before the diagnosis of lung cancer), and 2 (2%) were nonsmokers.

Follow-up data of the study population were obtained by direct patient contact. Follow-up occurred at 2-month intervals for the initial 2 years and at 4-month intervals afterward. Recurrences were detected by computed tomography scans or scintigrams and confirmed by pathological examination, using biopsy specimens. Patients were categorized as alive with evidence of disease, alive without disease, and dead as a result of lung carcinoma. No patient in this series died of cancer-unrelated causes. Time in days from the date of the operation to the date of follow-up or death was recorded. The median follow-up in the series of patients examined was 55 months (range, 7–94 months).

RNA Extraction and cDNA Synthesis. Total RNA was extracted from frozen tumor and normal tissue specimens by using a commercial kit, TRIzol (Life Technologies, Inc.), according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis through agarose gels stained with ethidium bromide. Total RNA (200 ng) was reverse transcribed in a total volume of 50 μl containing 1× TaqMan buffer (5.5 mM $MgCl_2$, 1 mM deoxynucleotides, 2.5 μM random hexamers, 20 units RNase inhibitor, 62.5 units murine leukemia virus reverse transcriptase). The samples were incubated at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min.

PCR Amplification. PCR was performed in a total volume of 50 μl containing 1× TaqMan buffer (5.5 mM $MgCl_2$; 200 μM dATP, dCTP, dGTP, and 400 μM dUTP; 300 nM each primer; 100 nM probe; 0.5 units of AmpErase UNG; 1.25 units AmpliTaq Gold; and 10 μl of cDNA). Both β-actin and *HIN-1* amplification were done in duplicate for each sample. The thermal cycling conditions included 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reagents used for RT-PCR were purchased from Applied Biosystems (Foster City, CA).

Primers and Probes. The primer probe set for HIN-1, obtained from Krop *et al.* (5) was as follows: forward primer, 5′-GAGCATCTACACCTGAGGACAAGAC-3′; reverse primer, 5′-TTTTGCTCTTAACCACGTTTATTGA-3′; and Taqman probe, (FAM)-CACCCGCGAGGGCTGAAAACC-(TAMRA). Primers and probe for β-actin mRNA (GenBank accession number X00351) were chosen using a computer program, Primer Express (Applied Biosystems, Foster City, CA). Primers and probe for β-actin mRNA were as follows: forward primer, 5′-TCCTTCCT-GGGCATGGAG-3′; reverse primer, 5′-AGGAGGAGCAAT-GATCTTGATCTT-3′; TaqMan probe 5′(FAM)-CCTGTGGCA-TCCACGAAACTACCTTC-(TAMRA)3′. Probes were purchased from Applied Biosystems.

Real-Time RT-PCR Analysis. *HIN-1* expression in tumors and matching normal lung samples was measured by real-time quantitative RT-PCR, based on TaqMan methodology, using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). This technique allows, by fluorescence emission, the location of the cycling point when PCR product is detectable (*Ct* value or threshold cycle). As reported previously, the *Ct* value correlates to the starting quantity of target mRNA (14).

To normalize the amount of total RNA present in each reaction, we amplified the housekeeping gene β-actin, which is assumed to be constant in both normal samples and lung carcinomas. Our results are expressed as relative levels of *HIN-1* mRNA, referred to a sample called calibrator chosen to represent 1× expression of this gene. The calibrator used was a lung carcinoma of the tissue collection under study, arbitrarily se-
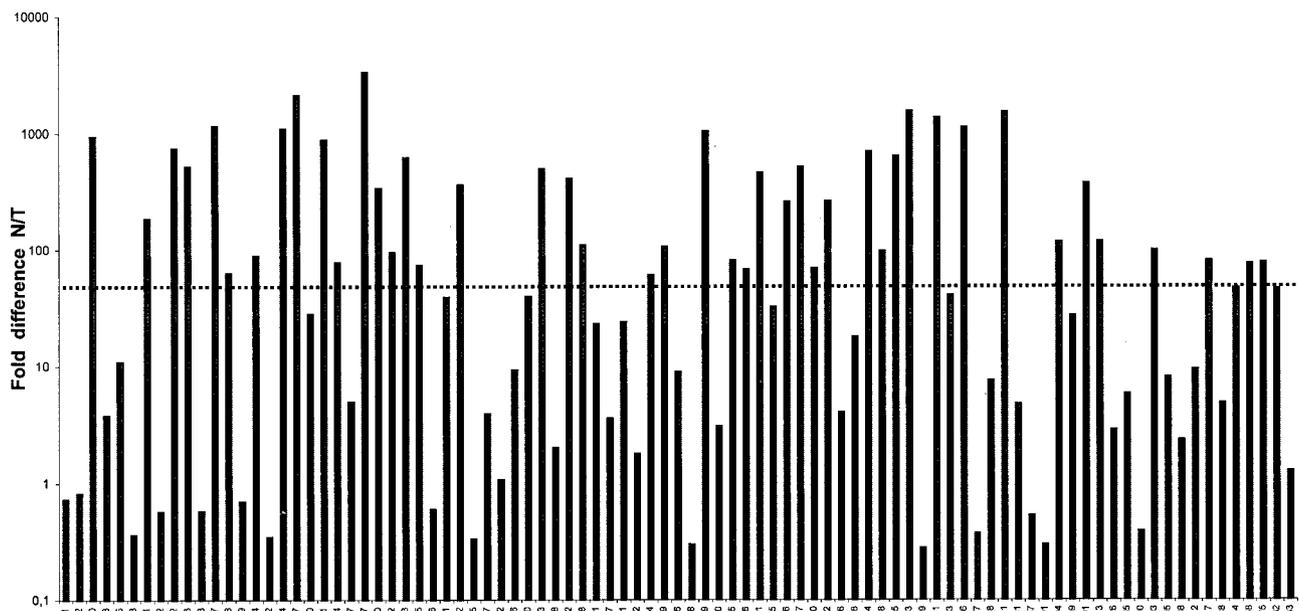
Fig. 1 Real-time quantitative reverse transcription-PCR analysis of HIN-1 expression in the 91 non-small cell lung carcinomas and matching normal lung samples. Each *bar* corresponds to a patient. Fold difference indicates the ratio of HIN-1 levels in normal and neoplastic tissue. The *dashed line* corresponds to the median value of *HIN-1* mRNA reduction.

lected, that was analyzed on every assay plate with the unknown samples. All of the analyzed tumors express *n*-fold *HIN-1* mRNA relative to the calibrator.

The amount of target normalized to an endogenous reference (β-actin) and relative to the calibrator is definite by the $\Delta\Delta Ct$ method as described by Livak K (Sequence Detector User Bulletin 2; Applied Biosystems, Foster City, CA). Specifically, the formula is applied as follows: target amount $= 2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = \{[Ct$ (*HIN-1* sample) $- Ct$ (β-actin sample)] $-$ [*Ct* (*HIN-1* calibrator) $- Ct$ (β-actin calibrator)]$\}$.

This method is based on the assumption that the target (*HIN-1*) and the reference (β-actin) display equal amplification efficiencies. To verify this condition, we checked ΔCt (*Ct* HIN-1 $- Ct$ β-actin) variations according to template dilution. To this end, we prepared a standard curve, composed of five different dilutions of total RNA obtained from a normal lung tissue (100, 25, 6.25, 1.6, 0.4 ng). The slope of this curve was 0.041. To assure the appropriate amplification efficiency, the slope of the standard curve should be <0.1.

Statistical Methods. The relationships between *HIN-1* expression and clinicopathological parameters were assessed by Fisher's exact test or χ^2 test as appropriate. The survival curves were estimated using the Kaplan-Meier method, and differences among them were evaluated by the log-rank test. Disease-free survival was defined as the period between surgery and the first local recurrence, the evidence of distant metastasis, or the end of the study. Overall survival was defined as the period from surgery to the patient's death. Cox's proportional hazards regression model was used to assess the impact of *HIN-1* expression on disease-free and overall survival after adjustment for tumor size (T_1 *versus* T_2), histological type (squamous carcinoma *versus* other histotypes), and tumor grade (G_{1-2} *versus*

G_3). The assumptions of the proportional hazards model were checked by plotting the log of the cumulative hazard function. A $P < 0.05$ was considered as significant.

RESULTS

HIN-1 Expression in Normal Lung and NSCLC Samples. To assess quantitatively the expression of *HIN-1* in early stage NSCLC, we analyzed, by real-time RT-PCR, tumors and matching normal lung tissues from 91 NSCLC patients at stage I. In all of the normal lung tissues examined, *HIN-1* mRNA was expressed at high levels. In 20 (22%) tumors we found a *HIN-1* mRNA expression comparable to that observed in the corresponding normal lung tissues. Seventy-one (78%) tumors showed a >2-fold reduction of *HIN-1* mRNA compared with the normal counterpart. The range of reduction of *HIN-1* expression varied greatly, from -2 n to -3350 n. Setting a cutoff at -46 n, which represented the median value of the reduction of *HIN-1* mRNA expression, 46 cases (51%) had markedly reduced *HIN-1* levels, and 45 cases (49%) showed normal or slightly reduced *HIN-1* levels. The extent of reduction of *HIN-1* expression in all of the tumors examined, expressed by the ratio of HIN-1 mRNA levels in normal and neoplastic tissue, is shown in Fig. 1.

Clinicopathological Correlations. No correlation was observed among decreased *HIN-1* expression and age, smoking habits, tumor grade, and tumor histotype. A statistically significant association between low expression levels of *HIN-1* and tumor extension, as measured by the T pathological staging, was present. A normal *HIN-1* expression was observed in 42% of T_1 tumors whereas T_2 tumors showed low *HIN-1* expression in 78% of cases, $P = 0.036$. Results are detailed in Table 1.

Table 1 Comparison of clinical and pathological variables with HIN-1 expression in stage I NSCLC[a] patients

| Variable | HIN-1 expression | | *P* |
	Reduced	Normal	
Age (mean $\pm$ SD, yr)	63.3 $\pm$ 7.1	63.1 $\pm$ 6.3	NS (0.7324)
Smoking habits[b]			
Smoker	23 (61%)	22 (55%)	
Former smoker	15 (39%)	16 (40%)	NS (0.367)
Non-smoker	0 (0%)	2 (5%)	
Tumor size			
T_1	10 (22%)	19 (42%)	
T_2	36 (78%)	26 (58%)	0.036
Histological type			
Squamous carcinoma	27 (59%)	23 (51%)	NS (0.3707)
Adenocarcinoma	16 (35%)	21 (47%)	
Large cell carcinoma	1 (6%)	1 (2%)	
Tumor grade			
G_1	8 (17%)	16 (36%)	
G_2	21 (46%)	15 (33%)	NS (0.1390)
G_3	17 (37%)	14 (31%)	

[a] NSCLC, non-small cell lung carcinoma; NS, not significant.
[b] Smoking history was available for 78 patients.

Table 2 Statistical analysis of prognostic markers and clinical outcome in stage I NSCLC[a] patients

Factor	Disease-free survival *P*	Overall survival *P*
Tumor size	0.3220 (NS)	0.4739 (NS)
Histologic grade	0.5870 (NS)	0.8682 (NS)
Histotype	0.3092 (NS)	0.3049 (NS)
HIN-1 expression	0.0122	0.0095

[a] NSCLC, non-small cell lung carcinoma; NS, not significant.

Survival Analysis. According to the Kaplan-Meier survival curves, the 5-year survival rate in the series of patients examined was 76%. Differences in survival of the patients were not significant in terms of T status, histotype, and histological grade of the tumor (Table 2). The median time to recurrence was 22 months (range, 3–56 months); the recurrence rate was 31 of 91 (34%), and the recurrences were initially located at a distant site in 24 cases (26%) or within the ipsilateral hemi-thorax in 7 cases (8%).

Univariate survival curves (Fig. 2), estimated using the method of Kaplan and Meier, defined a significantly worse prognosis for patients with low *HIN-1* expression. The five-year disease-free survival rates for normal-*HIN-1* and low-*HIN-1* patients were 82% and 56% (*P* = 0.0122), respectively. *HIN-1* expression proved to affect overall survival as well. The five-year survival rates were 84% for the patients with normal *HIN-1* expression and 60% for the patients with low *HIN-1* expression (*P* = 0.0095). There was no significant correlation between disease-free survival and T status, histological type, and histological grade of the tumor (Table 2). The joint effect of tumor size, histological type, and histological grade were examined using stepwise Cox regression. The multivariate analysis confirmed that *HIN-1* expression was the only independent and significant factor to predict poor prognosis, for both disease-free (*P* = 0.03) and overall survival (*P* = 0.02; Table 3).

DISCUSSION

Lung cancer is a complex multifactorial multistep disease wherein genetic and epigenetic alterations represent crucial regulators of initiation, promotion, and progression. The resulting clinico-pathological heterogeneity of this disease makes current diagnostic and therapeutic strategies inappropriate for an optimal treatment of each patient. Recent studies by global gene expression analysis might allow for the deciphering of the molecular complexity of cancer, providing opportunities to identify new diagnostic and prognostic markers and new therapeutic targets (15–17). In one of these studies, conducted by serial analysis of gene expression, several genes differentially expressed in neoplastic cells were found. *HIN-1*, a member of the secretoglobin family, is one of the most promising genes to have emerged from this study (5).

We have examined the expression levels of *HIN-1* by real time RT-PCR in 91 NSCLCs and matching normal lung tissues from a well-characterized series of consecutive operable patients. These patients were subjected to surgery in a single
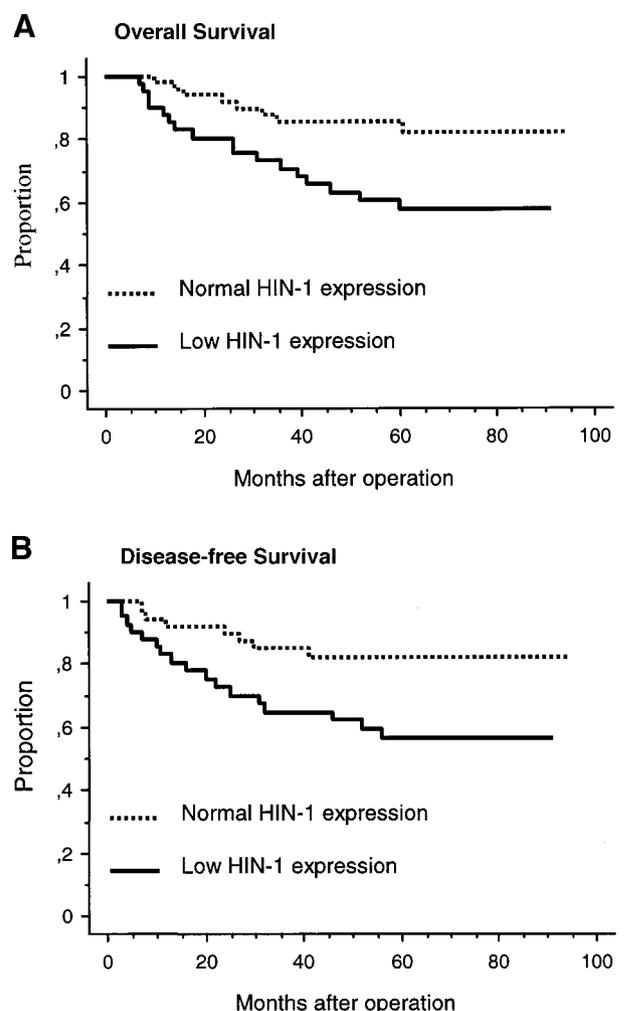

Fig. 2 Overall (*A*) and disease-free (*B*) survival curves in the 91 stage I non-small cell lung carcinoma patients according to the HIN-1 expression status in primary tumors.

Table 3 Multivariate analysis of prognostic variables for disease-free and overall survival

Variable	Overall survival				Disease-free survival			
	β^a	SE	HR (95% CI)	P	β	SE	HR (95% CI)	P
Tumor differentiation G_1-G_2/G_3	0.89	0.62	2.43 (8.26–0.72)	NS (0.15)	0.96	0.62	2.60 (8.85–0.77)	NS (0.12)
Histological type SCC/non-SCC	0.59	0.41	1.82 (4.02–0.82)	NS (0.14)	0.53	0.40	1.70 (3.75–0.77)	NS (0.18)
Tumor size T_1/T_2	0.08	0.46	0.93 (2.29–0.37)	NS (0.87)	0.04	0.46	0.96 (2.36–0.39)	NS (0.93)
HIN-1 Low/High	1.03	0.45	2.79 (6.80–1.15)	0.02	0.97	0.45	2.63 (6.33–1.09)	0.03

[a] β, β coefficient; SE, standard error; HR, hazard ratio; CI, confidence interval; NS, not significant; SCC, squamous cell carcinoma.

institution, had standardized therapy (surgical resection only), long-term complete follow-up, and did not have any confounding outcome variables, such us poor performance status, positive lymph nodes, or distant metastasis.

Elevated *HIN-1* mRNA levels were detected in all of the normal lung samples examined. This confirmed previous data obtained in adult and developing mouse and in human tissues indicating that *HIN-1* is highly expressed in normal lung (5, 6). Indeed, in both mouse and human subjects, the highest expression of *HIN-1* has been shown in lung tissue. A substantial expression of *HIN-1* has been reported also in other organs composed of branching ductal epithelia including breast, prostate, and salivary glands, in keeping with the hypothesis that *HIN-1* may be involved in regulating epithelial cell proliferation, differentiation, and morphogenesis (5, 6, 18, 19).

In almost 80% of the primary lung tumors examined, we found a decreased expression of *HIN-1*. These data are in agreement with results reported by Krop *et al.* (5) in a series of 32 primary breast carcinoma samples evaluated by real time RT-PCR. *HIN-1* expression was found to be decreased in 88% of cases. In this latter study, similar data were reported by using a dot blot expression array in a series of 40 primary lung tumors. In addition, it has been shown that the loss of *HIN-1* expression in breast and lung tumors is mainly caused by epigenetic mechanisms (5, 20, 21). The promoter region and the first exon of this gene harbor an ≈1500-bp CpG island containing 138 potential methylation sites. In most breast and lung tumors with decreased expression of *HIN-1*, the promoter region of the gene was found to be hypermethylated by using a methylation-specific PCR assay. Moreover, the treatment of breast cancer cell lines with a DNA methyl-transferase inhibitor led to a dramatic re-expression of *HIN-1* to levels found in normal mammary epithelial cells, and the reintroduction of *HIN-1* into breast cancer cells inhibits cell growth (5). Taken together, these results suggest that *HIN-1* is a putative tumor suppressor gene playing a role in multiple cancer types.

When tumors examined in this study were subdivided into two groups with low and high *HIN-1* expression, according to the median value of expression distribution, a significant association was observed between *HIN-1* mRNA levels and tumor size (*P* = 0.036). This may indicate that expression of *HIN-1* is somehow related to tumor progression. However, a reduced expression of the *HIN-1* gene was present in 10 (22%) T_1 tumors suggesting that alterations of this gene can occur from the earliest phases of the neoplastic development. This is in keeping with the observation that in breast carcinoma *in situ* and in atypical ductal hyperplasia, *HIN-1* levels can be significantly decreased (5).

A trend indicating an association between *HIN-1* and tumor grade has emerged, but data were not statistically significant. In particular, G_1 (histologically well-differentiated) tumors showed the lowest percentage (17%) of *HIN-1* reduction. This is interesting, considering that in mouse normal tissues HIN-I is mainly expressed in terminally differentiated epithelial cells (6). In this view, it is tempting to hypothesize that decreased levels of *HIN-1* expression could induce loss of differentiation, increased cell growth, and a more aggressive tumor phenotype. No association was observed between *HIN-1* expression and other clinico-pathological parameters such us age, smoking habits, and tumor histotype.

When results were compared with follow-up data, *HIN-1* expression was found to be a significant predictor of overall and disease-free survival (*P* = 0.0095 and *P* = 0.0122, respectively). Other pathological variables evaluated (including tumor size, histological type, and histological grade of the tumors) were not significantly associated with prognosis. A Cox proportional hazards model that included *HIN-1* expression and three other pathological variables (tumor size, tumor histology, and histological grade) still showed that *HIN-1* mRNA expression had a significant independent predictive power for both overall survival (*P* = 0.02) and disease-free survival (*P* = 0.03). To our knowledge, this is the first time that *HIN-1* mRNA levels have been accurately quantified in NSCLC samples and that *HIN-1* expression has been associated with patient outcome. In our judgement, the fact that all of the patients were treated at a single institution and received a long follow-up after surgery makes survival analysis quite reliable.

In conclusion, our data indicate that *HIN-1* is highly expressed in normal lung tissue and frequently down-regulated in lung cancer, irrespective of tumor histotype. Our findings, together with those published previously concerning breast carcinoma patients, suggest that *HIN-1* plays a role in different forms of human malignancies. Moreover, *HIN-1* expression may represent an important prognostic factor that should be validated in future prospective multi-institutional trials of adjuvant therapy for high risk stage I NSCLC patients.

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